Conserved Amino Acids in Each Subunit of the Heteroligomeric tRNA $\text{m}^1\text{A58}$ Mtase from *Saccharomyces cerevisiae* Contribute to tRNA Binding

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Conserved amino acids in each subunit of the heteroligomeric tRNA m\(^1\)A58 Mtase from *Saccharomyces cerevisiae* contribute to tRNA binding

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**ABSTRACT**

In *Saccharomyces cerevisiae*, a two-subunit methyltransferase (Mtase) encoded by the essential genes *TRM6* and *TRM61* is responsible for the formation of 1-methyladenosine, a modified nucleoside found at position 58 in tRNA that is critical for the stability of tRNA\(^{\text{Met}}\). The crystal structure of the homotetrameric m\(^1\)A58 tRNA Mtase from *Mycobacterium tuberculosis*, TrmI, has been solved and was used as a template to build a model of the yeast m\(^1\)A58 tRNA Mtase heterotetramer. We altered amino acids in *TRM6* and *TRM61* that were predicted to be important for the stability of the heteroligomer based on this model. Yeast strains expressing trm6 and trm61 mutants exhibited growth phenotypes indicative of reduced m\(^1\)A formation. In addition, recombinant mutant enzymes had reduced *in vitro* Mtase activity. We demonstrate that the mutations introduced do not prevent heteroligomer formation and do not disrupt binding of the cofactor S-adenosyl-L-methionine. Instead, amino acid substitutions in either Trm6p or Trm61p destroy the ability of the yeast m\(^1\)A58 tRNA Mtase to bind tRNA\(^{\text{Met}}\), indicating that each subunit contributes to tRNA binding and suggesting a structural alteration of the substrate-binding pocket occurs when these mutations are present.

**INTRODUCTION**

Modified nucleosides are abundant and diverse in transfer RNA (tRNA), and influence translation accuracy (1), reading frame maintenance (2), recognition by aminoacyl-tRNA synthetases (3), and tRNA structure (4). The modifications found in tRNAs occur post-transcriptionally and range from simple base or ribose methylations to more complex multi-step additions (5). Various types of modifications are found throughout a tRNA molecule, but the greatest assortment is found in the anticodon region (6). In most tRNAs from *Saccharomyces cerevisiae*, the modified nucleoside 1-methyladenosine (m\(^1\)A) is found at position 58 in the T-loop. This modification results from the transfer of a methyl group from the cofactor S-adenosyl-L-methionine (AdoMet) to the N\(^1\) position of adenosine. While many tRNA modifications are not required for growth, m\(^1\)A58 has been found to be essential in yeast (7). Previous work has shown that m\(^1\)A58 is necessary to maintain the stability of one tRNA, initiator methionine tRNA (tRNA\(^{\text{Met}}\)) (7). The occurrence of m\(^1\)A58 in tRNA is widespread, as it is found in bacterial, archaeal and eukaryotic tRNAs. The tRNA m\(^1\)A58 methyltransferase (Mtase) has been characterized in organisms representing each of these domains, and the crystal structure of the *Mycobacterium tuberculosis* tRNA m\(^1\)A58 Mtase, TrmI, with AdoMet bound has been solved (8–12). These studies have shown that the bacterial and archaeal tRNA m\(^1\)A58 Mtases are composed of one subunit and are found as...
homotetramers; however, the known eukaryotic tRNA m'\text{A}58 Mtases consist of two subunits and are believed to function as heterotetramers. In yeast, the two subunits of the tRNA m'\text{A}58 Mtase are encoded by TRM6 and TRM61. Trm61p contains AdoMet-binding motifs and shows obvious and extensive sequence similarity to the bacterial and archaeal tRNA m'\text{A}58 Mtases. TRM6 has close homologs only in eukaryotic organisms, and does not share evident sequence similarity with any proteins other than orthologs (13). However, based on fold-recognition analyses, it has been postulated that Trm6p and Trm61p have a common core structure, and it has been hypothesized that a duplication of a trmI-like ancestral gene, followed by divergent evolution, resulted in the creation of TRM6 and TRM61 (13).

There have been only a limited number of studies regarding structure–function relationships in tRNA modification enzymes. Structure–function analyses have been performed for the Mtases responsible for formation of 1-methylguanosine 37 (TrmD) (14), 2'-O-methylguanosine 18 (TrmH) (15), and 7-methylguanosine 46 (TrmB) (16), and further insight into the mechanics of these enzymes has been gained from their crystal structures (17–19). However, these enzymes do not have heteroligomeric structures and, as a result, these studies do not give us insight into how Trm6p and Trm61p may interact with each other and a tRNA substrate. Additionally, studies of TrmD and TrmH are not entirely applicable to studies of the m'\text{A}58 Mtase, as these proteins are members of the SPOUT family of Mtases (17,18), while the Trm6p/Trm61p complex is predicted to belong to the larger and structurally distinct Rossmann-fold Mtase family. Lastly, no structure–function studies have been performed for TrmI.

The tRNA m'\text{A}58 Mtase from \textit{S. cerevisiae} is intriguing—tRNA modification enzymes are rarely essential, but TRM6 and TRM61 are both essential (20); tRNA Mtases are usually single subunit enzymes, but the yeast tRNA m'\text{A}58 Mtase is composed of two subunits (21), and Rossmann-fold Mtases are mostly monomeric, but Trm6p/Trm61p complexes are oligomeric (22). Because of these peculiarities and our lack of knowledge about this enzyme, we decided to perform a structure–function analysis of the yeast tRNA m'\text{A}58 Mtase. In order to guide our characterization of this enzyme, we created a model of a Trm6p/Trm61p heterotetramer using bioinformatics. Trm6p and Trm61p subunits were modeled individually based on alignments to known Trm1 structures obtained by protein fold-recognition analyses. The two subunits were then superimposed onto the structure of the \textit{M. tuberculosis} Trm1 homotetramer. Using this model, we introduced mutations into TRM6 and TRM61, targeting conserved amino acids predicted to be important for protein–protein interactions between the subunits of the enzyme. We expected that the resulting mutant enzymes would be defective in Mtase activity due to an inability to form heteroligomers. Surprisingly, the mutations made in Trm6p and Trm61p had no effect on oligomerization and, furthermore, did not inhibit AdoMet binding. Instead, we found that tRNA binding was affected. Loss of tRNA binding resulted from the corresponding homologous mutations in either Trm6p or Trm61p, indicating that both subunits make contributions to protein–substrate interactions. This is the first study to identify the amino acids in a heteroligomeric tRNA Mtase that are required for substrate binding.

**MATERIALS AND METHODS**

**Yeast strains and media**

Strain Y350 was created by exchanging the high-copy LEU2 marked plasmid bearing IMT4 in strain Y146 (7) for a high-copy UR3 marked plasmid bearing IMT4(C-50) (23). Transformation of yeast strains was done as described (24). Y350 was transformed with YCplac111 (Y351) (25) or the following plasmids described below: p317 (Y353), p326 (Y354), p325 (Y360) or p334 (Y367). Selection for 5-FOA resistance was performed on Sc-leucine plates containing 0.1% 5-FOA. The 5-FOA resistant strains of trm6-504 (Y361) and trm6-420 (Y368) were isolated by streaking the initial 5-FOA resistant papillae to another plate containing 5-FOA and waiting for colonies to form. Y261 (trm61-2) is strain Hm296 (26). Y261 was transformed with pRS316 (Y428) (27) or the following plasmids described below: pJA148 (Y429), pJA149 (Y430) or p430 (Y431). Gap repair was used to identify the mutation present in the trm6-504 strain. A plasmid containing TRM6 (pJA117) was digested with BsrGI and HindIII to create a gap ~500 nt long at the 3' end of the open reading frame. This plasmid was used to transform strain H2457 (7). Repaired plasmids were isolated and two independent isolates were sequenced. A point mutation was found resulting in a P431R substitution. The BsrGI/HindIII fragment from one of the repaired plasmids was moved into the same sites of pJA117 and used to transform H2457. Transformants failed to complement the temperature sensitive and 3-aminotriazole resistant phenotypes of trm6-504, insuring no other mutations were present in trm6-504.

**Plasmid construction**

For expression in yeast, single copy TRM6 with a histidine/Flag tag was moved from pLPYGCD10His Flag (10) as a Spfi/XbaI fragment into the single copy LEU2 marked plasmid YCplac111 (25) digested with the same enzymes to give p317. Quik Change site-directed mutagenesis (Stratagene) was used to introduce the trm6-416 (p326), trm6-504 (p325) and trm6-420 (p334) mutations into p317. The entire TRM6 open reading frame in p326 and p334 was sequenced to confirm the presence of the desired mutations and absence of any other mutations. Plasmids carrying TRM61 (pJA148) and trm61-3 (pJA149) have been described (10). To create a plasmid carrying trm61-255, mutagenesis was performed on TRM61 in plasmid pAK001 (described below) to give p420. An AgeI/HpaI fragment of TRM61 containing the trm61-255 mutation was moved from p420 to AgeI/ HpaI digested pJA148 to give p430. The presence of the trm61-255 mutation was confirmed by DNA sequencing.
For co-expression of TRM6 and TRM61 in Escherichia coli, both reading frames were initially cloned separately. First, pAK001 was created by inserting a Ndel/BamHI fragment containing TRM61 with a C-terminal Flag tag into pET11α (Novagen). The Ndel/BamHI fragment came from pJA166, created by PCR amplification of TRM61 from pJA148 and cloning of the product into pET3α (Novagen). pAK002 was constructed by cloning a Ndel/BamHI fragment containing TRM6 from pJA165 into pET15b (Novagen) cut with the same enzymes, giving TRM6 an N-terminal 6×His tag. pJA165 consists of TRM6, which was amplified from pMG107 (28) using the PCR and inserted into pET14b (Novagen). The final plasmid for co-expression (p328) was created by inserting a BglII(blunted)/AatII fragment containing TRM6-His from pAK001 into pAK001 that had been digested with HindIII(blunted)/AatII. Using Quik Change site-directed mutagenesis (Stratagene), the trm6-416 (p330), trm6-504 (p331) and trm6-420 (p338) mutations were created in p328. The TRM6 and TRM61 open reading frames were sequenced to show the presence of the desired mutations and verify that no other mutations had been introduced. The trm61-3 mutant was created by transferring an AgeI/NheI fragment from pJA149 into p328, producing p358. The trm61-255 mutant was created by transferring an AgeI/NheI fragment from p420 to AgeI/NheI digested p328 to give p426, and confirmed by DNA sequencing. To create trm6-416/trm61-255, this same AgeI/NheI fragment was transferred to p350, giving p427, and verified using DNA sequencing. BL21(DE3) cells were transformed with these plasmids to give the following strains used for expression and purification of recombinant Trm6p/Trm61p complexes: TRM6/TRM61 (B329), trm6-416 (B332), trm6-504 (B333), trm6-420 (B343), trm61-3 (B360), trm61-255 (B428), trm6-416/trm61-255 (B429).

Structure prediction and protein modeling

(For a detailed description of this process, please see Supplementary Data S1) Fold recognition analyses run via the GeneSilico MetaServer (29) were used to identify the best structural templates. Models of Trm6p and Trm61p subunits, excluding regions of predicted disorder, were built using the ‘FRankensteins’s monster’ approach (30). The model of the Trm6p/Trm61p heterotetramer was constructed by superimposing two copies of Trm6p and Trm61p onto the Trm1 homotetramer.

Enzyme purification

Trm6p/Trm61p complexes were purified from E. coli using TALON metal affinity resin (Clontech). Cells were grown to OD$_600$ = 0.2 at 37°C and induced with 0.5 mM IPTG at 30°C for 3 h, then harvested and the pellets frozen at −20°C. Pellets were thawed on ice and resuspended in equilibration buffer (50 mM sodium phosphate, pH 7, 1 M NaCl) with complete protease inhibitor cocktail, EDTA-free (Roche). After sonication and centrifugation (12,000g, 20 min, 4°C), clarified cell extract was incubated at 4°C for 2 h with TALON resin in equilibration buffer. Enzyme purification was carried out at 4°C following the manufacturer’s instructions for batch/gravity-flow column purification, using 20 mM imidazole in wash buffer and 200 mM imidazole in elution buffer.

In vitro activity assays

Mtase activity assays of purified enzymes were conducted as described (10,11) using Mtase buffer (100 mM Tris, pH 7.6/0.1 mM EDTA/10 mM MgCl$_2$/100 mM NH$_4$Cl/1 mM DTT). A standard assay contained 15 nM enzyme, 150 nM in vitro transcribed tRNA$^{Met}$ (11), and 30 μM S-adenosyl-l-[methyl-$^3$H]methionine (GE Healthcare). The amount of radioactive incorporation into tRNA$^{Met}$ was determined by first collecting the acid-insoluble material from the reaction on a GN-6 metriecel membrane disc filter (Pall) using a vacuum manifold, and then quantitating the radioactivity on the filter using liquid scintillation counting.

Gel filtration chromatography

To analyze Trm6/Trm61p complexes from yeast, cells were washed with 1 × Tris buffered saline (TBS) (31), resuspended in breaking buffer [1 × TBS, 1 mM DTT, 1 × complete protease inhibitor cocktail (Roche)], and lysed using a French press. Cell extract was subjected to centrifugation (107,000 g, 1 h, 4°C) and the clarified extract injected onto a Superose 12 HR 10/30 column (GE Healthcare) equilibrated with 1 × TBS. The fractions collected from the column were precipitated with acetone, subjected to SDS-PAGE, and transferred to nitrocellulose for western blotting. Protein standards (Sigma) were analyzed similarly, but were not precipitated and were detected by Coomassie blue staining. Antibodies to either Trm6p or Trm61p were used to visualize the elution patterns of the proteins by immunoblot analysis.

Recombinant enzyme from E. coli, purified as described above, was also analyzed using gel filtration. Purified enzyme (200 μg) in elution buffer (50 mM sodium phosphate, pH 7, 1 M NaCl, 200 mM imidazole) was injected onto a Superose 12 HR 10/30 column (GE Healthcare) equilibrated with 50 mM sodium phosphate, pH 7, 1 M NaCl. Proteins recovered in the fractions collected from the column were separated by SDS-PAGE and visualized using Coomassie blue staining. Protein standards (Sigma) were analyzed in the same way.

STD-NMR spectroscopy

Purified recombinant enzymes were exchanged into an NMR buffer comprised of 20 mM sodium phosphate, pH 7.0 (uncorrected for isotope effect) and 200 mM NaCl in $D_2$O, using PD-10 gel filtration columns (GE Healthcare). Saturation difference (STD) spectra of enzymes at 7 μM were obtained on a 600 MHz Varian NMR System at 8°C using the cycloneoe program (Varian pulse sequence), with low power (−6 dB) irradiation performed for 4 s using a train of 100 ms rectangular pulses. Two interleaved spectra were obtained, one with irradiation of upfield shifted protein methyl resonances (at 0 p.p.m.) and a second control spectrum with off-resonance irradiation at −10 p.p.m., and these spectra were subtracted to give the STD spectrum. AdoMet...
signals that were monitored included resonances at 6 p.p.m. (ribose) and 8.2 p.p.m. (adenine ring), which were well-resolved from residual protein signals. Control STD experiments were done in the absence of protein or in the absence of AdoMet, and they gave no false positive STD signals. Enzymes (wt or mutant) were titrated with 0, 100, 200, 300, 400, 600 or 800 μM AdoMet by repeated addition of AdoMet immediately before performing the STD experiment (acquisition time = 1 h, 512 acquisitions). All spectra were printed with the same vertical scale and the height of the resonance for an adenine ring proton of AdoMet was measured for the STD signal. Data were fitted to Equation (1) using SigmaPlot (Systat):

\[ \text{STD} = \frac{\text{STD}_{\text{max}} - \text{STD}_{\text{max}}(1 + [\text{AdoMet}] / K_d)}{1} \]

where STD is the intensity change for AdoMet protons, at a given AdoMet concentration ([AdoMet]), STD_{max} is the corresponding signal when enzyme is fully bound with AdoMet and \( K_d \) is the dissociation constant for AdoMet binding to enzyme. STD signals were effectively adjusted with the amplification factor (32). Errors for the fitted \( K_d \) values represent SDs from the nonlinear least squares fitting process.

tRNA-binding assays

tRNA-binding was studied by incubating 500 nM enzyme with ~1 nM \(^{32}\)P end-labeled tRNA\textsubscript{Met} in Mtase buffer in a total volume of 20 μl. The tRNA\textsubscript{Met} used was previously purified from a trm6A strain (Y146) (10). Reactions were incubated for 20 min at room temperature and then put on ice for 5 min. tRNA\textsubscript{Met} bound to Trm6p/Trm61p was collected on a GN-6 metricel membrane disc filter (Pall) using a vacuum manifold. Filters were washed with Mtase buffer, dried and counted by liquid scintillation. To determine the \( K_d \) of the wild-type enzyme, 1 nM \(^{32}\)P end-labeled tRNA\textsubscript{Met} was incubated with 50 nM, 100 nM, 250 nM, 500 nM, 1 μM, 5 μM or 10 μM enzyme and the assay carried out as described above. The \( K_d \) value was obtained by performing a nonlinear least squares fit of the data using SigmaPlot (Systat).

RESULTS

Trm6p/Trm61p heterotetramer modeling

To provide a structural platform for sequence–function analyses, we decided to construct a model of the Trm6p/Trm61p enzyme using bioinformatics. First, the Protein Data Bank was searched with the Trm6p and Trm61p sequences to find close homologs that could serve as modeling templates. We ran a set of fold-recognition (FR) methods via the GeneSilico MetaServer (29) to obtain target-template alignments. The best structural templates reported by the FR methods for both Trm6p and Trm61p were the prokaryotic Trm1 family members from \( \textit{M. tuberculosis} \) (19g) and \( \textit{Thermotoga maritima} \) (1o54). An alignment of these templates with Trm6p and Trm61p reveals long insertions and terminal extensions in Trm6p and Trm61p (60–185, 308–339 and 450–478 in Trm6p and 1–11, 266–342 and 370–384 in Trm61p) that are not present in prokaryotic homologs (Figure 1A) and algorithms for disorder prediction suggested these regions are flexible and lack a defined 3D structure. The alignment also illustrates that many of the residues predicted to be important for tetramer formation in Trm1, such as E229, R233, W235 and P244, are conserved in the two eukaryotic proteins (highlighted in Figure 1A). Importantly, these residues are located in the same positions in our Trm6p/Trm61p tetramer model as in the Trm1 structure.

Based on the crystal structure of Trm1, salt bridges between residues E229 and R233 from each subunit are predicted to provide crucial strength for the tetramer (12). As these residues are highly conserved in the entire m\(^A\) Mtase family, including both TRM6 and TRM61 in yeast (E416 and R420 in Trm6p; E255 and R259 in Trm61p, Figure 1A), we hypothesized that these amino acids may play an important role in formation or stabilization of quaternary structure (Figure 1B and Figure S1). The crystal structure of Trm1 also shows that W235, P244 and H251 form a three-layer sandwich, comprised of W235 and P244 from one subunit and H251 from another, that may contribute to the stability of the homotetramer. In both human and yeast Trm6p and Trm61p, as well as many other organisms, the aromatic nature of W235 is conserved by the presence of tyrosine (Y422 in Trm6p and Y261 in Trm61p); therefore, we predicted this amino acid may also be important for the stability of the eukaryotic heterotetramer (Figure 1B and Figure S1). While P244 is not conserved in Trm61p (substituted by methionine), it is conserved in Trm6p (P431); conversely, H251 is conserved in Trm61p (H354), but not in Trm6p (substituted by glycine). The preservation of this H–P pair suggested that it may contribute to the stability of the interface between subunits (Figure 1B and Figure S1).

In order to study the role of these conserved amino acids in yeast Trm6p/Trm61p protein–protein interactions, we introduced alanine substitutions to remove side chains. The Trm6p mutants created are trm6-416, which has alanine substitutions at positions E416, R420 and Y422, and trm6-420, which has an alanine substitution at position R420 (Table 1). A spontaneous Trm6p mutant that was previously isolated, trm6-504 (33), was also included in these studies because we found that this mutant has an arginine substitution at P431. A Trm61p mutant was also created with mutations corresponding to those of trm6-416. This mutant, trm6-255, has alanine substitutions at E255, R259 and Y261 (Figure 1A). Finally, the mutations in trm6-416 and trm6-255 were combined to create trm6-416/trm6-255 (Table 1).

Mutations in TRM6 and TRM61 result in growth defects

Although TRM6 is an essential gene, a trm6 deletion (trm6A) strain is viable when a high-copy plasmid containing IMT4, which encodes tRNA\textsubscript{Met}*, is present (7). To demonstrate whether or not mutations in Trm6p affect m\(^A\)58 Mtase activity, a trm6A strain that over-expresses tRNA\textsubscript{Met} from a plasmid marked with URA3 (Y350) was transformed with a single copy LEU2 marked plasmid containing either TRM6 (Y353),
Expression of Trm6p was found to be similar between the wild-type and mutant strains (data not shown). These strains were then evaluated on plates containing 5-fluoroorotic acid (5-FOA), which selects against \( URA3 \). Patches of cells grown on synthetic complete media lacking uracil and leucine (Sc-ura-leu) were replica printed to Sc-leu plates containing 5-FOA. Under these conditions, expression of \( TRM6 \) permitted growth throughout the patch, indicating the \( URA3 \) marked plasmid encoding tRNA\(_{\text{Met}}\) had been readily evicted from these cells (Figure 2A).

Table 1. \( trm6/61 \) mutants constructed

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amino acid substitutions</th>
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<tr>
<td>( trm6-416 )</td>
<td>E416A, R420A, Y422A</td>
</tr>
<tr>
<td>( trm6-420 )</td>
<td>R420A</td>
</tr>
<tr>
<td>( trm6-504 )</td>
<td>P431R</td>
</tr>
<tr>
<td>( trm61-3 )</td>
<td>G118A, G120A</td>
</tr>
<tr>
<td>( trm61-255 )</td>
<td>E255A, R259A, Y261A</td>
</tr>
<tr>
<td>( trm6-416/trm61-255 )</td>
<td>E416A, R420A, Y422A, E255A, R259A, Y261A</td>
</tr>
</tbody>
</table>

Figures 1A and 1B show the predicted structure of the Trm6p/Trm61p tetramer. Figure 1A: Modeling templates aligned with Trm6p and Trm61p sequences. The shaded background designates chemically similar residues while the black background designates identical residues among at least three sequences. Residues mutated in this study (E416, R420, Y422 and P431 in Trm6p and E255, R259 and Y261 in Trm61p) are indicated by asterisks. TrmI is the m\(^{1}A\)58 Mtase from \( M.\) \( \text{tuberculosis} \). \( 1o54 \) is a predicted AdoMet-dependent Mtase from \( T.\) \( \text{maritima} \). (B) The predicted Trm6p/Trm61p tetramer is shown with Trm6 subunits in teal and Trm61p subunits in blue. AdoMet (tan, spacefill) is shown bound to both Trm61p subunits. The amino acids that were mutated in this study (E416, R420, Y422 and P431 in Trm6p and E255, R259 and Y261 in Trm61p) are shown with side chains in fuchsia. Flexible loops lacking defined structure are not shown. Figure generated with PyMOL (41).
trm6-504 and trm6-420 were able to form papillae, but not a confluent patch, indicating only occasional eviction of the URA3 plasmid. The trm6-416 strain and the trm6Δ strain carrying an empty vector (Y351) were unable to grow (Figure 2A). The growth of cells containing TRM6 is expected because a functional m1A58 Mtase would be present and over-expression of tRNA^Met would no longer be required. The limited growth of the trm6-504 and trm6-420 mutants suggests the m1A58 Mtase has reduced activity, while the complete lack of growth of the trm6-416 strain suggests the enzyme is no longer functional. The phenotypes observed using 5-FOA selection were the first indication that the mutations created in Trm6p had a detrimental effect on m1A58 Mtase activity.

It has been previously noted that a trm6-504 mutant strain is temperature-sensitive (Ts-) at 37°C due to decreased levels of mature tRNA^Met, thought to result from a decrease in the stability of tRNA^Met tertiary structure from the absence of m1A58 (7,33). Because the Ts- phenotype can serve as a measure of mature tRNA^Met levels in trm6 mutant strains, the 5-FOA resistant cells containing trm6-504 (Y361) or trm6-420 (Y368) were tested for temperature sensitivity. The trm6-420 strain exhibited slow growth at 30 and 37°C, while the trm6-504 displayed slow growth at 30°C and a Ts- phenotype at 37°C (Figure 2B). This result suggests that the trm6-504 and trm6-420 strains have reduced amounts of mature tRNA^Met, presumably due to decreased m1A58 Mtase activity.

Because the three amino acids mutated in trm6-416 are also found in a topologically similar position in Trm6p, we created a Trm6p mutant with the corresponding mutations to see if these amino acids were also important for activity. This mutant, trm6Δ-255, was tested for its ability to complement a trm6Δ-2 strain (Y261), which has a temperature-sensitive phenotype (26,34). The trm6Δ-2 strain was transformed with an empty plasmid (Y428) or the same plasmid carrying either TRM6 (Y429), trm6Δ-255 (Y431) or trm6Δ-3 (Y430). The trm6Δ-3 mutant has two conserved glycine residues in its predicted AdoMet-binding motif changed to alanines, causing a null mutant by inactivating the enzyme (10). Only expression of TRM61 was able to complement the growth defect of the trm6Δ-2 strain (data not shown), suggesting that Trm61-255p does not form an active m1A58 Mtase in the presence of Trm6p. We conclude from these studies that the conserved sequence of E, R and Y residues in both the Trm6p and Trm6p subunits is crucial for Mtase activity.

**Figure 2.** trm6 mutants exhibit growth defects. (A) A trm6Δ strain over-expressing tRNA^Met and containing empty vector (Y351) or single copy TRM6 (Y353), trm6-416 (Y354), trm6-504 (Y360) or trm6-420 (Y367) was patched to a Sc-leu plate, grown, replica printed to a Sc-leu plate containing 5-FOA and incubated at 30°C for 3 days. (B) trm6-420 and trm6-504 strains that evicted the high-copy IMT4 plasmid (Y368 and Y361, respectively) were grown on YPD (Yeast extract/Peptone/Dextrose) at either 30 or 37°C for 3 days.

Mutations in Trm6p result in reduced m1A Mtase activity

The growth phenotypes described above suggest that Trm6-416p, Trm6-420p and Trm6-504p cannot form fully functional m1A58 Mtases in the presence of Trm6p. To confirm that tRNA from these strains was lacking m1A, we used high performance liquid chromatography (HPLC) to determine the modified nucleoside content of total tRNA from a trm6Δ strain over-expressing tRNA^Met and containing single copy TRM6 (Y353), trm6-416 (Y354), trm6-504 (Y360), trm6-420 (Y367) or empty vector (Y351) (Figure 3). In order to control for the amount of sample loaded onto the HPLC column, modified nucleoside levels in each sample were normalized to the amount of pseudouridine (Ψ) detected in that sample. Ψ was used for this purpose as it is a modified nucleoside and its formation does not depend on the presence of m1A58 in tRNA (35). The amount of m1A or Ψ detected was determined using the area of the peak from the HPLC chromatogram. In contrast to the equivalent levels of the modified nucleoside N2, N2-dimethylguanosine detected in all samples (data not shown), the trm6 mutants contained reduced levels of m1A (Figure 3). The m1A levels in the trm6 mutants are reported as a percentage of the amount found in the TRM6 strain, which was set to 100%. The level of m1A was lowest in the trm6-416 strain, which had only 5% of the amount found in the TRM6 strain, while the trm6-420 and trm6-504 strains contained greater amounts of m1A, 35 and 19%, respectively (Figure 3). The HPLC data supports our interpretation of the growth studies, illustrating that the trm6 mutants have reduced m1A Mtase activity in vivo. In addition, the magnitude of the growth defects seen for the mutant strains are mirrored by the m1A levels, as the lowest levels of m1A are found in the strains with the most severe growth defects.

In order to produce large quantities of mutant enzymes and be able to combine mutations in both Trm6p and Trm6p, we reconstructed each trm6 mutant in a vector that allows co-expression of TRM6 and TRM61 in bacteria. TRM61 was expressed in E. coli together with either TRM6 (B329), trm6-416 (B332), trm6-420 (B343) or trm6-504 (B333). Using a polyhistidine tag on Trm6p, protein was purified from soluble E. coli extract using affinity chromatography. Trm6p co-purified with...
both wild-type Trm6p and Trm6-416p, Trm6-420p and Trm6-504p in apparent stoichiometric amounts (Figure 4A). Purified recombinant enzymes were incubated with S-adenosyl-L-[methyl-3H]methionine (3H-AdoMet) and in vitro transcribed tRNA\textsubscript{Met}. The incorporation of 3H into tRNA\textsubscript{Met} was monitored by liquid scintillation counting. Under optimal conditions, the wild-type enzyme has been found to convert a maximum of 50% of tRNA substrate to product. In the assays reported in this study, for which the results are shown as the counts per minute of 3H detected, the wild-type enzyme converted 40% of substrate to product, but the mutant enzymes lacked Mtase activity (Figure 4B). Since the trm61-3 and trm61-255 mutants were not able to complement a trm61-2 strain, we also reconstructed these mutants and co-expressed them in bacteria with TRM6 (B360 and B428, respectively). In addition, we created a mutant, called trm6-416/trm61-255 (B429), which has the three mutations in trm6-416 combined with the three mutations in trm61-255. All of these enzymes also lacked in vitro Mtase activity (Figure 4B).

While we did not observe in vitro Mtase activity for the trm6-420 and trm6-504 mutants, the HPLC analysis of tRNA from yeast expressing these Trm6p mutants had shown some m\textsuperscript{1}A was still formed in vivo, although far less efficiently than wild-type enzyme (Figure 4B). Therefore, we suspected that the low level of activity of these mutants was undetectable in our in vitro assay or indistinguishable from a negative control. Therefore, we increased the concentration of enzyme and tRNA in the assay 10-fold to try and amplify the signal-to-noise ratio and make low-level activity detectable. Under these conditions, the trm6-504 and trm6-420 mutant enzymes consistently showed Mtase activity (Figure 4B). Trm6p-420/Trm61p complexes consistently displayed more potent Mtase activity than the other Trm6p mutants, in accord with the less severe growth defect and higher level of m\textsuperscript{1}A seen in yeast expressing this mutant.

Mutations in Trm6p and Trm61p do not prevent heteroligomer formation

Based on its crystal structure, the m\textsuperscript{1}A58 Mtase from M. tuberculosis was described as a dimer of two dimers (12). The interactions between the two subunits that form one dimer are extensive, while the interactions between all four subunits are limited to a central barrel structure (12). As described above, we created alanine substitutions in Trm6p and Trm61p in order to destabilize protein–protein interactions and found that the mutant enzymes had reduced Mtase activity, although all of the mutant
enzymes were still capable of forming heterodimers (Figure 4A). We wanted to determine whether this loss of activity was due to disruption of the predicted heterotetrameric structure of the enzyme. To size mutant Trm6p/Trm61p complexes, purified recombinant enzyme was analyzed by gel filtration chromatography. The fractions that contained Trm6p and Trm61p were determined using SDS-PAGE followed by Coomassie blue staining. Since the molecular weight of Trm6p is 55 kDa, determined using SDS-PAGE and western blotting to visualize Trm6p and Trm61p. Again, the wild-type and mutant enzymes were found to have the same elution patterns (Figure 5B). In addition to gel filtration chromatography, limited proteolysis was used to try to detect structural differences between wild-type and mutant enzymes. After digestion of the wild-type and trm6-416, trm6-420, trm6-504, trm61-3, trm6-255 and trm6-416/trm61-255 enzymes with non-specific proteases for set periods of time, no differences in the cleavage pattern and rate were observed between wild-type and mutant complexes (data not shown). Overall, these experiments imply that alteration of conserved amino acids predicted to be involved in protein–protein interactions do not prevent oligomerization or cause drastic changes in the overall structure of the yeast m^1A58 Mtase. Therefore, the reduced Mtase activity observed is the result of a different defect caused by the mutations—such as loss of either substrate or co-factor binding.

**Trm6p mutants are not defective for AdoMet binding**

Previously, it was noted that TRM61 contains binding motifs for AdoMet (34), and later it was found that mutation of two conserved glycine residues in motif I destroys Mtase activity (10). However, TRM6 does not have these motifs; therefore, we did not expect the mutations present in trm6-416, trm6-420 and trm6-504 to affect AdoMet binding. Nevertheless, we wished to eliminate this as a possible reason for the reduced activity observed for these mutants, and used a ligand-binding assay which does not rely on enzymatic activity. To this end, saturation transfer difference-nuclear magnetic resonance (STD-NMR) spectroscopy was used to measure AdoMet binding to protein. STD-NMR is able to measure binding to a protein, based on transfer of magnetization from protein (which is irradiated with R_{A} energy) to any ligand that comes into contact with the protein (32,36,37). The STD signal that is measured increases in proportion to the fractional saturation of protein-binding sites with ligand. Using this method, AdoMet binding was detected when magnetization was transferred from irradiated purified protein to AdoMet. Control STD spectra collected when the protein was not irradiated were subtracted from those obtained when the protein was irradiated, so that the resulting STD spectrum would reflect only AdoMet molecules that had been in contact with the protein. The resonances corresponding to AdoMet protons were identified based on known ^1H NMR chemical shifts (38). Recombinant purified enzymes were incubated with a range of AdoMet concentrations made in Trm6p and Trm61p to the predicted protein–protein interface of the yeast Mtase disrupted oligomerization.

To be confident that the results obtained using recombinant purified enzymes reflected the structure of the enzyme in yeast, gel filtration chromatography was also performed using soluble whole cell extract from yeast strains expressing either TRM6 (Y353), trm6-416 (Y354), trm6-504 (Y360) or trm6-420 (Y367). The fractions collected from the gel filtration column were subjected to SDS-PAGE and western blotting to visualize Trm6p and Trm61p. Again, the wild-type and mutant enzymes were found to have the same elution patterns (Figure 5B). In addition to gel filtration chromatography, limited proteolysis was used to try to detect structural differences between wild-type and mutant enzymes. After digestion of the wild-type and trm6-416, trm6-420, trm6-504, trm61-3, trm6-255 and trm6-416/trm61-255 enzymes with non-specific proteases for set periods of time, no differences in the cleavage pattern and rate were observed between wild-type and mutant complexes (data not shown). Overall, these experiments imply that alteration of conserved amino acids predicted to be involved in protein–protein interactions do not prevent oligomerization or cause drastic changes in the overall structure of the yeast m^1A58 Mtase. Therefore, the reduced Mtase activity observed is the result of a different defect caused by the mutations—such as loss of either substrate or co-factor binding.

**Figure 4.** Purification and characterization of recombinant Trm6p/Trm61p complexes. (A) Wild-type and mutant enzymes were purified from E. coli whole cell extract via a polyhistidine tag on Trm6p. Samples were analyzed by SDS-PAGE and stained with Coomassie blue. (B) Fifteen nanomolar of purified recombinant enzyme was incubated with 30 μM ^3H-AdoMet and 150 nM in vitro transcribed tRNA Met (standard reaction), or 150 nM enzyme was incubated with 30 μM ^3H-AdoMet and 1.5 μM tRNA Met (10x reaction). After precipitation with 5% trichloroacetic acid, insoluble material was collected on nitrocellulose filters and ^3H was measured using liquid scintillation. The data reported is the average of duplicate experiments.

<table>
<thead>
<tr>
<th>TRM6/61 Allele</th>
<th>Standard reaction (counts/min. ^3H)</th>
<th>10X Reaction (counts/min. ^3H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRM6</td>
<td>2,542</td>
<td>26,352</td>
</tr>
<tr>
<td>trm6-416</td>
<td>69</td>
<td>147</td>
</tr>
<tr>
<td>trm6-504</td>
<td>98</td>
<td>232</td>
</tr>
<tr>
<td>trm6-420</td>
<td>80</td>
<td>598</td>
</tr>
<tr>
<td>trm61-3</td>
<td>64</td>
<td>220</td>
</tr>
<tr>
<td>trm61-255</td>
<td>64</td>
<td>62</td>
</tr>
<tr>
<td>trm6-416/trm61-255</td>
<td>57</td>
<td>88</td>
</tr>
<tr>
<td>No Enzyme</td>
<td>62</td>
<td>167</td>
</tr>
</tbody>
</table>
and then subjected to STD-NMR spectroscopy in order to generate a binding curve and an estimated $K_d$ (see Materials and Methods section) For wild-type enzyme, the $K_d$ was found to be 376 ± 73 μM (Table 2). The $K_d$ values obtained for the mutant enzymes were not considerably different, at 206 ± 35 μM for trm6-504 and 634 ± 73 μM for trm6-416 (Table 2). trm6-420 was not tested because the mutation present in trm6-420 is also found in trm6-416.

Table 2. AdoMet $K_d$ values

<table>
<thead>
<tr>
<th>TRM6/61 Allele</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRM6</td>
<td>376 ± 73</td>
</tr>
<tr>
<td>trm6-416</td>
<td>634 ± 73</td>
</tr>
<tr>
<td>trm6-504</td>
<td>206 ± 35</td>
</tr>
<tr>
<td>trm61-3</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*ND: The $K_d$ value could not be determined as only very low levels of binding were seen at the highest concentrations of AdoMet tested.

**tRNA binding is diminished in Trm6p and Trm61p mutants**

Because mutations made in Trm6p that are predicted to be located at the Trm6p/Trm61p interface did not affect oligomerization or AdoMet binding, we wanted to determine whether or not tRNA binding was altered. First, increasing amounts of purified recombinant wild-type enzyme were incubated with radiolabeled tRNA$\text{Met}^i$ purified from a trm6D strain (Y146) (7). tRNA$\text{Met}^i$ bound by Trm6p/Trm61p complexes was trapped on a nitrocellulose filter and quantitated by liquid scintillation counting. A binding curve generated from this data (Figure 6A) was used to determine the $K_d$ for tRNA to be 330 ± 80 nM. To test the tRNA-binding ability of trm6 mutant enzymes, purified enzymes (500 nM) were assayed as described above. The trm6-416, trm6-420 and trm6-504 mutants did not bind considerably more tRNA than a control reaction lacking enzyme (Figure 6B). In addition, we did not detect tRNA binding when the concentration of trm6-416 and trm6-504 complexes used was increased 10-fold (5 μM), and, therefore, could not create binding curves for these enzymes. Because the mutations in trm61-255 and trm6-416/trm61-255 are predicted to lie in a structurally similar region as the trm6 mutations, and as these enzymes form oligomers but lack activity, we tested their ability to bind tRNA. Similar to trm6-416, trm6-420 and trm6-504 mutants, the trm61-255 and trm6-416/trm61-255 mutants did not bind tRNA$\text{Met}^i$ (Figure 6B). Importantly, the trm61-3 mutant, which we have shown is defective in AdoMet binding, is able to bind tRNA$\text{Met}^i$ as effectively as the wild-type
enzyme. We conclude that the reduced m^1A levels observed in vivo and the lack of Mtase activity seen in vitro result from the inability of trm6^-416, trm6^-420, trm6^-504, trm6^-255 and trm6^-416/trm6^-255 mutants to effectively bind their tRNA substrate.

DISCUSSION

Because of the lack of information that currently exists regarding structure–function relationships of heterologomeric tRNA Mtases, we have performed a structure–function analysis of the yeast m^1A58 Mtase using a model we created as a guide. We designed a set of mutations of residues predicted to be involved in protein–protein interactions in Trm6p and Trm6lp that are also preserved in TrmI, and evaluated the effects of these mutations on enzyme function. Although we predicted these mutations would destabilize the interactions between the subunits, all mutants retained the ability to form heteroligomers, even a variant with three substitutions in Trm6p (E416A, R420A and Y422A) and three substitutions in Trm6lp (E255A, R259A and Y261A), i.e. a protein that lacked 12 functional groups in the protein–protein interface (Figure 1B and Figure S1). We then tested two tm^6^i mutants to see if AdoMet binding was affected by these mutations, and found it was not. Furthermore, Trm6p does not appear to play a role in AdoMet binding, as the trm^6^-i-3 enzyme, which has wild-type Trm6p subunits, did not bind AdoMet.

Although heterologomerization and AdoMet binding were not affected by the mutations we introduced, these mutants did exhibit strong defects in Mtase activity, which led us to discover that tRNA binding was compromised. This was an unexpected finding, considering that all of the mutated residues are predicted to be buried inside the protein and not exposed at the surface, where they could potentially interact with the tRNA substrate. In addition, our studies show that both Trm6p and Trm6lp contribute to tRNA binding, as mutations in Trm6lp alone abolished tRNA^Met binding. Previous studies had shown that Trm6lp could not bind tRNA^Met without Trm6p, leading to the hypothesis that Trm6p is responsible for tRNA binding (10). However, the data presented here suggests that both subunits help to create the architecture necessary for substrate binding. We also observed that wild-type Trm6p/Trm6lp complexes did not show an increased affinity for tRNA in the presence of S-adenosyl-l-homocysteine (data not shown). Similarly, the trm^6^-i-3 enzyme, which cannot bind AdoMet, binds tRNA as efficiently as wild-type enzyme, suggesting that AdoMet binding is not required for tRNA binding. Finally, the K_d values for AdoMet and tRNA^Met we report for the wild-type enzyme in this study are ~100-fold greater than the K_m values we reported previously, which were determined by measuring product formation in Mtase activity assays (10). It should be noted that these data are not contradictory, as K_d and K_m constants are not equivalent except in cases when the conversion of enzyme–substrate complexes to enzyme and product is a rate-limiting step. Since both K_d values are greater, we presume the conversion of enzyme–substrate to product is not a rate-limiting step.

Based on the crystal structure of TrmI, it was proposed that the tRNA substrate is bound in two clefts, one of which is found in the interface between the subunits and is lined with positively charged residues (12). We have calculated the electrostatic surface potential of the equivalent surface of the Trm6/Trm6lp tetramer, and have found that it is negatively charged (Figure S2); however, the flexible extensions that are present in Trm6p and Trm6lp but absent in TrmI (in particular regions comprising residues 265–345 in Trm6lp and 455–478 in Trm6p, Figure S2) are positively charged. This suggests that the yeast m^1A58 Mtase uses a different mechanism for tRNA recognition and binding than its prokaryotic counterpart. Alternatively, the positively charged regions of Trm6p and Trm6lp, which we cannot accurately position in our model, could be located in the interface between subunits. It would be interesting to determine whether or not the mutations that abolished tRNA
binding by the yeast Mtase would cause this same defect if present in Trm1.

Previously, it has been shown that the tRNA modification enzyme pseudouridine 55 synthase undergoes substantial conformational changes upon binding an RNA substrate (39). Unfortunately, the existing methodology does not allow modeling of large conformational changes, such as the induced fit of flexible protein loops upon the formation of a Mtase–tRNA complex. While the mutations we introduced into Trm6p and Trm61p are not exposed to the surface, they could bring about a conformational change of residues directly involved in tRNA binding. In the case of large systems such as Trm6p/Trm61p tetramer model or a model with E416, bond lengths and dihedral angles (40). A wild-type electrostatic interactions, while constraining bond angles, hydrogen bonds and van der Waals and circular contacts to minimize the potential energy due to mutated interface of Trm6p and Trm61p using the assessment of the relative stability of the wild-type and of this study. Therefore, we carried out only a preliminary assessment of the relative stability of the wild-type and mutated interface of Trm6p and Trm61p using the modeling system Sculpt (40). Sculpt optimizes intramolecular contacts to minimize the potential energy due to torsions, hydrogen bonds and van der Waals and electrostatic interactions, while constraining bond angles, bond lengths and dihedral angles (40). A wild-type Trm6p/Trm61p tetramer model or a model with E416, R420 and Y422 residues in Trm6p and E255, R259, and Y261 in Trm61p truncated beyond Cα atoms was subjected to energy minimization, with most of the molecule ‘frozen’, and only the regions 413–441 in Trm6p and 253–265 and 343–357 in Trm61p ‘thawed’ (data not shown). Using this analysis, which allowed the protein to shift to a minimum energy conformation, the structure of the mutant enzyme changed more than that of the wild-type enzyme, which changed very little. The backbone of the mutant protein is less stable because of the extra space created by the alanine substitutions, and because the interactions between side chains have been lost.

Although the above-mentioned simulation must be regarded as very preliminary and will have to be validated by more advanced computational methods and perhaps also by biophysical measurements, it generally agrees with our experimental finding that mutations of residues at the Trm6p/Trm61p interface do not disrupt oligomerization, but interfere with tRNA binding. Thus, we propose that the presence of salt bridges in the yeast m’A58 Mtase (Trm61p-E255 and Trm6p-R420, and between Trm6p-E416 and Trm61p-R259), and most likely also in bacterial Trm1 Mtases (e.g. E299-R233 in Rv2118c), serves to establish the structure of the tRNA-binding region rather than to promote binding of subunits to each other. We hypothesize that the mutations reported in this work could have long-range structural effects on the conformation of the positively charged loop (residues 265–345) in the Trm61p subunit, which may be involved in tRNA binding. By identifying amino acids involved in tRNA binding, this study has provided a foundation on which further studies can be built, such as experiments to address our hypothesis regarding tRNA binding and to determine how the yeast m’A58 Mtase is able to recognize substrate tRNAs amongst the cellular tRNA pool.

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Conflict of interest statement. None declared.

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