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Structure of *Mycobacterium tuberculosis* Phe-tRNA synthetase reveals ligand binding and two-stage tRNA recognition

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Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis*, responsible for ~1.5 million fatalities in 2018, is the deadliest infectious disease. Global spread of multidrug resistant TB is a public health threat. Aminoacyl-tRNA synthetases are plausible candidates as potential targets because they play pivotal roles in translating the DNA code into protein sequence by attaching specific amino acid to their cognate tRNAs. One of the best characterized synthetases is specific for L-Phe and tRNA^{Phe}. Here we report structures of *M. tuberculosis* Phe-tRNA synthetase complexed with precursor tRNA and either phenylalanine or a phenylalanine adenylate analog, 5'-O-(N-phenylalanyl)sulfamoyl-adenosine. Crystallographic models for the first time reveal two modes of interaction with tRNA: an initial recognition via the anticodon loop and stem only and productive binding with interaction of the 3' end of tRNA^{Phe} with the adenylate site. We biochemically characterize the enzyme and provide the highest resolution, most complete view of the Phe-tRNA synthetase/tRNA^{Phe} system to date.

Introduction

With ~1.5 million people killed in 2018, *Mycobacterium tuberculosis*, the etiological agent of the tuberculosis (TB)¹ remains the deadliest pathogen worldwide ¹ and the leading cause of morbidity and mortality among HIV-infected patients. It is estimated that one third of the world's population is at risk of developing active TB disease, especially in low and middle income countries. Extensively drug resistant TB (XDR-TB) and multidrug-resistant tuberculosis (MDR-TB) is a growing global public health threat ². The existing TB multi-drug treatments are long, particularly for XDR-TB and MDR-TB infections. Discovery of new drugs with novel mechanisms of action are critical to treat infections with existing drug resistant strains and to shorten TB treatment ³.

Protein translation is an essential processes and the target of Linezolid an approved drug for TB treatment and several other compounds in clinical trials for TB ^{4,5}. One of the crucial steps in protein synthesis involves the enzymatic attachment of a specific amino acid to its cognate tRNA ⁶, carried out by aminoacyl-tRNA synthetases (aaRS). In addition, some aaRSs deploy proofreading mechanisms to ensure correct pairing. The synthesis of aminoacyl-tRNA is accomplished by the 2' or 3'-esterification of tRNA with an appropriate amino acid in a two-step reaction. First, the aminoacyl-adenylate is formed by reaction between ATP and an amino acid, sometimes in a tRNA-dependent manner ⁷, followed by ligation to the adenosine of tRNA.

FRS, which is specific for L-phenylalanine and tRNA^{Phe}, has been studied extensively biochemically, structurally and computationally for the past 50 years ⁸⁻¹¹. Several FRS structures at moderate resolution are available in Protein Data Bank (PDB) from bacteria, archaea and eukaryotes (Table 1). FRS belongs to the class IIC aaRS but it is considered an outlier and shows similarity to class IID enzymes SRS and ancient GRS and ARS ⁶. Typically, class II aaRSs aminoacylate tRNA on 3'OH of ribose, but FRS does so on 2'OH. The bacterial, archaeal and human cytoplasmic enzymes consist of α (PheS) and β subunits (PheT) that via domain swapping form highly intertwined ($\alpha\beta$)₂ heterotetramers ^{10,12-16}. The α subunit recognizes L-Phe and 3' terminal adenosine of tRNA and carries out key catalytic activities ¹⁷. The β subunit recognizes tRNA^{Phe} and provides editing function assuring that the L-Phe is attached to tRNA^{Phe} ¹⁸.

¹ Abbreviations used: aaRS, aminoacyl-tRNA synthetase; F-AMS, 5'-O-(N-phenylalanyl)sulfamoyl-adenosine; FRS, phenylalanyl-tRNA synthetase; hmFRS, human mitochondrial FRS; hcFRS, human cytosolic FRS; TB, tuberculosis;

As essential enzymes, aaRS have been for a long time considered as drug targets ^{19,20}. For TB, an inhibitor of LRS has recently completed Phase 1 clinical trials ⁴. FRS has been recognized as a high-priority antimicrobial drug target ^{18,20} and several inhibitors have been reported. Phenyl-thiazolylurea-sulfonamides are selective and potent inhibitors of both Gram-negative and Gram-positive bacterial FRS orthologs ⁸. Other bacterial FRS inhibitors include ethanolamine derivatives for *Staphylococcus aureus* ²¹, gossypol for *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* ²² and some natural products for *MtFRS* ²³. However, no FRS inhibitor has reached advanced therapeutic development stages.

Thus far, *M. tuberculosis* FRS has not been characterized, hampering structure-based drug discovery approaches. Here, we address this major knowledge gap and present four crystal structures of *MtFRS* in complex with a specific tRNA^{Phe} precursor (*MttRNA*^{Phe}). For the first time we show tRNA recognition and binding occurring in two stages. The “initial tRNA recognition” state was determined with either bound L-Phe (in two crystal forms), or with an inhibitor – a nonhydrolyzable phenylalanine adenylate analog 5'-O-(N-phenylalanyl)sulfamoyl-adenosine (F-AMS). The structure of productive tRNA aminoacylation state that is predisposed for acceptance of an activated amino acid was determined in complex with F-AMS. These structures offer the most complete picture of the FRS-tRNA^{Phe} complex to date and combined with biochemical characterization of the enzyme and F-AMS binding provide the bases for rational structure-based drug design of selective inhibitors of *MtFRS*.

Results and Discussion

tRNA^{Phe} and F-AMS binding

To investigate structure-function relationship of *MtFRS*, we have co-expressed PheS (α) and PheT (β) subunits of *MtFRS* and purified the functional heterotetrameric complex. In addition, we have prepared unmodified *M. tuberculosis* tRNA^{Phe} precursor specific for the 5'UUC codon – iso-acceptor *MttRNA*^{Phe} with the GAA3' anticodon. This synthetic *MttRNA*^{Phe} lacks post-transcriptional modifications. The actual nature of genus-specific modifications is unknown but in other organisms tRNA^{Phe} has been shown to carry many variations: m²G, D, Cm³, Gm³, Cm, Gm, 2-thiomethyl-N⁶-(delta²-isopentenyl)A, m¹G, m⁵C, Ψ , m¹C, 3-(3-carboxypropyl)U, T, m¹A and in eukaryotic tRNAs also large wybutosine ²⁴. The majority of these variations have little effect on aminoacylation by

aaRSs but are important for codon – anticodon interactions, translational rates, reading frame maintenance, co-translational folding dynamics and other cellular processes ^{25,26}.

To confirm enzymatic activity of the protein, we have independently tested both reaction steps, L-Phe adenylation and tRNA aminoacylation, with three substrates: synthetic *Mt*tRNA^{Phe} precursor as well as *E. coli* and yeast tRNA^{Phe}. Unmodified *Mt*tRNA^{Phe} can be almost completely (>99%) charged by *Mt*FRS with L-Phe (Fig. 1). *Mt*FRS can also aminoacylate mature tRNA^{Phe} molecules isolated from *E. coli* and yeasts (Fig. 1). *Mt*FRS, like all known FRS, is able to activate its amino acid substrate in the absence of tRNA (Fig. 1A-1C), though the addition of tRNA stimulates this reaction.

Aminoacyl-sulfamoyl adenosine have been previously been reported as inhibitors of bacterial aaRS ²⁷⁻²⁹. Here, we report strong inhibition of *Mt*FRS by the non-hydrolyzable phenylalanine adenylate analog, 5'-*O*-[*N*-(phenylalanine)sulfamoyl] adenosine (F-AMS). Earlier studies have demonstrated that aminoacyl-sulfamoyl-adenosines can efficiently inhibit *E. coli* ERS (K_i 2.8 nM) ²⁷, murine liver ERS (K_i ~70 nM) ²⁷, *E. coli* QRS (K_i 1.3 μ M), *S. aureus* IRS (IC₅₀ 4 nM) ²⁹, RRS (IC₅₀ 4.5 nM), HRS (IC₅₀ 130 nM) and TRS (IC₅₀ 15 nM) ²⁸. F-AMS acts as a competitive inhibitor with a K_i of 33 nM and IC₅₀ value of approximately 36 nM (Fig. 2).

Overall structure

To understand structural basis of aminoacylation reaction and tRNA recognition, we have crystallized *Mt*FRS with *Mt*tRNA^{Phe} and either L-Phe or F-AMS. Four structures have been determined - two representing different crystal forms of the *Mt*tRNA^{Phe}/L-Phe complex (*Mt*FRSt/F1, and *Mt*FRSt/F2) and two corresponding to different forms of the *Mt*tRNA^{Phe}/F-AMS complex (*Mt*FRSt/F-AMS1 and *Mt*FRSt/F-AMS2) (Fig. 3, Table 2). The former three structures illustrate the initial tRNA recognition state, where the enzyme binds primarily to the anticodon region, with only partial contacts with D- and T-loops of tRNA. In these structures the electron density for the acceptor end of tRNA is poorly defined and cannot be modeled. The *Mt*FRSt/F-AMS2 structure corresponds to the enzymatically productive tRNA binding, where the complex of *Mt*FRS with tRNA^{Phe} is predisposed for acceptance of the activated amino acid. *Mt*FRSt/F2 and *Mt*FRSt/F-AMS1 are isomorphous and contain a single $\alpha\beta$ unit in the asymmetric unit (ASU), while *Mt*FRSt/F1 and *Mt*FRSt/F-AMS2 have an entire $(\alpha\beta)_2$ heterotetramer in the ASU of different unit cells. The crystal lattice or the tRNA binding status do not seem to affect the protein interactions with L-Phe, F-AMS and tRNA ligands.

The structures of enzyme with specific *MttRNA*^{Phe} precursor shows the previously described tetrameric assembly (Fig. 3). The electron density for protein, tRNA, L-Phe, F-AMS is excellent and with a few exceptions, as discussed earlier and below, the entire structure can be modeled. The ($\alpha\beta$)₂ assembly has a complex shape that can fit into rectangular prism with overall dimensions 70 Å x 110 Å x 170 Å. The individual subunits are composed of several domains, which structurally and functionally make sense only when assembled into highly intertwined heterotetramer of this intricate molecular machine. Each tRNA molecule interacts with all four polypeptide chains.

Our high-resolution structures allow us to improve domain assignment and their boundaries. The smaller α subunit (modeled residues 3 - 341) is very extended (~130 Å) and has two distinct domains, $\alpha 1$ and $\alpha 2$, connected by a 14-residue linker (Fig. 4, Supplemental Fig. S2). The N-terminal $\alpha 1$ all helical domain binds the tRNA D-loop region and the C-terminal $\alpha 2$ domain performs aminoacylation. The larger β subunit (modeled residues 1 - 831) can be subdivided into three globular domains $\beta 1$ -3, two of which have subdomains (Fig. 4, Supplemental Fig. S2). $\beta 1$ has four distinct subdomains: $\beta 1s1$, $\beta 1s2$ and $\beta 1s3$ corresponding to the editing domain that shares fold with $\alpha 2$ catalytic domain (CATH domain 3.30.930.10), and $\beta 1s4$. The latter, a small α/β subdomain together with $\beta 1s1$ interacts with the $\alpha 2$ aminoacylation domain via direct protein-protein contacts and a conserved magnesium ion. The $\beta 1s1$, $\beta 1s3$ and $\beta 1s4$ subdomains form a core of $\beta 1$ and $\beta 1s2$ is on its periphery. $\beta 1s4$ is followed by a 13-residue linker connecting to the $\beta 2$ domain, which consists of two subdomains – larger $\beta 2s1$ and smaller $\beta 2s2$. $\beta 2$ interacts extensively with aminoacylation domain $\alpha 2$ (surface area ~2,150 Å²) contributing significantly to the complex interface. $\beta 2$, via a 15-residue linker, leads to the C-terminal domain $\beta 3$ that extends out of the main body of the protein and is responsible for tRNA anticodon binding.

***MttRNA*^{Phe} structure**

The complete *MttRNA*^{Phe} molecules are modeled only in the *MtFRSt*/F-AMS2 complex, where the only atoms missing from the model correspond to the linkage between U22 and G23. Notably, this structure has been determined at high magnesium concentration and several Mg²⁺ ions have been identified – some of them are highly hydrated and bind exclusively to the nucleic acid via electrostatic interactions rather than direct metal ion coordination. The complete structure of *MttRNA*^{Phe} is similar to the partial structure of the *MttRNA*^{Phe} in the initial recognition structure (see below) and other structures: the mature yeast equivalent carrying the Y base in the anticodon region (PDB id 4TNA),

T. thermophilus precursor tRNA^{Phe} in complex with FRS (PDB id 2IY5) and unmodified tRNA^{Phe} from *E. coli* (PDB id 3L0U). Sequence identity of these tRNAs varies between 45 to 60% and their superposition indicates that root mean square deviations (RMSD) between them varies between 2.3 to 3.2 Å, with the largest differences observed for *MttRNA*^{Phe} and *TtRNA*^{Phe}. There is one magnesium site in the D-loop region conserved between yeast, *E. coli* and *MttRNA*^{Phe}. As expected, the largest differences are in the 3' and 5' ends, anticodon, T- and D-loops. Some of the discrepancies may result from the fact that we compare free tRNA (*E. coli* and yeast) with tRNA bound to protein (*M. tuberculosis* and *T. thermophilus*). In fact, anticodon region of these last two tRNAs are more similar to each other. The anticodon loop after the U34 base forms a canonical U-turn stabilized by an internal hydrogen bond of U34 N3 to phosphoryl group of A37 and 2'OH to N7 of A36. This element is conserved in all four structures. In *MttRNA*^{Phe}, six consecutive purine bases in the anticodon region (G35 to G40) stack together and form a rigid structure important for tRNA recognition (see below), while *E. coli*, *T. thermophilus* and yeast tRNAs have a block of five stacked purine bases. Majority of bases are involved in base-pairing/stacking, but curiously a number of bases project into solvent in different tRNAs and they correspond to this same RNA regions. For example: C17, U20, U48 (*M. tuberculosis*), U20, U45 (*E. coli*), dhU17, U47 (yeast), U17, U47 (*T. thermophilus*). These structural features must be conserved for functional reasons.

Ligand binding

The active site of FRS adenylation domain of the α subunit contains subpockets responsible for recognition of L-Phe and ATP substrates. Our structures illustrate how L-Phe and its adenylylate analog bind to their respective sites (Fig. 5). In the phenylalanine-dedicated subpocket (Fig. 5), α Phe255, α Phe257, α Ala305, α Arg201, and α Thr258 interact with the aromatic ring of the ligand in both *MtFRSt/F1* and *MtFRSt/F2* structures. α Arg201 and α Gln215 form hydrogen bonds with the carboxyl group while the amino group is bonded to α His175 and, via a water molecule, to α Gln215 and α Glu217. The latter residue participates in an extended network of hydrogen bonds which, besides α Gln215, includes α Ser177 and α Gln180. The organization of the L-Phe binding subpocket is virtually identical in our two L-Phe complex structures. Some discrepancies are observed in the neighborhood that does not directly participate in the amino acid recognition: the conformations of some side chains facing the adenine binding site differ between the two structures and FRSt/F2 contains Mg²⁺ ion bound nearby the pocket, interacting with α Glu263, α Glu279 and a water

molecule.

An analogous pattern of interaction with phenylalanyl portion of the F-AMS ligand is observed in the *MtFRSt*/F-AMS1 and *MtFRSt*/F-AMS2 complexes. Additional bonds are formed with sulfamoyl group, ribose and adenine moieties. The former interacts with the guanidinium group of α Arg201 and sometimes Glu263 (via a water molecule) while the sugar ring forms hydrogen bonds with α Gln215, main chain carbonyl oxygen atom of α Trp280 and carboxyl group of α Glu279 (in chain A of *MtFRSt*/F-AMS2). The nucleotide base is anchored between α His209 and α Phe213 and participates in several hydrogen bonds: (i) N1 with main chain nitrogen atom of α Thr210, (ii) N3 via a water molecule with main chain of α Arg312 and α His214, and side chain of α Glu311, (iii) N7 with α Arg201 and α Asp203 via another molecule, and (iv) N6 with main chain carbonyl group of α Thr210 and carboxyl group of α Asp203. Most of these interactions are conserved between the two F-AMS complexes, regardless of the tRNA binding status, with the exception of α Glu279, α Glu263 and N7-mediated contact. The adenine subpocket, in the absence of a biological molecule, is occupied by the glycerol molecule.

The conformation of α 2 domain is susceptible to the type of ligand, as clear rearrangements are observed when L-Phe and F-AMS complexes are compared. Specifically, in the presence of F-AMS, the loop connecting β 4 and β 5 elements from the β 4 $\uparrow$ β 5 $\downarrow$ β 9 $\uparrow$ β 8 $\downarrow$ β 7 $\uparrow$ β 6 $\downarrow$ sheet moves towards the β 8 $\downarrow$ β 7 $\uparrow$ β 6 $\downarrow$ half closing over the nucleotide moiety. This movement enables α His209 and α Asp203 to approach the F-AMS base ring (Fig. 5). The β 8 $\downarrow$ β 7 $\uparrow$ β 6 $\downarrow$ section, especially the β 7- β 8 hairpin also comes closer to the β 4- β 5 loop.

Initial tRNA^{Phe} recognition

In the assembly, each of the tRNA molecules contacts all four subunits through mostly α 1' and β 3' domains and to a lesser extent α 2, β 1, and β 2 (Fig. 3). Remarkably, tRNA recognition is accomplished by one $\alpha\beta$ unit while aminoacylation and editing are completed by the complementary $\alpha'\beta'$ dimer. In the initial recognition state, the anticodon-, D- and T- stem and loop regions are well-ordered. On the other hand, the 3' and 5' ends of tRNA are disordered and consequently only 5-70 tRNA bases could be modeled. In these structures, access to the aminoacylation site is blocked by the 3_{10} -helix region of the α 2 domain (residues 152-157, discussed below).

The tRNA selectivity is accomplished by enzyme interacting with the anticodon region of

tRNA^{Phe} via anticodon binding domain $\beta 3'$ (Fig. 6). The rigid 6-purine stack (G35 to G40) seems to be the key structural feature recognized by protein that provides a shallow surface with several specific contact points. The conserved β 'Phe780 (FRSs have either phenylalanine or tyrosine in this position) interact via π - π stacking with the G35 guanine wobble anticodon base, extending the GAAAAG RNA stack. In addition, G35 forms several direct hydrogen bonds. Specifically, β 'Asp778 hydrogen bonds to G35 N1 and N2, β 'Arg830 to O6 and N7, and β 'Gln784 interacts with G35 phosphoryl group or ribose ring oxygen atom (observed in two structures of the initial recognition state). Both β 'Asp778 and β 'Arg830 are highly conserved residues. There are two direct hydrogen bonds to A36, one from β 'Ser747 to N6 and second from β 'Thr793 to N1. A37 interacts with the carboxylate group of conserved β 'Asp745 via ribose 2'OH. The A38 ribose 2'OH also interacts with β 'Asp745.

The interactions with D-loop vary across all structures in the initial recognition state, indicating the dynamic nature of these contacts. Here, the $\alpha 1'$ domain participates in hydrogen bonds to G19 and/or U20 via α 'Arg45 and/or α 'Arg56, α 'Asp37. Inconsistent pattern of interactions is also present in binding with the T-loop. This region primarily implicates $\alpha 1'$ Asn64 residues and, in some instances, $\alpha 1'$ Ala57 and/or $\alpha 1'$ Arg45 that form hydrogen bonds with C57 and G58.

tRNA^{Phe} binding in preaminoacylation complex

The MtFRSt/F-AMS2 structure illustrates the tRNA binding where the entire tRNA molecule is ordered, with the anticodon region firmly bound and the acceptor arm approaching the active site containing the F-AMS – a phenylalanine adenylylate analog (Fig. 6). In this state the enzyme continues to interact with the anticodon region via domain $\beta 3'$, maintaining all interactions described above for the initial recognition state. D-loop G19 and U20 bind to the $\alpha 1'$ domain with an inconsistent pattern of interactions seen in the four structural representations. The T-loop is engaged as described above and variable loop contributes one stabilizing interaction via the G46 - α 'Thr32 hydrogen bond.

New features are observed as well in the investigated complex. For example, the phosphoryl group of A39 interacts with two cations. One of them links to β 'Glu807 and two water molecules, the other binds to β 'Phe743 and two water molecules. This latter region bridges the anticodon loop with the D-loop arm, as the magnesium-coordinating water molecules and β 'Phe743 main chain amide group bind to phosphoryl group of G27 and $\beta 3'$ Glu807 main chain interacts with the phosphoryl group of C26. C26 and G25 2'OH groups interact with main chain carbonyl oxygen atoms

of β' Ala741 and β' Leu805. Additional hydrogen bonds link β' Pro738 to 2'OH of C11 and β' Thr804 to G25, with the latter OG1 – N2 interaction being the only one involving a D-loop base. Then, three hydrogen bonds connect the backbone of D-loop (G10, C11 and U12) to the $\beta 2s1$ subdomain (β Ser578 and β Arg579). All the contacts with the D-loop arm (G10, C11, U12, G25, C26) exist only in the productive binding of the tRNA molecule.

The most significant structural change, besides tighter contacts with the D-loop arm, is the stabilization of the acceptor arm and 3' end. This region becomes ordered and the CCA3' motif is inserted into acylation site. This is possible because the $\alpha 2$ domain rearranges (Fig. 6). Specifically, the 3_{10} -helix $\eta 2$ and its short flanking sections (residues 151-159) unfold into a loop-like structure. Residues α Gln158, α Glu157 and α Asp159 that in other structures face the CCA binding pocket dramatically change their positions providing access to the otherwise blocked active site. Thus, this section of the protein serves as a 3' end gate. Another residue that moves significantly is α His152, which relocates closer to the binding pocket. There are two consequences of these shifts – the first movement, particularly that of α Asp159, creates a new magnesium binding site, while the second positions α His152 for the interaction with the RNA backbone.

The very well-ordered magnesium ion is hexagonally coordinated by five water molecules and the carboxyl group of α Asp203. The water molecules bridge the protein and the tRNA 3' end, as they interact with α Thr202, α Gln158 (main chain), α Asp159 (main chain and side chain) as well as all three CCA bases. This rich network of water-mediated contacts appears to be crucial for tRNA anchoring in the active site as there are only two direct hydrogen bonds contributing to the binding: A77 phosphoryl group interacts with α His152 and its purine N6 atom binds to the α Asp159 main chain. The magnesium ion might be therefore essential for proper positioning of the A76 ribose, which approaches the imino moiety of the ligand within the 3.0 Å distance of nitrogen atom to 2'OH. The observed geometry between the acyl donor (F-AMS) and acceptor (tRNA), observed for the first time for any aaRS, mimics the reaction stage where the 2'OH attacks the carbonyl carbon atom and is fully consistent with the accepted mechanism for FRS^{30,31}.

MtFRS vs other bacterial homologs

FRS homologs from *E. coli* (*EcFRS*), *S. haemoliticus* (*ShFRS*), *P. aeruginosa* (*PaFRS*), and the most extensively studied from *T. thermophilus* (*TtFRS*) have been characterized structurally as

functional heterotetramers (Table 1). Overall organization of these proteins is identical to that of *MtFRS*, however superpositions of the entire chains lead to high RMSDs due to the movements of the individual subdomains.

Several features of the adenylation active site are common between the four proteins. In particular, the residues interacting with the L-Phe or adenylate ligands are mostly conserved and superpose well, especially in the vicinity of the amino acid moiety. The exceptions include α His175 substitution by α Gln169 in *EcFRS* associated with a different confirmation of the L-Phe ligand and inhibitor-induced adjustments to the equivalents of Gln180 in *ShFRS* and *PaFRS*. The region binding the nucleotide is less aligned. Interestingly, the reorganization of the $\alpha 2$ domain upon adenylate binding vs Phe binding varies between the homologs. While in *EcFRS*, *PaFRS*, and *ShFRS* the conformation of ligand binding fragment of the $\alpha 2$ domain appears to follow the *MtFRS* pattern, being a more compact in the presence of a nucleotide (*E. coli*) and an open without it (*P. aeruginosa*, *S. haemoliticus*), such a change is less pronounced in *TtFRS*. A small shift is only observed in the $\beta 4$ - $\beta 5$ loop, the $\beta 8 \downarrow \beta 7 \uparrow \beta 6 \downarrow$ subsection of the central β -sheet remains intact (Fig. 6). The structure is not much different when *TtFRS* is in complex with adenylate and tRNA.

The editing domain, encompassed within the $\beta 1$ s4 subdomain, also bears high level of similarity (Fig. 6). The residues lining the amino acid binding pocket, *Mt* β His275, *Mt* β Leu300, *Mt* β Gly325, *Mt* β Glu344 and *Mt* β Pro273 are conserved in *TtFRS*, *EcFRS*, *PaFRS*, and *ShFRS*. *Mt* β Gly324, *Mt* β Met327, *Mt* β Ala366 and *Mt*Tyr370 are sometimes replaced by similar residues (Supplemental Fig. S1). The most striking discrepancy is the presence of *Mt* β Trp348. This residue is unique to *M. tuberculosis* homolog, all other sequences contain Phe. The indole nitrogen atom is perfectly positioned to form a hydrogen bond with the ligand, possibly switching the amino acid recognition from repulsive to attractive interactions and providing distinct opportunities for selective inhibitors targeting the editing domain.

T. thermophilus is the only homolog allowing for comparisons of the tRNA binding. As in *MtFRS*, the anticodon recognition is achieved by binding to the $\beta 3'$ domain, but the interactions with tRNA vary between the two proteins due to sequence variations. *Mt* β 'Ser747 and *Mt* β 'Thr793 and are both replaced by alanine residues (*Tt* β 'Ala698 and *Tt* β 'Ala744, respectively), leading two the loss of direct hydrogen bonds to A36 equivalent (*Tt*A35) and a potential (water molecules are not modeled in the *TtFRSt* structures) water-mediated contact with G35 (*Tt*G34). Alanine residues in these positions are

more common in homologs than Thr and Ser (Supplemental Fig. S1). Additional hydrogen bond bridging to the RNA backbone is eliminated by another non-conserved substitution *Mtβ*'Gln784/*Ttβ*'Pro735. Residues *Mtβ*'Asp745, *Mtβ*'Phe777, *Mtβ*'Asp778 and *Mtβ*'Arg830 are conserved (*Ttβ*'Asp696, *Ttβ*'Phe728, *Ttβ*'Asp729 and *Ttβ*'Arg780) and bind the tRNA molecule, albeit in somewhat different fashion, and *Ttβ*'Tyr731 stacks against G34 as *Mtβ*'Phe780 does against G35.

In the two *Tt*FRS models with tRNA, the 3' end appears to be inserted into the active site of the acylation domain. However, neither of the modeled states illustrates the reaction-ready position of the tRNA molecule observed in *Mt*FRSt/FAMS2. In the *Tt*FRSt complex, the 3' end adenine moiety occupies the adenylate binding pocket, it is of note, however, that the electron density map for the acceptor arm is poor and therefore the accuracy of the model raises some concerns. In the *Tt*FRSt/adenylate structure the 3' end nucleotide faces the solvent. In neither of the structures, the region equivalent to the 3' end gate is significantly reorganized to facilitate tRNA binding. It thus suggests that in the observed states, the *Tt*FRS 3' end gate remains closed, preventing the proper positioning of CCA. Of note, while the η 2 sequence is conserved in FRSs, the following three amino acids are not: *Mt*FRS contains the GEQ segment (residues 156-158) while *Tt*FRS has DMW. It is not only folded differently, but also is positioned closer to the ligand binding pocket, with α Trp149 facing the lumen and overlapping with the 3' end adenine of tRNA. Another feature distinct for *Tt*FRS is the replacement of *Mtα*Asp203 with *Tt*Glu206, indicating that the potential magnesium binding site created during the aminoacylation reaction may look somewhat different. In other bacterial homologs the sequence of 3' end gate region varies and adopts either the *Tt*FRS state (*Ec*FRS) or *Mt*FRSt/FAMS2 state (*St*FRS, *Pa*FRS). While they preserve Asp corresponding to *Mtα*Asp203, neither binds a metal cation in the magnesium site. Given the lack of any obvious common patterns guiding the conformation of the 3' end gate, such as the tRNA presence, magnesium coordination, ligand binding or sequence motifs, it appears that its state might be governed by cellular conditions these enzymes must function in.

***Mt*FRS vs human FRSs**

Humans have two FRSs, cytoplasmic and mitochondrial, both of which have had their structures determined (Table 1). The former enzyme (hcFRS) resembles *Mt*FRS ($\alpha\beta$)₂ heterotetramer but it

lacks the anticodon binding domain and contains an extra domain in the α subunit. The mitochondrial variant (hmFRS), characterized in several states, is a monomeric chimera of the bacterial $\alpha 2$ catalytic module and anticodon binding domain $\beta 3$. HmFRS exists in two states: closed in the absence of tRNA, where the $\beta 3$ domain blocks the access to $\alpha 2$, and open, which mimics the domains arrangement seen in bacterial $(\alpha\beta)_2$ complexes¹⁰. While overall organization and sequences of the human enzymes are distinct from *MtFRS*, the corresponding domains superpose well. The aminoacylation domain of hmFRS adopts conformation similar to that of *MtFRSt/FAMS2*, with the $\beta 7$ - $\beta 8$ hairpin equivalent closed over the pocket and the 3' end gate open regardless of the ligand/tRNA binding status. The cytosolic homolog, characterized in the presence of L-Phe, remains more open, as seen in the *MtFRSt/F* complexes, with its 3' end gate open as well.

The aminoacylation sites of the human enzymes, especially hmFRS, share high similarity with *MtFRS* (Fig. 7). Several residues lining the adenylate binding site are conserved. In hmFRS the only exception include replacement of *Mt* α Val286, sitting deep in the L-Phe pocket, by hmMet258. HcMtFRS is more variable, with several substitutions in the L-Phe binding site: *Mt* α Phe257/hc α Tyr412, *Mt* α Phe255/hc α Asn410, *Mt* α Val286/hc α Phe438, *Mt* α Gly282/hc α Asn434, and *Mt* α Gln180/hc α Ser332. These residues are located in the L-Phe binding cavity, which is generally better aligned between the homologs than the adenine-dedicated section.

The editing domain of hcFRS is differently organized in terms of the secondary structure composition, and consequently some of the residues lining the cavity come from different structural elements than in *MtFRS*, making the architecture of this site substantially different. In particular, the side chain of *Mt* β Tyr370 is in three-dimensional context replaced by hc β Arg118 and the role of *Mt* β Trp348 is played by hc β Thr256 and the surface created by *Mt* β Ala366 is lined with hc β Arg154. Also, some residues contributed by analogous secondary structures differ: *Mt* β His275 is replaced by hc β Gly160 while *Mt* β Gly328 is mutated to hc β Asn238.

The tRNA anticodon interactions with hmFRS mostly resemble those found in *MtFRS* including recognition of the wobble base and the key residues are conserved or replaced by similar side chains (*Mt* β 'Thr793 to hmCys377 or *Mt* β 'Phe777 to hmIle363). The hydrogen bond linking the protein with the RNA backbone via *Mt* β 'Gln784 (replaced by hmLys370) is eliminated due the conformational rearrangement of this protein segment.

Functional implications

All tRNAs must have similar shape to fit into ribosomal translation machinery and have identical CCA3' end to deliver amino acid to the ribosome active site. The tRNAs seem to have common origin and have significant sequence conservation, as they were generated from repeating sequences and inverted repeats³². During evolution these tRNAs differentiated into over 20 amino acid specific groups. It is believed that aaRSs emerged together with tRNAs and they coevolved, making aaRSs one of the oldest enzymes to arise. Today's aaRS utilize different scaffolds, tRNA recognition strategies and catalytic mechanisms to catalyze aminoacylation reaction. In bacteria, each enzyme must specifically recognize tRNA(s) specific for a single amino acid from a pool of some 30 plus tRNAs.

So how aaRSs enzymes make the connection? The amino acid specific isoacceptor tRNAs may have up to six different codons and these tRNAs are aminoacylated by a single enzyme. At the same time, amino acid must be delivered to adenosine ribose at 3' end, about 65 Å away from the anticodon. Therefore, for aaRSs codon recognition may not be the easiest solution to accomplish this.

It was proposed that a code exist equivalent to the “genetic code” in the interactions between aaRS and tRNAs. This code was linked to amino acid properties. One hypothesis proposed that aaRS emerged first as ancestor urzyme-like synthetase that initially recognized only the acceptor stem and later evolved to include other parts of tRNA to increase specificity. Urzymes correspond to conserved cores of both classes of aaRSs lacking their anticodon-binding domains. Surprisingly, urzymes catalyze amino acid activation and acyl transfer with affinities similar to full length aaRS³³. The argument for first aaRS to recognize tRNA acceptor stems rather than anticodons, is in agreement with the idea that originally proteins were coded by an “operational RNA code” located in the acceptor stem^{34,35}.

Structural studies of aaRS complexes with tRNA showed, that there are two groups of enzymes, one that recognizes anticodon loop and stem to discriminate between cognate and non-cognate tRNA and the other that does not. Earlier studies of the yeast FRS binding to tRNA^{Phe} identified five bases that were important for selectivity in positions 20, 35, 36, 37 and 73³⁶, implying that FRS belongs to the former category. Two codons code for L-Phe, 3'CUU and 3'UUU, however typically only one isoacceptor tRNA^{Phe} with 5'GAA anticodon is required to decode both of these two codons, as the wobble G base can decode both C and U. This is the case for *M. tuberculosis*, as it has only one tRNA^{Phe}. Our structures showed direct FRS interactions only with U20, G35, A36 and A37, with U20

and A37 having very minor contributions. Therefore, the role of A37 and A73 must be indirectly affecting the structure of tRNA that allows it to make the other interactions.

Our data confirm the interaction of FRS with the anticodon loop and stem, yet no anticodon sequence is recognized. The key feature of the initial detection is binding to the G35 wobble base, the least important in codon-anticodon pairing. With the most extensive interactions with $\beta 3'$ domain residues G35 appears to be the primary discriminator with some additional selectivity provided by A36, A37, G19 and U20. We believe that the recognition of the anticodon loop and stem is governed by the structural motif of stacked six purine bases (unique for tRNA^{Phe} in *M. tuberculosis*), which positions tRNA and FRS to make additional contacts, including D- and T-loops. This interface is preserved across the FRS family, including much simplified version of the enzyme found in human mitochondria. FRS does not interact with the acceptor stem at all, in fact in the initial recognition step the acceptor stem is disordered and the access to active site is often blocked by the enzyme itself. Completion of the recognition process allows for conformational changes in the 3' gate permitting binding of the CCA3' region into the active site.

It seems FRS lost its need to read the CCA stem sequence. This is because other parts of tRNA^{Phe} provide unique signatures sufficient to allow FRS to distinguish it from other tRNAs. FRS is a large, multidomain enzyme that provides many opportunities for interactions and specificity. tRNA molecules also offer a variety of surfaces for interactions. Our results suggest that recognition of the CCA stem might have been essential for ancestral aaRSs to discriminate between tRNAs, but later in the evolution of the protein genetic code new specificity determinants were added while previous were lost. This indirect recognition is one of the most important highlights of biological systems.

Therapeutics development

With a multidomain structure rich in pockets, equally complex reaction and essentiality FRS represents a golden mine for drug discovery. Structural differences between the active sites of bacterial and human FRS could be exploited to design narrow spectrum inhibitors that would have low toxicity for humans, other animal hosts and their microbiomes. Admittedly, close similarity of the adenylate binding cavity between *Mt*FRS and mitochondrial homolog present a formidable challenge for medicinal chemistry. Therefore, targeting the editing domain, substantially distinct from the cytoplasmic variant and absent in the mitochondrial enzyme may offer an untapped opportunity.

Methods

Cloning and expression of M. tuberculosis FRS subunits, PheS and PheT

The *pheS* and *pheT* genes were amplified from genomic DNA of *M. tuberculosis* H37Rv using the following pairs of primers, respectively: 5'-TACTTCCAATCCAATGCCATGTTGTCGCCGGAGGCATTGA, TTATCCACTTCCAATGTTAGGCACCCACCCCGAACGG and 5'-TACTTCCAATCCAATGCCATGCGGCTACCCTACAGCTGG, TTATCCACTTCCAATGTTAGCCACGCAGCACGGCACC. PCR product was purified and treated T4 polymerase in presence of dCTP according to Y. Kim et al.³⁷. *PheS* gene was cloned to pMCSG53, while *pheT* was cloned to pMCSG120. Both vectors contain identical cloning site, pMCSG53 carries ampicillin resistance and ColE1 origin of replication, pMCSG120 retains resistance to kanamycin and has RSF origin of replication. In the final construct the *PheS* sequence is amended on N-terminus with His₆ affinity tag and Tobacco Etch Virus (TEV) protease cleavage site.

Plasmids *PheS*-pMCSG53 and *PheT*-pMCSG120 were co-transformed to *E. coli* BL21-Gold (DE3) and grown overnight in LB medium supplemented with 40 mM K₂HPO₄ at 37°C/220 rpm with selection against ampicillin (150 µg/mL) and kanamycin (100 µg/mL). The following morning the culture was diluted 1:100 into LB media with the same concentration of antibiotics and grown at 37°C/190 rpm till OD₆₀₀ reached about 1.0 followed by cooling of media to about 20°C. At this point the media was supplemented with: IPTG 0.25 mM, K₂HPO₄ 40 mM, glucose 0.5%. The culture was grown for another 20 hours at 18°C/190 rpm followed by cells harvest and resuspension in buffer A (50 mM HEPES/NaOH, pH 8.0, 0.5 M NaCl, 20 mM imidazole, pH 8.0, 5% glycerol, 1 mM TCEP). The cell suspension was frozen and stored at -80°C.

Synthesis of M. tuberculosis tRNA^{Phe}(GAA) precursor

The isoacceptor tRNA^{Phe}(GAA) recognizing specifically UUC codon and its truncated derivatives lacking the 3'-terminal adenosines were prepared as described previously by Sherlin et al.³⁸. Oligonucleotides used for construction of the tRNA^{Phe} gene are: 5'-AATTCCTGC

AGTAATACGACTCACTATAGGCCAGGTAGCTCAGTCGGTATGAGC-3', 5'-mUmGG-TGGCCAGGGGCGGGATCGAACCGCCGACCTTCCGCTTTTCAGGCGGACGCTCATA-CCGAC-3' and 5'-mGmGTGGCCAGGGGCGGGATCGAACCGCCGACCTTCCGCTTTTC-AGGCGGACGCTCATAACCGAC-3', where mU and mG represent the 2'-O-methyl nucleotides and the underlined portions represent the overlapped segment. Bold type indicates the T7 RNA polymerase promoter. To synthesize these transcripts, 10 µg/mL duplex DNA template in a solution containing 200 mM HEPES/KOH (pH 7.5), 30 mM MgCl₂, 2 mM spermidine, 40 mM DTT, 6 mM each ATP, CTP, GTP and UTP was incubated with 100 µg/mL T7 RNA polymerase, 5 U/mL inorganic pyrophosphatase, 50 U/mL SUPERase·In RNase Inhibitor (Invitrogen) in a 8 mL reaction for 5 h at 37°C. DNA template was removed by addition of 50 U/mL RQ1 RNase-free DNase (Promega) and incubation for 1 h at 37°C, followed by phenol-chloroform extraction. The tRNA was recovered from the aqueous phase by ethanol precipitation and then fractionated on DE-52 (Whatman) column as described before ³⁸. Integrity of the purified tRNA was confirmed by electrophoresis on a 10% denaturing polyacrylamide gel. The tRNA was dialyzed into 20 mM HEPES/KOH (pH 7.5) 150 mM KCl and 5 mM MgCl₂ buffer prior to further use. The fraction of tRNA capable of being aminoacylated by *Mt*FRS was determined by assays under conditions giving maximal amino acid incorporation (approximately ten-fold molar excess of enzyme over tRNA).

FRS purification and crystallization

Frozen cells were thawed and sonicated (5 min total time, 130 W power output) and spun at 30,000 × g at 4°C for 1 h. The initial Ni²⁺ affinity purification step was performed using a 2.5-cm × 10-cm Flex-Column connected to a Van-Man vacuum manifold (Promega) ³⁹. Briefly: supernatant was loaded on 3 mL Ni²⁺ Sepharose (GE Healthcare Life Sciences) equilibrated with buffer A and mixed thoroughly with the resin. Vacuum of 15 p.s.i. was used to speed removal of supernatant as well as wash out of unbound proteins (150 mL buffer A supplemented to 50 mM imidazole). The FRS complex was eluted with 15 mL buffer A supplemented with 500 mM imidazole, pH 8.0. The eluate was concentrated to about 2 mL and loaded on a Superdex 200 16/70 size exclusion column (GE Healthcare Life Sciences) equilibrated with buffer A. Fractions containing the FRS complex were collected, and buffer A was replaced with crystallization buffer (20 mM HEPES/NaOH, pH 8.0, 150 mM KCl) on Amicon 30-kDa-cutoff concentrators (Millipore) followed by overnight incubation at 4°C with synthetic tRNA^{Phe}. The molar ratio of tRNA^{Phe} to FRS heterotetramer (αβ)₂ was 2:1.

Protein-tRNA complex was concentrated to ~20 mg/mL of complex. The ligands were added at molar ratios 4:1. Crystallization experiments were performed at 16°C using the sitting-drop vapor-diffusion method in 96-well CrystalQuick plates (Greiner Bio-One, Monroe, NC) with the help of the Mosquito liquid dispenser (TTP LabTech, Cambridge, MA). Crystallizations trials were performed using protein-tRNA to matrix ratio 1:1 (800 nL drops). Crystallization and cryocooling conditions are given in Table 1.

X-ray data collection, structure determination, refinement and analysis

Prior to data collection at 100 K, all cryoprotected crystals were flash-cooled in liquid nitrogen. The x-ray diffraction experiments were carried out at the Structural Biology Center 19-ID beamline at the Advanced Photon Source, Argonne National Laboratory. The diffraction images were recorded on the PILATUS3 X 6M detector. The data sets were processed with the HKL3000 suite⁴⁰. Intensities were converted to structure factor amplitudes in the Ctruncate program^{41,42} from the CCP4 package⁴³. The data collection and processing statistics are given in Table 1.

The initial FRS structure was solved by molecular replacement using monomeric units of *E. coli* FRS as a search template (PDB id 3PCO) against the dataset from low resolution apo crystals of *Mt*FRS. The FRSt/F1 structure was determined using the partially built apo model. The search of tRNA molecule from *T. thermophilus* FRS complex structure (PDB id 1EIY) failed so the entire nucleic acid molecule has been built manually from scratch. The subsequent structures were determined using the FRSt/F1 model. The structures were refined by manual corrections in Coot⁴⁴ and crystallographic refinement in Refmac⁴⁵ and Phenix⁴⁶. The refinement statistics are given in Table 1. The structure has been analyzed using Chimera⁴⁷, SNAP (for protein-RNA interactions, snap.x3DNA.org), and the RNA-align server (for RNA superposition)⁴⁸. Some of the atoms that appeared to be cations could not be confidently identified and have been modeled as unknown atoms UNX. The atomic coordinates and structure factors have been deposited in the PDB under accession codes 7KA0, 7KAB, 7K9M, 7K98.

Aminoacylation of precursor of *M. tuberculosis* tRNA^{Phe}

Purified *M. tuberculosis* tRNA transcripts lacking the 3'-terminal adenosine were ³²P-labeled with yeast ATP/CTP tRNA nucleotidyl transferase in the presence of [α -³²P]ATP as described in Wower

et al. ⁴⁹. Aminoacylations of the 3'- ³²P-labeled *M. tuberculosis* tRNA^{Phe} transcript were carried out in 50 µL of 50 mM HEPES/KOH, pH 7.5- 20 mM KCl- 10 mM MgCl₂- 100 µg/mL BSA- 5 mM DTT- 4 mM ATP- 0.2 mM L-Phe and 50 nM tRNA transcript. Reactions were initiated by addition of a purified *M. tuberculosis* phenylalanyl-tRNA synthetase to final concentration of 0.7 µM and incubated for 30 min at 37°C. The extent of aminoacylation was analyzed by gel-shift electrophoresis according to Varshney et al. ⁵⁰. Prior to electrophoresis on a 10% acid/7 M urea polyacrylamide gel, 10 pmoles of uncharged and aminoacylated tRNA (Phe-tRNA) were completely digested with 2 U of RNase T1 (Worthington) in 10 mM sodium acetate (pH 4.5). The amount of radioactivity in the bands corresponding to uncharged and aminoacylated CCACCA oligonucleotides was quantitated by phosphorimaging and/or liquid scintillation counting of the excised strips of the polyacrylamide gel.

The value of IC₅₀ was determined at 1.5 µM concentration of phenylalanine over a range of inhibitor concentration from zero to 200 nM. According to the Cheng-Prusoff equation, IC₅₀ values approximate K_i when substrate concentration used in the assay is lower than K_m ⁵¹.

Aminoacylation of E. coli and yeast tRNA^{Phe}

Aminoacylation reactions were performed as described above with the following modifications: 3'- ³²P-labeled *M. tuberculosis* tRNA^{Phe} transcripts were replaced either by non-radioactive *M. tuberculosis* tRNA^{Phe} transcripts or fully modified *E. coli* and yeast tRNA^{Phe} preparations from Sigma. [α -³²P]ATP from PerkinElmer was used instead of non-radioactive ATP. All reactions were quenched in 400 mM sodium acetate (pH 5.2) with 0.1% SDS. Aliquots were spotted on prewashed polyethyleneimine-cellulose TLC plates (Macherey and Nagel) and developed in 100 mM ammonium acetate, 5% (v/v) acetic acid.

MtFRS inhibition by F-AMS

Aminoacylation assays were carried out by a very sensitive assay that has been described by Bullock et al. ⁵². The assay was performed by 10 min incubation of L-Phe with purified enzyme in 50 mM HEPES/KOH (pH 7.5)- 20 mM KCl- 10 mM MgCl₂ – 4 mM ATP/20 µM [α -³²P]ATP - 0.1% dimethyl sulfoxide in a final volume of 20 µL at 37°C. K_m for L-Phe and K_i were determined by varying concentrations of amino acid and inhibitor from 0.5 – 25 mM and from 15 – 125 nM, respectively. The concentration of FRS was 6 nM. The reactions were stopped by adding 2.0 µL of the reaction mixtures to 3.0 µL of a solution containing 400 mM sodium acetate (pH 5.0) and 0.1%

SDS. 2.0 μ L aliquots of this mixture were spotted on dry polyethyleneimine (PEI) cellulose plates that have been prewashed in water. Phe-[32 P]AMP were separated from [α - 32 P]ATP using 0.1 M ammonium acetate/5% acetic acid as developing solution, visualized by autoradiograph and quantified by phosphorimaging analysis.

Synthesis of phenylalanine adenylate analog

5'-O-[N-(phenylalanine)sulfamoyl] adenosine, Phe-AMS, has been prepared in six steps as previously described ⁵³.

Conflict of Interest Statement

All authors declare no competing interest

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Table 1. FRS structures in the Protein Data Bank.

PDB ID	Source	Structure	Res (Å)	% Sequence identity to <i>Mt</i> (α/β) ^a	reference
1B70	<i>T. thermophilus</i>	(α/β) ₂ complex with L-Phe in α	2.70	39/34	Reshetnikova <i>et al.</i> ¹⁷
1B7Y	<i>T. thermophilus</i>	(α/β) ₂ complex with adenosine-5'-[phenylalaninol-phosphate] in α	2.50		Reshetnikova <i>et al.</i> ¹⁷
1EIY	<i>T. thermophilus</i>	(α/β) ₂ complex with tRNA ^{Phe}	3.30		Goldur <i>et al.</i> ⁵⁴
1JJC	<i>T. thermophilus</i>	(α/β) ₂ complex with adenosine-5'-[phenylalaninyl-phosphate] in α	2.60		Fishman <i>et al.</i> ⁵⁵
1PYS	<i>T. thermophilus</i>	(α/β) ₂ complex	2.90		Mosyak <i>et al.</i> ¹²
2AKW	<i>T. thermophilus</i>	(α/β) ₂ complex with p-Cl-Phe in α	2.80		Kotik-Kogan <i>et al.</i> ¹⁸
2ALY	<i>T. thermophilus</i>	(α/β) ₂ complex with 5'-O-[N-L-tyrosyl)sulfamoyl]adenosine in α	2.60		Kotik-Kogan <i>et al.</i> ¹⁸
2AMC	<i>T. thermophilus</i>	(α/β) ₂ complex with L-Tyr in β	2.70		Kotik-Kogan <i>et al.</i> ¹⁸
2IY5	<i>T. thermophilus</i>	(α/β) ₂ complex with tRNA ^{Phe} and adenosine-5'-[phenylalaninol-phosphate] in α	3.10		Moor <i>et al.</i> ⁵⁶
3HFZ	<i>T. thermophilus</i>	(α/β) ₂ complex with m-L-Tyr in α	2.90		Klipcan <i>et al.</i> ⁵⁷
3TEH	<i>T. thermophilus</i>	(α/β) ₂ complex with 3,4-dihydroxyphenylalanine in α	2.85		Moor <i>et al.</i> ⁵⁸
4TVA	<i>T. thermophilus</i>	(α/β) ₂ complex with puromycin in β and L-Phe in α	2.60		Tworowski <i>et al.</i> ⁵⁹
2RHQ	<i>S. haemolyticus</i>	(α/β) ₂ complex with 1-{3-[(4-pyridin-2-yl)piperazin-1-yl)sulfonyl]phenyl}-3-(1,3-thiazol-2-yl)urea in α , 6 mutations in β	2.20	41/31	Evdokimov <i>et al.</i> ¹⁴
2RHS	<i>S. haemolyticus</i>	(α/β) ₂ complex with 1-{3-[(4-pyridin-2-yl)piperazin-1-yl)sulfonyl]phenyl}-3-(1,3-thiazol-2-yl)urea in α , 5 mutations in β	2.20		Evdokimov <i>et al.</i> ¹⁴
3PCO	<i>E. coli</i>	(α/β) ₂ complex with L-Phe and AMP in α	3.02	45/31	Mermershtain <i>et al.</i> ¹⁶
4P71	<i>P. aeruginosa</i>	(α/β) ₂ complex	2.79	43/32	Abibi <i>et al.</i> ⁶⁰
4P72	<i>P. aeruginosa</i>	(α/β) ₂ complex with compound 2-{3-[(4-chloropyridin-2-yl)amino]phenoxy}-N-methylacetamide in α	2.62		Abibi <i>et al.</i> ⁶⁰
4P73	<i>P. aeruginosa</i>	(α/β) ₂ complex with compound 1-{3-[(4-pyridin-2-yl)piperazin-1-yl)sulfonyl]phenyl}-3-(1,3-thiazol-2-yl)urea in α	3.03		Abibi <i>et al.</i> ⁶⁰
4P74	<i>P. aeruginosa</i>	(α/β) ₂ complex with compound N-[(3S)-1,1-dioxidotetrahydrothiophen-3-yl]-2-[(4-methylphenoxy)methyl]-1,3-thiazole-4-carboxamide in α	2.70		Abibi <i>et al.</i> ⁶⁰
4P75	<i>P. aeruginosa</i>	(α/β) ₂ complex with compound 3-(3-methoxyphenyl)-5-(trifluoromethyl)-1H-pyrazole in α	2.96		Abibi <i>et al.</i> ⁶⁰
3ICA	<i>P. gingivalis</i>	β subunit fragment	2.44	36/27	Fan <i>et al.</i> , unpublished
3IG2	<i>B. fragilis</i>	β subunit fragment	2.09	38/28	Stein <i>et al.</i> , unpublished
2CXI	<i>P. horikoshii</i>	β subunit fragment, 9 mutations	1.94	19/13	Sasaki <i>et al.</i> ¹³
3CMQ	<i>H. sapiens</i>	Mitochondrial monomer with adenosine-5'-[phenylalaninyl-phosphate]	2.20	27, 28 ^b	Klipcan <i>et al.</i> ⁶¹
3HFV	<i>H. sapiens</i>	Mitochondrial monomer with m-L-Tyr	2.60		Klipcan <i>et al.</i> ⁵⁷

3TEG	<i>H. sapiens</i>	Mitochondrial monomer with 3,4-dihydroxyphenylalanine	2.20		Moor <i>et al.</i> ⁵⁸
3TUP	<i>H. sapiens</i>	Mitochondrial monomer with <i>TtrRNA</i> ^{Phe}	3.05		Klipcan <i>et al.</i> ¹⁰
5MGH	<i>H. sapiens</i>	Mitochondrial monomer, point mutant with L-Phe	1.87		Kartvelishvili <i>et al.</i> ⁶²
5MGU	<i>H. sapiens</i>	Mitochondrial monomer, point mutant with L-Phe	1.89		Kartvelishvili <i>et al.</i> ⁶²
5MGW	<i>H. sapiens</i>	Mitochondrial monomer, point. mutant with L-Phe	1.46		Kartvelishvili <i>et al.</i> ⁶²
5MGV	<i>H. sapiens</i>	Mitochondrial monomer, point mutant	2.05		Kartvelishvili <i>et al.</i> ⁶²
3L4G	<i>H. sapiens</i>	Cytosolic (α/β) ₂ complex with L-Phe in α	3.30	34/18	Finarov <i>et al.</i> ¹⁵

^a As determined by Emboss Needle⁶³.

^b The numbers correspond to *MtFRS* α subunit residues 108 – 341 aligned with human mitochondrial FRS residues 82-347 and *MtFRS* β subunit residues 739-831 with 360-451).

Table 2. Data processing and refinement statistics.

Data processing				
Structure	FRSt/F1	FRSt/F2	FRSt/F-AMS1	FRSt/F-AMS2
Crystallization conditions; cryoprotectant supplement	0.2 M Li ₂ SO ₄ , 0.1 M HEPES pH 7.5, 25% PEG3350; 10% glycerol	14.3% PEG20K, 150 mM TAPS pH 8.5, 5% 1,2- propanediol; 20% glycerol	0.2 M Na/K tartrate, 20% PEG3350; 15% glycerol	30% PEG400, 0.2 M MgCl ₂ , 0.1 M HEPES pH 7.5; No cryoprotectant
Wavelength (Å)	0.9793	0.9793	0.9793	0.9793
Resolution range (Å) ^a	2.40-50.00 (2.40-2.44)	2.50-30.00 (2.50-2.54)	2.50-50.0 (2.50-2.54)	2.20-30.0 (2.20-2.24)
Space group	<i>C</i> 2	<i>C</i> 2	<i>C</i> 2	<i>P</i> 2 ₁
Unit cell (Å, °)	a=292, b=110, c=128, β =100	a=127, b=110, c=148, β =105	a=128, b=110, c=147, β =103	a=147, b=65, c=193, β =109
Unique reflections (merged)	155,793 (7,677)	68,179 (3,404)	68,621 (3,274)	173,141 (8,263)
Multiplicity	6.4 (5.5)	7.0 (5.9)	6.2 (3.9)	6.4 (6.0)
Completeness (%)	98.9 (98.2)	99.3 (99.4)	99.0 (96.2)	96.9 (93.6)
Mean I/sigma(I)	14.2 (1.78)	17.9 (1.10)	13.8 (1.60)	21.5 (1.56)
Wilson B-factor (Å ²)	32.4	52.5	31.9	45.8
R-merge ^b	0.147 (1.207)	0.141 (1.374)	0.171 (0.832)	0.094 (1.223)
CC1/2 ^c	0.992 (0.673)	(0.487)	0.990 (0.522)	(0.596)
Refinement				
Resolution range (Å)	2.40-48.0	2.50-29.69	2.50-47.92	2.19-29.85
Reflections work/test	135,987/7,094	66,452/1,665	63,320/3,264	169,565/3,498
R _{work} /R _{free} ^d	0.176/0.215	0.187/0.213	0.179/0.215	0.178/0.224
Number of non-hydrogen atoms	21,651	10,440	10,722	22,017
macromolecules	20,563	10,237	10,292	21,241
ligands/solvent	184/904	53/150	108/322	101/675
Amino acid residues	339+835+336+835	336+835	339+834	343+834+343+836
Nucleic acid bases	66+66	66	66	77+77
RMSD(bonds) (Å)	0.003	0.002	0.002	0.007

RMSD(angles) (°)	0.599	0.478	0.504	0.915
Ramachandran favored ^c (%)	96.65	96.39	96.83	96.85
Ramachandran allowed (%)	3.09	3.26	3.08	3.02
Ramachandran outliers (%)	0.26	0.34	0.09	0.13
Rotamer outliers (%)	2.03	1.88	0.98	2.79
Clashscore	3.45	3.93	3.04	2.70
Average B-factor (Å ²)	65.58	99.46	56.72	61.11
macromolecules	66.51	100.05	57.36	61.46
ligands	67.43	92.47	54.38	49.86
solvent	44.14	61.43	36.99	51.67
Number of TLS groups	39	16	11	34
PDB ID	7KA0	7KAB	7K9M	7K98

^a Values in parentheses correspond to the highest resolution shell.

^b $R_{\text{merge}} = \sum_h \sum_j |I_{hj} - \langle I_h \rangle| / \sum_h \sum_j I_{hj}$, where I_{hj} is the intensity of observation j of reflection h .

^c As defined by Karplus and Diederichs ⁶⁴.

^d $R = \sum_h |F_o| - |F_c| / \sum_h |F_o|$ for all reflections, where F_o and F_c are observed and calculated structure factors, respectively. R_{free} is calculated analogously for the test reflections, randomly selected and excluded from the refinement.

^d As defined by Molprobit ⁶⁵.

Figure legends

Figure 1. *Mt*FRS activity. (A) Aminoacylation of unmodified *M. tuberculosis* tRNA^{Phe}. The 3'-³²P-labeled tRNA transcript was charged with L-Phe in the presence of purified *Mt*FRS. The extent of aminoacylation was analyzed according to Varshney *et al.*⁵⁰. Prior to polyacrylamide gel electrophoresis, tRNA and Phe-tRNA were digested with RNase T1 to yield 3'-³²P-labeled CCACCA and CCACCA-Phe, respectively. (+) and (-) indicate the presence and the absence of the enzyme. (B-D) Synthesis of the Phe-AMP intermediate by *Mt*FRS in the presence of unmodified *M. tuberculosis* tRNA^{Phe} (B), *E. coli* tRNA^{Phe} (C) and yeast tRNA^{Phe} (D). Phe-[³²P]AMP synthesis was monitored by thin layer chromatography. Phe-AMP and ATP denote ³²P-labeled compounds. F – full reaction; -E, -tRNA and -Phe – control reactions lacking the enzyme, tRNA or amino acid.

Figure 2. Inhibition of *Mt*FRS by F-AMS. (A) Competitive binding of inhibitor with respect to L-Phe. Inhibitor concentrations were as follows: 0 μ M (black line), 15 nM (green line), 50 nM (red line), and 125 nM. (B) Phe-[³²P]AMP synthesis in the presence of 15 nM inhibitor (corresponding to green line) monitored by thin layer chromatography. The rate of Phe-[³²P]AMP formation was determined at 37°C over 10 min. (C-D) Competitive inhibition of *Mt*FRS by the F-AMS shown in the form of a Lineweaver-Burk plot. K_m for Phe is 1.78 μ M (C). The secondary diagram was used to determine the inhibition constant K_i , which is 33 nM (D). (E) Plot of the dose-response curve shows the percentage of initial velocity reduced by various concentrations of the F-AMS. The insert illustrates the inhibition of Phe-[³²P]AMP synthesis monitored by thin layer chromatography. Dotted lines indicate that the inhibitor has IC_{50} value of approximately 36 nM.

Figure 3. Overall structure of the *Mt*FRS-tRNA^{Phe} complex. (A) *Mt*FRS-tRNA^{Phe} heterotetramer with initial tRNA binding (*Mt*FRS/F1) (B) *Mt*FRS-tRNA^{Phe} heterotetramer with productive tRNA binding (*Mt*FRS/F-AMS2). Subunits α shown in purple and coral, subunits β shown in grey and teal, tRNA represented as yellow and green ribbon models.

Figure 4. *Mt*FRS domains. (A) Subunit α . (B) Subunit β . (C) Domain boundaries with starting residues numbered above the sequence-representing bars and ending residues shown under the bars. Grey sections depict linkers.

Figure 5. *Mt*FRS interactions with ligands. (A) L-Phe binding to the aminoacylation site of FRSt/F1. The upper-left insert shows 2mFo-DFc electron density map contoured around L-Phe at 1.2σ . (B) Stereoview of F-AMS binding to the aminoacylation site of FRSt/F-AMS2. (C) Superposition of the aminoacylation site of FRSt/F-AMS2 (purple), FRSt/F-AMS1 (yellow), and FRSt/F1 (coral). (D) F-AMS ligand and its 2mFo-DFc electron density map from the FRSt/F-AMS2 complex contoured at 1.2σ . Red spheres represent water molecules, a green sphere indicates magnesium ion. Hydrogen bonds are shown as dashed lines.

Figure 6. *Mt*FRS interactions with tRNA. (A) tRNA molecule and interacting residues in *Mt*FRSt/F-AMS2. (B) tRNA molecule and its contacts with the polypeptide chain as seen in *Mt*FRS/F-AMS, (chain C). The interacting residues include those forming hydrogen bonds, participating in base/amino acid pairs and base/amino acid stacks, as defined by the SNAP program (<http://forum.x3dna.org/dna-protein-interactions/>). The C α atoms of the interacting residues are shown, colored as in A. Magnesium ions are colored green. Nucleotides have the following color-coding: G-purple, C-pink, A-green, U-orange. (C) Stereoview of *Mt*FRS interactions with the anticodon loop. (D) F-AMS (grey), tRNA 3' end (green) and magnesium ion (green sphere) binding in the *Mt*FRSt/F-AMS2 active site shown in 2DFo-Fc electron density map contoured at 1.2σ . (E) tRNA 3' end interactions in *Mt*FRSt/F-AMS2. (F) Superposition of the tRNA 3' end binding region of *Mt*FRSt/F-AMS2 (purple) and *Mt*FRSt/F-AMS1 (blue).

Figure 7. Comparison of *Mt*FRS with *T. thermophilus* FRS. (A, B) Comparison of the anticodon binding between *Mt*FRSt/F-AMS2 (green, chain C/E) and *Tt*FRSt/adenylate (yellow, PDB 2IY5). In (A) ribbon representation of the $\beta 3'$ domain and anticodon loop. In (B) atomic representation of the anticodon loop and interacting residues. For superposition only domain $\beta 3'$ was used. Labels in black correspond to *Mt*FRS, in yellow to *Tt*FRS. (C) Superposition of the aminoacylation domains from *Mt*FRSt/F (coral), *Mt*FRSt/F-AMS1 (blue), *Tt*FRS/F (grey, 1B70), and *Tt*FRS/adenylate (green, 1B7Y). Only select residues are labeled. Parentheses indicate *Tt*FRS. (D) Superposition of the 3' end binding region in *Mt*FRSt/F-AMS2 and *Tt*FRSt/adenylate. *Mt*FRS is shown in purple (protein), green (tRNA) and grey (F-AMS). *Tt*FRS is shown in yellow. (E) As in (D) but with the *Tt*FRSt complex (pink). Labels in black correspond to *Mt*FRS, in yellow or pink to *Tt*FRS. (F) Superposition of the

editing domains (β 1s4 subdomain) from *MtFRSt/F-AMS2* (grey) and *TtFRS/L-Tyr* complex (gold, 2AMC).

Figure 8. Comparison of *MtFRS* with human *FRSs*. (A-B) Superposition of the aminoacylation domains from *MtFRSt/F-AMS2* (purple) with human mitochondrial (hm) enzyme in complex with adenosine-5'-[phenylalaninyl-phosphate] (3CMQ, olive green) or with *T. termophilus* tRNA^{Phe}, hmFRSt (3TUP, coral). In (B) close-up view of the L-Phe subpocket is shown. (C-D). Superposition of the aminoacylation domains from *MtFRSt/F-AMS2* (purple) with human cytosolic enzyme (hcFRS) in complex with L-Phe (3L4G, green). In (D) close-up view of the L-Phe subpocket is shown. (E) Superposition of the anticodon binding region of *MtFRSt/F-AMS2* (grey) and hmFRSt (coral). Labels in parentheses correspond to the respective human homologs. (F) Superposition of the editing domains (β 1s4 subdomain) from *MtFRSt/F-AMS2* (grey) and hcFRS (3L4G, green). Gold L-Tyr from the *TtFRS/L-Tyr* complex marks the amino acid binding site.

Figure 1.

Figure 2.

Figure 3.

A

B

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Supplemental figures

Figure S1. Sequence alignment of FRSs. (A) Subunit α . (B) Subunit β . The species include: *M. tuberculosis*, *T. thermophilus*, *E. coli*, *P. aeruginosa*, *S. haemolyticus*, *K. pneumoniae*, *A. baumannii*, *S. aureus*, *C. difficile*, *L. pneumophila*, *E. faecium*. Crystallographic models for the biologically relevant FRS heterotetramers exist for the top 5 homologs.

1 10 20 30 40 50 60 70 80 90

Mtub MR LPYS WLR EVVAVGASGWD VTPGELE QTLRI GHEVEE VIPLGPVDPGP VTVGR VADIEELTG YKPI RACAVDIGRQYRE TICGATNF

Tter MRVFPFSLWKAYVP...ELESPEVLEERLAGLGFETDRIERVFPPIRG VVFARVLEAHPIPGT...R...LKRLVLDAAGRTVEVVS GAENA

Ecol MKFSEKWLRSWAN...PAIDSDALANOITMA GLEVDGVEPVAGSFGH VVVGEVVECAQHPNA...DKLRVTKVNVGGDRLLDI VCGAPNC

Paer MLFISENWLKDYVD...AGVKVEDLAERITRT GLEVDMDIDYSKDKN LVVGYI QSKKEHPDA...DKLNICQVDIGEEEPV VCGAPNV

Shae MKFSELWLREWVN...PAIDSEALSDQITMA GLEVDGVEPVAGSNG VVVGEVVECGQHPNA...DKLRVTKVNVGGERLLDI VCGAPNC

Kpne MLFISENWLRTWVN...PAIDSENTLS DQITML GLEVDDELAPVAKPTG VVVGEVLTVEQHPDA...DKLRVTKVNVGGERLLDI VCGAPNV

Abau MKFISENWLRTWVN...PAIDSENTLS DQITML GLEVDDELAPVAKPTG VVVGEVLTVEQHPDA...DKLRVTKVNVGGERLLDI VCGAPNV

Lmon MLFISENWLRTWVN...PAIDSENTLS DQITML GLEVDDELAPVAKPTG VVVGEVLTVEQHPDA...DKLRVTKVNVGGERLLDI VCGAPNV

Saur MLFISENWLRTWVN...PAIDSENTLS DQITML GLEVDDELAPVAKPTG VVVGEVLTVEQHPDA...DKLRVTKVNVGGERLLDI VCGAPNV

Cdif MLFISENWLRTWVN...PAIDSENTLS DQITML GLEVDDELAPVAKPTG VVVGEVLTVEQHPDA...DKLRVTKVNVGGERLLDI VCGAPNV

Lpne MKVSKLWLREWVN...FSLTEQELAEQLTMA GLEVDVAVNPVAGQTFH VIVAEVLTNTKHPDA...DKLITLCEVNNINKDKPL VCGAPNV

Efae MLVSYKWLNRVYVDL...SNVTPKEIADKMSVT GEEVEGTVPEEGLKKI VVGEVKECVPHDS...DHSICQVDIGEEDELSSQ VCGAPNV

100 110 120 130 140 150 160 170

Mtub AV GDLVVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Tter RK GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Ecol RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Paer RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Shae RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Kpne RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Abau RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Lmon RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Saur RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Cdif RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Lpne RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

Efae RQ GIGVVALP GATLP GSGFTTSARKAYGRNSD CMICSAALNLN GA...DHSGLVLP PGC...AEP GADGAGV GDDVVFHAI TTP

180 190 200 210 220 230 240 250 260

Mtub DR GYMSVR GLARELACAYDLD FVD PASNSRVPLPIEGPAWPTITVQ PETGVR RFALRPVIG TDPAAV SPW LQRLL LCGT RATCPAVD

Tter NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Ecol NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Paer NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Shae NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Kpne NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Abau NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Lmon NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Saur NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Cdif NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Lpne NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

Efae NR PDALGLI GLARDLHALGYA...LVEPEAAKAEAL...PLPFLATKVEDEPEGAFHTLGYAFGLR...VAP SPW LQRLL LCGT RATCPAVD

270 280 290 300 310 320 330 340

Mtub VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Tter VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Ecol VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Paer VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Shae VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Kpne VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Abau VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Lmon VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Saur VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Cdif VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Lpne VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

Efae VT NVVMBL GH PPHAHDRNRIS.GT GVRRARSGETAVT LGIERK LDTADVL TVD...DAATAAIG GVMCAAS TEVRADST D VLL EAA

350 360 370 380 390 400 410 420 430

Mtub IMDPAAVSRTQRRLH LPSEAAR RYERTVD PAISVAAL DRCARLIAD TAGGEVSP TTDWRGDPPCDDWSPPP RMGVDVFPDRIAGVAYPQ

Tter GQDPAVSRRTQRRLH LPSEAAR RYERTVD PAISVAAL DRCARLIAD TAGGEVSP TTDWRGDPPCDDWSPPP RMGVDVFPDRIAGVAYPQ

Ecol FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Paer FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Shae FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Kpne FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Abau FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Lmon FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Saur FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Cdif FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Lpne FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

Efae FFDPSLITGRARRHGLHTDASH RYERGVD PALQHAMERATRLTLDI CGGEAGPV DITNEAT...LPKRATITLRRSKLDRILGHIAID

440 450 460 470 480 490 500 510 520

Mtub GTIARRLAQT GAVVTH.DGDTLT VTFPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Tter GTIARRLAQT GAVVTH.DGDTLT VTFPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Ecol EQVTDILRRGCG.EVTEGKDEWQAVVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Paer AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Shae AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Kpne AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Abau AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Lmon AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Saur AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Cdif AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Lpne AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

Efae AEIVRLQLALELTVVADGEQWVGVPSWRPDLRQ PADLVEEVARLRCLEVITP SLPPA...PAGRGTLA...GQRRRTIGRSIALSGYVE

530 540 550 560 570 580 590 600 610

Mtub ILPTPTF PAGVFDLWGLEADSSRMTRVLN FLEADRPGLATILLPALLEAVRNVSRGL VDVAFALFAIAQVQPTTEGTGRGVLPVDRR

Tter VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Ecol VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Paer VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Shae VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Kpne VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Abau VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Lmon VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Saur VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Cdif VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Lpne VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

Efae VYTPFMPDPEDARRFRID...PPRLLLN FLAPLEAKAALRTHLPGLVRLKENLDLDRPERALLFEVGRVRE...PPEAIPFRFPEYANRLLGTSYPE

	620	630	640	650	660	670	680	690
Mtub	PTDDEIAMLDASLP	QPHVAAVL	AGLREPRGP	FWGPGGRP	VEAADAFEAVRI	IAR..ASRV	VTLRP.AQYLP	WHPGRCAQVFV
Tter	REETHLAG	LLFEGVG	LFWAKE	RLSGYFL	LKGYLEAL	FARLG	LA..FRVEA	EAFPTWHPGV
Ecol	LGIRQDLML	AGVICGNRYEEH	WNLAKET	VDFYDL	LKGDLES	VLDLTGKLN	EVEFRA	EANPAWHPGQ
Paer	EGLKQEA	MLAGAICGKRL	PEGWANGRDG	VDFD	AKADVEAV	LASAGALG	DFS	FVP.GEHPA
Shae	ELPDVEY	LSGILTGEY	VVNAAWQKKKEE	IDFFI	AKGVDR	VAEKLNL	..EFSYKA	GKIEG
Kpne	LGIRQDVML	AGICGNRYEEH	WTLAKET	VDFYDL	LKGDLEA	VLDLTGKLAD	IEFRA	EATTAWHPGQ
Abau	QDLKQIP	TTLTLVAVGS	REPESWHAKPQP	MDFFD	FKGEVEE	ILAAAG..RVK	VEYVR	SERPW
Lmon	NLPIEQEH	LAGLITGN	NWHIADWQKTPKP	ADFFV	LKGI	VEGLVNKL	GKSE	ELHWKQ
Saur	ELPDQVEY	LSGILTGDY	VVNQWQKKKET	VDFYLA	KGVVDR	VSEKLN	..EFSYRR	ADIDG
Cdif	ELPKEENR	MCVGM.....	..YGKD	VDFE	ALKGAI	ETILVN	VGFEG	YEIEPQ
Lpne	GQLKERS	CIAGLLMGEQGN	LNWSESARL	DFDYDL	LKGDLS	LFAS.LKLN	VEFIQ	SSHHA
Efae	HLPQEIKH	AAIAISGI	IWEERKD	QTKAQN	VDFET	LKGLLEN	LNFEQL	GIHED
								DIHYQT
								VTEIKE
								MHPGR
								TAATY
								LGQFI
								CGF

	700	710	720	730	740	750	760	770	780
Mtub	AGQLHP	AVIERS	GLPKGTCA	VELNLDA	IPCSA..PLP	APRVSPY	PAVFD	VSLVV	AADIPAQA
Tter	IGALHP	ETIAQEL	ELPP.VHLF	ELRLPLP	..DK..PLAF	QDPSRH	PAAFRD	LAVVVP	PAPT
Ecol	VGVVHP	ELERKLD	INGRTL	VFELEW	NKLADRV..VPQ	AREISR	PANRRD	IAVVVA	ENVP
Paer	IGALHP	ELAKKL	DLEQPV	FLFELL	LAEVVDGH..LPK	FERLSR	PEVRRD	LALLVD	QDVPAQD
Shae	IGELHP	QVAADND	LK.RTYVF	ELNYDAM	QVAVGYIN	YEIQIPK	PGVTRD	IALEVNH	DVPSS
Kpne	IGVVHP	ELERKLD	INGRTL	VFELEW	NKLADRV..VPQ	ARDISR	PANRRD	IAVVAEN	VAAADV
Abau	IGRLHP	SELENEL	DIST.TWVA	ELDQA	AVLQSY..VSN	FTELSR	PSVRRD	IALLS	DNINVRD
Lmon	IGALHP	AVEANY	DLK.ETYVF	ELINVA	ALLEATK	EVVYHPI	PRYP	EMTRD	LALLVD
Saur	IGELHP	TAAADND	LK.RTYVF	ELNFDAL	MAVSVGYIN	YQPIPR	PGMSRD	IALEV	DQNI
Cdif	IGELHP	DVIENYN	LGQRVYVA	EIDIDF	VFDNSDR	TKNYVPL	PKYP	STSRD	IALIV
Lpne	IGVLHP	RLSDAF	DLQDVV	LFELN	LES	LINPT..IPLY	KPISKY	PQIRRD	LSFLV
Efae	VGQVHP	TLAKSY	DLIP.ETYVA	ELDLSA	VVEAAQ	KEIVEAVT	KEPAVS	RDIALLV	KETV
									TNQEL
									TEV
									HS.AAGR
									FLTDI
									QLFDV
									YQGEN

	790	800	810	820	830
Mtub	IGEH	RKSL	TFA	LRFRAPD	RTLT
Tter	LEPG	HKSL	AFH	LRFRHPK	RTL
Ecol	VAEGY	KSLAIS	LILQD	TSRTL	EEEEIA
Paer	IDPH	RKSL	AVGLT	WQHPSRTL	NDDDEV
Shae	LEKGG	KSLAIS	LILQD	TSRTL	DERVSKI
Kpne	VAEGY	KSLAIS	LILQD	TSRTL	EEEEIA
Abau	VEEGK	SLAF	ALLWQHPS	RTL	EDAETK
Lmon	LEGEN	KSLAY	TTLTFL	DSERTL	VEEDVQ
Saur	LEKGG	KSLAIS	LILQD	TSRTL	DERVSKV
Cdif	IEVGY	KSLAYS	ITYRS	KDRTL	DEDVAK
Lpne	IPEDK	KSLAV	AMTLQDD	TRLV	DAEIN
Efae	LEKGG	KSLAYS	LYT	VNPE	ATL
					DEET
					NKAMD
					VTKA
					LTETL
					GSIR

Fig. S2. Domain assignment in *MtFRS*.

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Figures

Figure 1

MtFRS activity. (A) Aminoacylation of unmodified *M. tuberculosis* tRNAPhe. The 3'- 32P-labeled tRNA transcript was charged with L-Phe in the presence of purified MtFRS. The extent of aminoacylation was analyzed according to Varshney et al. 50. Prior to polyacrylamide gel electrophoresis, tRNA and Phe-tRNA were digested with RNase T1 to yield 3'- 32P-labeled CCACCA and CCACCA-Phe, respectively. (+) and (-) indicate the presence and the absence of the enzyme. (BD) Synthesis of the Phe-AMP intermediate by MtFRS in the presence of unmodified *M. tuberculosis* tRNAPhe (B), *E. coli* tRNAPhe (C) and yeast tRNAPhe (D). Phe-[32P]AMP synthesis was monitored by thin layer chromatography. Phe-AMP and ATP denote 32P-labeled compounds. F – full reaction; -E, -tRNA and -Phe – control reactions lacking the enzyme, tRNA or amino acid.

Figure 2

Inhibition of MtFRS by F-AMS. (A) Competitive binding of inhibitor with respect to LPhe. Inhibitor concentrations were as follows: 0 μM (black line), 15 nM (green line), 50 nM (red line), and 125 nM. (B) Phe-[³²P]AMP synthesis in the presence of 15 nM inhibitor (corresponding to green line) monitored by thin layer chromatography. The rate of Phe-[³²P]AMP formation was determined at 37°C over 10 min. (C-D) Competitive inhibition of MtFRS by the F-AMS shown in the form of a Lineweaver-Burk plot. K_m for Phe is 1.78 μM (C). The secondary diagram was used to determine the inhibition constant K_i , which is 33 nM (D). (E) Plot of the dose-response curve shows the percentage of initial velocity reduced by various concentrations of the F-AMS. The insert illustrates the inhibition of Phe-[³²P]AMP synthesis monitored

by thin layer chromatography. Dotted lines indicate that the inhibitor has IC₅₀ value of approximately 36 nM.

Figure 3

Overall structure of the MtFRS-tRNAPhe complex. (A) MtFRS-tRNAPhe heterotetramer with initial tRNA binding (MtFRS/F1) (B) MtFRS-tRNAPhe heterotetramer with productive tRNA binding (MtFRS/F-AMS2). Subunits a shown in purple and coral, subunits b shown in grey and teal, tRNA represented as yellow and green ribbon models.

Figure 4

MtFRS domains. (A) Subunit a. (B) Subunit b. (C) Domain boundaries with starting residues numbered above the sequence-representing bars and ending residues shown under the bars. Grey sections depict linkers.

Figure 5

MtFRS interactions with ligands. (A) L-Phe binding to the aminoacylation site of FRSt/F1. The upper-left insert shows 2mFo-DFc electron density map contoured around L-Phe at 1.2s. (B) Stereoview of F-AMS binding to the aminoacylation site of FRSt/F-AMS2. (C) Superposition of the aminoacylation site of FRSt/F-AMS2 (purple), FRSt/F-AMS1 (yellow), and FRSt/F1 (coral). (D) F-AMS ligand and its 2mFo-DFc electron density map from the FRSt/F-AMS2 complex contoured at 1.2s. Red spheres represent water molecules, a green sphere indicates magnesium ion. Hydrogen bonds are shown as dashed lines.

Figure 6

MtFRS interactions with tRNA. (A) tRNA molecule and interacting residues in MtFRSt/F-AMS2. (B) (B) tRNA molecule and its contacts with the polypeptide chain as seen in MtFRS/F-AMS, (chain C). The interacting residues include those forming hydrogen bonds, participating in base/amino acid pairs and base/amino acid stacks, as defined by the SNAP program (<http://forum.x3dna.org/dna-protein-interactions/>). The Ca atoms of the interacting residues are shown, colored as in A. Magnesium ions are colored green. Nucleotides have the following colorcoding: G-purple, C-pink, A-green, U-orange. (C) Stereoview of MtFRS interactions with the anticodon loop. (D) F-AMS (grey), tRNA 3' end (green) and (E) (F) MtFRS interactions with the anticodon loop.

magnesium ion (green sphere) binding in the MtFRSt/F-AMS2 active site shown in 2DFo-Fc electron density map contoured at 1.2s. (E) tRNA 3' end interactions in MtFRSt/F-AMS2. (F) Superposition of the tRNA 3' end binding region of MtFRSt/F-AMS2 (purple) and MtFRSt/F-AMS1 (blue).

Figure 7

Comparison of MtFRS with *T. thermophilus* FRS. (A, B) Comparison of the anticodon binding between MtFRSt/F-AMS2 (green, chain C/E) and TtFRSt/adenylate (yellow, PDB 2IY5). In (A) ribbon representation of the b3' domain and anticodon loop. In (B) atomic representation of the anticodon loop and interacting residues. For superposition only domain b3' was used. Labels in black correspond to MtFRS, in yellow to

TtFRS. (C) Superposition of the aminoacylation domains from MtFRSt/F (coral), MtFRSt/F-AMS1 (blue), TtFRS/F (grey, 1B70), and TtFRS/adenylate (green, 1B7Y). Only select residues are labeled. Parentheses indicate TtFRS. (D) Superposition of the 3' end binding region in MtFRSt/F-AMS2 and TtFRSt/adenylate. MtFRS is shown in purple (protein), green (tRNA) and grey (F-AMS). TtFRS is shown in yellow. (E) As in (D) but with the TtFRSt complex (pink). Labels in black correspond to MtFRS, in yellow or pink to TtFRS. (F) Superposition of the 27 editing domains (b1s4 subdomain) from MtFRSt/F-AMS2 (grey) and TtFRS/L-Tyr complex (gold, 2AMC).

Figure 8

Comparison of MtFRS with human FRSs. (A-B) Superposition of the aminoacylation domains from MtFRSt/F-AMS2 (purple) with human mitochondrial (hm) enzyme in complex with adenosine-5'-[phenylalaninyl-phosphate] (3CMQ, olive green) or with *T. termophilus* tRNAPhe, hmFRSt (3TUP, coral). In (B) close-up view of the L-Phe subpocket is shown. (C-D). Superposition of the aminoacylation domains from MtFRSt/F-AMS2 (purple) with human cytosolic enzyme (hcFRS) in complex with L-Phe (3L4G, green). In (D) close-up view of the L-Phe subpocket is shown. (E) Superposition of the anticodon binding region of MtFRSt/F-AMS2 (grey) and hmFRSt (coral). Labels in parentheses correspond to the respective human homologs. (F) Superposition of the editing domains (b1s4 subdomain) from MtFRSt/F-AMS2 (grey) and hcFRS (3L4G, green). Gold LTyr from the TtFRS/L-Tyr complex marks the amino acid binding site.