The Temperature Sensitivity of a Mutation in the Essential tRNA Modification Enzyme tRNA Methyltransferase D (TrmD)*

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Background: Although temperature-sensitive (ts) mutations of TrmD exist, deficient in converting G37 to m1G37-tRNA, the basis of their phenotype is unknown.

Results: The ts-S88L mutation, while conferring thermal lability, caused a stronger defect on catalysis.

Conclusion: The catalytic defect of the ts-S88L mutation reduced the quantity and quality of tRNA methylation.

Significance: ts mutations leading to catalytic defects are useful for studying enzyme mechanism.

Conditional temperature-sensitive (ts) mutations are important reagents to study essential genes. Although it is commonly assumed that the ts phenotype of a specific mutation arises from thermal denaturation of the mutant enzyme, the possibility also exists that the mutation decreases the enzyme activity to a certain level at the permissive temperature and aggravates the negative effect further upon temperature upshifts. Resolving these possibilities is important for exploiting the ts mutation for studying the essential gene. The trmD gene is essential for growth in bacteria, encoding the enzyme for converting G37 to m1G37 on the 3’ side of the tRNA anticodon. This conversion involves methyl transfer from S-adenosyl methionine and is critical to minimize tRNA frameshift errors on the ribosome. Using the ts-S88L mutation of Escherichia coli trmD as an example, we show that although the mutation confers thermal lability to the enzyme, the effect is relatively minor. In contrast, the mutation decreases the catalytic efficiency of the enzyme to 1% at the permissive temperature, and at the nonpermissive temperature, it renders further deterioration of activity to 0.1%. These changes are accompanied by losses of both the quantity and quality of tRNA methylation, leading to the potential of cellular pleiotropic effects. This work illustrates the principle that the ts phenotype of an essential gene mutation can be closely linked to the catalytic defect of the gene product and that such a mutation can provide a useful tool to study the mechanism of catalytic inactivation.

Essential genes encode critical cellular functions that are not supported by redundant pathways. Because of their indispensability, essential genes have been studied and functionally controlled by using temperature-sensitive (ts) alleles. Such alleles are typically mis-sense mutations, which preserve the gene function at permissive and low temperatures but inactivate the gene function upon temperature upshifts. The study of ts phenotypes is fundamental and important for insight into gene essentiality. However, the molecular basis of ts phenotypes of essential genes remains poorly understood; although it is generally assumed that such phenotypes result from thermal inactivation of gene products, the possibility that they arise from inherent functional defects that become lethal at higher temperatures is often overlooked. In the latter case, ts mutations giving rise to severe functional defects at the permissive temperature can be powerful tools to study essential genes and to correlate phenotypes with mechanisms of gene inactivation.

In Escherichia coli, systematic exploration and profiling of single-gene knockouts has suggested that ~7% of the ~4,000 protein-coding genes are essential (1, 2). One example of an essential gene is trmD, broadly conserved in all bacterial species, encoding the tRNA methyltransferase for conversion of G37 to m1G37 necessary to prevent frameshift errors on the 3’ side of the anticodon (3–6). In addition to E. coli, the growth essentiality of trmD has also been shown for Streptococcus pneumoniae and Bacillus subtilis (7, 8). In one genetic study of Salmonella typhimurium, several ts mutants of trmD were isolated for the deficiency in m1G37-tRNA synthesis, resulting in changes in the thiamine biosynthesis pathway while activating expression in an alternative pathway. This study showed that the trmD deficiency at the restrictive temperature of these mutants reduced the m1G37-tRNA level, such that ribosome translation at codons normally read by the modified tRNA was slow, thus inactivating gene expression in the normal thiamine biosynthesis pathway while activating expression in an alternative pathway. This study showed that the deficiency of trmD had altered the profile of global gene expression, consistent with increasing evidence indicating that tRNA modifications often have regulatory and stress-response roles at genome-wide levels (10).

Although the isolated ts-trmD mutants had been confirmed for the deficiency in m1G37-tRNA synthesis (9), none had been characterized at the structural level for locations, at the molecular level for thermal instability, or at the enzyme level for catalytic defect. To gain insight into these mutations, we have mapped their locations onto the known structure of E. coli TrmD (EcTrmD) in complex with S-adenosyl homocysteine.
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(AdoHcy), the product after methyl transfer (11). From this mapping, we selected for further analysis of the S88L mutation, which is placed closely adjacent to the S-adenosyl methionine (AdoMet)-binding site. Remarkably, TrmD binds AdoMet in a topologically knotted trefoil fold, involving the protein backbone making three passes in and out of a tightly folded loop (11, 12). The location of the S88L mutation within the trefoil knot is attractive for interrogating the molecular basis of its temperature sensitivity.

We report here that although the S88L mutation indeed confers susceptibility to thermal denaturation, the effect is rather minor. Instead, the mutation markedly decreases the enzyme activity relative to the native sequence at the permissive temperature, and it causes further deterioration of activity upon temperature upshifts. We further show that the decrease in the enzyme activity is correlated with decrease in the level of tRNA methylation and the quality of tRNA recognition, suggesting the possibility of accumulating frameshift errors during protein synthesis and altering global gene expression. We suggest that it is the loss of the catalytic efficiency of TrmD by the S88L mutation, and consequently the increase of protein synthesis errors, that is the driving force for the lethal phenotype at the restrictive temperature. This work demonstrates that ts phenotypes can be closely correlated with catalytic defects of an essential gene product and that such correlation can provide unique insight into the function of the essential gene in vivo and the mechanism of action in vitro.

MATERIALS AND METHODS

Construction of the E. coli S88L-trmD-Kan Strain—The S88L-trmD mutation was isolated with a ts phenotype in Salmonella (25). We introduced this mutation to the chromosomal trmD gene of E. coli Xac strain, using the λ Red recombinase-mediated gene disruption method (16). We inserted the E. coli wt-trmD gene to the pQE-30 vector and introduced the ts-S88L mutation, by QuikChange site-directed mutagenesis (Stratagene). The modified coding sequence was amplified by PCR using the following primer set: 5'-ATGTGGATTG-GCATATAATTAGCTGTTTCC (forward) and 5'-GGACCTTCAGCAGCTCAGG ATAAGCTATCAGCCATTCATGTTATG-3' (reverse). The PCR product was purified and amplified again according to the megaprimer method (34) in a mixture containing the first PCR product, the kanamycin resistance cassette plasmid pKD4, and the third primer, 5'-ATCTGTTGGTTACAGTATCCTGGGGGGCATGGGATTAAGCCATGGCCATGAT-3'. The final PCR product, together with pKD46 (the λ Red recombinase plasmid), was introduced to E. coli Xac strain and colonies were selected on LB-Kan plates. The mutation was verified by sequence analysis, and the temperature sensitivity and curing of pKD46 was confirmed. In parallel, a wt-trmD-Kan construct was made as a control.

Measurement of Kf (AdoMet) by Fluorescence—The quenching of the intrinsic tryptophan fluorescence of EcTrmD by AdoMet binding was determined as described (17). The WT enzyme was titrated at 0.2 μM with AdoMet ranging from 0.2 to 4.5 μM, whereas the S88L mutant enzyme was titrated at 2.0 μM with AdoMet ranging from 1.5 to 22 μM. Enzyme fluorescence was excited at 280 nm in the standard buffer (100 mM Tris-HCl, pH 8.0, 4 mM DTT, 0.1 mM EDTA, 6 mM MgCl2, and 100 mM KCl), and emission was monitored at 320–340 nm at room temperature. Inner filter corrections were calculated by the equation: 

\[ F / F_0 = \frac{1}{1 + [E]_{0} / K_{M}} \]

where \( F \) is the corrected dilution-corrected fluorescence, \( F_0 \) is the observed fluorescence, and \( K_{M} \) and \( A_{0} \) are the absorbencies at the excitation and emission wavelengths, respectively. Nonspecific quenching was measured by titrating AdoMet against a solution of L-Trp. Data corrected after inner filter effects and nonspecific quenching were fit to a hyperbolic equation: 

\[ y = Ax / (S + K_y) \]

where \( y \) is the change in fluorescence from the reference point, \( A \) is the maximum change in amplitude, and \( S \) is the AdoMet concentration.

Measurement of Cell Growth and Viability—E. coli cells harboring wt or S88L-trmD were grown in LB medium overnight at 30 °C. A fresh culture was inoculated with the overnight culture at 1:100-fold dilution and continued to grow for 3–4 h at 30 °C until \( A_{600} = \sim 0.3 \). The culture was split into two, with one shifted to 43 °C by mixing with an equal volume of LB at 55 °C while the other was maintained at 30 °C by mixing with an equal volume of LB at 30 °C. Aliquots of each culture were sampled up to 24 h. Cell viability was determined by directly plating the cell culture at each time point with appropriate dilution. The plates were incubated at 30 or 43 °C, respectively, for additional 12–14 h. The number of colonies formed was normalized by \( A_{600} \) and shown as relative to the time of split.

Measurement of Intracellular TrmD Activity—E. coli cells were grown as above. Each aliquot of cell culture was spun down, and the cells were disrupted by sonication to separate the lysate from debris. The lysate was directly used to measure enzyme activity in the TrmD reaction buffer (100 mM Tris-HCl, pH 8.0, 24 mM NH4Cl, 4 mM DTT, 100 μM EDTA, 6 mM MgCl2, 0.024 mg/ml BSA, 40 units/sample RNasin (Promega)) at 30 or 43 °C, using Ec-tRNAPro as the substrate at 5-fold or lower concentration of K\text{cat}. The A37 mutant of Ec-tRNAPro, which was not a TrmD substrate, was used to measure methyl transfer to non-G37 positions. [3H]-methyl]AdoMet (25 μM) was used, and the synthesis of [3H]mG37-tRNA was monitored by acid precipitation (35). The initial rate of mG37-tRNA synthesis was normalized by \( A_{600} \) and shown as relative to the time of culture split.

Western Blot Analysis—Because the endogenous level of TrmD is very low in E. coli (28), we used E. coli strain S13009 overexpressing the WT or S88L-TrmD from the plasmid pQE30. Cells were grown in LB medium at 30 °C for an hour and then split into three cultures, each at 30, 37, or 43 °C. After 8 h of growth, when the mutant TrmD was inactivated at the restrictive temperatures (see Fig. 5), cells were harvested and sonicated, the soluble cell lysates were collected, and protein concentrations were determined by the Bradford method. Total protein (10 μg) of each cell lysate was loaded to a 12% SDS-PAGE, transferred to Immobilon PVDF membrane (Millipore), and reacted with the primary antibody raised against StTrmD (given by Dr. Glenn Björk). The membrane was incubated with the anti-rabbit IgG secondary antibody conjugated with peroxidase (Sigma-Aldrich), and the signal was detected using SuperSignal West Pico (Thermo Scientific) and quantified by ImageQuan (GE Healthcare). Two independent exper-
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A Temperature-sensitive Mutation in TrmD—A genetic study led by Björk and Nilsson (9) isolated a group of ts mutations in *S. typhimurium* *TrmD* (StTrmD) that conferred altered thiamine metabolism at a restrictive temperature (P58L/L94F, S88L, G117S, G117N, G117Q, S165L, P184L, G199R, G214D, W217D, and E243K). A separate study by Li and Björk (13) isolated additional ts mutants (E243K, L94F, P184L, G140S, and A145T) that displayed elevated levels of frameshift errors caused by the deficiency in m1G37-tRNA. The two groups of mutants showed overlapping amino acid substitutions at the protein level and collectively occupied 12 positions in the StTrmD enzyme structure. Because these mutations were isolated before the structure of TrmD was available, nothing was known about their structural context.

We now mapped these mutations onto the crystal structure of EcTrmD in complex with AdoHcy (11), which was a logical model based on the over 92% homology in the primary sequence between the *S. typhimurium* and *E. coli* enzymes. Although TrmD exists as an obligated homodimer, with each subunit featuring an N-terminal domain (residues 1–159), a flexible linker (residues 160–169), and a C-terminal domain (residues 170–250), the active site is built between the N-terminal domain of one subunit and the flexible linker and the C-terminal domain of the other. In this intriguing cross-subunit active site, AdoMet is bound to the trefoil knot fold in the subunit active site, whereas those localized to the C-terminal domain of one are near the AdoMet binding site, whereas those localized to the C-terminal domain were in helical regions (Fig. 1).

We focused on mutations near the AdoMet binding site to provide a structural framework for interpreting mutational effects. In this framework, AdoMet is bound to the trefoil knot fold, which is initiated with a β strand (β4) in the central β sheet structure of the N-terminal domain. The β4 curves around through α4 and β5 and turns into α5 and then into β6, which makes a circular insertion into the loop (Fig. 1A). Within this knot, AdoMet adopts a distinctively bent conformation that places the adenosine and methionine moieties facing each other, which is rare among AdoMet-dependent methyltransferases (12, 14). During methyl transfer, the
AdoMet-binding site is presumably “capped” by the flexible linker provided by the C-terminal domain of the other monomer. Proteins with a knotted fold, such as the trefoil knot in TrmD, are rare but are known to have an inherent rigidity not present in unknotted proteins (15).

Of the mutations localized to near the AdoMet site, some were discovered together in one mutant (e.g., P58L and L94F), whereas others were discovered in multiple mutants each with a distinct substitution (e.g., G117S, G117N, and G117Q). We chose to study the S88L mutation, which was discovered in one mutant at a single position. The natural S88 residue is at a position highly conserved among Gram-negative TrmD enzymes but not conserved in Gram-positive (Fig. 2A), indicating the possibility of differential roles in the two classes of bacteria. In the EcTrmD structure, S88 is placed in the beginning of the loop emanating from the central β4 that stabilizes the knot (Fig. 2B). The S88L mutation is therefore attractive for interrogating the molecular basis of its ts phenotypes, because on the one hand it is expected to interfere with AdoMet binding, because of the replacement of the smaller side chain of serine with the bulkier side chain of leucine, whereas on the other hand it is located at a key position of the knot such that the mutation might disrupt the rigidity of the knot and confer sensitivity to thermal denaturation.

FIGURE 1. Mapping of ts mutations onto the structure of EcTrmD in complex with AdoHcy (Protein Data Bank code 1P9P) drawn in PyMOL. The ts mutations were isolated from two previous genetic studies (9, 13), and each is marked in purple; AdoHcy is marked in blue by elements. A, ts mutations localized to the N-terminal domain (residues 1–159). B, ts mutations localized to the C-terminal domain (residues 170–250). Note that A and B were made separately in different orientations to clearly display residues of interest. The flexible linker between the two domains is not visible in the structure. The trefoil knot fold in the N-terminal domain is marked with the initiating β4, followed by α4, β5, and α5, and ending with β6 making a circular insertion into the loop created by β4, α4, β5, and α5.

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A. ts mutations localized to the N-terminal domain (residues 1–159). B, ts mutations localized to the C-terminal domain (residues 170–250). Note that A and B were made separately in different orientations to clearly display residues of interest. The flexible linker between the two domains is not visible in the structure. The trefoil knot fold in the N-terminal domain is marked with the initiating β4, followed by α4, β5, and α5, and ending with β6 making a circular insertion into the loop created by β4, α4, β5, and α5.

FIGURE 2. The S88L mutation in EcTrmD. A, sequence alignment of the region in TrmD harboring S88L. The alignment is as follows: E. coli (YP_490830), Enterobacter cloacae (YP_004953267), Haemophilus influenzae (NP_438371), Helicobacter pylori (NP_207939), Pseudomonas aeruginosa (NP_252432), Salmonella enterica (NP_461604), B. subtilis (ZP_03591325), Clostridium botulinum (YP_001254944), Enterococcus faecalis (ZP_07107430), Mycobacterium tuberculosis (YP_005309161), Staphylococcus aureus (YP_040627), and Streptococcus pneumoniae (YP_816173). The alignment was generated using the ClustalW program (38). B, the position of S88 in the binary crystal structure of EcTrmD in complex with AdoHcy, showing secondary structural elements β4, α4, β5, α5, and β6 that form the trefoil knot fold (Protein Data Bank code 1P9P). The fold is localized in the N-terminal domain of TrmD, which is connected to the C-terminal domain via an invisible flexible linker.
Introduction of the ts-S88L-trmD Mutation to *E. coli*—The S88L-TrmD mutation was originally isolated from chemical mutagenesis of *S. typhimurium* (9). The mutation was identified and confirmed in the *trmD* gene by complementation analysis using plasmids. Because the mutation occurred at a highly conserved position in Gram-negative TrmD enzymes (Fig. 2A), we tested its broader significance by introducing it to *E. coli*. This was achieved by replacing the chromosomal wt-*trmD* locus in *E. coli* with a plasmid carrying a cassette of the S88L-*trmD* gene linked to the Kan resistance marker (S88L-trmD-Kan), using the λ Red approach (16), such that the reading frame of *trmD* was preserved. This consideration was necessary because *trmD* is present in an operon, consisting of four genes in the order of *rpsP* (encoding ribosomal protein S16), *rimM* (encoding a 21-kDa protein that processes 16 S rRNA), *trmD*, and *rplS* (encoding ribosomal protein L19) (Fig. 3A). As a control, the Kan resistance cassette of the native *trmD* sequence (wt-*trmD*-Kan) was introduced to *E. coli* to create an isogenic wt strain. This wt-*trmD* and ts-S88L-*trmD* pair was compared for the rest of the study. Growth analysis by serial dilution revealed thermal sensitivity of the ts-S88L-*trmD* allele at 43 °C (Fig. 3B), demonstrating that the S88L mutation also gave rise to a ts phenotype in *E. coli*. Thus, consistent with the broad sequence conservation of *trmD* between *S. typhimurium* and *E. coli*, the S88L mutation conferred a thermal sensitivity of growth in both bacteria.

The structural and functional significance of the S88L mutation, which was mapped to near the AdoMet-binding site, was confirmed by a fluorescence analysis of enzyme binding to the methyl donor. Both the WT and S88L enzymes were purified from *E. coli* by a metal affinity resin, followed by anion exchange on an FPLC column to remove residual nucleic acids. Using a fluorescence titration assay developed previously, showing that AdoMet binding quenches the intrinsic fluorescence of EcTrmD in a concentration-dependent manner (17), we monitored fluorescence changes, corrected for inner filter effects and nonspecific quenching, and determined the equilibrium *K*_d*(AdoMet) for these enzymes. Although the *K*_d*(AdoMet) for the WT enzyme was 0.8 ± 0.3 μM (Fig. 4A), closely similar to the value obtained previously (1.1 ± 0.2 μM) (17), the *K*_d*(AdoMet) for the S88L mutant was 7 ± 1 μM (Fig. 4B), almost 10-fold higher. Thus, the S88L mutant was indeed deficient for AdoMet binding, possibly because of the bulkier leucine blocking the methyl donor from accessing the active site. Although this AdoMet binding deficiency of the mutant was detected at room temperature by fluorescence analysis, the effect of temperature shifts on the deficiency is unknown.

**FIGURE 3. The *E. coli* wt and S88L-*trmD* alleles.** *A*, the operon structure harboring wt-*trmD*-Kan or S88L-*trmD*-Kan. The operon consists of four genes in the order of *rpsP* (the ribosomal protein S16), *rimM* (a 21-kDa protein that processes 16 S rRNA), *trmD*, and *rplS* (the ribosomal protein L19). A Kan-resistant cassette containing wt-*trmD*-Kan or S88L-*trmD*-Kan was introduced to replace the chromosomal *trmD* in the operon. *B*, growth analysis by a dilution series. *E. coli* cells harboring wt-*trmD*-Kan or S88L-*trmD*-Kan were spotted on an LB-Kan plate (Kan at 50 μg/ml) through serial dilutions and incubated at 30 or 43 °C overnight.

**FIGURE 4. Determination of *K*_d*(AdoMet) by fluorescence.** *A*, intrinsic fluorescence of WT TrmD enzyme upon titration of AdoMet (0.2–4.5 μM), showing the *K*_d*(AdoMet) of 0.8 ± 0.3 μM. *B*, intrinsic fluorescence of S88L TrmD enzyme upon titration of AdoMet (1.5–22 μM), showing the *K*_d*(AdoMet) of 7 ± 1 μM. Each trace was the average of at least two independent measurements at room temperature. The error bars are standard deviations.
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Cellular Consequences of the S88L-trmD Mutation—The arrest of cell growth by the S88L-trmD mutation at the restrictive temperature prompted us to determine the timing of cell death to correlate with intracellular activity of TrmD and levels of m'G37-tRNA at the time of cell death. This correlation was necessary to gain insight into the mechanism of cell death. The timing of cell death was determined by monitoring cell viability over time rather than cell density, because the latter lacked the sensitivity once cells entered the stationary phase. E. coli cells harboring the wt-trmD or ts-trmD allele were first grown at 30 °C to a mid-log phase and were then split into two cultures, with one continuing growth at 30 °C while the other shifted to growth at 43 °C. After this split, analysis of colony-forming units (colony-forming units/ml) over time relative to the time of split showed that although both wt-trmD and ts-trmD cells were viable at 30 °C (Fig. 5, A and B), they gradually lost viability at 43 °C (Fig. 5, C and D). However, although the viability of both began to decay after 7 h at 43 °C, the mutant decayed significantly faster than the wt (Fig. 5, C and D). We marked the onset of the decay, at 7 h after the shift to 43 °C, as the beginning of cell death.

To determine the intracellular TrmD activity, we measured the intracellular enzyme activity in cell lysates as we described (18), using [3H]-methyl)AdoMet as the methyl donor to monitor the synthesis of [3H]-m'G37-tRNA in acid-precipitable counts. The transcript of EctRNA^Pro was used as the substrate, because the synthesis of m'G37 on this tRNA had been shown to be essential for bacterial cell growth (19), and key features of the tRNA for the synthesis had been identified (20). The tRNA substrate was maintained at low concentrations relative to the respective K_m for the WT and mutant enzymes (Table 1), so that the methyl transfer activity was measured near the k_cat/K_m condition. The measured acid-precipitable counts were then corrected by removing counts from the methyl transfer activity to non-G37 positions on the tRNA, such as methyl transfer to synthesize m'G46 by the enzyme TrmB, which was also present in cell lysates (21). For the correction, we used the single-substitution mutant A37-EctRNA^Pro as the substrate, which would not be recognized by TrmD (22) but would allow AdoMet-dependent methylation to all but G37 positions on the tRNA. Analysis of the G37-specific activity in each cell lysate relative to the activity at the time of split showed that, although the enzyme remained active in both wt-trmD and ts-trmD cells at 30 °C (Fig. 5, A and B), it began to deteriorate at 43 °C (Fig. 5, C and D). This deterioration occurred instantly for the mutant but with a 4-h delay in the wt cells (Fig. 5, C and D), showing the temperature sensitivity of the mutant enzyme in cell lysates. After 7 h at 43 °C, when the cell viability began to decline in both cultures, the mutant enzyme retained 30% activity, whereas the wt enzyme retained ~80% (Fig. 5, C and D). After 24 h at 43 °C, when the cell viability stalled at 30% for the wt cells and at 10% for the mutant, the wt enzyme retained 30% activity, whereas the mutant retained 10%. These results demonstrate a clear correlation between the decline of cell viability and the decrease of intracellular TrmD activity.

We confirmed that the rapid decline of intracellular TrmD activity in the mutant was not due to the instability of the enzyme as the temperature shifted upward. Western analysis using an anti-TrmD antibody showed that both enzymes maintained similar protein levels throughout the temperature range (Fig. 5E). Specifically, whole cell lysates from cells overexpressing...
ing WT or mutant enzymes at 30, 37, and 43 °C were prepared and probed at a constant amount of total protein. Although the signal for the WT enzyme was generally stronger than that of the mutant at a specific temperature, the relative level of each enzyme remained within 83% for the WT and 88% for the mutant. Thus, despite the possibility of activating proteases by higher temperatures (23), both the WT and mutant enzyme were considerably resistant to proteolysis.

Upon inactivation of TrmD, cells would contain a mixture of pre-existing m1G37-tRNA and newly synthesized unmodified G37-tRNA. Although the m1G37 state would support normal protein synthesis, the G37 state would introduce frameshift deviations. The ratio of the two states would be a determinant of cell viability in the restricted condition. Our data further show that, after 7 h at 43 °C, marking the onset of cell death; intracellular TrmD activity in mutant cells was reduced to 30% of the normal level, and intracellular m1G37-tRNA level was reduced to 40% of the normal level.

**Temperature Denaturation of TrmD in Vitro**—The decrease of the intracellular TrmD activity in both wt and S88L mutant cells raised the possibility of structural changes in both enzymes. This possibility was addressed by CD analysis of these enzymes at 30 and 43 °C. CD spectra of a buffer-only sample scanned from 260 to 200 nm revealed stable signals and a sufficiently low dynode voltage (<500 V) to permit detecting signal with high sensitivity. These qualities were maintained in a temperature scan of 15–75 °C at 222 nm and throughout experiments.

CD spectra of the WT enzyme displayed peaks at 208 and 222 nm at both temperatures, indicative of the presence of α-helices (Fig. 6A). The degree of α-helices, determined from the degree of ellipticity, was 24% at 30 °C, similar to values obtained for *Thermus thermophilus* TrmH, a close structural homolog of TrmD for AdoMet-dependent synthesis of G37 (27). This degree of α-helices dropped to 21% at 43 °C, indicating the loss of 3% helical structure. These features were reproduced in the mutant enzyme; CD spectra revealed stable α-helical structure at 26% at 30 °C, which was dropped to 18% at 43 °C (Fig. 6B), indicating the loss of 8% helical structure upon the shift. Thus, whereas both the WT and mutant enzymes were subject to a small degree of thermal denaturation, the mutant was ~3-fold more sensitive. This was confirmed in a temperature scan analysis of helical elements, showing that the mutant enzyme was generally more thermal labile than the WT and that as the temperature shifted from 30 to 43 °C, the mutant was more prone to lose helical structure by 3-fold (Fig. 6C), consistent with results of spectral analysis.

**Enzyme Activity of TrmD in Vitro**—To understand the basis of the thermal inactivation, we performed kinetic analysis of the mutant relative to the WT enzyme at three temperatures: 30,
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37, and 43 °C. Native gel analysis confirmed that both enzymes migrated to a molecular weight corresponding to a dimer (data not shown), indicating that the S88L mutation, although localized near the dimer interface of TrmD, did not disrupt the dimer structure. The transcript of EctRNA^{Leu/CAG}, after heat denaturation and reannealing, was used as the substrate and shown with a capacity of methylation to 80–90% levels in extended time courses. Steady-state parameters were determined and summarized in Table 1. AdoMet was maintained at 25 μM, which was saturating for both the WT and mutant enzymes (k_{cat} = 0.8 ± 0.3 and 7 ± 1 μM; Fig. 4). For the WT enzyme, the parameters at 37 °C (K_m (tRNA), k_{cat} and k_{cat}/K_m (tRNA)) were closely similar to published data (11, 17). As the temperature shifted downward or upward, the k_{cat} value maintained relatively constant, whereas the K_m value varied, showing a small decrease when measured at 30 °C relative to 37 °C and a significant increase at 43 °C. These variations resulted in changes in the k_{cat}/K_m value for catalytic efficiency. Using the k_{cat}/K_m value at 30 °C as the reference, the catalytic efficiency of the WT enzyme increased by 1.5-fold at 37 °C but decreased by 3.4-fold at 43 °C. However, the S88L mutant enzyme was severely defective even at 30 °C, showing a 100-fold decrease in k_{cat}/K_m relative to WT because of a defect in both K_m (tRNA) and k_{cat}. As the temperature increased to 37 and 43 °C, the K_m defect worsened progressively, whereas the k_{cat} defect remained relatively unchanged, a trend consistent with the WT enzyme (Table 1). Overall, the S88L mutant showed a decrease in k_{cat}/K_m by 1.8-fold at 37 °C and by 10-fold at 43 °C. Thus, except for the 100-fold difference in their catalytic efficiency at 30 °C, the WT and mutant enzymes reacted similarly to thermal inactivation, both displaying a temperature-dependent increase in K_m (tRNA) and an overall decrease in k_{cat}/K_m by 3–10-fold as the temperature shifted from 30 to 43 °C. As a result of the inherent catalytic defect and the temperature-dependent decline of activity, the mutant at 43 °C was only acting at 0.1% capacity of the WT capacity at 30 °C.

**FIGURE 6. CD spectra of TrmD enzymes.** A, CD spectra of the WT enzyme at 30 °C (solid line) and 43 °C (dotted line). B, CD spectra of the S88L mutant enzyme at 30 °C (solid line) and 43 °C (dotted line). C, CD spectra of the WT and mutant enzymes over a temperature scan. The spectrum of each enzymes at 0.1 μM in the CD buffer was recorded from 15 to 75 °C at 222 nm, with temperature rise at 24 °C/h, response time at 32 s, and bandwidth at 1 nm. In A and B, the content of α-helix at each temperature was calculated and shown as a percentage.

**TABLE 2**

**Steady-state discrimination of EctRNA^{Leu/CAG} by TrmD**

|                | WT-TrmD       | S88L-TrmD    |
|----------------|---------------|--------------|
|                | 30 °C         | 43 °C        | 30 °C       | 43 °C         |
| k_{cat}/K_m (μM^{-1}·s^{-1}) |               |              | (2.4 ± 0.3) × 10^{-4} | (2.5 ± 0.6) × 10^{-5} |
| G36-tRNA       | (2.0 ± 0.4) × 10^{-2} | (6 ± 1) × 10^{-3} | (1.6 ± 0.1) × 10^{-6} | (2.8 ± 0.6) × 10^{-7} |
| C36-tRNA       | (3.3 ± 0.2) × 10^{-5} | (2.1 ± 0.1) × 10^{-5} |              |              |
| Fold discrimination G36/C36 | 615 | 287 | 158 | 87 |
| Relative to 30 °C | 1.0 | 0.47 | 1.0 | 0.55 |
similar to the 2-fold relaxation of the WT enzyme. However, despite the lack of amplification of the adverse effect by temperature shift, the mutant was acting with a 7-fold less stringency at 43 °C relative to WT at 30 °C, representing a reduction to ~15% in the quality of tRNA discrimination.

**DISCUSSION**

Insight into the mechanism of temperature sensitivity of macromolecules caused by a mutation has been limited by the lack of biochemical assays to directly correlate the loss of activity with the susceptibility to thermal denaturation. In many studies, ts phenotypes are inferred only from thermal instability. Here we use the S88L mutation of the essential enzyme TrmD as an example to illustrate the correlation of thermal instability with loss of activity for both the WT and mutant enzymes. We show by CD analysis that although the mutation does confer a higher degree of thermal instability relative to the native sequence, the effect is rather mild on the order of ~3-fold from 30 to 43 °C. This mild effect is not detected at protein levels by Western analysis, and it is consistent with activity analysis of TrmD upon the temperature shift (Table 1), showing that although both the WT and mutant enzymes decrease in activity, the extent of decrease of the mutant (10-fold) is 3-fold more severe relative to the WT (3-fold). However, whereas the mutant is indeed more prone to thermal inactivation both in structure and in activity, it has catalytic deficiency acting at 1% of the WT capacity at 30 °C, which deteriorates to 0.1% capacity at 43 °C (Table 1). This decreased catalytic efficiency is accompanied by the reduced quality of tRNA discrimination to 15% of the WT (Table 2). For TrmD, the residual 0.1% capacity of catalytic efficiency and 15% quality of tRNA recognition is likely the major cause of cell death at 43 °C. This model is supported by the observation that the decline of intracellular TrmD activity over time is closely correlated with the decline of cell viability. At the onset of cell death, we show that cells with S88L-TrmD maintain m^1G37-tRNA only at 40% level of the normal.

Is the 0.1% catalytic capacity and 15% quality of TrmD lethal to cells? We have shown that the intracellular concentration of EcTrmD in wt cells is already low (~0.1 μM) (18) relative to its cellular tRNA substrates (~60 μM). The low enzyme level is consistent with a previous study showing that translation of trmD is the lowest among the four genes in the operon (28). This presents a challenging situation, implying that the enzyme must possess high levels of efficiency and quality to rapidly screen for, bind to, and catalyze methyl transfer to all of its tRNA substrates to provide m^1G37-tRNA for cell survival. Given that the concentration difference between TrmD and its tRNA substrates inside an E. coli cell is already large (~600-fold), a decrease of the enzyme catalytic efficiency to 0.1% and quality to 15% will further aggravate the difference by leaving many substrate tRNA molecules unmodified and in the highly error-prone G37 state.

Is the residual 40% level of m^1G37-tRNA lethal to cells? Some tRNA substrates for TrmD have a strong propensity to induce frameshift errors on the ribosome, such as EcTrnA^Pro/GGG (GGG: the anticodon sequence), which reads CC(C/U) codons (29). Deficiency of m^1G37 in this tRNA increases +1 frameshift errors, particularly at slippery sequences such as CC(C/U)(C/U), which we estimate occurs at a notable frequency of ~0.4% among E. coli protein-coding sequences. A larger impact, however, is that accumulation of frameshift errors can generate translational stress at global levels, such as the earlier observation that TrmD deficiency forced bacteria to switch to an alternative thiamine biosynthesis pathway (9). An emerging theme has been that tRNA modifications are intimately involved in cellular stress response. For example, the deficiency of Trm9 affects cellular response to oxidative stress, because translation of stress-response genes requires Trm4-dependent methylation in m^6C34 of tRNA^Leu/CAA (32). These examples suggest a role of tRNA modifications in translational control of gene expression. For the deficiency of m^1G37-tRNA, whereas the full extent of global impact on protein synthesis is unknown, the deficiency may affect the modification pattern of other tRNA molecules, based on a genome wide analysis showing that the synthesis of m^1G37, m^6G46, and Gm^18 appears interdependent (33). This interdependence can further expand the impact of m^1G37 deficiency to levels of gene expression involving m^6G46 and Gm^18. The potential of multiplicity and collateral damage may explain the observation of cell death at 40% level of m^1G37-tRNA.

The isolation of S88L-TrmD by ts phenotypes has identified a mutation that induces a mild thermal denaturation relative to the wt sequence while more importantly generating a significant catalytic defect that further aggravates upon temperature upshifts. The catalytic defect of this mutation at the permissive temperature now provides a basis to probe its perturbation of the structure and mechanism of TrmD and to link the perturbation to cellular deficiencies. This work demonstrates the benefits of isolating ts mutations of an essential gene and using the mutations to correlate gene functions in vivo and in vitro.

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