Characterization of the Human Mitochondrial Methionyl-tRNA Synthetase†

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ABSTRACT: Human mitochondrial methionyl-tRNA synthetase (human mtMetRS) has been identified from the human EST database. The cDNA encodes a 593 amino acid protein with an 18 amino acid mitochondrial import signal sequence. Sequence analysis indicates that this protein contains the consensus motifs characteristic of a class I aminoacyl-tRNA synthetase but lacks the Zn^{2+} binding motif and C-terminal dimerization region found in MetRSs from various organisms. The mature form of human mtMetRS has been cloned and expressed in *Escherichia coli*. Gel filtration experiments indicate that this protein functions as a monomer with an apparent molecular mass of 67 kDa. The kinetic parameters for activation of methionine have been determined for the purified enzyme. The K_M and k_{cat} for aminoacylation of *E. coli* initiator $tRNA_f^{Met}$ are reported. The kinetics of aminoacylation of an in vitro transcript of human mitochondrial $tRNA^{Met}$ ($mtRNA^{Met}$) have been determined. To address the effects of the modification of mtRNA on recognition of the mitochondrial tRNA by human mtMetRS, the kinetics of aminoacylation of native bovine $mtRNA^{Met}$ and of an in vitro transcript of the bovine $mtRNA^{Met}$ have also been investigated.

The fidelity of protein synthesis is dependent on the attachment of the correct amino acid to its cognate tRNA by the appropriate aminoacyl-tRNA synthetase (aaRS).[1] These enzymes catalyze two stepwise reactions. In the first reaction, an activated aminoacyl adenylate intermediate is formed which, in the second reaction, is transferred to the 2′ or 3′ hydroxyl on the terminal adenosine of the tRNA. The 20 aaRSs fall into two classes depending on specific functional and structural similarities. Class I synthetases are characterized by two signature sequences, HIGH and KMSKS. They contain a Rossman fold and add the amino acid to the 2′ hydroxyl of the cognate tRNA. Class I synthetases are further divided into three subclasses depending on the nature of an insertion, called the connective peptide, located between the two halves of the Rossman fold. Methionyl-tRNA synthetase (MetRS) belongs to the class Ia synthetase group, which includes IleRS, ValRS, LeuRS, CysRS, and ArgRS (*1*). Class II synthetases are characterized by a catalytic core of antiparallel β-sheets and three consensus motifs (*2*).

There is a considerable amount of structural and biochemical data on the bacterial MetRS. The crystal structures of both *Escherichia coli* and *Thermus thermophilus* MetRS have been solved at atomic resolution, including the structure of the *E. coli* enzyme in complex with methionine and ATP (*3*−*6*). *E. coli* MetRS functions as a homodimer carrying one zinc ion per subunit (*6*). However, the enzyme remains active in monomeric form after removal of a C-terminal region required for dimerization (*1*). The crystal structure of the monomeric form of *E. coli* MetRS indicates that the catalytic core of the enzyme folds into four discrete domains: (1) the classical Rossman fold, (2) the connective peptide, (3) the KMSKS signature peptide, and (4) the C-terminal domain (Figure 1A) (*6*).

While bacterial aaRSs have been studied extensively, there is limited information concerning mitochondrial aaRSs, especially those from mammals. The human mitochondrial PheRS, LeuRS, and IleRS and the bovine mitochondrial SerRS have been studied biochemically (*7*−*10*). The mitochondrial MetRSs from *Saccharomyces cerevisiae* and the pathogenic fungus *Candida albicans* have been identified and characterized as well (*11*, *12*). Mitochondrial synthetases are encoded in the nucleus and contain mitochondrial import sequences for transport into the organelle (*2*). These genes are usually distinct from their cytoplasmic counterparts and are essential for mitochondrial translation (*2*).

Mammalian mitochondrial DNA encodes 22 tRNAs, one for each amino acid and two for serine and leucine, respectively (*13*). There is no evidence for the import of tRNAs into mammalian mitochondria, so these species represent all the tRNAs available for the translational apparatus. One distinct feature of animal mitochondria is the presence of a single gene for $tRNA^{Met}$, which must function as fMet-tRNA for initiation and Met-tRNA for chain elongation. This phenomenon has not been observed in other systems, including the mitochondria of most lower eukaryotes. The nucleotide sequence of purified bovine liver mitochondrial $tRNA^{Met}$ ($mtRNA^{Met}$) reveals the presence of

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; mtMetRS, mitochondrial methionyl-tRNA synthetase; $mtRNA^{Met}$, mitochondrial $tRNA^{Met}$; TCA, trichloroacetic acid; AMP, adenosine monophosphate; PP_i, inorganic pyrophosphate; f^5C, 5-formyl cytidine; BSA, bovine serum albumin.

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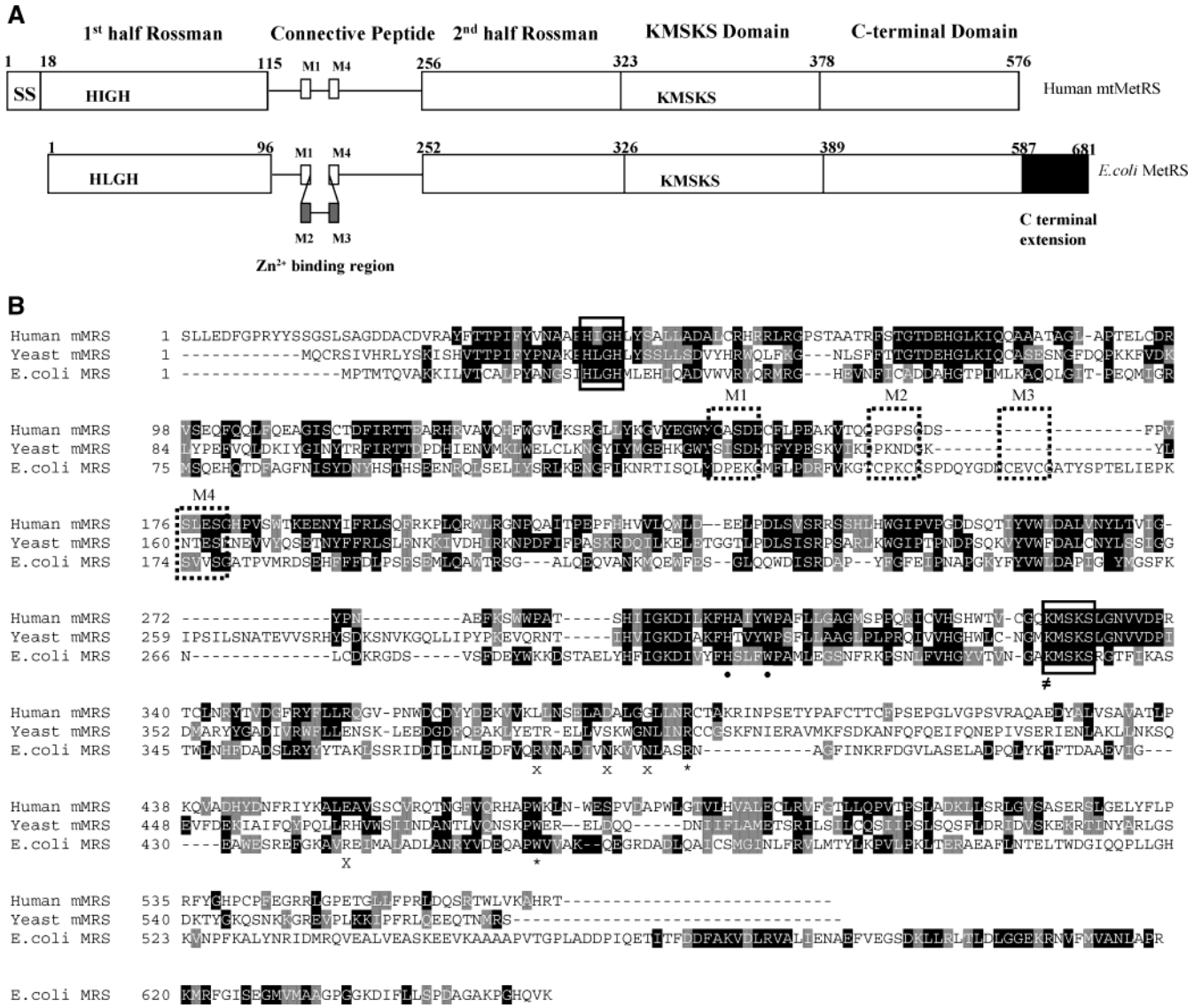

FIGURE 1: Panel A shows a schematic diagram of the domain organization of human mtMetRS compared with that of *E. coli* MetRS. The four domains that compose the catalytic core of MetRSs are shown with the amino acid residue numbers in each domain indicated above the figure. The location of the class I signature sequences are shown in their respective domains. The 18 amino acid mitochondrial signal sequence (SS) of human mtMetRS is indicated but is not present in the mature enzyme. The C-terminal extension of *E. coli* MetRS is shown in black. The zinc-binding region in the connective peptide domain is designated with boxes, which correspond to the motifs assigned in ref *6* (M1−M4). Motifs 1 and 4 form the proximal zinc-binding knuckle, and motifs 2 and 3 form the distal knuckle. The shaded boxes indicate the zinc-binding knuckle of *E. coli* MetRS. The white boxes indicate knuckles devoid of zinc. Panel B shows the sequence alignment of MetRSs from *E. coli* and yeast and human mitochondria. The signature motifs, HIGH and KMSKS, of a class I synthetase are boxed in solid black, and the four possible motifs for zinc binding (M1−M4) are boxed in dotted lines. The residues involved in the activation of methionine are indicated with a •. Other important functional residues are highlighted as follows: anticodon recognition (∗) and both interaction with the 3′ end of the tRNA and methionine activation (≠). The residues in *E. coli* MetRS that are involved in the interaction with $tRNA^{Met}$ but are not conserved in human mtMetRS are indicated with an X. The alignment was generated using CLUSTALW on Biology Workbench. Accession numbers are indicated in Table 1.

the unusual modified base, 5-formyl cytidine (f^5C) in the first position of the anticodon (*14*). This minor base may be responsible for the unique ability of this tRNA to decode both the AUG and AUA codons, which specify methionine in mammalian mitochondria (*14*−*16*). This modification is present in the mitochondrial $tRNA^{Met}$ of a number of species (*15*, *17*). In the present work, we have identified, expressed, and characterized human mitochondrial MetRS (human mtMetRS).

MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB). [^{32}P]PP_i, [^{3}H]-Met, and [^{35}S]Met were obtained from NEN Life Science Products. *E. coli* tRNA was purchased from Roche. *E. coli* $tRNA_f^{Met}$ was partially purified from crude *E. coli* tRNA as described (*18*). Oligonucleotides were prepared at the Lineberger Comprehensive Cancer Research Center at the University of North Carolina at Chapel Hill. Crude bovine mitochondrial tRNA was kindly provided by Senyene Eyo Hunter (University of North Carolina at Chapel Hill). The purification of T7 RNA polymerase was adapted from reference *19*.

Cloning the Gene for Human mtMetRS. The primers were designed from the human EST database (accession no. NM_138395), and a part of the coding region of human

mtMetRS was amplified by RT-PCR. Total RNA from growing HL-60 cells and an oligo(dT) primer were used for the first strand cDNA synthesis. The primers for the PCR step were forward 5′-GATGATGCTTGTGATGCGCGC-CTAC-3′ and reverse 5′-GGTCCGGTGGGCTTTCAC-CAGCCAAG-3′. Full-length cDNA was obtained by the combination of 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE, using the RACE system (Gibco BRL) according to the manufacturer's instructions. Primers used in 5′ RACE were forward 5′-GCTGCTGGAACTGCTC-3′ for the first strand DNA synthesis, 5′-CTGCTGCCTGCT-GAATCTTC-3′ for the first PCR, and 5′-GCCCGT-GCTCGTCGGTACC-3′ for the nested PCR. Primers used in 3′ RACE were 5′-GCTGGTGAAAGCCCACCGGACC-3′ for the first PCR reaction and 5′-CATTTCTCTGTGAC-CATTGATC-3′ for the nested PCR. The resulting cDNA was inserted into the pET28 expression vector (Novagen) at the *Nde*I and *Not*I restriction sites.

The initial clone obtained was transformed into *E. coli* DH5α cells and sequenced at the Automated DNA Sequencing Facility at the University of North Carolina at Chapel Hill. The inserted DNA (nucleotides 89−1900 of accession no. NM_138395) contained a point mutation (C186T) resulting in a proline to leucine amino acid substitution at position 50 (amino acid numbering by precursor). Expression of human mtMetRS from this construct was unsuccessful. Therefore, the mutation was corrected using a site-directed mutagenesis approach similar to the QuikChange method (Stratagene).

Mutagenesis reactions (50 μL) contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/mL BSA, 50 ng of pET28-human mtMetRS, 250 μM dNTPs, 125 ng of primer no. 1 (5′-TACTTCACCACACCCATTTTCTACGTGAACGC-3′), 125 ng of primer no. 2 (5′-CACGTAGAAATGGGTGTGGT-GAAGTAGGCGCGCAC-3′), and 2.5 U of *Pfu* DNA polymerase (Stratagene). Immediately following temperature cycling, 20 U of *Dpn*I (NEB) was added to the reaction, which was incubated at 37 °C for 2 h. The mutated DNA (5 μL) was then directly transformed into *E. coli* DH5α competent cells, and clones were verified by DNA sequencing. The recombinant DNA was transformed into *E. coli* BL21-Codon Plus(DE3)-RIL competent cells (Stratagene) to circumvent the codon bias problem when expressing mammalian proteins in *E. coli*.

Expression and Purification of Human mtMetRS. Saturated cultures of cells harboring the pET28-human mtMetRS construct grown in 2YT media (40 mL) were used to inoculate 6 L of 2YT media (4 × 1.5 L), which were grown to an A_{600} of 0.8 at which time expression of human mtMetRS was induced by the addition of 50 μM isopropyl-β-D-thiogalactopyranoside. The cultures were grown overnight at 25 °C after induction. Cells were harvested, resuspended in 120 mL of buffer containing 10 mM Tris-HCl (pH 7.6), and collected by low-speed centrifugation.

The cell pellets were resuspended in 90 mL of buffer I (50 mM Hepes-KOH (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonylfluoride) supplemented with lysozyme (0.03% w/v final) and 0.1% Triton X-100 before sonication (60 W) on ice for 12 min with 1 s bursts followed by 4 s cooling periods. The lysed cells were then supplemented with 5 μg/mL DNase I and subjected to centrifugation at 100 000 × *g* for 1 h at 4 °C in a Beckman type 60 rotor. Human mtMetRS was purified from the supernatant using 1.5 mL of Ni−NTA resin (50% slurry in buffer I). The resin was washed three times with 50 mL of buffer II (buffer I containing 10 mM imidazole). The protein was eluted with 4.5 mL of buffer III (buffer I containing 150 mM imidazole) for 20 min at 4 °C. The sample was then dialyzed twice against 500 mL of buffer IV (20 mM Hepes-KOH (pH 7.6), 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 50 mM KCl, 7 mM β-mercaptoethanol) for 45 min and diluted to ∼29 mL with buffer IV. The enzyme was further purified by ion exchange. DEAE-Sephadex resin (4.5 mL, 50% slurry in buffer IV) was added to the partially purified protein sample and incubated for 30 min at 4 °C with rocking. The resin was removed by centrifugation, and the purified protein was recovered in the supernatant. Protein concentrations were determined by the Bradford method (Bio-Rad). Human mtMetRS was stored at −80 °C in small aliquots until use.

Gel Filtration Chromatography. The molecular mass of recombinant human mtMetRS was determined from its sequence to be 67 kDa. To determine whether human mtMetRS is monomeric or multimeric, gel filtration chromatography at 4 °C was performed on a Sephacryl S-200 column (1.5 cm × 60 cm) equilibrated with buffer IV. The column was calibrated with Blue Dextran, alcohol dehydrogenase (M_r = 160 000), bovine serum albumin (BSA; M_r = 66 000), and horse cytochrome *c* (M_r = 12 500). A sample of human mtMetRS (2 mL, 52 μg of protein) supplemented with 25 mg of BSA was applied to the column, and fractions of 150 drops (∼4 mL) were collected at a flow rate of ∼0.4 mL/min. The fractions were analyzed for human mtMetRS activity in the aminoacylation assay described below. The molecular weight was assigned according to plots of the log-(M_r) versus elution volume.

ATP/PP_i Exchange Reactions. All ATP/PP_i exchange reactions were performed as previously described (*8*, *9*). Reaction mixtures (100 μL) containing 50 mM Tris-HCl (pH 8.2), 2.5 mM $MgCl_2$, 0.2 mM spermine, 8.4 mM KF, 0.2 mg/mL BSA, 200 nM human mtMetRS, and 2 mM [^{32}P]PP_i at 60 cpm/pmol were incubated at 37 °C. KF was added to the exchange reactions to minimize the possibility of hydrolysis of pyrophosphate. In all reactions in which the ATP concentration was varied, the methionine concentration was held constant at 2 mM and vice versa. The [^{32}P] exchange from PP_i into ATP for determining the kinetic parameters for methionine was measured from 2.5 to 15 min while the methionine concentration was varied from 10 to 200 μM. The [^{32}P] exchange from PP_i into ATP to determine the kinetic parameters for ATP was measured between 1 and 12.5 min while the ATP concentration was varied from 25 to 300 μM. The exchange reactions were stopped by spotting 2 μL of the reaction mixtures onto Selecto Scientific PEI cellulose thin-layer chromatography (TLC) plates (prewashed with H_2O and dried overnight). To separate ATP and PP_i, 4 M urea, 0.75 M KP_i at pH 3.5 was used as the mobile phase (*8*, *9*, *20*). All radiolabeled species (P_i, PP_i, and ATP) were quantified by phosphor-imaging with a Molecular Dynamics Storm 840 and the ImageQuant software. The exchange assays were carried out with two negative controls, one containing [^{32}P]PP_i with no enzyme and the other [^{32}P]ATP with no enzyme. The second control was used to locate

labeled ATP on the developed TLC plates. There was no ATP/PP_i exchange observed with the negative controls.

$tRNA^{Met}$ Aminoacylation Assays. The aminoacylation reactions were performed essentially as described (*8*, *9*). Reaction mixtures (100 μL) containing 50 mM Tris-HCl (pH 8.2), 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.2 mM spermine, 100 μM [^{3}H]Met at 60 cpm/pmol, 0.2 mg/mL BSA, 100 nM human mtMetRS, and 0.18−7.32 μM *E. coli* $tRNA_f^{Met}$. The concentration of $tRNA_f^{Met}$ was determined by aminoacylation assays at 37 °C for 20 min with 22 μg of partially purified *E. coli* $tRNA_f^{Met}$ and increasing amounts of human mtMetRS up to 200 nM until saturation was reached (data not shown). The amount of chargeable *E. coli* $tRNA_f^{Met}$ was 604 pmol/A_{260}. For experiments with mitochondrial $tRNA^{Met}$, reaction mixtures (50 μL) contained 50 mM Tris-HCl (pH 7.8), 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.2 mM spermine, 100 μM [^{35}S]-Met at 2500 cpm/pmol, 0.2 mg/mL BSA, 30−38 nM human mtMetRS, and 0.03−0.3 μM human $mtRNA^{Met}$ transcript, 0.06−0.24 μM native bovine mitochondrial $tRNA^{Met}$, or 0.03−2.5 μM bovine $mtRNA^{Met}$ transcript. The concentration of mitochondrial $tRNA^{Met}$ was determined by the maximum amount of [^{35}S]Met-tRNA formed after incubation at 37 °C for 15 min using either 3 pmol of the human $mtRNA^{Met}$ transcript, 3.8 pmol of the bovine $mtRNA^{Met}$ transcript, or 1.2 μg of crude mitochondrial tRNAs and increasing amounts of human mtMetRS up to 230 nM until saturation was reached (data not shown). Typical transcript preparations were 30% active in aminoacylation. For determination of kinetic constants, the aminoacylation reactions were incubated at 37 °C for 1−5 min (except when the concentration of tRNA was less than 0.06 μM when the incubation time was extended up to 30 min). For each time point, 20% of the reaction was removed and the labeled Met-$tRNA^{Met}$ was precipitated by adding 3 mL of ice cold 5% trichloroacetic acid (TCA). After a 10 min incubation at 0 °C, the radiolabeled Met-tRNA formed was collected on nitrocellulose filters (Millipore HA 0.45 μm). All filters were washed with 2 × 3 mL of cold 5% TCA and dried for 10 min at 100 °C. The amount of radiolabeled Met-tRNA was determined by scintillation counting (Minaxi Tri-Carb 4000 series). A negative control (no enzyme or no tRNA) was used to represent the background for each experiment. Its value was subtracted from the measured values in each experiment.

Preparation of Mitochondrial $tRNA^{Met}$ Transcripts. A DNA encoding the sequence of bovine mitochondrial $tRNA^{Met}$ was prepared in a construct containing the hammerhead ribozyme between a strong T7 RNA polymerase promoter and the tRNA gene (*21*) using the following oligonucleotides: mtRNAM1, 5′-pAGCTTAATACGACTCACTATAGGGAGATACTCTGATGAGTCCGTGAGGACGAAACGGTACCCGGTACCGTCAGTAAG-3′; mtRNAM2, 5′-pGTCAGCTAATTAAGCTATCGGGCCCATACCCCGAAAATGTTGGTTTATATCCTTCCCGTACTACCAGGT-3′; mtRNAM3, 5′-pGCTGACCTTACTGACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGAGTATCTCCCTATAGTGAGTCGTATTA-3′; mtRNAM4, 5′-pCTAGACCTGGTAGTACGGGAAGGATATAAACCAACATTTTCGGGGTATGGGCCCGATAGCTTAATTA-3′. The oligonucleotides were purified on a 12% polyacrylamide gel (16 × 20 × 1.5 cm^3) using UV-shadowing to visualize the bands. Oligonucleotides were hybridized by combining ∼5 pmol of each purified oligonucleotide in hybridization buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM spermidine, 1 mM ATP) in a final volume of 4.5 μL. The oligonucleotide mix was heated at 90 °C for 5 min and then allowed to equilibrate to room temperature for 5−10 min. The oligonucleotide mixture was ligated using 200 U of T4 DNA ligase (NEB) for 16 h at 16 °C. A fraction (1/10) of the ligation reaction was used as a template for PCR amplification using the following primers: mtRNA FP (5′-CCCCAAGCTTAATACGACTCACTATAGGG-3′) and mtRNA RP (5′-CTAGTCTAGACCTGGTAGTACGGGAAGG-3′). The resulting DNA fragment was cloned into pUC19 using the restriction enzymes *Hin*dIII and *Xba*I (NEB). The positive clones were verified by DNA sequencing at the Automated DNA Sequencing Facility at the University of North Carolina at Chapel Hill.

To prepare the human mitochondrial $tRNA^{Met}$ transcript, the bovine mitochondrial $tRNA^{Met}$ gene was mutated using the QuikChange protocol as described above. Primers for the T5048 deletion and T5052C mutation (numbering according to the bovine $tRNA^{Met}$ gene) were 5′-CCGAAAATGTTGGTTATACCCTTCCCGTACTACC-3′ and 5′-TAGTACGGGAAGGGTATAACCAACATTTTCGGGG-3′ with the pUC19-bovine $tRNA^{Met}$ construct as the template. The mutated DNA (5 μL) was directly transformed into *E. coli* ER2267 competent cells, and clones were verified by DNA sequencing. The T5012A mutation was made using the primers 5′-AGTAAGGTCAGCTAAATAAGCTATCGGGCC-3′ and 5′-GGGCCCGATAGCTTATTTAGCTGACCTTAC-3′, and the DNA template containing the T5048 deletion and T5052C mutation. The resulting DNA was then directly transformed into *E. coli* ER2267 competent cells, and clones were verified by DNA sequencing.

Transcription of $mtRNA^{Met}$. Template DNA was linearized by digestion with *Bst*NI for 4 h at 60 °C. SDS and proteinase K were then added at 0.5% and 100 μg/mL, respectively, and the reaction was incubated at 50 °C for 1 h. The template DNA was purified by phenol and chloroform extractions followed by ethanol precipitation. Transcription reactions contained 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM spermidine, 10 mM dithiothreitol, 10 μg of linearized template DNA, 4 mM each NTP, 25 mM GMP, 0.05 mg/mL BSA, 10 units of SUPERase•In (Ambion), and saturating amounts of T7 RNA polymerase. Transcription reactions were performed at 37 °C for 4 h. DNase I (3.2 units, Ambion) was then added to the reaction, and incubation was continued at 37 °C for 1 h. Transcription products were analyzed on an 8% denaturing polyacrylamide gel.

Greater than 50% of the transcript was cleaved following transcription as determined by gel electrophoresis. However, to obtain full cleavage of the transcript by the hammerhead ribozyme, the transcription reaction was diluted to 500 μL with buffer containing 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 10 mM dithiothreitol and incubated at 55 °C for 1 h. The RNA was then purified by phenol and chloroform extractions followed by ethanol precipitation. The concentration of $mtRNA^{Met}$ was determined by aminoacylation assays using saturating concentrations of human mtMetRS.

Modeling of Human mtMetRS. The structure of human mtMetRS was modeled using the Swiss-Model program in the first approach mode given the pdb coordinates of *E. coli*

Table 1: Comparison of Human mtMetRS with MetRSs from Various Organisms

species	accession number	% identity[a]	probability of mt import[b]
Rattus norvegicus mt	XM_237156	88	0.97
Mus musculus mt	AK050196	87	0.99
Takifugu rubripes mt	AJ290422	61	0.77
Drosophila melanogaster mt	NM_142091	44	0.89
Schizosaccharomyces pombe mt	Z98978	39	0.96
Caenorhabditis elegans mt	NM_170952	38	0.44
Rhodospirillum rubrum	ZP_00015030	37	
Geobacter sulfurreducens	NP_953281	36	
Mycobacterium tuberculosis	AE006986	36	
Neurospora crassa mt	XM_323060	35	0.98
Saccharomyces cerevisiae mt	X14629	34	0.86
Thermus thermophilus	M64273	34	
Candida albicans mt	AB006140	33	0.81
Bacillus stearothermophilus	X57925	32	
Homo sapiens cyto	BC011849	23	
Saccharomyces cerevisiae cyto	CAA69086	22	
Escherichia coli	X55791	19	

[a] Represents sequence identity to the mature form of human mtMetRS, determined using the CLUSTALW program on Biology Workbench. [b] Determined by MitoProtII.

MetRS (1QQT) and the entire protein sequence of the human enzyme. When these parameters were used, only a partial model of human mtMetRS (aa 171−361) was generated. To obtain coordinates for missing portions of human mtMetRS, smaller regions of higher sequence identity (aa 23−161, aa 280−392, aa 325−534) were submitted independently to Swiss-Model and modeled in the first approach mode using the pdb coordinates of both *E. coli* MetRS (1QQT) and *T. thermophilus* MetRS (1A8H), respectively. The final model was generated by compiling each section of the pdb coordinates obtained from Swiss-Model.

RESULTS

Sequence Analysis of Human mtMetRS. The gene for human mtMetRS was identified from the human EST database, and primers were designed to clone the cDNA from HL-60 cells. The coding sequence for human mtMetRS was inserted into the pET28 expression vector, which provides an N-terminal His-tag to facilitate purification of the recombinant protein. The open reading frame of human mtMetRS encodes a 593 amino acid protein with a 98% probability of mitochondrial localization. MitoProtII and PSORT indicate that human mtMetRS contains an 18 amino acid mitochondrial import signal sequence. The portion of the gene corresponding to the mature protein beginning at amino acid 19 was present in the recombinant protein (67 kDa).

A number of mitochondrial MetRSs can be identified with a BLAST search using the human mtMetRS sequence. The mammalian mitochondrial MetRSs share a high degree of sequence conservation with human mtMetRS; however human mtMetRS is less well conserved with the corresponding enzymes from the lower eukaryotes (Table 1). The closest bacterial homologue to human mtMetRS currently in the databases is *Rhodospirillum rubrum.* Surprisingly, human mtMetRS is only 19% identical to its *E. coli* homolog. It is interesting to note, however, that MetRSs are not highly conserved among some bacterial species. For example, the *E. coli* and *T. thermophilus* MetRSs possess only 25% sequence identity to each other. Finally, the human mtMetRS does not share considerable sequence similarity to the human cytoplasmic MetRS.

MetRSs from all species are thought to possess a conserved catalytic core composed of the Rossman fold, the connective peptide, the KMSKS domain, and the C-terminal domain (Figure 1A) (*6*). Comparison of the domain organization of *E. coli* MetRS and human mtMetRS indicates that the catalytic core is conserved between these two factors with significant differences observed in the connective peptide region and the C-terminal end (Figure 1A).

MetRSs, despite their functional similarity, are a diverse group of enzymes in terms of sequence and quaternary structure. Much of the sequence diversity of MetRSs comes from N- or C-terminal extensions (or both) from the catalytic core, which is common among all MetRSs (*1*). Little is known about the role of N-terminal extensions in MetRS. Yeast cytoplasmic MetRS has an N-terminal extension of 185 amino acids that is not required for enzymatic activity but mediates its interaction with other proteins (*1*, *22*). MetRSs from various organisms exist as either monomers or homodimers, and in the cytoplasm of some higher eukaryotes, MetRS exists as part of a multisynthetase complex (*1*). For many MetRSs, a C-terminal extension plays a role in this dimerization (*1*). For example, *E. coli* MetRS contains a C-terminal extension of approximately 100 amino acids that is responsible for dimerization (Figure 1A).

Sequence alignments indicate that human mtMetRS does not contain N- or C-terminal extensions and, thus, is not likely to form quaternary structure. Gel filtration chromatography was used to analyze whether human mtMetRS functions as a monomer or multimer. Human mtMetRS activity, as measured by aminoacylation of $tRNA_f^{Met}$, eluted from a Sephacryl S200 column as a single peak with an apparent molecular mass of 67 kDa as determined by a plot of the log(M_r) versus elution volume of a series of protein standards (data not shown). These data strongly suggest that human mtMetRS functions as a monomer.

The MetRSs in different species differ in the ability to bind Zn^{2+} ions. MetRSs have been classified according to four motifs for Zn^{2+} binding located in the connective peptide region and are grouped accordingly (*23*). These four motifs structurally form two knuckles, each of which can potentially bind one Zn^{2+} ion (*6*). Sequence analysis of MetRSs reveals four groups ranging from two knuckles with two zinc binding sites to one knuckle with no zinc site (*23*). Human mtMetRS falls into the classification of having one knuckle and no Zn^{2+} since it lacks the zinc-binding ligands in the knuckle (Figure 1). Included in this group are the MetRSs from *S. cerevisiae* and *C. albicans* mitochondria (*12*, *23*) and the bacterium *Mycobacterium tuberculosis* (*24*).

Alignment of the sequence of human mtMetRS with *E. coli* MetRS and *S. cerevisiae* mitochondrial MetRS shows that the class I signature sequences HIGH and KMSKS are conserved in the mitochondrial protein (Figure 1B). A number of other residues important for the activity of *E. coli* MetRS either in activation of the amino acid or in $tRNA^{Met}$ recognition are also conserved to a large extent in the human mtMetRS (Figure 1B).

Expression and Purification of Human mtMetRS. The best expression of the recombinant human mtMetRS in *E. coli* was obtained in overnight cultures grown at 25 °C (data not shown). Purification of the expressed histidine-tagged human

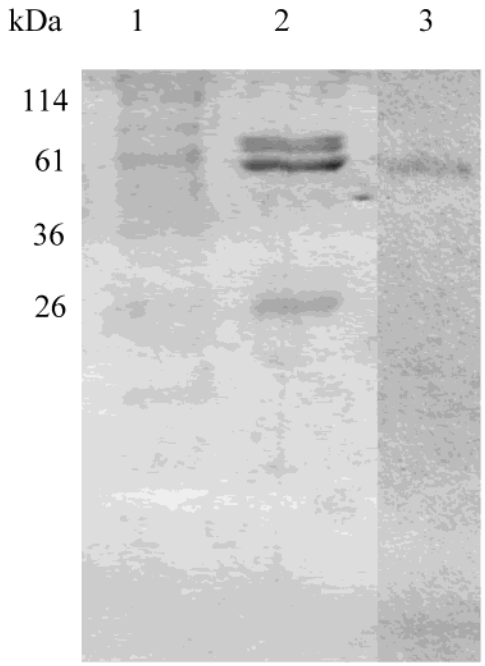

FIGURE 2: SDS−PAGE analysis of purified human mtMetRS. All samples were analyzed on a 10% (w/v) SDS−polyacrylamide gel stained with Coomassie Blue: (lane 1) BenchMark protein ladder; (lane 2) Ni−NTA purified human mtMetRS (3.2 μg); (lane 3) DEAE-Sephadex purified human mtMetRS (1.1 μg).

mtMetRS with metal ion affinity chromatography on Ni−NTA resin resulted in reasonably pure enzyme (Figure 2, lane 2). Remaining impurities were removed in a second step with ion exchange chromatography on DEAE-Sephadex to give highly purified enzyme (Figure 2, lane 3). It was important to keep the protein concentration of the human mtMetRS sample relatively dilute (0.04 mg/mL) to reduce the tendency of the enzyme to precipitate.

Enzymatic Properties of Human mtMetRS−ATP/PP$_i$ Exchange. Aminoacylation is a two-step process in which the amino acid is covalently attached to its cognate tRNA as indicated:

$$\text{mtMetRS} + \text{Met} + \text{ATP} \rightleftharpoons [\text{mtMetRS/Met−AMP}] + \text{PP}_\text{i} \quad (1)$$

$$[\text{mtMetRS/Met−AMP}] + \text{tRNA}^{\text{Met}} \rightleftharpoons \text{Met−tRNA}^{\text{Met}} + \text{AMP} + \text{mtMetRS} \quad (2)$$

In eq 1, mtMetRS condenses its cognate amino acid methionine and ATP to form the intermediate aminoacyladenylate, Met−AMP, and releases pyrophosphate. The second step (eq 2) involves the formation of the ester bond between the 2′ hydroxyl group of the terminal adenosine of tRNA$^{\text{Met}}$ and the carboxyl group of the methionine. Since the formation of the aminoacyladenylate by MetRS is independent of tRNA and is completely reversible in the absence of tRNA, the kinetic parameters for the interaction of human mtMetRS with its substrates methionine and ATP can be analyzed by monitoring the exchange of radiolabeled pyrophosphate into ATP. By measuring the initial rate of the exchange reaction, the values for the kinetic parameters K_M and k_cat and the catalytic efficiency (k_cat/K_M) have been determined for human mtMetRS with methionine and ATP. The Michaelis−Menten constant (K_M) and the maximum velocity, V_max, for methionine were determined using a Lineweaver−Burk plot. The K_M and k_cat values for methionine are 18 μM and 0.41 s^{-1}, respectively (Table 2).

The kinetic parameters in amino acid activation of several MetRSs from various species and a limited number of mammalian mitochondrial aminoacyl-tRNA synthetases have been characterized (Table 2). The K_M value for methionine obtained for human mtMetRS is comparable to that observed with the bacterial MetRS enzymes, but the human mitochondrial enzyme has a lower k_cat. This value of k_cat is, however, within 7-fold of the values observed with other human mitochondrial aaRSs characterized to date, and the catalytic efficiency of the human mtMetRS is within a factor of 2.5 of the values obtained with other mitochondrial synthetases (Table 2). It is interesting to note that, despite sharing similar K_M values for their cognate amino acids with the bacterial synthetases, the mitochondrial synthetases exhibit lower values for k_cat. A similar observation has been made for the catalytic efficiencies of the synthetases when kinetic parameters are measured for ATP and aminoacyl-tRNA (see below). The tendency for mitochondrial synthetases to have lower turnover numbers may reflect the lower rates of translation in mitochondria compared with both bacterial and cytoplasmic translational systems (*25*). Animal mitochondria synthesize only 13 polypeptides. Incorporation of amino acids into proteins in mitochondria occurs at only about 2% the rate observed in prokaryotes (*25*). There is no indication that there is any selective advantage for mitochondrial translation to occur with the same efficiency as that in bacteria. Presumably the lower catalytic efficiencies generally observed with the mitochondrial aaRSs reflect the lack of selective pressure for rapid protein synthesis in this system.

For reactions used to analyze the kinetic parameters for the interaction of ATP with human mtMetRS, the ATP concentration was varied from 25 to 300 μM with the methionine concentration held constant at 2 mM. The K_M and V_max were determined using a Lineweaver−Burk plot. The values for K_M and k_cat are 85 μM and 0.033 s^{-1}, respectively (Table 2). The K_M for ATP varies by over 20-fold among the MetRSs characterized, and the value observed for human mtMetRS is quite reasonable given this range. This value is somewhat lower than the K_M values observed with several other mammalian mitochondrial aaRSs (Table 2). Among the mitochondrial factors, only human mtLeuRS has a comparable K_M for ATP. The other values are significantly higher, especially human mtPheRS. The k_cat values for ATP for the mitochondrial synthetases, including human mtMetRS, are generally lower than their bacterial counterparts, supporting the notion that the lower activity of the mitochondrial synthetases may correlate with lower translation rates (Table 2 and references therein).

Aminoacylation−Effects of Spermine, Cation Concentration, BSA, and pH. Previous work on human mitochondrial aaRSs showed that the enzymes were sensitive to different reaction conditions, and thus, it was important to optimize the aminoacylation reaction for human mtMetRS prior to determination of the K_M for tRNA (*8*, *9*). Polyamines are known to play a role in stabilizing the conformation of negatively charged nucleic acids (*26*). In this study, the optimal catalytic activity of human mtMetRS with *E. coli*

Table 2: Comparison of the Kinetic Parameters for Methionine and ATP of Human mtMetRS with MetRSs from Various Organisms

	Met			ATP			
MetRS	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	refs
E. coli	20	76	3.8	20	42	2.1	*47*, *48*
T. thermophilus	27, 18[a]			180, 1600[b]			*49*, *50*
B. stearothermophilus	8			11			*35*
H. sapiens mt[c]	18	0.41	0.023	85	0.033	0.00038	this work

	amino acid			ATP			
mt aaRSs	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	refs
H. sapiens LeuRS	45	2.7	0.05	90	0.8	0.0088	*8*
H. sapiens PheRS	33	1.1	0.033	2500	2.9	0.0012	*9*
H. sapiens TrpRS	27	1.2	0.044	400	1.4	0.0035	*51*

[a] Values represent independent data from different laboratories. [b] Values represent the biphasic nature of kinetics. [c] Uncertainties in the values reported are estimated to be approximately 5−10%.

$tRNA_f^{Met}$ was observed at a spermine concentration of about 0.2 mM. However, spermine did not stimulate the aminoacylation of mitochondrial $tRNA^{Met}$ significantly (data not shown). Aminoacylation is also sensitive to the Mg^{2+} ion concentration (*8*, *9*), so the effects of various concentrations of $MgCl_2$ (0−10 mM) were analyzed. To reach the zero concentration for Mg^{2+}, 0.2 mM EDTA was added to the reaction mix. Optimal results were achieved with a Mg^{2+} concentration of 2.5−3 mM. The effect of monovalent cations was analyzed with various concentrations of KCl and optimal activity of human mtMetRS was achieved at very low concentrations (2.5 mM) and decreased at higher KCl concentrations (data not shown).

The effect of BSA (0.2 mg/mL) on the aminoacylation activity of human mtMetRS was also analyzed, since BSA was shown to stabilize the human mtLeuRS enzyme at low enzyme concentrations (<100 nM) (*8*). BSA had no significant affect on human mtMetRS activity but was added at a final concentration of 0.2 mg/mL to all aminoacylation reactions as a precaution. The effect of different buffers on the aminoacylation reaction was analyzed by using Hepes-KOH and Tris-HCl based buffers at various pHs. The pH with Hepes-KOH based buffers was varied at 7.0 and 7.6, and Tris-HCl based buffers were tested at pH 7.6, 8.0, 8.2, and 8.5. Tris-HCl buffers in the pH range from 7.6 to 8.2 yielded optimal aminoacylation activity. The optimal conditions for aminoacylation were determined to be 50 mM Tris-HCl (pH 7.8−8.2), 2.5−3 mM $MgCl_2$, 0.2 mM spermine, 0.2 mg/mL BSA, and low KCl concentrations. The slightly basic pH required for optimal human mtMetRS activity is consistent with a pH of 8 inside the mitochondrial matrix (*27*).

Enzymatic Properties of Human mtMetRS—Aminoacylation of $tRNA^{Met}$. The transfer of methionine to the 2′ hydroxyl group of the terminal adenosine of $tRNA^{Met}$ and the release of AMP occur during step 2 of the aminoacylation reaction. This step was assayed using various $tRNA^{Met}$ substrates. To obtain an estimate of the K_M for aminoacylation of $tRNA^{Met}$, partially purified *E. coli* initiator $tRNA_f^{Met}$ was initially used as a substrate since human mtMetRS was active in aminoacylating the *E. coli* tRNA. It should be noted that much of the literature on mitochondrial synthetases report the kinetic parameters for aminoacylation of *E. coli* tRNAs due to the difficulty in obtaining reasonable amounts of mitochondrial tRNAs (*8*, *9*). A large amount of biochemical data has shown that the primary determinant for MetRS recognition of tRNA is the anticodon sequence, CAU (*28*−*30*). *E. coli* $tRNA_f^{Met}$ also contains a number of other important determinants for aminoacylation by MetRS, notably the acceptor arm and discriminator base (Figure 3A) (*31*, *32*).

The K_M and k_{cat} values for the aminoacylation of *E. coli* $tRNA_f^{Met}$ were determined to be 2.1 μM and 11 s^{-1}, respectively, with a catalytic efficiency of 5.2 μM^{-1} s^{-1} (Table 3). The observed K_M value is comparable to the bacterial enzymes but 6−9-fold below the values observed for other human mitochondrial aaRSs with *E. coli* tRNAs (Table 3). The observed k_{cat} is 100 times higher than the known literature values for human mitochondrial aaRSs and 2−12 times higher than the values reported for bacterial MetRSs. The catalytic efficiency is similar to that reported for *E. coli* MetRS but is significantly higher than that observed for other MetRSs and for several other mitochondrial aaRSs (Table 3). It is surprising that the human mtMetRS is so efficient at aminoacylating the heterologous *E. coli* tRNA substrate since the other mitochondrial synthetases studied have relatively low turnover numbers and catalytic efficiencies with *E. coli* tRNAs.

The experiments described above were carried out using *E. coli* $tRNA_f^{Met}$ as a substrate. More important, however, are the kinetics governing aminoacylation of mitochondrial $tRNA^{Met}$. We, therefore, tested the kinetics of aminoacylation of a transcript of human mitochondrial $tRNA^{Met}$ (Figure 3B). It was also of interest to study the effects of modified nucleotides on the recognition of tRNA by human mtMetRS. To this end, we studied the aminoacylation kinetics of both native and an in vitro transcript of bovine mitochondrial $tRNA^{Met}$. The transcript lacks the three modified nucleotides found in the native $mtRNA^{Met}$, two pseudouridines and a f^5C found in the 1st position of the anticodon (Figure 3C). The human and bovine $tRNA^{Met}$ genes are 96% identical with only three nucleotide changes in the D or T loops, respectively. The change in the D loop is A16 in human $mtRNA^{Met}$ to U16 in bovine $mtRNA^{Met}$ (Sprinzl numbering system used (*33*)). The differences in the T loop are an insertion of a U at position 56 in bovine $mtRNA^{Met}$ and the substitution of a C at position 60 in human $mtRNA^{Met}$ for a U in the bovine $mtRNA^{Met}$ (Figure 3B,C).

For transcription of the unfavorable 5′ terminal sequence of both the human and bovine mitochondrial $tRNA^{Met}$ genes, a hammerhead ribozyme sequence was introduced between a strong T7 RNA polymerase promoter and the gene sequence of $mtRNA^{Met}$ (Figure 3D). Transcription and

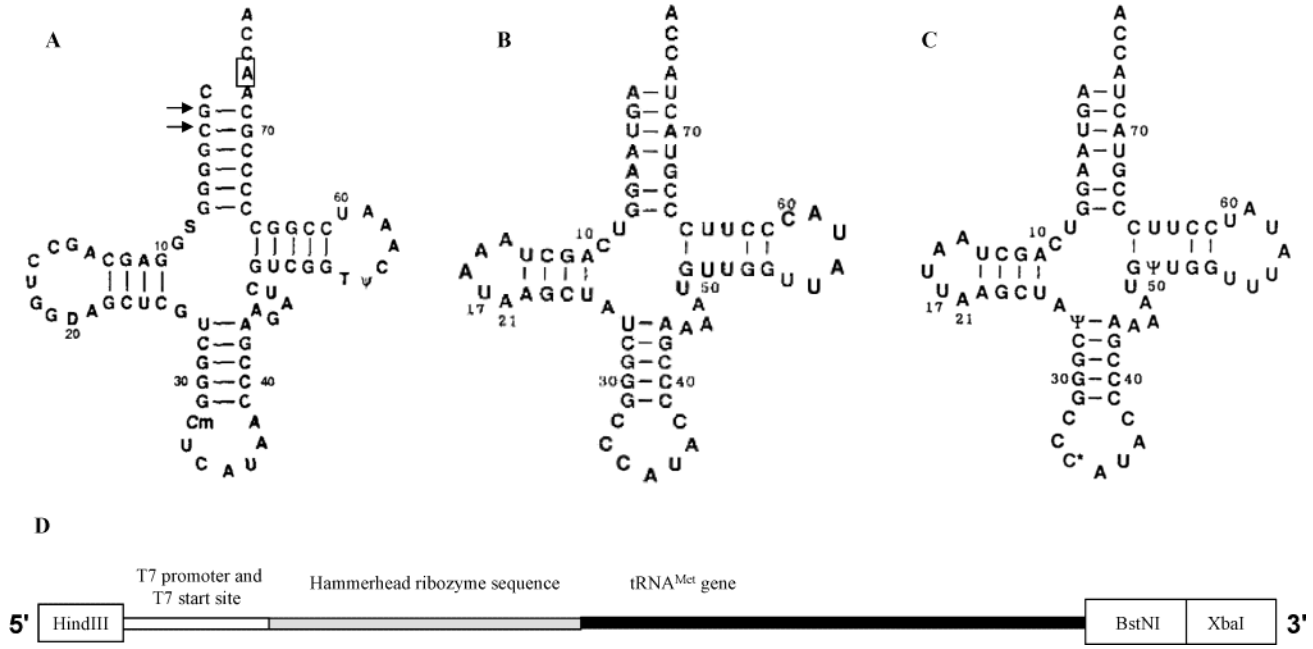

FIGURE 3: tRNA^{Met} cloverleaf structures: (A) *E. coli* tRNA$_f^{Met}$—important determinants for aminoacylation by *E. coli* MetRS include the anticodon CAU, the discriminator base A73 (boxed), and the GC pairs in the acceptor stem (arrows); (B) human mitochondrial tRNA^{Met}; (C) native bovine mitochondrial tRNA^{Met}—the asterisk indicates the presence of a f^{5}C modification; (D) hammerhead ribozyme construct for the preparation of the transcript of mtRNA^{Met}.

Table 3: Kinetic Parameters for Aminoacylation of tRNAs by MetRS from a Variety of Organisms and Aminoacylation Kinetics of Other Mitochondrial aaRSs

	tRNA^{Met}				
MetRS	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\,s^{-1}$)	tRNA^{Met} source	refs
H. sapiens mt	2.1	11	5.2	*E. coli* initiator	this work
	0.16 ± 0.08	0.021 ± 0.009	0.13	*H. sapiens* mt transcript	
	0.079 ± 0.035	0.018 ± 0.014	0.23	*B. taurus* mt transcript	
	0.15 ± 0.07	0.019 ± 0.006	0.13	*B. taurus* mt	
E. coli	1	6.85	6.85	*E. coli* initiator	*24*
T. thermophilus	1.4			*E. coli* initiator	*49*
M. tuberculosis	3.2	2.83	0.88	*E. coli* initiator	*24*
B. stearothermophilus	1.5	0.91	0.61	*E. coli* initiator	*35*
S. cerevisiae mt	0.4	0.2	0.5	*S. cerevisiae* cytoplasmic initiator	*23*
A. suum mt	0.12			*A. suum* mt	*34*

	tRNA^{aa}				
mt aaRSs	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	tRNA^{aa} source	refs
H. sapiens LeuRS	14	0.12	0.008	*E. coli*	*8, 52*
	2.3	0.02	0.009	*H. sapiens* mt transcript (UUR)	
H. sapiens IleRS	4.3	0.13	0.03	*H. sapiens* mt transcript	*10*
H. sapiens PheRS	18	0.11	0.006	*E. coli*	*9*
B. taurus SerRS	0.37	0.35	0.95	*B. taurus* mt (GCU)	*7*
	0.22	0.63	2.86	*B. taurus* mt (UGA)	

subsequent enzymatic cleavage by the ribozyme yields a mtRNA^{Met} transcript starting with the correct 5′ nucleotide.

The K_M for the aminoacylation of the in vitro transcript of human mtRNA^{Met} is 0.16 ± 0.08 μM with a k_{cat} of 0.021 ± 0.009 s^{-1} (Table 3). Not surprisingly, the K_M for the in vitro transcript of bovine mtRNA^{Met} is 0.079 ± 0.035 μM with a k_{cat} of 0.018 ± 0.014 s^{-1} (Table 3). The native bovine mitochondrial tRNA^{Met} was aminoacylated with a K_M of 0.15 ± 0.07 μM and a k_{cat} of 0.019 ± 0.006 s^{-1}. This latter observation clearly indicates that the K_M values for native bovine mtRNA^{Met} and the bovine mtRNA^{Met} transcript are remarkably similar suggesting that the f^{5}C in the anticodon of the tRNA does not act as a determinant for the human mtMetRS. The modifications of the human mtRNA^{Met} are unknown but are likely to be similar to those observed with bovine mtRNA^{Met}.

Interestingly, the K_M for aminoacylation of the bovine mitochondrial tRNA^{Met} is more than an order of magnitude lower than that observed with *E. coli* tRNA$_f^{Met}$ (Table 3). However, the k_{cat} is over 500 times slower with mitochondrial tRNA^{Met} than with *E. coli* tRNA$_f^{Met}$. The net result is a catalytic efficiency that is an order of magnitude lower for the mitochondrial tRNA^{Met}. The K_M difference was rather unexpected since the *E. coli* tRNA$_f^{Met}$ shares many features with the mitochondrial tRNA^{Met}, especially the anticodon, the most important determinant for tRNA^{Met} recognition by

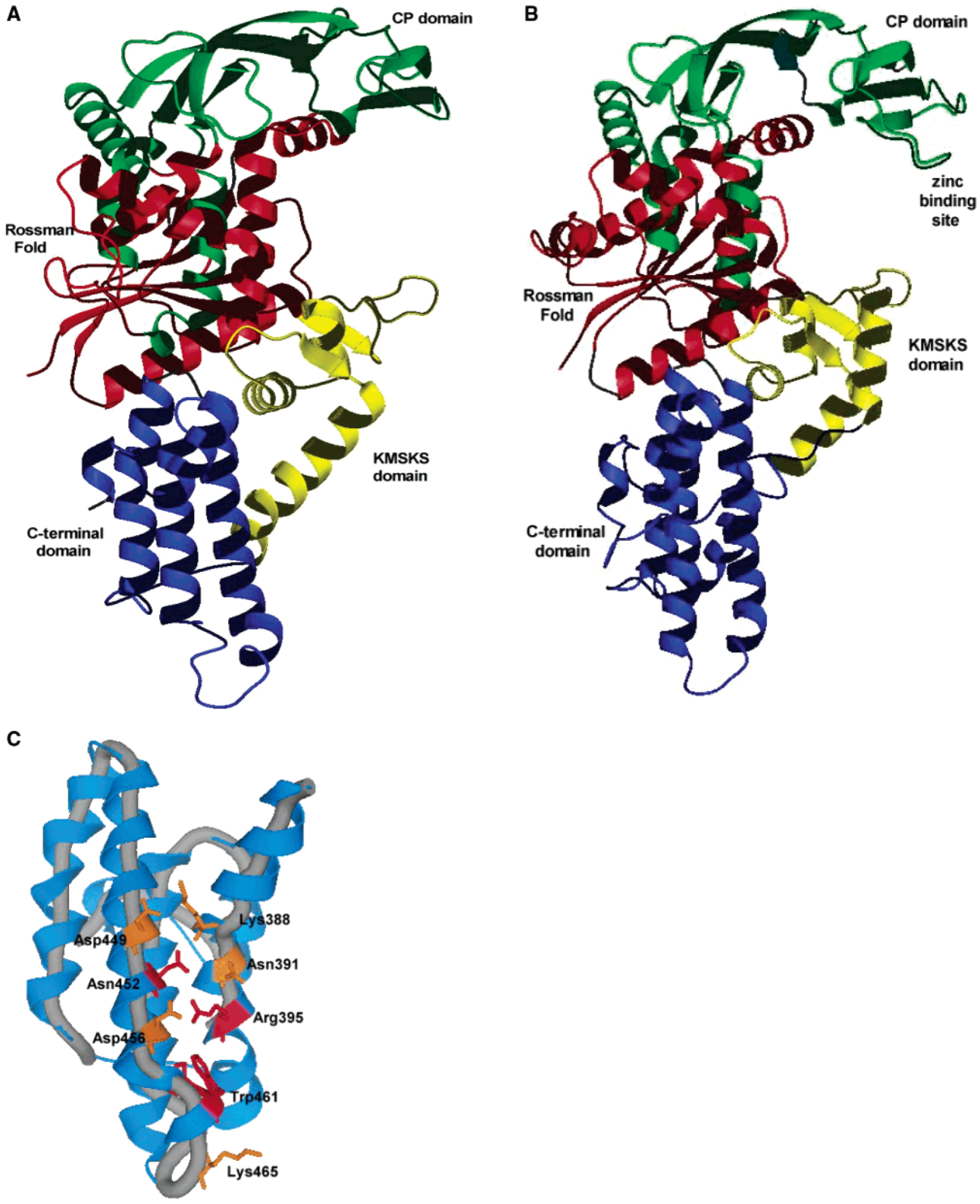

FIGURE 4: Structural representations of MetRS: (A) Swiss-Model (*45*, *46*) of human mtMetRS with the domains colored according to ref *6*, Rossman fold in red, connective peptide domain in green, KMSKS domain in yellow, and C-terminal domain in blue; (B) crystal structure of *E. coli* MetRS (pdb file 1QQT) (*6*) with domains colored as indicated in panel A and the zinc binding region in the connective peptide domain labeled to highlight missing regions in human mtMetRS structure; (C) close-up view of the anticodon binding site of MetRS. *E. coli* MetRS is displayed as ribbons and is colored blue. The side chains of the residues that have been shown to affect the aminoacylation of $tRNA^{Met}$ are indicated and are colored as follows: residues conserved with human mtMetRS in red and residues not conserved in orange. The portion of the anticodon binding site of human mtMetRS that was modeled is shown as a gray backbone superimposed on the *E. coli* MetRS structure.

the bacterial MetRS (*28*). The catalytic efficiency in aminoacylation of native mitochondrial $tRNA^{Met}$ is within an order of magnitude of bovine mitochondrial SerRS in aminoacylation of its cognate native mitochondrial tRNAs.

It should also be noted that the K_M values for the aminoacylation of native and in vitro transcripts of the two mitochondrial serine isoacceptor tRNAs are similar, as observed of the $tRNA^{Met}$ in this study. Finally, the K_M for aminoacylation of *Ascaris suum* mitochondrial $tRNA^{Met}$ by *A. suum* MetRS is 0.12 μM, which is very similar to the value observed here (*34*).

DISCUSSION

The domain organization of human mtMetRS most closely resembles that of the *S. cerevisiae* and *C. albicans* mitochondrial MetRSs. Human mtMetRS lacks the approximately 100 amino acid C-terminal extensions found in many bacterial MetRSs and thus functions as a monomer, similar to the characterized mitochondrial MetRSs (*11*, *12*). Despite the lack of a high degree of sequence similarity to *E. coli* MetRS (Table 1), Swiss-Model was able to model a significant region of the catalytic core of human mtMetRS based on the crystal structure of both *E. coli* and *T. thermophilus* MetRS (Figure 4A). This region includes most of the primary functional regions of MetRSs: the HIGH signature sequence, the methionine binding site, the KMSKS signature sequence, and the two tRNA binding sites (*35*). The most prominent functional regions lacking in the model are the Zn^{2+} binding site in the connective peptide region, a helical segment in the 2nd half of the Rossman fold, and a 32 amino acid region at the start of the C-terminal domain. These regions represent sequences that are absent in human mtMetRS or share low sequence identity with *E. coli* MetRS (Figure 1B). Specifically, human mtMetRS does not contain the Zn^{2+} binding site motifs in the connective peptide domain of *E. coli* MetRS (Figure 4B). In addition, the C-terminal domain is less well conserved overall among the MetRSs, and this region could not be completely modeled for human mtMetRS. However, a number of amino acids that are critical for tRNA recognition by the bacterial MetRS have been conserved in the C-terminal region of the protein.

Many of the residues in *E. coli* and *Bacillus stearothermophilus* MetRS important for methionine activation and tRNA aminoacylation that have been proposed to contribute to a universal core MetRS in all organisms are conserved in human mtMetRS (*35*) (Figure 1B). Particularly, the residues Tyr15, Trp253, His301, Trp305, and Lys335 (*E. coli* MetRS numbering) implicated in methionine binding or activation are conserved in human mtMetRS (*5*, *35*−*37*). In addition, a few of the residues important for aminoacylation of $tRNA^{Met}$ including Lys335, Arg395, and Trp461 are conserved (*38*−*40*). However, several residues (Arg380, Asn387, Asn391, Lys442, and part of the region surrounding Lys465) that have been shown to affect the aminoacylation activity of bacterial MetRS are not conserved in mammalian mitochondrial MetRS. Of these residues, Asn387 and Asn391 have been mapped to interact with the anticodon. Asn391, which interacts with the 3′ end of the anticodon (*41*), is conserved in all prokaryotic and lower eukaryotic mitochondrial MetRSs but is a glycine at the corresponding position in the MetRS of higher eukaryotes.

Like several of the characterized mitochondrial aaRSs, human mtMetRS can aminoacylate the heterologous *E. coli* tRNA. This result was expected since the anticodon is the primary determinant for aminoacylation. In fact, the turnover number for aminoacylation of *E. coli* $tRNA_f^{Met}$ was significantly greater than that for $mtRNA^{Met}$. The efficiency of the enzyme was 20-fold greater for the *E. coli* $tRNA_f^{Met}$ despite the lower K_M for the $mtRNA^{Met}$.

Strikingly, *E. coli* MetRS can aminoacylate the bovine $mtRNA^{Met}$ transcript efficiently. However, this bacterial enzyme fails to aminoacylate the native bovine mitochondrial $tRNA^{Met}$ efficiently (data not shown). Since *E. coli* MetRS recognizes the CAU anticodon of its cognate tRNA, its discrimination against the native bovine $mtRNA^{Met}$ probably reflects the presence of the f^5C in the first position of the anticodon in $mtRNA^{Met}$. Two acidic residues, Asp449 and Asp456 (Figure 4C), in *E. coli* MetRS act as negative determinants toward the binding of noncognate tRNA anticodons (*42*). A hydrogen bonding network formed by these residues is important to form a "rigid" anticodon binding pocket in *E. coli* MetRS and may reduce the affinity of *E. coli* MetRS for the native modified mitochondrial $tRNA^{Met}$. In comparison, human mtMetRS lacks many of the residues involved in forming the hydrogen bonding framework, in particular, Asn391, Asp449, and Asp456 (Figure 4C). It may, therefore, have a more flexible anticodon binding pocket allowing better recognition of the modified anticodon of the native $mtRNA^{Met}$.

In addition to the anticodon, there are several other regions of the tRNA that appear to be of varying importance for aminoacylation by MetRS. The size and sequence of the anticodon loop (*43*, *44*), the discriminator base (*31*), base pairs 2:71 and 3:70 in the acceptor stem and residues 32, 33, and 37 near the anticodon loop (*32*) all play a role in the aminoacylation of $tRNA^{Met}$. Comparisons of *E. coli* $tRNA_f^{Met}$ and mitochondrial $tRNA^{Met}$ indicate that these tRNAs are remarkably similar yet contain a number of differences that may account for the different K_M and k_{cat} values. Of the critical regions of tRNA for aminoacylation by MetRS, the mitochondrial tRNA differs from the *E. coli* $tRNA_f^{Met}$ significantly in the absence of a run of strong G·C base pairs in the acceptor stem. This region of the tRNA has been postulated to influence the rate of the catalytic step of amino acid activation by positioning the 3′ end of the tRNA (*32*). The lower stability of the acceptor stem of $mtRNA^{Met}$ may contribute to the lower k_{cat} values observed with this tRNA.

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