A Disease-causing Point Mutation in Human Mitochondrial tRNA\textsuperscript{Met} Results in tRNA Misfolding Leading to Defects in Translational Initiation and Elongation*

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The mitochondrial tRNA genes are hot spots for mutations that lead to human disease. A single point mutation (T\textsuperscript{4409}C) in the gene for human mitochondrial tRNA\textsuperscript{Met} (hmtRNA\textsuperscript{Met}) has been found to cause mitochondrial myopathy. This mutation results in the replacement of U8 in hmtRNA\textsuperscript{Met} with a C8. The hmtRNA\textsuperscript{Met} serves both in translational initiation and elongation in human mitochondria making this tRNA of particular interest in mitochondrial protein synthesis. Here we show that the single 8U→C mutation leads to a failure of the tRNA to respond conformationally to Mg\textsuperscript{2+}. This mutation results in a drastic disruption of the structure of the hmtRNA\textsuperscript{Met}, which significantly reduces its aminoacylation. The small fraction of hmtRNA\textsuperscript{Met} that can be aminoacylated is not formuloylated by the mitochondrial Met-tRNA\textsuperscript{Met} transformylase preventing its function in initiation, and it is unable to form a stable ternary complex with elongation factor EF-Tu preventing any participation in chain elongation. We have used structural probing and molecular reconstitution experiments to examine the structures formed by the normal and mutated tRNAs. In the presence of Mg\textsuperscript{2+}, the normal tRNA displays the structural features expected of a tRNA. However, even in the presence of Mg\textsuperscript{2+}, the mutated tRNA does not form the cloverleaf structure typical of tRNAs. Thus, we believe that this mutation has disrupted a critical Mg\textsuperscript{2+}-binding site on the tRNA required for formation of the biologically active structure. This work establishes a foundation for understanding the physiological consequences of the numerous mitochondrial tRNA mutations that result in disease in humans.

Human mitochondria are subcellular organelles that produce more than 90% of the energy required by the cell. The mitochondrial genome encodes 13 proteins necessary for energy production, two tRNAs and all of the 22 tRNAs required for the synthesis of these proteins (1, 2). Mammalian mitochondrial tRNAs have several unusual features that distinguish them from canonical tRNAs. In many cases, they lack a number of the conserved or semi-conserved nucleotides that play important roles in creating the L-shaped tertiary structure of prokaryotic and eukaryotic cytoplasmic tRNAs (3). There is little detailed structural information on these tRNAs. No data are currently available that examine the structure of mammalian mitochondrial tRNAs with single nucleotide resolution. However, chemical and enzymatic probing has lead to the idea that these tRNAs have retained the basic cloverleaf structure of canonical tRNAs but that they lack several conserved tertiary interactions leading to a weaker three-dimensional structure (4–8). In particular, a number of the long range interactions between the D- and T-arms of the tRNAs appear to be missing.

All 22 tRNAs that function in mammalian mitochondria are encoded in the mitochondrial DNA. Considerable interest in mitochondrial tRNAs centers on the occurrence of diseases arising from mutations in their genes that lead to maternally inherited genetic disorders (9–12). The diseases associated with mitochondrial tRNA mutations may arise from failure in the processing of the tRNA (13), from reduced stability of the tRNA (14, 15), from a reduction in aminoacylation (12, 16, 17), from a reduced ability of the mutated aminoacyl-tRNA to interact with mitochondrial elongation factor Tu (EF-Tu\textsubscript{mt})\textsuperscript{3} (the corresponding prokaryotic factor is also designated EF1A) (16), and from the failure of the tRNA to be correctly modified leading to translational defects (18).

Normally, protein biosynthetic systems have two tRNA\textsuperscript{Met} species. One is used solely for initiation, and the other functions in polypeptide chain elongation. Animal mitochondria are quite unusual in that they contain a single gene for tRNA\textsuperscript{Met}, which functions in both polypeptide chain initiation and chain elongation. As a result of this dual role, mitochondrial Met-tRNA\textsuperscript{Met} must be recognized by the mitochondrial Met-tRNA\textsuperscript{Met} transformylase (MTF\textsubscript{mt}) and be brought as fMet-tRNA\textsuperscript{Met} to the ribosome for translational initiation (19). In addition, Met-tRNA\textsuperscript{Met} must interact with elongation factor EF-Tu\textsubscript{mt}

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\textsuperscript{3}The abbreviations used are: EF-Tu\textsubscript{mt}, mitochondrial elongation factor Tu; hmtRNA\textsuperscript{Met}, human mitochondrial tRNA\textsuperscript{Met}; MetRS, methionyl-tRNA synthetase; hmMetRS, human mitochondrial methionyl-tRNA synthetase; MTF, methionyl-tRNA transformylase; SHAPE, selective 2′-hydroxyl acylation analyzed by primer extension; PMSF, phenylmethylsulfonyl fluoride; \(\beta\)ME, \(\beta\)-mercaptoethanol; 1M7, 1-methyl-7-nitroisatoic anhydride.
and bind to the A-site of the ribosome during translational elongation. Thus, this tRNA\textsuperscript{Met} is of central importance in mitochondrial translation.

Human tRNA\textsuperscript{Met} has a number of interesting features (Fig. 1A). The D-loop is somewhat small and lacks the G residues at positions 18 and 19 that facilitate interactions with the T-loop in the tertiary structure. The first position of the anticodon contains the rare modified base 5-formylcytidine. This modification may play a role in the unusual codon recognition requirements of this tRNA, which must recognize both AUG and AUA codons. The minor loop is short lacking the usual pairs (U-U and U-G47, and the T-stem has two adjacent pyrimidine:pyrimidine pairs (U-U and U-W). Furthermore, the T-loop lacks the ΨΣC sequence and contains only six nucleotides instead of the normal seven. These unusual structural features suggest that human mitochondrial tRNA\textsuperscript{Met} may have an intrinsically weak tertiary structure.

Three interesting point mutations (T4409C, A4435G, and G4450A) occur in the gene for human tRNA\textsuperscript{Met} (hmtRNA\textsuperscript{Met}). The T4409C mutation (Fig. 1A) results in a U8 to C change at the corner of the acceptor stem and D-stem of hmtRNA\textsuperscript{Met}. This mutation leads to mitochondrial myopathy resulting in dystrophic muscles and exercise intolerance (20). The A4435G mutation leads to the change of A37 to G37 in the anticodon loop of the tRNA (21). This mutation acts as a modulator of Leber’s Hereditary Optic Neuropathy increasing the severity of this condition when it arises because of other mutations in the mitochondrial DNA. The G4450A mutation leads to loss of the final base pair in the T-stem (Fig. 1A). This mutation presents as splenic lymphoma, is largely confined to lymphocyte cells, and results in severely abnormal mitochondria leading to serious defects in energy production (22). A systematic examination of the structural and biochemical consequences of these mutations is lacking. Here we examine the structure of human mitochondrial tRNA\textsuperscript{Met} and probe the effects of the 8U→C mutation on the structure and function of this tRNA.

**EXPERIMENTAL PROCEDURES**

**RNA Synthesis**—Human mitochondrial tRNA\textsuperscript{Met} transcripts for aminoacylation experiments were prepared by in vitro transcription using the hammerhead ribozyme construct described previously (23). The hmtRNA\textsuperscript{Met} was purified by denaturing (10%) PAGE (29:1 acrylamide:bisacrylamide prepared with 7 M urea, 90 mM Tris borate, 2 mM EDTA), visualized by UV shadowing, excised from the gel, and recovered by passive elution in water followed by ethanol precipitation. hmtRNA\textsuperscript{Met} transcripts for selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) experiments were prepared in the context of the structure cassette as described (24). D- and T-half-molecules were chemically synthesized (Dharmacon), purified, and analyzed as described previously (25).

**Purification of E. coli Methionyl-tRNA Synthetase (MetRS) and Human Mitochondrial MetRS (hmMetRS)—**A saturated overnight culture of JM109 cells carrying the pQE60-Escherichia coli MetRS plasmid construct (kindly provided by Uttam RajBhandary, Massachusetts Institute of Technology) was grown at 37 °C in 2× YT media (20 ml) supplemented with 50 μg/ml ampicillin and used to inoculate 2 liters of 2× YT media (50 μg/ml ampicillin). The cells were grown at 37 °C for 4 h (A\textsubscript{600} = 0.6), induced with 50 μM isopropyl β-d-thiogalactopyranoside and then grown at 37 °C for 4 h post-induction. The cells were harvested by centrifugation at 4,000 rpm for 30 min. The cell pellet was resuspended in 500 ml of 10 mM Tris-HCl, pH 7.6, and then re-collected by low speed centrifugation. The cell pellet was fast frozen and stored at −80 °C until use.

The cell pellet (7 g) was resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM MgCl\textsubscript{2}, 200 μM phenylmethylsulfon fluoride (PMSF), 0.1% Triton X-100, and 7 mM β-mercaptoethanol (βME)) and sonicated on ice for 7 min with 10-s bursts followed by 50-s cooling periods. The cell lysate was centrifuged at 15,000 rpm for 30 min at 4 °C. E. coli MetRS was purified from the supernatant using 400 μl of a 50% nickel-nitritroliacetic acid (Qiagen) slurry in wash buffer (100 mM Tris-HCl, pH 7.6, 1 M KCl, 10 mM MgCl\textsubscript{2}, 10 mM imidazole, 200 μM PMSF, and 7 mM βME). The resin was washed with 200 ml of wash buffer. The protein was eluted with 4 ml of elution buffer (100 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM MgCl\textsubscript{2}, 150 mM imidazole, 200 μM PMSF, and 7 mM βME). The protein sample was dialyzed against 2 volumes of 500 ml of dialysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 2.5 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 10% glycerol and 7 mM βME) for 1 h.

Cells carrying a plasmid encoding the His\textsubscript{6}-tagged human mitochondrial MetRS were grown as described (23). The cells were lysed as described above, and the hmMetRS was further purified as described (23).

**Purification of Bovine Mitochondrial Methionyl-tRNA Transformylase (MTF\textsubscript{mt})—**E. coli BL21 cells, carrying the pET15-bovine MTF\textsubscript{mt} plasmid construct, were grown as described (19). Cells were harvested and lysed as described for E. coli MetRS above. The protein was purified as described for the E. coli MetRS except that the buffers contained 50 mM Tris-HCl, pH 7.6. The purified protein sample was dialyzed against 2 volumes of 500 ml of MTF dialysis buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 10% glycerol, and 3 mM βME) for 1 h, fast-frozen, and stored at −80 °C.

**Assay for the Aminoacylation of Human Mitochondrial tRNA\textsuperscript{Met}—**The aminoacylation reactions for both the normal and 8U→C mutated tRNA\textsuperscript{Met} transcripts were performed essentially as described (23). Reaction mixtures (100 μl) contained 50 mM Tris-HCl, pH 7.6, 2.5 mM Mg\textsubscript{2+}, 2.5 mM ATP, 0.2 mM spermine, 200 μg/ml bovine serum albumin, 0.2 units/μl SUPERase\textsuperscript{In} RNase inhibitor, 40 μM [\textsuperscript{35}S]methionine (4,000 cpm/pmol), 50 nm human mitochondrial MetRS or 8 nm E. coli MetRS, and 1 μM U8 or 8U→C hmtRNA\textsuperscript{Met}. The amount of aminoacylated tRNA formed was determined by trichloroacetic acid-precipitable counts at the indicated times.

**Preparative Aminoacylation of [\textsuperscript{35}S]Met-tRNA\textsuperscript{Met}—**Reaction mixtures (2 ml) were prepared as described above except that 20 μM [\textsuperscript{35}S]methionine (20,000 cpm/pmol), 0.5 μM U8, or 8U→C hmtRNA\textsuperscript{Met}, and saturating amounts of human mitochondrial MetRS were used. Reactions were incubated for 15 min at 37 °C, followed by phenol/chloroform extraction. The tRNA\textsuperscript{Met} was collected by ethanol precipitation and then dissolved in 10 mM potassium succinate, pH 6.0, before use.
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Formylation of Human Mitochondrial Met-tRNA<sup>Met</sup>—Formylation reactions (5 μl) contained 20 mM Tris-HCl, pH 7.6, 100 μM EDTA, 150 mM KCl, 7 mM MgCl₂, 10 mM BME, 125 μM folic acid (Sigma), 100 nM normal or 8U→C mutated [³²P]Met-hmtRNA<sup>Met</sup> and 8 mM MTf<sub>me</sub>. Reactions were performed at 37 °C for 0–8 min (0-min time point was taken in the absence of enzyme). At the indicated time, 83 mM NaOH (1 μl of 500 mM) was added, and incubation was continued at 37 °C for 30 min. The [³²P]Met and [³5S]Met in 5 μl of each reaction were separated on Partisil LKSD TLC plates (Whatman) with a butanol:acetic acid:water (4:1:1) mixture. TLC plates were visualized by phosphorimaging (GE Healthcare) and the spots were analyzed using the ImageQuant program.

Binding of Human Mitochondrial Met-tRNA<sup>Met</sup> to Bovine Mitochondrial EF-Tu (EF-T<sub>u</sub>)—EF-T<sub>umt</sub> was prepared as described (26), except that the cells were lysed as described above for E. coli MetRS, and the high speed centrifugation step was omitted. Where indicated the normal U8 or 8U→C mutated hmtRNAs were phosphorylated with cold ATP using polynucleotide kinase (New England Biolabs) prior to large scale aminoacylation.

To measure ternary complex formation, reaction mixtures (50 μl) were prepared as reported (27) except that 20 mM Hepes-KOH, pH 7, and the indicated amounts of EF-T<sub>umt</sub> were used. The reactions were incubated for 15 min at 0 °C or 6 min at 37 °C as indicated. Free [³5S]Met-hmtRNA<sup>Met</sup> was digested by a 30-s incubation with 10 μg of RNase A, and the reaction was terminated by the addition of cold 5% trichloroacetic acid. Following a 10-min incubation on ice, the [³5S]Met-hmtRNA<sup>Met</sup> precipitate was collected on nitrocellulose filters (Merck) and 32P-labeled using polynucleotide kinase (New England Biolabs) prior to large scale aminoacylation.

Structural Studies of hmtRNA<sup>Met</sup> Half-molecules—Reconstitution of hmtRNA<sup>Met</sup> from U8 and 8U→C mutant tRNAs, the two data sets were normalized to the reactivity of the -CCA end nucleotides. The reactivity of each nucleotide was assigned a value between 0 and 1. Nucleotides fall into one of four categories (32, 33) as follows: unreactive (0.000–0.055), low reactivity (0.055–0.110), moderately reactive (0.110–0.220), or highly reactive (0.220–1.000).

Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE) Analysis of Normal U8 and 8U→C Mutated hmtRNA<sup>Met</sup> Transcripts—Normal U8 or 8U→C mutated hmtRNA<sup>Met</sup> (12 pmol, 0.33 μM) in 36 μl of nuclease-free water (Ambion) was incubated at 50 °C for 2 min and then cooled on ice for 2 min. The RNA was divided into 2 aliquots of 4 pmol (12 μl) and 8 pmol (24 μl). Folding buffer (6 μl; 333 mM Hepes-KOH, pH 8, 333 mM NaCl) was added to the 4 pmol of RNA, and folding buffer with 20 mM Mg<sup>2+</sup> (12 μl) was added to the 8 pmol of RNA, and the two samples were incubated at 37 °C for 20 min. To 1 μl of 100 mM 1-methyl-7-nitroisotoic anhydride (1M7) in anhydrous DMSO or 1 μl of anhydrous DMSO (control), 9 μl (2 pmol) of folded RNA was added and allowed to react at 37 °C for 70 s (5 half-lives). The balance of the RNA folded in the presence of Mg<sup>2+</sup> (18 μl) was divided into 2 aliquots of 2 pmol (9 μl) each and stored at 37 °C for sequencing. MgCl₂ (1 μl; 64 mM) was added to the RNA treated with the folding buffer in the absence of Mg<sup>2+</sup>. Radiolabeled oligonucleotide (0.3 μM; 3 μl; 5·³²P-GAACCGGACCGAAGCCCG) was added from the Nucleic Acids Core Facility at University of North Carolina) was added to the 1M7-treated, DMSO-treated, or untreated RNA (2 pmol), and the samples were incubated at 65 °C for 5 min and then at 35 °C for 20 min for primer annealing. To each reaction, reverse transcription buffer (6 μl; 250 mM KCl, 167 mM Tris-HCl, pH 8.3, 17 mM dithiothreitol, and 0.42 mM each dNTP) was added. Then either ddCTP or ddTTP (1 μl; 5 mM; Amersham Biosciences) was added to the untreated RNA. After heating to 52 °C, reverse transcriptase (1 μl; 200 units; Superscript III, Invitrogen) was added, and the primer extension reactions were performed at 52 °C for 5 min. Reactions were quenched with 4 M NaOH (1 μl) and heated at 95 °C for 5 min. For gel analysis, a gel loading solution (29 μl; 138 mM unbuffered Tris-HCl, 73% (v/v) formamide, 2 mM Tris borate, 86 mM EDTA, pH 8, with xylene cyanol and bromphenol blue) was added, and the samples were heated at 95 °C for an additional 5 min. The cDNA products from the + and −1M7 and sequencing reactions were separated by denaturing gel electrophoresis (10% polyacrylamide). Samples on gels (21 cm × 40 cm × 0.4 mm) were subjected to electrophoresis at 1400 V for −2.5 h. Gels were visualized by phosphorimaging (GE Healthcare). The + and −1M7 band intensities were quantified using SAFA (30) and corrected for signal drop-off (31). SHAPE reactivities were normalized by subtracting intensities for the −1M7 control from the +1M7 reaction and dividing each by the average reactivity of the most reactive 7% of the nucleotides. To facilitate comparison of the normal U8 and 8U→C mutant tRNAs, the two data sets were normalized to the reactivity of the -CCA end nucleotides. The reactivity of each nucleotide was assigned a value between 0 and 1. Nucleotides fall into one of four categories (32, 33) as follows: unreactive (0.000–0.055), low reactivity (0.055–0.110), moderately reactive (0.110–0.220), or highly reactive (0.220–1.000).
recorded with a temperature change of 1 °C per min from 4 to 90 °C. The one most inconsistent of the 10 melting transitions (either a denaturation or renaturation) was discarded from each set, and the resulting data were averaged on a point-by-point basis.

For UV melts of the hmtRNA$^{\text{Met}}$ transcripts, the normal U8 and the 8U→C hmtRNA$^{\text{Met}}$ were dialyzed against water using 10-kDa cutoff dialysis cups (Stratagene). The U8 and 8U→C hmtRNA$^{\text{Met}}$ transcripts were diluted to 0.5 µM in a buffer containing 10 mM NaCl and 10 mM Hepes-KOH, pH 8.0. The thermal denaturation of the tRNAs was monitored by UV absorbance at 260 nm using a Cary 3 spectrophotometer. Data points were recorded once per min from 4 to 95 °C with a temperature change of 1 °C per min. Following thermal renaturation, 6 mM Mg$^{2+}$ was added to the U8 and 8U→C transcripts, and the UV-monitored thermal denaturation experiments were repeated.

RESULTS

Aminoacylation of the Normal and 8U→C Mutated tRNA$^{\text{Met}}$—

Previous studies (23) have shown that the transcript of mitochondrial tRNA$^{\text{Met}}$ has aminoacylation properties similar to those observed with the native tRNA. Thus, it was possible to use the normal transcript and a transcript containing the 8U→C mutation for studies on the effect of the mutation on the properties of the tRNA. The 8U→C mutation leads to a myopathy presumably arising from a reduction in translational activity in mitochondria. To determine the biochemical consequence of the 8U→C mutation, the abilities of the U8 and 8U→C hmtRNA$^{\text{Met}}$ transcripts to be aminoacylated by the human mitochondrial methionyl-tRNA synthetase (hMfMetRS) were tested. Aminoacylation is an early step required for the tRNA to be used in either the elongation or initiation phase of protein synthesis and is thus of central importance for protein synthesis in mitochondria. The normal U8 transcript was aminoacylated as expected (23); however, the 8U→C mutation caused a significant reduction in the rate of aminoacylation of the tRNA by hmMetRS (Fig. 1B). This observation provides one clear rationale for the failure of this tRNA to function in mitochondrial protein biosynthesis.

Not unexpectedly, the normal hmtRNA$^{\text{Met}}$ was aminoacylated by the E. coli MetRS (Fig. 1C). Interestingly, whereas the 8U→C hmtRNA$^{\text{Met}}$ was poorly aminoacylated by the hmMetRS, it was not aminoacylated at all by the E. coli MetRS (Fig. 1C) suggesting that the mutated tRNA had a significantly altered structure. The hmMetRS is believed to be both structurally and functionally homologous to its prokaryotic counterpart (23). However, this work demonstrates that the hmMetRS is less discriminatory than E. coli MetRS for the structure of the tRNA. Because a major determinant in the recognition of tRNA$^{\text{Met}}$ by the MetRS is thought to lie in the anticodon sequence that is unchanged (36), the weak aminoacylation most likely reflects significant structural alterations in the tRNA as a result of the mutation.

Formylation of Normal and 8U→C Mutated Met-tRNA$^{\text{Met}}$—

The defective aminoacylation of the 8U→C hmtRNA$^{\text{Met}}$ made it difficult to assess the effects of the mutation on additional steps in protein biosynthesis. However, small amounts of the aminoacylated 8U→C mutated hmtRNA$^{\text{Met}}$ could be isolated, permitting a limited investigation of additional steps in translation.

In the mammalian mitochondrial system, the Met-tRNA$^{\text{Met}}$ must be formylated by the mitochondrial transformylase (MTF$_{\text{mt}}$) to be used in initiation (19, 37). The abilities of the U8 and 8U→C Met-tRNA$^{\text{Met}}$ to be formylated were tested by incubation of the $[^{15}S]$Met-tRNA with the bovine MTF$_{\text{mt}}$. For UV melts of the hmtRNA$^{\text{Met}}$, the normal U8 and 8U→C (U8C) transcripts (squares) of hmtRNA$^{\text{Met}}$ by hmMetRS, C, aminoacylation of normal (circles) and 8U→C (U8C) mutated (squares) hmtRNA$^{\text{Met}}$ by E. coli MetRS.

FIGURE 1. The sequence of the normal and 8U→C hmtRNA$^{\text{Met}}$ and the effect of the mutation on the aminoacylation of the tRNA. A, primary sequence of hmtRNA$^{\text{Met}}$ indicating the position of the 8U→C mutation. The Sprinzl numbering system is used throughout (53). B, aminoacylation of the normal (circles) and 8U→C (U8C) transcripts (squares) of hmtRNA$^{\text{Met}}$ by hmMetRS. C, aminoacylation of normal (circles) and 8U→C (U8C) mutated (squares) hmtRNA$^{\text{Met}}$ by E. coli MetRS.
formation of the ternary complex with *E. coli* EF-Tu is not affected by the 5'-phosphate (40), but no studies were available on the importance of this group for EF-Tumt. The first issue addressed was whether the ability of EF-Tumt to bind the aminoacylated tRNA in the ternary complex was affected by the presence of the 5'-phosphate. For this experiment, the aminoacylated transcript carrying the 5'-H11032-OH was tested in ternary complex formation and compared with an aminoacylated transcript that carried a 5'-phosphate following phosphorylation by polynucleotide kinase. As indicated in Fig. 3A, the Met-tRNA transcript carrying the 5'-phosphate formed a ternary complex with EF-Tumt efficiently. However, the transcript with the 5'-OH was noticeably less active in interacting with EF-Tumt. This observation indicates that, unlike *E. coli* EF-Tu, the interaction of EF-Tumt with Met-tRNA benefits from contact with the phosphate at the 5'-end of the aminoacyl-tRNA. Examination of the residues in the binding pocket for the aminoacyl-tRNA in the bacterial and mitochondrial factors reveals that Glu-287 in *E. coli* EF-Tu is replaced by the oppositely charged residue Arg-335 in EF-Tumt (27). This change alters the charge balance in the region of EF-Tu in close proximity to the 5'-end of the tRNA in the ternary complex and may provide an additional stabilizing interaction enhancing the binding of EF-Tumt to the conformationally fragile mitochondrial tRNAs.

Analysis of the binding curve between EF-Tumt and the phosphorylated Met-tRNA\textsubscript{Met} indicates a binding constant of 27 \pm 6 nM at 0 °C. This value is in good agreement with the \( K_d \) value observed for the binding of several other mitochondrial aminoacyl-tRNAs to EF-Tumt (41).

The interaction of the U8\textrarr;C Met-tRNA\textsubscript{Met} was then investigated at 0 °C using the phosphorylated transcript (Fig. 3B). Rather surprisingly, the mutated tRNA was as effective as the normal transcript in interacting with EF-Tumt under these conditions suggesting that the U8\textrarr;C C Met-tRNA had adopted a tRNA-like conformation recognized by this factor. This observation appeared to be in conflict with the poor activity of the U8\textrarr;C hmtRNA\textsubscript{Met} in aminoacylation and formylation. However, the previous assays were carried out at 37 °C, whereas the ternary complex assay was carried out at 0 °C. Hence, ternary complex formation was tested at 37 °C with both the phosphorylated U8 and U8\textrarr;C hmtRNA\textsubscript{Met} species (Fig. 3C).

At this higher temperature, the U8\textrarr;C C Met-tRNA\textsubscript{Met} was almost inactive in ternary complex formation, whereas the U8 Met-tRNA\textsubscript{Met} had significant activity in ternary complex formation. These data suggest a temperature-dependent destabilization of the structure of the U8\textrarr;C mutated tRNA. Structural probing provided insight into this question as described below.
Chemical Probing of the Structure of the Normal U8 tRNA\textsuperscript{Met} in the Presence and Absence of Mg\textsuperscript{2+} — The structures of the normal U8 and 8U→C mutant hmtRNA\textsuperscript{Met} transcripts were probed using SHAPE chemistry at 37 °C (24, 42). SHAPE chemistry relies on the differential reactivity of the nucleotide 2'-OH to the small molecule 1M7 (24, 42). Nucleotides in flexible, single-stranded regions are preferentially modified by the reagent, revealing different conformational states of residues within the RNA. The chemically treated tRNA transcripts were reverse-transcribed using a radiolabeled primer. Sites of modification were identified based on their ability to obstruct reverse transcription. The cDNA products were analyzed on a denaturing polyacrylamide gel. When residues in unstructured or flexible regions of the tRNA are modified, the reverse transcriptase stops, leading to a labeled band observed on the gel one residue shorter than the position of the modification. Comparison of the position of these bands to a sequencing ladder allows the identification of residues susceptible to modification (32). The reactivity of each nucleotide toward 1M7 was quantified, and the relative reactivity of each nucleotide was assigned a value between 0 and 1. The higher the value, the greater the propensity the nucleotide has to be in an unstructured region. This information allowed us to develop a model of the tRNA structure (43).

The reactivity of 1M7 is not Mg\textsuperscript{2+}-sensitive; hence, SHAPE chemistry was used to examine the influence of Mg\textsuperscript{2+} on the structure formed by the normal and 8U→C mutant hmtRNA\textsuperscript{Met} transcripts. In the presence of Mg\textsuperscript{2+}, a number of distinct structural features were observed for the normal hmtRNA\textsuperscript{Met} (Fig. 4A and B). The 2'-OH of residues predicted to form the acceptor stem and the D-stem were not accessible to the reagent. This indicated that they were largely paired as expected. Only a single residue in the D-loop was highly reactive suggesting that most of the residues in this region of the tRNA were involved in tertiary interactions that reduced their reactivity. The anticodon stem was clearly protected, but as expected residues in the anticodon loop were available for modification. The variable loop again showed limited modification indicating tertiary interactions. These interactions were expected to take place primarily with residues in the D-loop as would be expected from tertiary contacts observed in canonical tRNAs. The T-stem apparently formed despite the presence of the U-U pairs. However, in contrast to other tRNAs (33), residues in the T-loop were quite reactive indicating that they were accessible in the tertiary structure of this tRNA.

The folding of the hmtRNA\textsuperscript{Met} structure required the presence of Mg\textsuperscript{2+} (Fig. 4A, lanes 1 and 3 compared). Mg\textsuperscript{2+} not only stabilizes the negatively charged backbone but is known to bind to specific sites of canonical cytoplasmic tRNAs to aid in folding their tertiary structures. In the absence of Mg\textsuperscript{2+}, the hmtRNA\textsuperscript{Met} structure was open and highly reactive to 1M7, indicating that both secondary and tertiary interactions had been lost (Fig. 4A, lane 1).

A more thorough analysis of the SHAPE data through quantitation of the individual nucleotide reactivities provided additional insight into the three-dimensional structure formed by the unusual hmtRNA\textsuperscript{Met}. The SHAPE reactivity pattern was suggestive of a folded cloverleaf-shaped tRNA and was similar to the reaction pattern observed with other tRNAs (33) (Fig. 5A). Superimposition of the nucleotide reactivities on the L-shaped tRNA structure (43) (Fig. 5B) provided additional information about possible tertiary interactions within the tRNA. For clarity, the nucleotides have been colored according to their level of reactivity. As expected, the most reactive portion of the tRNA was the anticodon loop. The reactivity data support a structure in which many of the conserved canonical tertiary interactions are preserved. For example, nucleotides U8, A14, and A21 which, in canonical tRNAs form a triple base
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Pair, were all modestly reactive or nonreactive in hmtRNAMet. Other possible preserved tertiary interactions are shown in Fig. 5B and include the expected interaction between nucleotides in the D-loop and the variable loop such as between A15 and U48.

Despite the likely presence of many conserved tertiary interactions in hmtRNAMet, other interactions are probably not occurring because of the shortened sequence of this tRNA. For example, interactions between the D- and T-loops may be different from canonical tRNAs because the D-loop is short and lacks the common GG sequence, whereas the T-loop is only six nucleotides instead of the universal seven nucleotides found in classical tRNAs. These deviations from classical tRNAs indicate that hmtRNAMet cannot have the G18-U55 and G19-C56 interactions that normally stabilize the corner of the L-shaped structure (3). Also, A58 in the T-loop was not reactive, whereas its expected partner U54 showed significant reactivity. This suggests that the canonical tertiary interaction of U54 with A58 may not occur. The tertiary interactions occurring in the T-loop of the hmtRNAMet may be significantly different from that of the canonical cytoplasmic tRNAs.

Chemical Probing of the Structure of the 8U → C Mutated tRNA in the Presence and Absence of Mg^{2+}—SHAPE analysis of the 8U → C mutated hmtRNAMet carried out at 37 °C in the presence of Mg^{2+} indicated that all of the nucleotides in the tRNA were susceptible to modification except the three G-C pairs in the anticodon stem. The single nucleotide mutation observed upon mutation of 8U → C results from a loss of stabilizing tertiary interactions that were present in the normal hmtRNAMet and that are dependent on the presence of Mg^{2+}. It should be noted that, alternatively, the tRNA could be folding into a dynamic mixture of transient conformations that do not reflect the structure of the normal U8 hmtRNAMet.

Analysis of the 8U → C mutated tRNA clearly showed a global increase in reactivity (Fig. 5, C and D). This increase was seen in all of the stems except the anticodon stem suggesting a loss of these secondary structural elements. Both the D- and T-loops also significantly increased in reactivity, suggesting a loss in stabilizing tertiary interactions. The variable loop, which makes extensive tertiary interactions in the U8 hmtRNAMet, became highly reactive in the 8U → C tRNA. Although the mutated 8U → C residue itself showed only a slight increase in reactivity, its tertiary interaction partners, A14 and A21, became highly reactive suggesting that they were unable to form the necessary stabilizing tertiary interactions in this region of the tRNA. When these reactivities were superimposed on the L-shaped hmtRNAMet structure, it was clear that changing the single U8 nucleotide to a C lead to a loss of both secondary and tertiary interactions resulting in a tRNA that was largely unstructured (Fig. 5D).

Stability of Normal and 8U → C Mutated hmtRNAMet in a Mitochondrial Extract—A number of mutated mitochondrial tRNAs are more rapidly degraded in vivo than are the normal (8U → C) at the corner of the acceptor stem and D-stem of the hmtRNAMet thus resulted in a drastic loss of structure that is seen even in the presence of Mg^{2+} (Fig. 4C, lane 3). The regions of the D-loop, the variable loop, acceptor stem, and T-stem all exhibited an increased reactivity to the reagent 1M7. For example, all of the residues in the D-loop and most of the residues in the variable loop were now reactive. Furthermore, the residues in the acceptor stem, which were base-paired in the normal U8 tRNA, were reactive. These changes indicated that the tertiary structure and a large portion of the secondary structure had been lost because of the mutation. As expected, the 8U → C mutant hmtRNAMet had essentially no structure in the absence of Mg^{2+}, as was observed for the normal hmtRNAMet (Fig. 4C, lane 1). Even the anticodon stem had lost its base-paired structure. Clearly, the folding of the mutated tRNA was not responding to the presence of Mg^{2+} and thus may have lost one or more Mg^{2+}-binding sites critical to the structure. It is likely that the loss in structure...
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![Graph](image)

**FIGURE 6. Stability of the normal and 8U→C mutated hmtRNA\textsuperscript{Met} in a mitochondrial extract.** The percentage of trichloroacetic acid-precipitable counts for the normal U8 transcript (diamonds) and the 8U→C mutated (squares) hmtRNA\textsuperscript{Met} remaining after incubation of \(3^2\)P-labeled transcripts with increasing amounts of a mitochondrial matrix extract.

tRNAs (43, 44). To determine how the 8U→C mutation might affect the stability of the tRNA within mitochondria, the degradation of the normal and 8U→C mutated hmtRNA\textsuperscript{Met} was monitored in an extract prepared from the mitochondrial matrix. For this analysis, the U8 and 8U→C transcripts were labeled at the 5’-end with \(32\)P-phosphate and incubated with varying concentrations of the mitochondrial extract. The amount of the tRNA remaining intact was determined by precipitation with trichloroacetic acid. The 8U→C mutated tRNA was more readily degraded by the mitochondrial nucleases than the normal U8 tRNA (Fig. 6). When visualized on a sequencing gel, it was apparent that the normal tRNA was degraded in discrete locations corresponding to loops, whereas the mutated tRNA was degraded throughout the body of the tRNA (data not shown). The differential degradation of the two tRNAs was in agreement with the SHAPE data showing an overall lack of agreement with the SHAPE data showing an overall lack of

dation of the normal and 8U→C transcribed hmtRNA\textsuperscript{Met} was demonstrated in a complex (Fig. 7, A-F). This was surprising considering that the T-stem contained two adjacent U-U mismatches (Fig. 1A). However, tandem U-U mismatches form one of the most stable internal loops in RNA (45, 46). These results suggested that the 8U→C D-half-molecule had lost the ability to bind one or more structurally important Mg\textsuperscript{2+} ions. The hmtRNA\textsuperscript{Met} transcripts were subjected to gel electrophoresis under native conditions and in the presence and absence of Mg\textsuperscript{2+} (data not shown). In the presence of Mg\textsuperscript{2+}, the 8U→C mtRNA\textsuperscript{Met} transcript migrated more slowly than the normal transcript. In the absence of Mg\textsuperscript{2+}, only a slight difference in migration was observed. These data again indicated that the 8U→C D-half-molecule and the normal T-half-molecules could not form a complex (Fig. 7, C and D).

**Thermal Denaturation of the Mutated tRNA**—The thermal stabilities of the U8 and 8U→C D-half-molecules and the normal T-half-molecule were also determined in the presence and absence of Mg\textsuperscript{2+}. The addition of countercations, particularly Mg\textsuperscript{2+}, to RNA stabilizes its structure. The PAGE results suggested that the 8U→C D-half-molecule had lost the ability to bind one or more critical Mg\textsuperscript{2+}. The U8 half-molecule gave different melting profiles in the presence and absence of Mg\textsuperscript{2+} (Fig. 8A). In contrast, the 8U→C D-half-molecule had the same thermal melting profile in both the presence and absence of Mg\textsuperscript{2+} (Fig. 8B) indicating that it was unable to respond to the addition of this counterion. This observation again argues that the mutation led to the loss of a Mg\textsuperscript{2+}-binding site.

The normal T-half-molecule exhibited a major thermal transition that was stabilized by the presence of Mg\textsuperscript{2+} (Fig. 8C). This was surprising considering that the T-stem contained two adjacent U-U mismatches (Fig. 1A). However, tandem U-U mismatches form one of the most stable internal loops in RNA and, despite the stem distortion that results, their presence actually stabilizes duplex RNA (45, 46).

Thermal denaturation of the intact U8 hmtRNA\textsuperscript{Met} transcript demonstrated the importance of Mg\textsuperscript{2+} for the correct folding of hmtRNA\textsuperscript{Met}. In the absence of Mg\textsuperscript{2+}, the U8 tRNA
began melting at low temperatures but demonstrated a major transition at around 30 °C (Fig. 9A, blue). In the presence of Mg²⁺, the 8U tRNA was stably folded until about 50 °C where a major transition occurred (Fig. 9A, pink). The shift in melting temperature in the presence of Mg²⁺ demonstrated that the structure was stabilized by the presence of Mg²⁺. Conversely, melting the 8U→C hmtRNAMet transcript did not show a strong dependence on Mg²⁺. In both the presence and absence of Mg²⁺, the 8U→C transcript failed to show a major thermal transition, although significant hyperchromicity was observed (Fig. 9B). Clearly the mutant 8U→C tRNA did not show the same structural response to Mg²⁺ as did the normal 8U tRNA. In addition, the lack of a major thermal transition for the 8U→C mutated tRNA agrees with the results of the SHAPE experiments, which indicated that the 8U→C mutated tRNA lacks significant structure.

**DISCUSSION**

Considerable information is now available on the sequences of mammalian mitochondrial tRNAs. With some exceptions, these tRNAs can be drawn as cloverleaf structures and are thought to be able to fold into a three-dimensional structure that resembles the classical L-shape of canonical tRNAs (43). In mammals, the mitochondrial tRNAs are A/U-rich reflecting the base composition of the genomes. In general, these tRNAs are shorter (71–72 nucleotides in length) than bacterial or eukaryotic cytoplasmic tRNAs (about 76 nucleotides for class I tRNAs). The D-loop tends to be smaller and most of them lack the GG sequence that is involved in tertiary interactions in normal tRNAs (8). The T-loop varies from five to nine residues deviating from the highly conserved seven residues found in cytoplasmic tRNAs. The cryo-EM study on the structure of the bovine mitochondrial ribosome (47) shows a tRNA tightly bound at the P-site. The structure of this tRNA can be fit into the crystallographic structure of a cytoplasmic tRNA except in the region of the elbow perhaps reflecting the smaller sizes of the T- and D-loops of many mitochondrial tRNAs. The D- and T-stems also vary significantly in size and often contain mismatches.

Searches for tertiary interaction networks corresponding to those found in other tRNAs have not been successful, and no compensating set of interactions can be deduced from sequence alignments (43). As discussed in more detail under “Results,” our data indicate that hmtRNAMet forms a cloverleaf structure but that D-loop and T-loop interactions cannot follow the pattern observed in canonical tRNAs.

More than 130 pathogenic mutations (see MITOMAP available online) have been observed in mitochondrial tRNA genes (48). Numerous studies have been carried out in efforts to delin-
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These mutations are thought to disrupt tertiary interactions and lead to the loss of aminocacylation of these tRNAs.

The data presented here indicate that the single point mutation 8U→C in hmtRNA<sup>Met</sup> causes the loss of stable structure at physiological temperatures. The inability of the 8U→C mutated tRNA to fold properly severely impedes aminocacylation, formylation, and interaction with EF-Tu<sub>met</sub>. Consequently, the mutated tRNA cannot function effectively in either translational initiation or elongation. With a significant proportion of the heterogeneous population of mitochondria having the 8U→C mutation, the result is the pathogenic manifestation of disease.

Residue U8 plays an important role in forming and stabilizing the tertiary structure of tRNA. Early work (51) indicated that Mg<sup>2+</sup> also plays a critical role in stabilizing the structure of the D-arm and its tertiary interactions. The crystal structure of yeast tRNA<sup>Phe</sup> clearly shows that Mg<sup>2+</sup> is tightly and site-specifically coordinated in binding pockets formed by tertiary interactions within the tRNA (52). It is believed that formation of these binding pockets precedes Mg<sup>2+</sup> binding, and that the subsequent binding of Mg<sup>2+</sup> then stabilizes the three-dimensional architecture of the tRNA (52). A single tightly coordinated Mg<sup>2+</sup> is bound at the elbow of tRNA<sup>Phe</sup>. The binding site for this ion is formed by nucleotides in the D-arm and at the corner of the D-stem and acceptor stem (Fig. 10A). The phosphate of U8 contacts this Mg<sup>2+</sup> through a bridging water molecule. U8 is also involved in a triple non-Watson-Crick pairing interaction with nucleotides A14 and A21 forming the binding pocket (Fig. 10B). Interestingly, in canonical tRNAs, U8 is highly conserved and is considered a universal nucleotide (Fig.
10C) underlying the critical role of this nucleotide in forming the site-specific Mg$^{2+}$ binding pocket that leads to a correctly folded, functionally L-shaped tRNA. Our work suggests that mutation of this single nucleotide to cytosine (U8→C) in hmtRNA$^{\text{Met}}$ prevents formation of this Mg$^{2+}$ binding pocket, resulting in a tRNA that fails to fold in the presence of Mg$^{2+}$. The loss of the structure of the tRNA could occur because of a disruption in the base pairing between nucleotides U8 and either A14 or A21, which in turn would destabilize the sharp turn and the Mg$^{2+}$ binding pocket. Alternatively, loss of structure could result from an inability to properly coordinate the Mg$^{2+}$ ion via a water molecule in the pocket. Without the stabilizing effect of Mg$^{2+}$ bound at this site, a drastic loss of structure results leading to a tRNA that does not effectively participate in protein biosynthesis. Although it is not possible to directly measure the specific binding of an individual Mg$^{2+}$ ion, the data presented here are in agreement with this basic hypothesis.

Of the 22 tRNAs present in human mitochondria, 16 of them have U at position 8 and 14 of them have the U8-A14-A21 combination seen in hmtRNA$^{\text{Met}}$ and canonical tRNAs. It is of interest to note that the most common mutation in human mitochondrial tRNAs (the A3243G mutation in the tRNA$^{\text{Leu}}$ gene associated with MELAS) is the mutation of A14 to G. The extremely deleterious effect of this mutation is also likely to arise from an inability to form the tertiary interaction involving the U8-A14-A21 base triple and Mg$^{2+}$ coordination. The absence of tertiary interactions between the D- and T-loop of the hmtRNA$^{\text{Met}}$ suggests a substantial role for U8 and its associated Mg$^{2+}$-binding site in stabilizing the three-dimensional structure of the mitochondrial tRNA.
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