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Biochemical Characterization of Pathogenic Mutations in Human Mitochondrial Methionyl-tRNA Formyltransferase*

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Background: Role of human mitochondrial MTF mutations causing Leigh syndrome is unknown.
Results: Mutation of certain conserved residues in MTF affects enzyme activity.
Conclusion: Strategic positioning of small aliphatic amino acids is required for normal MTF function.
Significance: This work presents the first characterization of human MTF mutants leading to poor formylation of mitochondrial methionyl-tRNA and thereby reduced mitochondrial translation efficiency, causing Leigh syndrome.

***N*-Formylation of initiator methionyl-tRNA (Met-tRNA^{Met}) by methionyl-tRNA formyltransferase (MTF) is important for translation initiation in bacteria, mitochondria, and chloroplasts. Unlike all other translation systems, the metazoan mitochondrial system is unique in using a single methionine tRNA (tRNA^{Met}) for both initiation and elongation. A portion of Met-tRNA^{Met} is formylated for initiation, whereas the remainder is used for elongation. Recently, we showed that compound heterozygous mutations within the nuclear gene encoding human mitochondrial MTF (mt-MTF) significantly reduced mitochondrial translation efficiency, leading to combined oxidative phosphorylation deficiency and Leigh syndrome in two unrelated patients. Patient P1 has a stop codon mutation in one of the MTF genes and an S209L mutation in the other MTF gene. P2 has a S125L mutation in one of the MTF genes and the same S209L mutation as P1 in the other MTF gene. Here, we have investigated the effect of mutations at Ser-125 and Ser-209 on activities of human mt-MTF and of the corresponding mutations, Ala-89 or Ala-172, respectively, on activities of *Escherichia coli* MTF. The S125L mutant has 653-fold lower activity, whereas the S209L mutant has 36-fold lower activity. Thus, both patients depend upon residual activity of the S209L mutant to support low levels of mitochondrial protein synthesis. We discuss the implications of these and other results for whether the effect of the S209L mutation on mitochondrial translational efficiency is due to reduced activity of the mutant mt-MTF and/or reduced levels of the mutant mt-MTF.**

One of the major energy-generating pathways in eukaryotic organisms involves the mitochondrion, where oxidation of NADH and $FADH_2$ is coupled to phosphorylation of ADP to synthesize ATP. Mitochondria have their own protein-synthesizing system, which is different from the one in the cytoplasm (1–3). Most mitochondrial DNAs, including mammalian mitochondrial DNAs, code for a total of 13 proteins, which are subunits of the membrane complexes I and III–V involved in oxidative phosphorylation (4–6), and code for rRNAs as well as tRNAs required for mitochondrial protein synthesis. All other proteins present in mitochondria, including the other subunits of the membrane complexes involved in oxidative phosphorylation, are encoded by nuclear DNA, synthesized in the cytoplasm, and imported into mitochondria. Mutations leading to defects of either the components of the mitochondrial protein-synthesizing system or of oxidative phosphorylation complexes can give rise to multiple oxidative phosphorylation deficiencies (7). These conditions are manifested in the form of a wide variety of human diseases associated with bioenergetic defects of mitochondria and generally affect the tissues that are mostly dependent on oxidative phosphorylation.

Protein synthesis is initiated universally with the amino acid methionine or its derivative formylmethionine (1, 8–10). Of the two classes of methionine tRNA (tRNA^{Met})[3] present in all kingdoms, the initiator tRNA^{Met} is used exclusively for the initiation of protein synthesis, whereas the elongator tRNA^{Met} is used for insertion of methionine internally. Bacteria and eukaryotic organelles, such as mitochondria and chloroplasts, initiate protein synthesis with formylmethionine, whereas the cytoplasmic protein-synthesizing systems of eukaryotes and archaea initiate protein synthesis with methionine. Following aminoacylation of the initiator tRNA^{Met} with methionine in bacteria and eukaryotic organelles, the methionyl-tRNA^{Met} (Met-tRNA^{Met}) formed is formylated to formylmethionyl-

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[3] The abbreviations used are: tRNA^{Met}, methionine tRNA; Met-tRNA^{Met}, methionyl-tRNA; fMet-tRNA^{Met}, formylmethionyl-tRNA^{Met}; tRNA$_2^{fMet}$, *E. coli* initiator tRNA species 2; MTF, methionyl-tRNA formyltransferase; mt-MTF, mitochondrial MTF; IF2, initiation factor 2; mt-IF2, mitochondrial IF2; mt-EF-Tu, mitochondrial elongation factor Tu; mt-tRNA^{Met}, human mitochondrial methionine tRNA; CAT, chloramphenicol acetyltransferase; FTHF, N^{10}-formyl tetrahydrofolate; MetRS, methionyl-tRNA synthetase; mt-MetRS, mitochondrial MetRS; MTF^s, suppressor mutant of *E. coli* methionyl-tRNA formyltransferase carrying a G41R mutation; NTA, nitrilotriacetic acid.

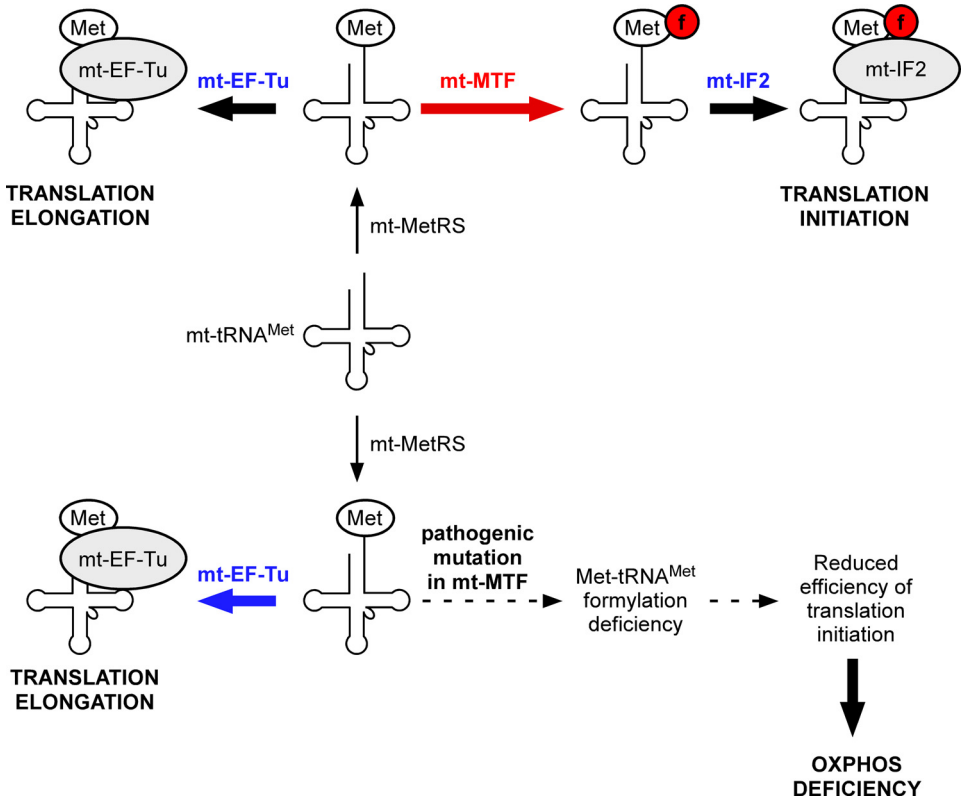

FIGURE 1. *Top, schematic representation* depicting the use of a single mt-tRNA^{Met} for both translation initiation and elongation. The mt-tRNA^{Met} is aminoacylated by mt-MetRS to form Met-tRNA^{Met}. A fraction of Met-tRNA^{Met} is formylated by mt-MTF to generate fMet-tRNA^{Met}, which interacts with mt-IF2 to participate in translation initiation. The remaining fraction of Met-tRNA^{Met} binds to mt-EF-Tu, which transports it to the mt-ribosome for use in translation elongation. *Bottom*, mutations in mt-MTF causing reduction in its activity can have a deleterious effect on translation initiation because mt-MTF and mt-EF-Tu compete for the same mitochondrial Met-tRNA^{Met}. Because of this competition, even small changes in the activity or levels of mt-MTF could potentially cause a significant reduction in mitochondrial translation initiation, leading to different forms of oxidative phosphorylation deficiency diseases.

tRNA^{Met} (fMet-tRNA^{Met}) by the enzyme methionyl-tRNA formyltransferase (MTF). In *Escherichia coli* and in many other organisms, the formyl group attached to methionine in the initiator tRNA plays an important role in initiation of protein synthesis by acting as a positive determinant for the initiation factor IF2 (11, 12) and as the second of two negative determinants for the elongation factor EF-Tu (13–15).

In contrast to the presence of two different methionine tRNA species in all organisms, metazoan mitochondria, including human mitochondria (1, 6), contain only a single species of methionine tRNA (mt-tRNA^{Met}). This striking exception, combined with the lack of evidence of import of any methionine tRNA from the cytoplasm into mitochondria, led to the hypothesis that the single species of mt-tRNA^{Met} acts both as an elongator in the form of Met-tRNA^{Met} and an initiator in the form of fMet-tRNA^{Met} (Fig. 1), with the Met-tRNA^{Met} binding to the mitochondrial elongation factor mt-EF-Tu and the fMet-tRNA^{Met} binding to the mitochondrial initiation factor mt-IF2. Recent work *in vitro* and *in vivo* has provided support for this hypothesis (16, 17). The dual role of the same tRNA in initiation and in elongation (Fig. 1, *top*), however, requires that the mitochondrial Met-tRNA^{Met} be a substrate for two competing proteins, mt-MTF and mt-EF-Tu (18). How mitochondria regulate the levels of Met-tRNA^{Met} and fMet-tRNA^{Met} necessary for their different roles in protein synthesis is unknown.

The importance of formylation of the mitochondrial Met-tRNA^{Met} for its activity in the initiation of protein synthesis in human mitochondria was demonstrated recently by the finding that mutations in the nuclear gene for mt-MTF led to a defect in mitochondrial translation and to pathogenic effects resulting in Leigh syndrome (Fig. 1, *bottom*) (17). Compound heterozygous mutations in the nuclear gene for human mt-MTF were identified as the causative agent in two unrelated patients (referred to as P1 and P2). These mutations resulted in significant impairment of mitochondrial protein synthesis in both patients and in dramatic decrease in levels of mitochondrial fMet-tRNA^{Met}. One of the mutant alleles in P1 replaces Ser-209 with leucine. This single mutation at the DNA level of C626 to T (c.626C→T) within the sequence GTTGTCAAG (mutated codon underlined) in exon 4 of the human MTF gene is also predicted to lead to alternative splicing of the pre-mRNA with skipping of exon 4 (19, 20), frameshifting of the reading frame, and a premature UGA termination codon leading to a protein truncated at amino acid 185 (p.R181sfs5) (17). The second allele of mt-MTF in P1 harbored a nonsense mutation c.382C→T at codon 128 leading to a protein truncated at amino acid Arg-128.

One of the mutant alleles in P2 had the same c.626C→T mutation at the DNA level found in P1. The second mutant allele had a c.374C→T mutation, which resulted in the replacement of a highly conserved serine to leucine (S125L) in the catalytic core of full-length mt-MTF. Thus, the only full-length mt-MTF protein present in P1 harbored an S209L mutation, whereas P2 could potentially have two different full-length

TABLE 1
Plasmids used in this study

Plasmids	Description	Reference/Source
pRSVCATam$_{1.2.5}$ *trnfM* U35A36/G72G73/QRS	pRSVCATam$_{1.2.5}$ *trnfM* U35A36/G72G73 with glutaminyl-tRNA synthetase (QRS) gene; ColE1 origin; amp^r	Ref. 55
pACD	*E. coli* vector containing p15A origin of replication, tet^r	Ref. 55
pACD MTF^s	pACD containing gene for *E. coli* MTF suppressor (MTF^s) under *lac* promoter	Ref. 27
pACD MTF^s A89S	pACD MTF^s with A89S mutation	This study
pACD MTF^s A89L	pACD MTF^s with A89L mutation	This study
pACD MTF^s A172S	pACD MTF^s with A172S mutation	This study
pACD MTF^s A172L	pACD MTF^s with A172L mutation	This study
pQE16 MTF	pQE16 expression vector (Qiagen) containing *E. coli* wild-type MTF gene; amp^r	Ref. 27
pQE16 MTF A89L	pQE16 MTF with A89L mutation	This study
pQE16 MTF A172L	pQE16 MTF with A172L mutation	This study
pET mt-MTF	pET19-based expression vector (Novagen) containing wild-type human mt-MTF; amp^r	This study
pET mt-MTF S125L	pET mt-MTF with S125L mutation	This study
pET mt-MTF S209L	pET mt-MTF with S209L mutation	This study

mutant mt-MTFs containing the S209L and the S125L mutations, respectively.

Quantitative RT-PCR and sequencing of the mt-MTF mRNAs showed that fibroblasts from patient P1 had only 9% of full-length mRNA coding for the S209L mt-MTF variant and the R128X truncated variant, whereas patient P2 had 56% of full-length mRNA coding mostly for the S125L variant (17). The reduced levels of mt-MTF mRNAs in the patients is probably due to the surveillance of mRNAs by the nonsense-mediated decay pathway operating in eukaryotes (21, 22). Despite the reduced levels of mRNAs coding for the mutant proteins, fibroblasts of both patients had residual mt-MTF enzyme activity, because mass spectrometric analysis of peptides from the mitochondrially synthesized cytochrome oxidase subunit I showed that the protein contained predominantly fMet at the N terminus (17).

The finding above that both patients had significantly reduced levels of mRNA for mutant forms of mt-MTF raised the question of whether the reduction in mitochondrial translation efficiency in patients is due to reduced amount of full-length mt-MTF transcripts, reduced activity of mutant enzymes, or both. There is the possibility that the mutations have only a marginal effect on mt-MTF activity as such and that the reductions in mitochondrial translational efficiency seen with the MTF mutants are more due to reductions in mt-MTF mRNA levels and/or the unusual situation of competition between mt-MTF and mt-EF-Tu for the same substrate. Analysis of the catalytic activities of the mt-MTF mutants compared with that of the wild-type MTF is, therefore, an important first step for a better understanding of the basis for reduced mitochondrial translational efficiency and the origin of residual mt-MTF activity in the patients and development of Leigh syndrome. Although recent papers have identified 12 new patients with pathological mutations in mt-MTF (23–25), including 10 more patients with the same S209L mutation found in patients P1 and P2, there have been no biochemical analyses of the effect of any of the mutations on the function of mt-MTF.

This paper describes two sets of experiments designed to study the effects of the S125L and S209L mutations in human mt-MTF on their activities. First, we transplanted these mutations into *E. coli* MTF to produce the corresponding *E. coli* MTF mutants (A89L and A172L, respectively) and analyzed their activities *in vitro* and *in vivo* using a genetic system that we had developed previously (26, 27). Second, we expressed the wild-type and mutant human mt-MTF proteins in *E. coli*, purified them, and measured their activities *in vitro* using *E. coli* initiator tRNA (tRNA$_2^{fMet}$) and a transcript of the human mt-tRNA^{Met} as substrates. Our results show that the S125L mutant mt-MTF (A89L in *E. coli* MTF), the predominant mt-MTF in fibroblasts of patient P2, has greatly reduced activity, with V_{max}/K_m lowered by factors of 107–653-fold for the human enzyme and 144-fold for the *E. coli* enzyme. The S209L mutation (A172L in *E. coli*) identified in patients P1 and P2 has a more moderate effect, lowering V_{max}/K_m by factors of 10–36 for the human enzyme and 4 for the *E. coli* enzyme. Thus, both patients P1 and P2 depend on activity of the S209L mutant mt-MTF to sustain a low level of translation in mitochondria.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and DNA Manipulations—E. coli DH5α (28) was used as host for cloning, and DH10B (Invitrogen) was used for *in vivo* assays. *E. coli* JM109 (29) and BL21 (DE3) (30) served as the host strains for overexpression of the recombinant forms of *E. coli* MTF and human mt-MTF variants, respectively. *E. coli* B105 (31) was used for overexpression and purification of *E. coli* initiator tRNA (tRNA$_2^{fMet}$). Plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown at 37 °C in 2× YT medium or on 2× YT agar. The following antibiotics were added to the media where appropriate: ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), and chloramphenicol (50 μg/ml). Transformation of competent *E. coli* cells and routine DNA manipulations were performed using standard procedures (32). Site-specific mutagenesis was done by QuikChange mutagenesis using high fidelity *Pfu* Turbo polymerase (Stratagene). All constructs were verified by DNA sequencing.

Preparation of Cell Extracts—A 5-ml culture of *E. coli* DH10B in 2× YT medium containing the appropriate antibiotics was inoculated with a fresh overnight culture of *E. coli* transformants and grown until mid-log phase at 37 °C. A cell pellet from 1.2 ml of this culture was resuspended in 120 μl of TME (25 mM Tris-HCl, pH 8.0, 2 mM β-mercaptoethanol, and 1 mM Na-EDTA) containing 0.1 mg/ml lysozyme, and the mixture was left at room temperature for 10 min. DNase I from New England Biolabs (2 units) and 10 mM $MgCl_2$ were added, and the mixture was incubated for another 5 min to digest chromosomal DNA. Cellular debris was removed by centrifugation at 4 °C for 10 min, and the supernatant was collected. Protein concentration

was estimated using Bradford reagent and BSA as the standard (33).

Assays for Chloramphenicol Acetyltransferase (CAT) and β-Lactamase Activity—The assays were done as described by Varshney and RajBhandary (26). For the CAT assay, the incubation mixture (100 μl) contained 470 mM Tris-HCl, pH 8.0, 0.8 mM acetyl CoA, 160 μM ^{14}C-chloramphenicol (specific activity 12.5 μCi/mol; PerkinElmer Life Sciences), and varying amounts of the cell extract. Incubation was at 37 °C for 15 min, after which the reaction was stopped by adding ethyl acetate. The ethyl acetate layer was evaporated to dryness and dissolved in a small volume of ethyl acetate, and an aliquot was spotted on a silica gel thin layer chromatography plate (J. T. Baker). CAT activity was normalized with β-lactamase activity to adjust for any fluctuations in plasmid copy number. The incubation mixture for assay of β-lactamase in cell extracts contained 1 ml of a 50 μg/ml solution of Nitrocefin (Calbiochem; in 100 mM sodium phosphate buffer pH 7.0, 1 mM Na-EDTA) and 1 μg of protein. Incubation was for 10 min at room temperature. The reaction was stopped by the addition of 110 μl of 10% SDS, and the absorbance was measured at 486 nm.

Immunoblotting—Total protein (2 μg) present in crude extracts was fractionated on 15% SDS-polyacrylamide gels, electroblotted onto polyvinylidene difluoride membrane (Millipore), and probed with specific antibodies. The antibodies used in this study include rabbit anti-CAT, rabbit anti-β-lactamase (both from 5 Prime 3 Prime, Inc.), mouse anti-tetra-His (Qiagen), and a rabbit polyclonal antibody raised specifically against purified recombinant *E. coli* MTF (Thermo Scientific). Specific bands were visualized using either HRP-conjugated goat anti-rabbit or anti-mouse IgG (Amersham Biosciences), and antibody-coupled horseradish peroxidase activity was detected with the ECL oxidase/luminol reagents (PerkinElmer Life Sciences).

Purification of Recombinant MTF Proteins—Recombinant *E. coli* MTF proteins were purified as described previously (27). Recombinant human mt-MTF proteins were purified from *E. coli* BL21 (DE3) transformed with pET19b (Novagen)-derived plasmids of mt-MTF. A 250-ml culture was grown at 37 °C in the presence of ampicillin until the A_{600} reached 0.4–0.5. The transcription of the mt-MTF gene was then induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside, and incubation was continued at 18 °C for 6 h. Following centrifugation, cells were resuspended in 20 ml of buffer 1 (50 mM Tris-HCl, pH 8.0, 500 mM KCl, 10 mM $MgCl_2$, 10 mM imidazole, and 20 mM β-mercaptoethanol). The cell suspension was passed twice through a French press cell (12,000 p.s.i.), and 0.5 ml of 100 mM phenylmethylsulfonyl fluoride was added to the lysate. The lysate was clarified by centrifugation (11,000 rpm for 40 min at 4 °C) and brought to room temperature, and 0.8 ml of freshly prepared 0.25 M ATP (pH 7.5 adjusted with NaOH) was added to a final concentration of 10 mM ATP. The lysate was then incubated for 40 min at room temperature with intermittent gentle mixing and added to 1 ml of slurry of Ni^{2+}-NTA affinity resin (Qiagen) pre-equilibrated at room temperature with buffer 1, and the mixture was further incubated for 30 min. The slurry was then poured into a column and washed with 15 ml of buffer 1. The column was then incubated with 10 ml of ATP wash buffer (20 mM Tris-HCl, pH 8.0, 1.5 M KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol supplemented with 10 mM ATP) on a nutator at room temperature, and the flow-through was collected. Washing of the column with 10 mM ATP was repeated once more, followed by two washings with NaCl wash buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl). The column was then transferred to a cold room, and all subsequent steps were carried out at 4 °C. The column was washed with 15 ml of buffer 1 and then with 8 ml of 20 mM imidazole and 50 ml (for wild-type) or 15 ml (for the mutants) of 50 mM imidazole at a flow rate of 0.5 ml/min. MTF was then eluted with 1.5 ml of buffer 1 containing 100, 150, 200, and 300 mM imidazole at the same flow rate. The purity of MTF proteins was monitored in each fraction of the eluate by SDS-PAGE. Fractions containing MTF were subsequently dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, and 50% glycerol), and the protein concentration was estimated by a Bradford assay with BSA as the standard. In general, 100–200 μg of recombinant purified mt-MTF proteins were recovered from a 250-ml culture.

tRNA and Aminoacylation Reactions—E. coli $tRNA_2^{fMet}$ was purified as described previously (34). T7 transcript of human mt-$tRNA^{Met}$ and purified recombinant mitochondrial methionyl-tRNA synthetase (mt-MetRS) were isolated as described previously (35, 36). Methionine acceptor activity of purified *E. coli* tRNA was determined by incubating the tRNA in a 30-μl reaction mixture containing 20 mM imidazole buffer, pH 7.6, 150 mM NH_4Cl, 10 mM $MgCl_2$, 10 μg/ml BSA, 0.1 mM Na-EDTA, 2 mM ATP, 0.5% (w/v) CHAPS, 0.5 μM [^{35}S]methionine (specific activity, 1175 Ci/mmol; PerkinElmer Life Sciences), 100 μM unlabeled L-methionine, and an excess of purified *E. coli* MetRS for quantitative aminoacylation. Incubation was at 37 °C, and aliquots (5 μl) taken at various time points were spotted on 3MM Whatman filter paper, which had been presoaked in 2% casamino acids and 10% trichloroacetic acid (TCA) and then dried. The filters were washed once for 15 min with 2% casamino acids and 10% TCA, twice with 5% TCA, and finally with ethanol. Acid-precipitable counts were determined by scintillation counting. Aminoacylation of the *E. coli* $tRNA_2^{fMet}$ was found to be essentially quantitative.

For the human mt-$tRNA^{Met}$, the tRNA transcript was first denatured by incubating it at 80–85 °C for 3 min. The tRNA transcript was then added to the same reaction mixture as described above and refolded by incubation at 37 °C for 15 min prior to the addition of an excess of mt-MetRS for aminoacylation. The extent of aminoacylation of the tRNA transcript (23–26%) was used to calculate the concentration of the tRNA substrate in formylation.

Measurement of Kinetic Parameters in Formylation of tRNA—The assay for formylation used a two-step reaction. tRNA was first aminoacylated with [^{35}S]methionine using an excess of MetRS. Subsequently, it was formylated using N^{10}-formyl tetrahydrofolate (FTHF) and purified MTF. FTHF was prepared from folinic acid (Sigma) according to the protocol described by Kahn *et al.* (37). Aminoacylation was allowed to proceed for 30 min at 37 °C in a total reaction volume of 10 μl under the same experimental conditions as described above to ensure maximal aminoacylation of native *E. coli* initiator

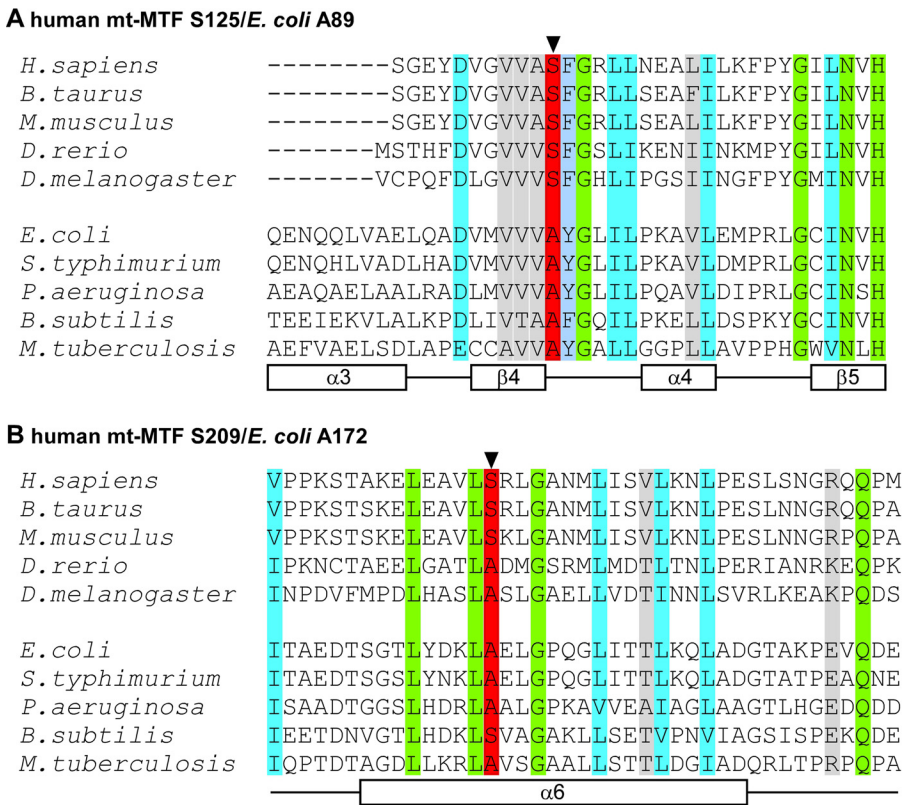

FIGURE 2. **Multiple sequence alignment of different eukaryotic and bacterial homologs of human mt-MTF highlighting the region harboring the recently identified mutations that form the genetic basis of oxidative phosphorylation disease and Leigh syndrome in humans (17).** The secondary structure elements for α-helices (designated as α) and β-sheets (designated as β) are indicated by *empty rectangles below* the aligned sequences and *numbered* based on the *E. coli* MTF crystal structure (38). Identical residues are *highlighted* in *green*, whereas similar residues are shown in *cyan*. The conserved residues Ser-125 (*A*) and Ser-209 (*B*), which are sites of mutations in patients with Leigh syndrome, are indicated in *red* and by *arrowheads*. The corresponding residues in *E. coli* MTF are Ala-89 and Ala-172, respectively. The numbering of residues in *E. coli* MTF starts with the serine residue at the N terminus of the mature protein. The numbering for human mt-MTF starts with the first amino acid methionine as encoded by the mt-MTF gene.

$tRNA_2^{fMet}$ or the mt-$tRNA^{Met}$ transcript. Subsequently, 2 μl of 0.3 mM FTHF and 3 μl of an appropriately diluted amount of MTF were added to initiate the formylation reaction. Control experiments showed that the aminoacyl-ester linkage in the aminoacyl-tRNA was completely stable during the course of the formylation reaction. The reaction was terminated at different time points by mixing aliquots of the reaction mix with an equal volume of 0.36 M $CuSO_4$ in 1.1 M Tris-HCl, pH 7.8, and further incubation for 10 min before spotting the mixture onto 3MM Whatman filter papers, presoaked in 2% casamino acids and 10% TCA. For calculation of initial velocity, acid-precipitable radioactivity was measured as described above.

Kinetic parameters (K_m and V_{max}) for the *E. coli* MTF were determined using Lineweaver-Burk plots. Because the activity of human mt-MTF was much lower than that of *E. coli* MTF, it was not possible to measure V_{max} and K_m individually. Instead, V_{max}/K_m of the wild-type and mutant mt-MTFs was calculated from the initial velocity of formylation using *E. coli* $tRNA_2^{fMet}$ or mt-$tRNA^{Met}$ transcript concentrations in which the initial rate of formylation of Met-$tRNA^{Met}$ is proportional to the tRNA or transcript concentration. At low tRNA substrate concentrations, when $[S] \ll K_m$, the Michaelis-Menten equation, $v = (V_{max}[S])/(K_m + [S])$, approximates to $v = (V_{max}[S])/K_m$, where v is initial velocity and [S] is substrate concentration. Under these conditions, $v \propto [S]$ and $v/[S] = V_{max}/K_m$.

RESULTS

Sequence Conservation between E. coli MTF and Human mt-MTF—The crystal structure of *E. coli* MTF showed that it contains two domains connected by a linker region (38). The structure of the N-terminal domain is highly homologous to that of glycinamide ribonucleotide formyltransferase, which, like MTF, uses FTHF as a formyl group donor in formylation reactions. Similar to glycinamide ribonucleotide formyltransferase and all other MTFs known to date, the N-terminal domain of *E. coli* MTF contains all of the amino acid residues needed to bind FTHF and for catalysis of formylation, such as Asn-108, His-110, Ser/Thr-137, and Asp-146 (Fig. 7*D*). The C-terminal domain of *E. coli* MTF is structurally homologous to some other tRNA-binding proteins and can bind tRNA on its own, although in a nonspecific manner (38).

Comparison of MTF sequences of a wide variety of organisms shows a significant level of sequence homology (*e.g.* ~30% sequence identity between *E. coli* MTF and human mt-MTF), including sequences around Ser-125 and Ser-209 mutated in the human mt-MTF of patients P1 and/or P2 (Fig. 2, *A* and *B*). Ala-89 of *E. coli* MTF (*highlighted* in *red* in Fig. 2*A*), corresponding to Ser-125 of human mt-MTF, is always conserved as alanine in bacteria and serine in eukaryotes and is part of the methionine binding pocket. Ala-172 of *E. coli* MTF (*highlighted*

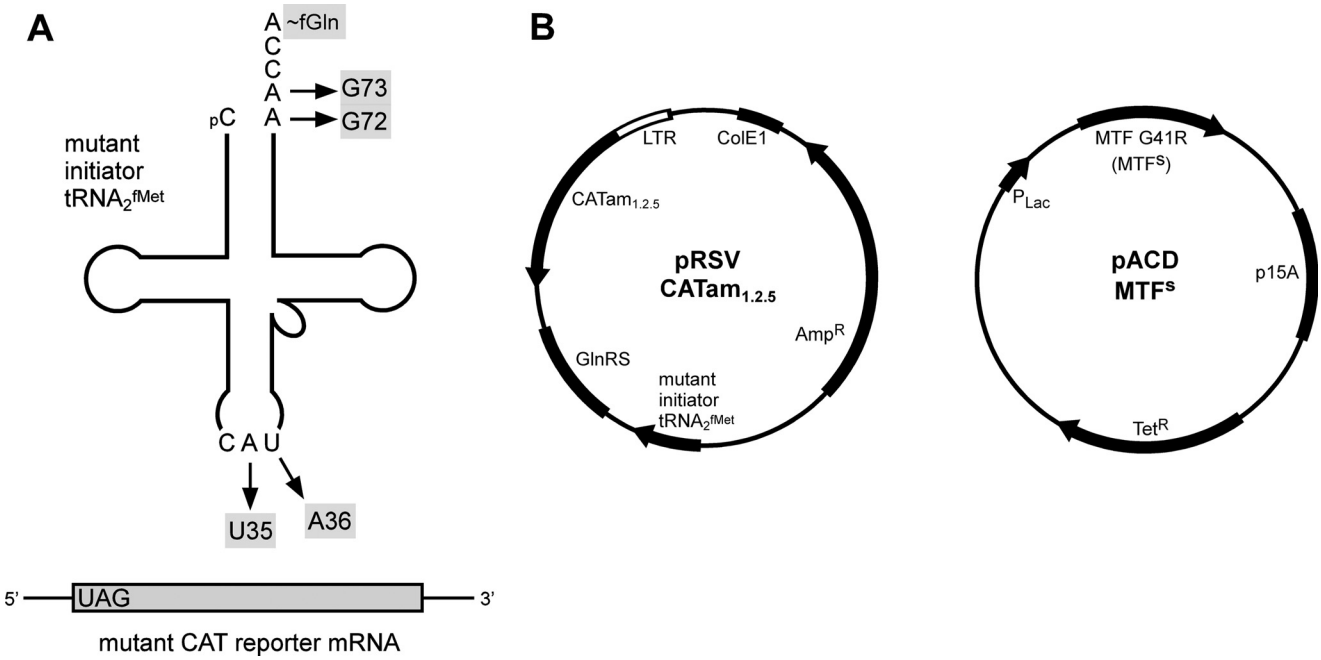

FIGURE 3. *A*, *schematic representation* of the genetic system used for functional analysis of MTF^s mutants in *E. coli*. The strategy for *in vivo* analysis of MTF^s activity relies on a mutant CAT reporter gene (CATam$_{1.2.5}$) coding for an mRNA transcript having UAG as the initiation codon. Translation of the mutant CAT mRNA requires the simultaneous presence of a mutant initiator tRNA with corresponding anticodon (U35A36) mutations. The inclusion of acceptor stem mutations (G72G73) makes the mutant initiator tRNA defective in formylation and, therefore, inactive in initiation. A suppressor mutation in MTF (MTF^s) was previously shown to rescue the defect (27) and to allow the synthesis of CAT *in vivo*. The sites of mutations in the initiator tRNA are indicated by *arrows*. *B*, plasmids used for analysis of MTF^s activity *in vivo*. pRSVCATam$_{1.2.5}$ (*left*) contains the genes for the mutant CAT reporter, the mutant initiator tRNA gene, and the *E. coli* glutaminyl-tRNA synthetase (*GlnRS*) gene. The CATam$_{1.2.5}$ gene has an amber mutation at codon 1 and additional mutations at codons 2 and 5. The U35A36 anticodon sequence change in the mutant initiator tRNA makes it a substrate for *E. coli* glutaminyl-tRNA synthetase (54). To ensure quantitative aminoacylation of the mutant tRNA *in vivo*, *E. coli* GlnRS was overproduced by cloning the glutaminyl-tRNA synthetase gene on the same plasmid. pACD MTF^s (*right*) contains the gene for *E. coli* MTF^s under the control of the P_{lac} promoter. CAT activities in extracts of cells expressing the Ala-89 or Ala-172 MTF^s mutants were compared to evaluate the effect of these mutations on MTF activity *in vivo*. *LTR*, long terminal repeat; *Amp^R*, ampicillin resistance; *Tet^R*, tetracycline resistance.

in *red* in Fig. 2*B*) is conserved as alanine or serine in most bacteria and eukaryotes and is part of a long α-helix (helix 6) in the N-terminal domain of MTF. Thus, the sites of the pathogenic substitution mutations in human mt-MTF being studied here consist of small amino acids. However, although *E. coli* MTF has been used extensively for studies of structure-function relationship and specificity in RNA-protein interaction, no mutants of Ala-89 or Ala-172 have been previously identified in genetic analyses or generated by site-specific mutagenesis (27, 39–41). Therefore, as a first step to study the effect of mutations in human mt-MTF and because of the feasibility of doing genetic and biochemical analyses of the mutants using *E. coli*, we transplanted the Ser-125 and Ser-209 human mt-MTF mutations into *E. coli* MTF and analyzed the properties of the mutant enzymes *in vivo* in *E. coli* and *in vitro*.

Genetic System for Analysis of MTF Activity in E. coli—We previously described a genetic system for analyzing the activities of mutants of *E. coli* MTF and *E. coli* initiator tRNA (tRNA$_2^{fMet}$) without interfering with activities of endogenous wild-type MTF and wild-type tRNA$_2^{fMet}$ (10). The system uses as reporter a mutant CAT gene, whose initiation codon AUG has been changed to UAG, a termination codon (Fig. 3*B*). Synthesis of CAT from this reporter gene (CATam$_{1.2.5}$) depends upon the presence of a mutant tRNA$_2^{fMet}$, whose anticodon sequence has been changed from CAU to CUA (the U35A36 mutant) and which can read the UAG initiation codon in the mutant CAT mRNA (26). Cells carrying the mutant CAT reporter gene and the U35A36 mutant initiator tRNA are therefore chloramphenicol-resistant. Introduction of further mutations in the acceptor stem of the U35A36 mutant tRNA$_2^{fMet}$ (*e.g.* G72G73) (Fig. 3*A*), makes the tRNA an extremely poor substrate for MTF and abolishes its activity in initiation (42). Consequently, *E. coli* cells carrying the U35A36/G72G73 mutant tRNA$_2^{fMet}$ (Fig. 3*A*) and the CATam$_{1.2.5}$ reporter gene cannot synthesize CAT and are sensitive to chloramphenicol. Using this system, we isolated previously a suppressor mutation in *E. coli* MTF (MTF^s) carrying a G41R change (27), which allowed CAT synthesis and conferred a chloramphenicol resistance phenotype to *E. coli* carrying the CATam$_{1.2.5}$ gene and the U35A36/G72G73 mutant tRNA$_2^{fMet}$ gene (Fig. 3*B*). Here, we have used the two-plasmid system shown in Fig. 3*B* to study the effect of introducing further mutations at Ala-89 or Ala-172 of the *E. coli* MTF^s on suppressor activity of MTF^s in *E. coli*.

Effect of Ala-89 and Ala-172 Mutations on Activity of E. coli MTF^s in Vivo—Ala-89 and Ala-172 were changed to serine with a small amino acid side chain or leucine with a large amino acid side chain, respectively. The effect of mutations on CAT synthesis *in vivo* was studied using three different assays: (i) growth on chloramphenicol-containing plates; (ii) measurement of CAT activity in cell extracts; and (iii) immunoblot analysis for CAT protein in cell extracts using a specific antibody.

E. coli DH10B carrying the pRSVCATam$_{1.2.5}$ plasmid was transformed with pACD vector containing either MTF^s or MTF^s mutants in which Ala-89 or Ala-172 had been changed to

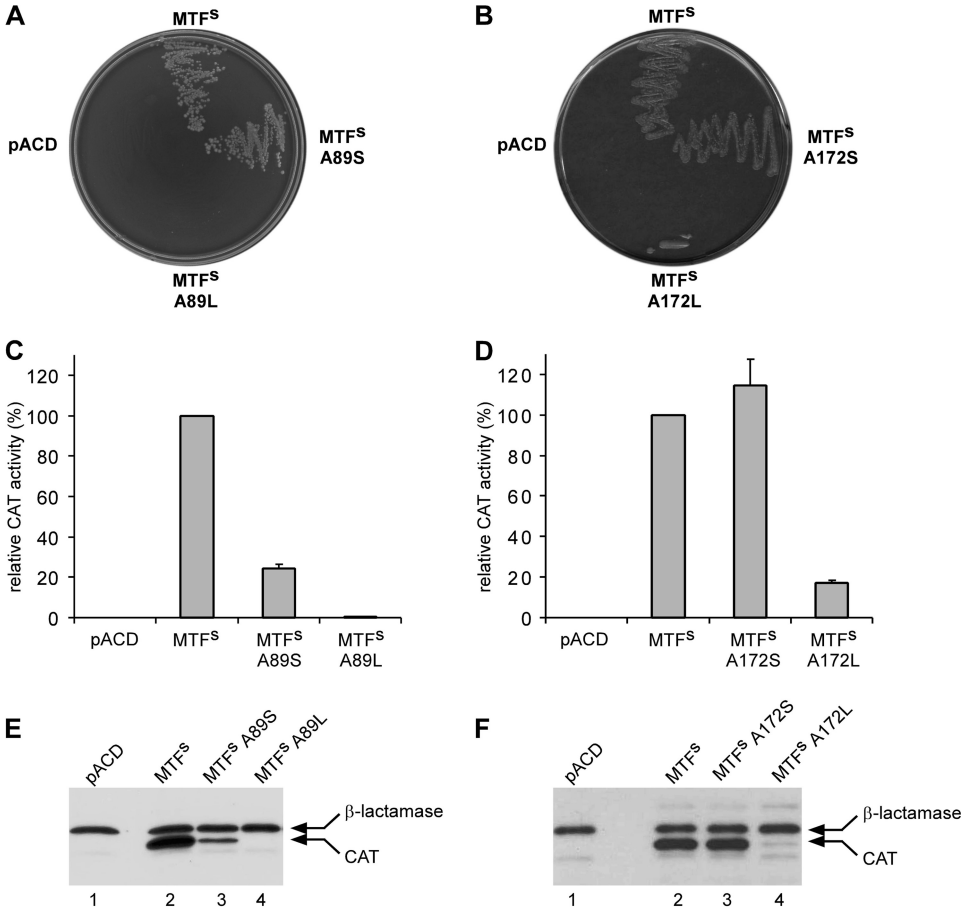

FIGURE 4. **Analysis of the effect of mutations in MTF^s *in vivo*.** *A* and *B*, growth of *E. coli* DH10B co-transformants, containing pRSVCATam$_{1.2.5}$ and one of the pACD MTF^s constructs, on 2× YT agar plates containing 50 μg/ml chloramphenicol. The growth phenotypes of cells carrying different mutations at position Ala-89 (*A*) or Ala-172 (*B*) are compared with those of cells carrying native MTF^s. pACD represents the empty vector control. *C* and *D*, relative CAT activities in cell extracts of different co-transformants are shown. CAT activities in cells expressing either Ala-89 (*C*) or Ala-172 (*D*) MTF^s mutant proteins were compared with those expressing MTF^s (set to 100%). CAT activities are normalized to β-lactamase activity in the same extracts to compensate for any fluctuations in plasmid copy number. Experiments were done in duplicate using extracts from two independent sets of transformations. *Error bars*, S.D. *E* and *F*, immunoblot analysis for CAT and β-lactamase in crude extracts (2 μg) prepared from co-transformants expressing either Ala-89 (*E*) or Ala-172 (*F*) MTF^s mutant proteins using anti-CAT and anti-β-lactamase antibodies. *Arrows*, positions of bands corresponding to CAT and β-lactamase proteins in different cell extracts.

serine or to leucine, and the co-transformants were monitored for growth on plates containing chloramphenicol. Fig. 4, *A* and *B*, shows that co-transformants harboring MTF^s grew normally upon streaking, whereas the one harboring the pACD vector control did not. This confirms earlier findings showing that formylation of the U35A36/G72G73 mutant $tRNA_2^{fMet}$ is carried out by MTF^s and not by the endogenous wild-type MTF (27, 42). The growth of cells carrying the MTF^s A89S mutant on chloramphenicol plates was comparable with that of MTF^s, suggesting that the ability of the mutant enzyme to formylate the U35A36/G72G73 mutant initiator $tRNA_2^{fMet}$ is not significantly affected by the A89S mutation. In contrast, transformants harboring the MTF^s A89L mutants did not grow in the presence of chloramphenicol (Fig. 4*A*). Analysis of the effect of the mutation at Ala-172 also showed that the growth of cells carrying the MTF^s with the A172S mutation remains unaffected, whereas transformants harboring the MTF^s A172L mutant showed poor growth in the presence of chloramphenicol, indicating that the mutation might have led to a reduction of enzyme activity (Fig. 4*B*).

The activity of MTF^s and its mutants in translation initiation was analyzed more quantitatively by measuring CAT activity in cell extracts of different co-transformants. To correct for possible variations in copy number of the pRSVCATam$_{1.2.5}$ plasmid, CAT activity in each extract was normalized to β-lactamase activity. Fig. 4, *C* and *D*, compares the relative CAT activities in various extracts, setting the activity measured in cell extracts carrying MTF^s as 100%. The data clearly show that the A89S mutation in MTF^s resulted in a small decrease of about 4-fold in CAT activity, whereas the MTF^s A89L mutation showed ~200-fold reduction in CAT activity. On the other hand, the MTF^s A172S mutant showed no discernible effect on the CAT activity compared with MTF^s, whereas the A172L mutation resulted in a reduction in CAT activity of about 6-fold.

Immunoblot analysis of total cell extracts with anti-CAT antibodies was also used to estimate the cellular levels of CAT in the various co-transformants. All in all, the results agree well with those based on the CAT assays. CAT expression is high in cells carrying MTF^s (Fig. 4, *E* and *F*; compare *lanes 1* and *2*). The MTF^s A89S mutant showed a moderate decrease in CAT expression level, whereas the A89L mutant failed to show any expression of the reporter CAT enzyme within the detection limits (Fig. 4*E*). Similarly, CAT protein levels were unchanged

TABLE 2

Steady state kinetic parameters of formylation of *E. coli* initiator $tRNA_2^{fMet}$ using wild-type and mutant *E. coli* MTF enzymes

Kinetic parameters were measured using Lineweaver-Burk plots. The kinetic parameters listed are the average and S.D. of three independent measurements.

E. coli MTF	K_m	V_{max}	V_{max}/K_m	Approximate -fold decrease in V_{max}/K_m[a]
	μM	$pmol \cdot min^{-1} \cdot \mu g^{-1}$		
Wild type	0.51 ± 0.08	4080.18 ± 635.91	8000.35	1
A89L	5.87 ± 2.06	326.78 ± 60.51	55.67	144
A172L	1.07 ± 0.39	1941.09 ± 180.17	1814.10	4

[a] Approximate -fold decrease in V_{max}/K_m is the ratio of V_{max}/K_m of wild-type MTF to that of the mutant MTF, setting wild-type MTF to 1.

in cells carrying the MTF^s A172S mutant compared with MTF^s. However, replacement of Ala-172 with leucine showed a marked reduction in the expression of the CAT protein consistent with measured CAT activities (Fig. 4*F*). Taken together, these observations corroborate the effects of these mutations on growth in the presence of chloramphenicol.

Measurement of Steady State Kinetic Parameters for E. coli MTF and Its Mutants—The genetic system for analysis of *in vivo* activity of *E. coli* MTF required the introduction of corresponding mutations in MTF^s, which used a mutant initiator tRNA as substrate. To investigate more directly the effect of the A89L or A172L mutation on the activity of *E. coli* MTF, we also introduced these mutations into wild-type *E. coli* MTF and measured the kinetic parameters of wild-type or the mutant *E. coli* MTF proteins using native *E. coli* $tRNA_2^{fMet}$ as a substrate in steady state enzyme kinetic assays. The recombinant *E. coli* MTF and wild-type initiator tRNA were purified to homogeneity as described under "Experimental Procedures." The assay for MTF activity takes advantage of selective protection of fMet-$tRNA^{Met}$ from $CuSO_4$-mediated cleavage of the ester bond linking methionine to tRNA in Met-$tRNA^{Met}$ (43, 44). The initial velocity was determined by measuring TCA-precipitable [^{35}S]methionine counts at various tRNA substrate concentrations ranging from 0.3 to 2 μM for wild-type or A172L mutant MTF and from 0.5 to 4.5 μM for the MTF A89L mutant. Kinetic parameters were determined using Lineweaver-Burk plots. Mean and S.D. values of different kinetic parameters were deduced from multiple replicates of steady state enzyme kinetic assays.

Table 2 compares the kinetic parameters in formylation of the wild-type initiator $tRNA_2^{fMet}$ by the purified wild-type and the mutant MTF enzymes. The V_{max}/K_m of MTF containing the A89L mutation was about 144-fold lower than that of the wild-type enzyme. The effect is due to both a decrease in V_{max} and an increase in K_m for the MTF A89L mutant compared with the wild-type MTF. With the A172L mutant MTF, there was only a 4-fold reduction in V_{max}/K_m with respect to the wild-type enzyme. In this case, the mutation has relatively minor effects on V_{max} and K_m, which together lead to moderate attenuation of enzyme activity. Overall, the effect of these mutations on wild-type *E. coli* MTF is similar to that of the corresponding mutations in MTF^s *in vivo*.

Purification of Recombinant Wild-type and Mutant Human mt-MTF Proteins—Analysis of the properties of the S125L and S209L human mt-MTF requires the isolation of wild-type and mutant proteins in homogenous form and, in particular, free of any MTF from *E. coli*. For this purpose, the genes for wild-type and mutant human mt-MTF were cloned in the plasmid vector pET19b as His-tagged recombinant proteins. The clones contain the open reading frame starting with the residue homologous to the mature form of bovine mt-MTF and lacking the first 29 residues that comprise the mitochondrial targeting sequence (18). The wild-type or mutant mt-MTF proteins expressed in *E. coli* were purified by chromatography on Ni^{2+}-NTA columns as described under "Experimental Procedures." Expression and purification of human proteins in *E. coli* are often hampered by problems due to insolubility, misfolding, or aggregation. Because of this, conditions for expression of recombinant human mt-MTF proteins in *E. coli* and their purification needed optimization. Initial attempts to purify recombinant human mt-MTF were complicated by co-purification of the chaperone GroEL (45) and poor yield of the recombinant proteins (data not shown). These problems were overcome using a combination of strategies, including (i) use of an N-terminal affinity tag with a longer stretch of histidine residues (His_{10} instead of His_6 tag); (ii) protein expression at low levels by inducing the culture with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, followed by growth at 18 °C for 6 h (instead of 12–16 h); (iii) incubation of cleared cell lysates with ATP at room temperature to facilitate dissociation of GroEL bound to recombinant protein (46); and (iv) multiple washings of the column material containing bound recombinant human mt-MTF with buffer supplemented with ATP (see "Experimental Procedures").

Although the yield was somewhat lower, the purity of mt-MTF proteins increased significantly under these conditions. SDS-PAGE analysis of the dialyzed fraction of each of these proteins showed that the human mt-MTF proteins were at least ~90% pure (Fig. 5*A*). Also, immunoblot analysis of these protein preparations with anti-His antibody detected a single band corresponding to the expected molecular mass (~43.2 kDa), further verifying the identity of these proteins as recombinant human mt-MTFs (Fig. 5*B*). To rule out the possibility of any contamination by endogenous *E. coli* MTF, mt-MTF protein preparations were also probed with a polyclonal antibody raised against recombinant *E. coli* MTF. The results showed the complete absence of detectable levels of *E. coli* MTF in recombinant mt-MTF preparations (Fig. 5*C*).

V_{max}/K_m of Wild-type and Mutant Human mt-MTF—Earlier studies on bovine mt-MTF, a closely related homolog of the human enzyme, showed that bovine mt-MTF formylates *E. coli* initiator Met-$tRNA^{fMet}$ with almost equal efficiency as the cognate mitochondrial Met-$tRNA^{Met}$ (18). We investigated the effect of S125L and S209L mutations on V_{max}/K_m of human mt-MTF using *E. coli* $tRNA_2^{fMet}$ or a T7 transcript of human mt-$tRNA^{Met}$ as substrate. Similar to bovine mt-MTF (47), the activity of human mt-MTF was substantially lower than that of *E. coli* MTF. Therefore, the effect of mutations on human mt-MTF activity was analyzed by comparing the V_{max}/K_m of the wild-type and mutant mt-MTFs at low tRNA substrate concentration (2 μM) in the presence of 0.5% (w/v) CHAPS (18) instead of measuring V_{max} and K_m individually. The tRNA concentration used is such that it falls within the range in which the initial rate of Met-$tRNA^{Met}$ formylation is proportional to tRNA con-

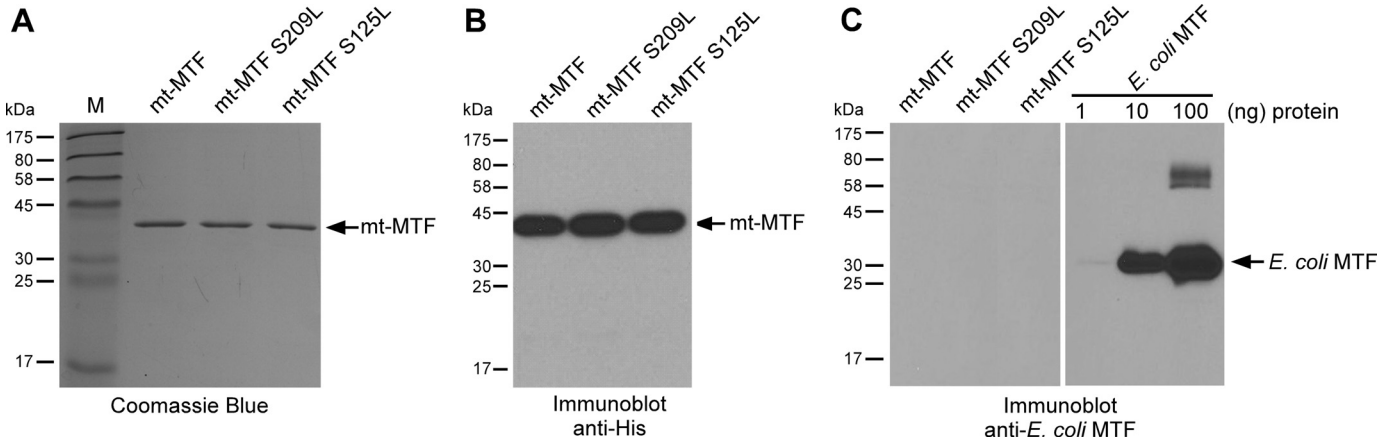

FIGURE 5. *A*, a 12% SDS-polyacrylamide gel representing the purified fractions of His_{10}-tagged recombinant human mt-MTF and mutants, as indicated. The recombinant proteins were expressed in *E. coli* and purified using Ni^{2+}-NTA immobilized metal affinity chromatography (see "Experimental Procedures"). The gel was stained with Coomassie Blue R-250. The *first lane* represents protein molecular mass markers, and *numbers* at the *left* indicate their molecular mass in kDa. *B*, immunoblot analysis of purified fractions of recombinant human mt-MTF or its mutants using an anti-His monoclonal antibody. The position and molecular mass of individual marker proteins (in kDa) are indicated at the *left*. *C*, immunoblot analysis of purified human mt-MTF wild-type and its mutants (2 μg) and *E. coli* MTF (1, 10, and 100 ng) using an anti-*E. coli* MTF polyclonal antibody.

TABLE 3

Kinetic parameters of formylation of *E. coli* Met-tRNA$_2^{fMet}$ or a transcript of human mitochondrial Met-RNA^{Met} using human wild-type and mutant mt-MTF enzymes

V_{max}/K_m listed is the average and S.D. of three independent measurements.

tRNA	mt-MTF	V_{max}/K_m	Approximate -fold decrease in V_{max}/K_m [a]
		$pmol \cdot min^{-1} \cdot \mu g^{-1} \cdot \mu M^{-1}$	
E. coli tRNA$_2^{fMet}$	Wild type	1.98 ± 0.181	1
	S125L	0.0185 ± 0.002	107
	S209L	0.201 ± 0.029	10
Human mt-RNA^{Met}	Wild type	3.46 ± 0.389	1
	S125L	0.0053 ± 0.0005	653
	S209L	0.096 ± 0.014	36

[a] Approximate -fold decrease in V_{max}/K_m is the ratio of V_{max}/K_m of wild-type MTF to that of the mutant MTF, setting wild-type MTF to 1.

centration. The data suggested that with the *E. coli* tRNA$_2^{fMet}$ as substrate, V_{max}/K_m for the S125L mutant was greatly reduced, by about 107-fold relative to wild-type mt-MTF (Table 3); for the S209L mutant, it was only about 10-fold lower than the wild-type mt-MTF. Thus, mutations at these positions affect the activity of human mt-MTF with *E. coli* tRNA$_2^{fMet}$ as substrate. As in the case of *E. coli* MTF, the S125L mutation had a substantially greater effect on V_{max}/K_m than did the S209L mutation.

The physiological substrate for human mt-MTF is the native mt-tRNA^{Met}, which is difficult to purify in significant amounts necessary for enzyme kinetic analyses. However, the ready availability, correct folding, and retention of function make the T7 transcript of mt-tRNA^{Met} a good alternative substrate (35, 36). The mt-tRNA^{Met} transcript lacks the three modified nucleotides found in the native mt-tRNA^{Met}, two pseudouridines and a 5-formyl cytidine (f^5C) found in the first position of the anticodon (Fig. 6) (48). Despite the absence of post-transcriptional modifications, the T7 transcript of human mt-tRNA^{Met} is functionally active in aminoacylation, formylation, and binding to mt-IF2 (16). Also, a lack of these three modifications is not likely to affect our studies in any significant manner, because the mt-MTF is not expected to interact with regions of the tRNA that contain these modifications.

The mt-tRNA^{Met} transcript was aminoacylated *in vitro* with [^{35}S]methionine using purified mt-MetRS (data not shown). The initial velocity of formylation for the wild-type and mutant mt-MTF enzymes was determined in the presence of low Met-tRNA^{Met} transcript concentration (2 μM). Comparison of V_{max}/K_m showed that, with the mt-tRNA^{Met} transcript as the substrate, S125L mutant mt-MTF showed a strong reduction (653-fold) in enzyme activity, whereas the S209L mutant was 36-fold less active compared with the wild-type mt-MTF (Table 3).

DISCUSSION

The work described here represents the first biochemical characterization of pathogenic mutations in human mt-MTF that lead to combined oxidative phosphorylation deficiency and Leigh syndrome. Of the two patients with compound heterozygous mutations in mt-MTF (17), P1 has a nonsense (stop codon) mutation at amino acid 128 in one of the MTF genes and an S209L mutation in the other MTF gene. P2 has an S125L mutation in one of the MTF genes and the same S209L mutation as P1 in the other MTF gene. Assuming that the stop codon mutation results in a complete loss of full-length mt-MTF, mitochondrial protein synthesis in P1 depends solely upon the function of the S209L mutant mt-MTF. Our result showing that the S125L mutant mt-MTF is 653-fold less active than wild-type mt-MTF suggests that mitochondrial protein synthesis in fibroblasts of P2 also depends essentially on the activity of the S209L mutant mt-MTF.

The S209L mutation also reduces mt-MTF activity, although the effect is more moderate, down by a factor of 36. This result would explain residual mitochondrial protein synthesis, initiated predominantly with formylmethionine in fibroblasts of P1 and P2 (17). It could also explain why the c.626C→T mutation in the MTF gene, leading to the S209L change in MTF, is the most prevalent one among patients identified so far (25). Subsequent to the report of the first mutations in mt-MTF in patients P1 and P2 in 2011 (17), of the 12 new patients born to non-consanguineous parents identified with mutations in the mt-MTF gene, 10 have the same c.626C→T mutation, with one

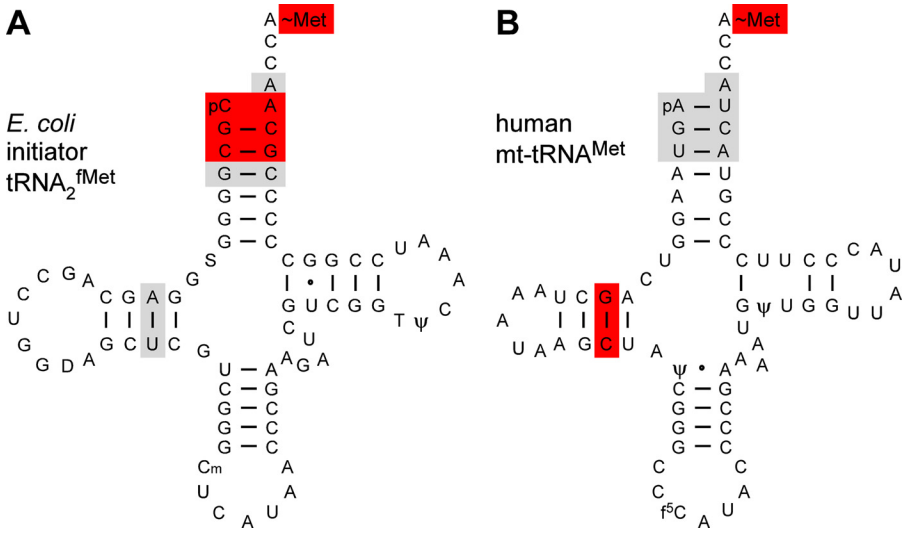

FIGURE 6. **Cloverleaf structures of *E. coli* initiator $tRNA_2^{fMet}$ (*A*) and human mt-$tRNA^{Met}$ (*B*) highlighting the known identity determinants for the corresponding MTF proteins.** *Red*, major determinants; *gray*, minor determinants. The assessment of major and minor identity determinants was as described earlier (10, 47). f^5C, 5-formyl cytidine.

of the 10 patients also being homozygous for the c.626C→T mutation. The patient homozygous for the c.626C→T mutation must clearly depend upon the function of the S209L mutant mt-MTF for mitochondrial protein synthesis, and it is likely that many of the other patients also do (24, 25).

It was shown previously that fibroblasts from wild-type cell lines contained uncharged mt-$tRNA^{Met}$ and mt-fMet-$tRNA^{Met}$ but no mt-Met-$tRNA^{Met}$, suggesting that mt-MTF is normally not limiting in mitochondria (17). It is interesting, therefore, that despite the relatively moderate effect of the S209L mutation on MTF activity, mitochondrial protein synthesis in fibroblasts of patients P1 and P2 is severely impaired (17). Also, steady state levels of fMet-$tRNA^{Met}$ are undetectable in mitochondria from patients P1 and P2, suggesting that fMet-$tRNA^{Met}$ levels are limiting and that any fMet-$tRNA^{Met}$ that is synthesized by the mutant MTF is used immediately for translation initiation. As pointed out above, because of the unique situation of the same $tRNA^{Met}$ being used for both initiation and for elongation in vertebrate mitochondria, mt-MTF and mt-EF-Tu compete for the same mt-Met-$tRNA^{Met}$. Thus, it is possible that the strong effect of the S209L mutation on mitochondrial translation is not only due to the reduced activity of the mutant MTF (36-fold lower) but also due to reduced amounts of mutant MTF synthesized and competition with mt-EF-Tu for the same Met-$tRNA^{Met}$ substrate in mammalian mitochondria.

In trying to assess the effect of the c.626C→T (p.S209L) mutation on the severity of mitochondrial dysfunction in patients, it is important to remember that the c.626C→T mutation has multiple effects at various levels. Besides the potential for producing the full-length S209L mutant MTF, the mutant pre-mRNA can also undergo alternative splicing (19, 20), involving skipping of exon 4, which leads to shifting of the mRNA reading frame and a premature stop codon (17). Use of this stop codon in turn results in degradation of much of the alternatively spliced mRNA by nonsense-mediated decay (21, 22). It is quite likely, however, that there are tissue-specific differences in the extent of alternative splicing (49), and because of this, some tissues in patients could have different levels of full-length S209L mutant MTF compared with other tissues, such as fibroblasts, that were analyzed in previous work with patients P1 and P2 (17). Our finding that the activity of the S209L mutant protein is not as severely impaired as that of the S125L mutant could be important in limiting the severity of mitochondrial dysfunction in patients carrying the S209L mutation only to certain tissues and/or to certain individuals (50).

Amino acid sequences in and around Ser-125 and Ser-209 of human mt-MTF, the sites of pathogenic mutations in P1 and P2, are highly conserved both in eukaryotic and in bacterial MTFs (Fig. 2, *A* and *B*). The amino acids corresponding to Ser-125 and Ser-209 in human mt-MTF are Ala-89 and Ala-172 in *E. coli*. Remarkably, Ser-125 of human mt-MTF is invariably serine in eukaryotes, and the corresponding position is invariably alanine in bacterial MTF. Analysis of A89S and A89L mutants of *E. coli* MTF[s] *in vivo* showed that mutation of Ala-89 to serine had a minor effect, whereas mutation to leucine had a major effect. In contrast, mutation of Ala-172 to serine or to leucine had only a small effect (Fig. 4). Direct analysis *in vitro* of the effect of A89L and A172L mutations on *E. coli* MTF activity showed that whereas the A89L mutation reduced V_{max}/K_m by 144-fold, A172L mutation reduced it by only 4-fold (Table 2). These results parallel those obtained with the corresponding human mt-MTFs (Table 3) and show that mutations of alanine to leucine at Ala-89 (corresponding to Ser-125 of mt-MTF) are more deleterious than at Ala-172 (corresponding to Ser-209). Combined with the fact that the human mt-MTF has ~30% sequence identity with the *E. coli* enzyme and, like all other MTFs known to date, has the highly conserved sequences possibly involved in FTHF binding and catalysis (Fig. 7*D*), these findings suggest that *E. coli* and human mt-MTF use similar mechanisms for substrate binding and catalysis (38, 39).

In trying to understand the molecular mechanism of the effect of the S125L and S209L mutations on the activity of human mt-MTF, we have superimposed these mutations on

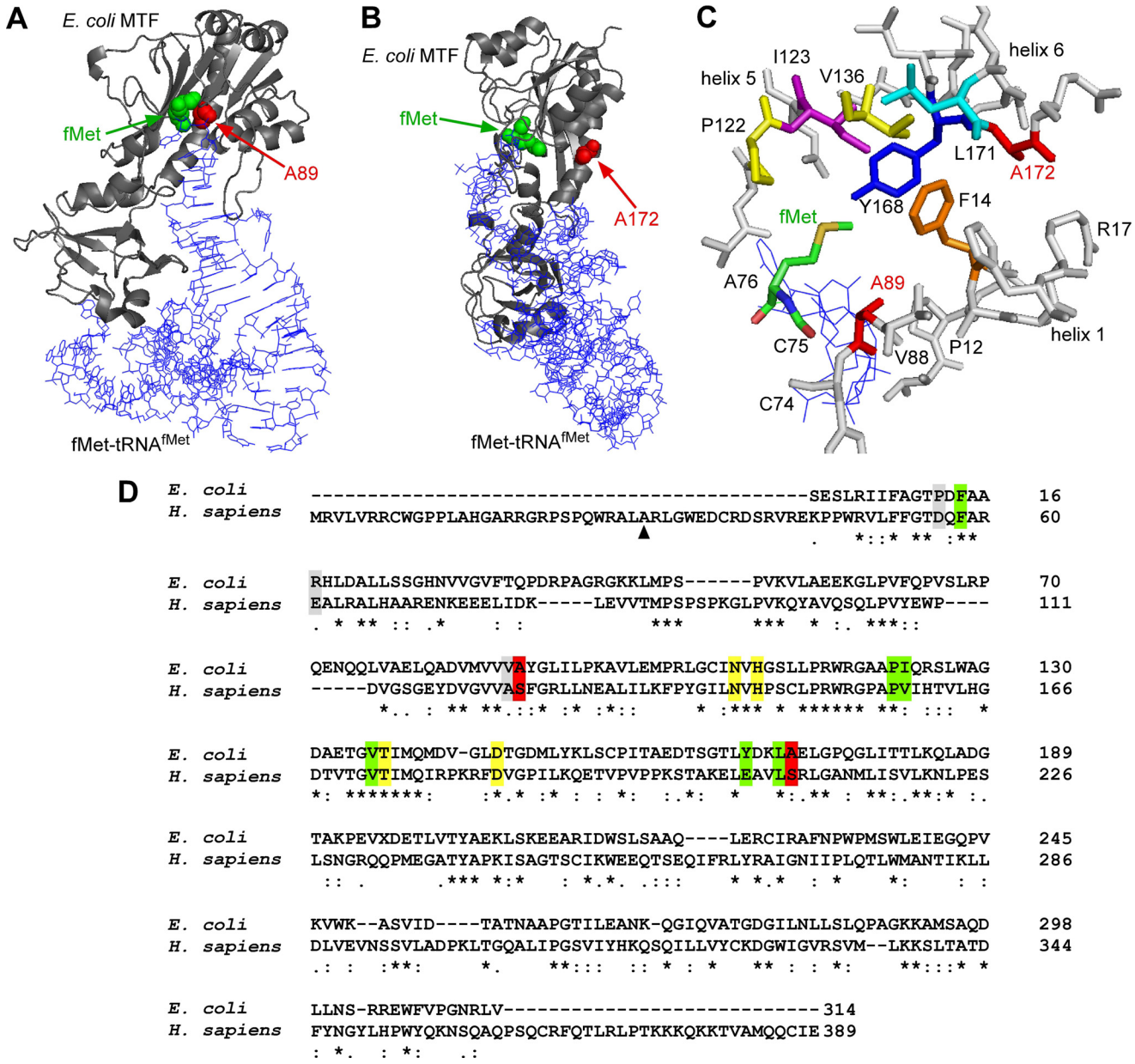

FIGURE 7. **Structural representation of Ala-89 and Ala-172 in the context of the *E. coli* MTF-fMet-tRNA^{fMet} complex.** Structures were drawn using PyMOL (Schrödinger, LLC, New York) and Protein Data Bank entry 2FMT. *A*, a backbone trace of the complex is depicted, where *E. coli* MTF is shown in a *ribbon representation* (*gray*) and the tRNA molecule is shown as *lines* (*blue*). The positions of the fMet moiety and Ala-89 in the methionine binding pocket are *highlighted* with *green* and *red spheres*, respectively. *B*, the same complex is shown from a different angle to highlight the positions of fMet and Ala-172, as indicated by *green* and *red spheres*, respectively. The Ala-172 residue is distal to the methionine binding pocket and belongs to helix 6. *C*, close-up view of amino acids (represented as *sticks*) lining the methionine binding pocket of *E. coli* MTF. The CCA end (Cys-74, Cys-75, and Cys-76) of the initiator tRNA is shown as *blue lines*. *D*, pairwise alignment of *E. coli* MTF and mt-MTF using ClustalW2. Ala-89 and Ala-172 (Ser-125 and Ser-209 in mt-MTF) are *highlighted* in *red*. *Green*, amino acids lining the methionine binding pocket (Phe-14, Ile-123, Val-136, Leu-171, Pro-122, and Tyr-168). Note that Ala-89 is part of the methionine binding pocket (39). *Yellow*, amino acids involved in FTHF binding and catalysis (Asn-108, His-110, Thr-137, and Asp-146) (38). *Gray*, additional amino acids of interest mentioned in this work (Pro-12, Val-88, and Arg-17). The numbering of residues in *E. coli* MTF starts with the serine residue at the N terminus of the mature protein. The numbering for human mt-MTF starts with the first amino acid methionine as encoded by the mt-MTF gene. ▲, start site of mt-MTF after removal of the mitochondrial targeting site.

the known structure of *E. coli* MTF complexed to fMet-tRNA^{fMet} (39). In *E. coli* MTF, the side chain of Ala-89 (corresponding to Ser-125) forms part of a hydrophobic pocket consisting of amino acids Phe-14, Ile-123, Val-136, Leu-171, Pro-122, and Tyr-168, which bind to methionine of the fMet-tRNA^{fMet} (Fig. 7*C*) (39). Of the seven amino acids lining the methionine binding pocket of *E. coli* MTF, the human mt-MTF has four amino acids that are identical (phenylalanine, proline, valine, and leucine) and two that are similar (serine instead of alanine and isoleucine instead of valine) (Fig. 7*D*). Therefore, the human mt-MTF probably has a binding pocket for methionine similar to that of *E. coli* MTF. The strong negative effect of mutation of Ala-89 to leucine on activity of *E. coli* MTF most likely occurs because replacement of this absolutely conserved alanine with leucine creates unfavorable interactions with Pro-14, Pro-12, Val-88, or the methyl group of methionine (Fig. 7).

Phe-14 is in the methionine binding pocket, and Pro-12 and Val-88 are nearby, and a clash of the leucine side chain with these residues and methionine would significantly perturb the methionine binding pocket of MTF.

Compared with the strong effect of the A89L mutation on *E. coli* MTF activity, the effect of the A172L mutation is relatively mild (4-fold). In contrast to Ala-89, which is present in the methionine binding pocket of *E. coli* MTF, Ala-172 is distal to the active site and is located in a long α helix (helix 6) closer to the C-terminal region of MTF, although Tyr-168 and Leu-171 in helix 6 are also part of the methionine binding pocket (Fig. 7, *B* and *C*). The small effect of the A172L mutation on *E. coli* MTF activity is presumably indirect. In the crystal structure of *E. coli* MTF, helix 6 is adjacent to helix 1, which contains Phe-14 that is close to the active site. It is possible that mutation of Ala-172 to leucine in *E. coli* MTF perturbs interactions between helix 1 and helix 6, leading to changes in the active site (51, 52).

Human mt-MTF has ~30% sequence identity and long stretches of sequence homology with the *E. coli* MTF in the regions surrounding Ala-89 and Ala-172 (Fig. 2, *A* and *B*). Therefore, conclusions reached above with the *E. coli* enzyme on the molecular basis of the effect of Ala-89 to leucine and Ala-172 to leucine mutations are likely to also hold for the corresponding Ser-125 to leucine and Ser-209 to leucine mutations in the human enzyme. The effect of the S209L mutation on the activity of the human enzyme (36-fold decrease) is, however, more deleterious than with the *E. coli* enzyme (4-fold decrease). This could be due to the fact that in addition to methionine attached to the tRNA (Fig. 6), *E. coli* MTF uses many sequence elements in the acceptor stem of the initiator tRNA as critical determinants for formylation (10), whereas the human mitochondrial enzyme uses the methionine attached to the tRNA and the purine 11:pyrimidine 24 base pair in the D stem as the two most critical determinants for formylation of Met-tRNA^{Met} (47). Therefore, the human mt-MTF could be more susceptible to relatively small changes in the methionine binding pocket and the tRNA D stem-binding region (39) of the mutant enzymes compared with the *E. coli* enzyme.

Finally, following the use of exome sequencing (53) for identification of pathogenic mutations in mt-MTF in patients P1 and P2 (17), the use of a similar approach has led to the identification of mutations in mt-MTF in 13 more patients, including several new mutations (23–25). It is hoped that the biochemical characterization of mutant mt-MTFs as described here will prove useful in studying the effects of newly identified mutations on activities of the mutant mt-MTFs.

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