Identification of Critical Residues for G_{-1} Addition and Substrate Recognition by $tRNA^{His}$ Guanylyltransferase[†]

Jane E. Jackman*[,‡] and Eric M. Phizicky

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

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ABSTRACT: The yeast $tRNA^{His}$ guanylyltransferase (Thg1) is an essential enzyme in yeast. Thg1 adds a single G residue to the 5′ end of $tRNA^{His}$ (G_{-1}), which serves as a crucial determinant for aminoacylation of $tRNA^{His}$. Thg1 is the only known gene product that catalyzes the 3′−5′ addition of a single nucleotide via a normal phosphodiester bond, and since there is no identifiable sequence similarity between Thg1 and any other known enzyme family, the mechanism by which Thg1 catalyzes this unique reaction remains unclear. We have altered 29 highly conserved Thg1 residues to alanine, and using three assays to assess Thg1 catalytic activity and substrate specificity, we have demonstrated that the vast majority of these highly conserved residues (24/29) affect Thg1 function in some measurable way. We have identified 12 Thg1 residues that are critical for G_{-1} addition, based on significantly decreased ability to add G_{-1} to $tRNA^{His}$ in vitro and significant defects in complementation of a *thg1*Δ yeast strain. We have also identified a single Thg1 alteration (D68A) that causes a dramatic decrease in the rigorous specificity of Thg1 for $tRNA^{His}$. This single alteration enhances the k_{cat}/K_M for ppp-$tRNA^{Phe}$ by nearly 100-fold relative to that of wild-type Thg1. These results suggest that Thg1 substrate recognition is at least in part mediated by preventing recognition of incorrect substrates for nucleotide addition.

$tRNA^{His}$ is unique among tRNA species in that it contains an additional G residue at its 5′ end that is normally unoccupied in all other sequenced tRNA species, with the exception of a single mitochondrial $tRNA^{Phe}$ that contains a U at this position (*1, 2*). This G_{-1} residue is an important determinant for aminoacylation by the histidyl-tRNA synthetase (HisRS),[1] both in yeast and in *Escherichia coli* (*3–6*). However, the mechanism by which G_{-1} is incorporated into $tRNA^{His}$ varies. In prokaryotes and in many archaebacteria, G_{-1} is genomically encoded and is retained following cleavage of the precursor tRNA transcript by RNase P (*7*). However, in eukaryotes, and possibly some archaebacteria, G_{-1} is added posttranscriptionally by a highly conserved enzyme, the $tRNA^{His}$ guanylyltransferase (Thg1) (*8–10*). The loss of growth that results from depletion of this essential enzyme in yeast is accompanied both by loss of G_{-1} and by loss of aminoacylation of $tRNA^{His}$, consistent with the requirement for G_{-1} for charging of $tRNA^{His}$ (*9, 11*).

The G_{-1} addition reaction catalyzed by Thg1 is highly unusual, and the molecular mechanism for the catalysis of this reaction is unknown. G_{-1} addition is formally a 3′−5′ nucleotide addition reaction that occurs in three steps, beginning with the monophosphorylated $tRNA^{His}$ (p-$tRNA^{His}$) produced by RNase P cleavage of the precursor $tRNA^{His}$ transcript (*9, 10*). First, Thg1 activates the p-tRNA by adenylylation using ATP. Second, this activated intermediate is attacked by the 3′-hydroxyl of GTP, yielding G_{-1} containing $tRNA^{His}$, with the additional residue linked to the tRNA via a standard phosphodiester bond. Third, pyrophosphate is released from the 5′-triphosphorylated G_{-1}-containing $tRNA^{His}$, yielding mature, monophosphorylated, G_{-1}-containing $tRNA^{His}$.

The three reactions that comprise G_{-1} addition include multiple types of chemical transformations and also require the recognition of different nucleotide substrates in various ways. To catalyze G_{-1} addition, Thg1 must interact with the 5′ end of monophosphorylated $tRNA^{His}$, the 5′-triphosphorylated end of ATP, the 3′-hydroxyl end of GTP, and the 5′-triphosphorylated end of the tRNA nucleotide addition product. The molecular mechanism by which Thg1 accommodates these multiple catalytic demands is not known, but the relatively small size of this enzyme (∼28 kDa) suggests a significant degree of economy of function within the Thg1 active site.

Thg1 also exhibits distinctive substrate recognition properties that account for the rigorous specificity of this enzyme for $tRNA^{His}$. Despite its small size, Thg1 specifically recognizes the $tRNA^{His}$ GUG anticodon, nearly 70 Å away from its site of activity at the top of the anticodon stem. Alteration of this anticodon sequence in p-$tRNA^{His}$ leads to at least 10000-fold decreased Thg1 activity (*12*). Moreover, introduction of the GUG anticodon into non-histidyl tRNA species is sufficient to confer recognition for G_{-1} addition,

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* To whom correspondence should be addressed at 742A Biological Sciences, The Ohio State University, 484 W. 12th Ave., Columbus, OH 43210. Phone: (614) 247-8097. Fax: (614) 292-6773. E-mail: jackman.14@osu.edu.

[‡] Current address: Department of Biochemistry, The Ohio State University, Columbus, OH 43210.

[1] Abbreviations: HisRS, histidyl-tRNA synthetase; Thg1, $tRNA^{His}$ guanylyltransferase; p-tRNA, 5′-monophosphorylated tRNA; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TLC, thin-layer chromatography; 5-FOA, 5-fluoroorotic acid; ppp-tRNA, 5′-triphosphorylated tRNA.

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raising the k_{cat}/K_M values for Thg1 catalysis with these substrates to within approximately 50-fold of the value for wild-type $tRNA^{His}$ (*12*). The mechanism by which Thg1 achieves this strict substrate specificity is also unknown but is also of great interest.

There is no identifiable sequence similarity between Thg1 and any known enzyme family that might lead to specific hypotheses regarding either the molecular mechanism of the G_{-1} 3′−5′ nucleotide addition or the enzyme's ability to specifically recognize $tRNA^{His}$. While the first two steps of the Thg1 reaction (adenylylation followed by nucleotide transfer) are chemically similar to reactions catalyzed by the well-studied ligase/nucleotidyltransferase family of enzymes (*13*), Thg1 exhibits none of the documented sequence motifs of these enzymes, such as the AMP-binding domain or ATP-dependent ligase signature sequences (*14*). Similarly, the anticodon recognition properties, site of action, and the chemical steps of the reaction catalyzed by Thg1 are highly reminiscent of those used by aminoacyl-tRNA synthetases (*12*), yet no sequence similarity is observed between Thg1 and synthetases. Therefore, we have sought to identify residues that are important for this reaction.

In this study, we investigated the role of individual amino acids in the reaction catalyzed by Thg1 by altering 29 highly conserved Thg1 residues to alanine. For each of the resulting variant Thg1 proteins, we measured G_{-1} addition activity, specificity for $tRNA^{His}$, and in vivo function using a yeast complementation assay. Of the 29 positions tested, 24 of these alterations affect Thg1 function in at least one of these assays. Twelve alterations lead to significant defects in both G_{-1} addition activity and complementation, demonstrating that these 12 residues play crucial roles in Thg1 catalysis. We have also identified an aspartate residue (D68) that, when altered to alanine, results in a dramatic loss of selectivity for $tRNA^{His}$, with at least 100-fold increased k_{cat}/K_M for G_{-1} addition to $tRNA^{Phe}$, relative to that with wild-type Thg1. These results constitute the first identification of residues that are involved in catalysis of this unusual reaction.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Mutagenesis was performed by QuikChange (Stratagene) according to the manufacturer's instructions. Reactions contained 50 ng of template plasmid DNA encoding yeast His_6-Thg1 (*9*) and 125 ng of each oligonucleotide (T_m > 78 °C, 35–58 nucleotides in length). After 18 rounds of temperature cycling, the resulting DNA was transformed into *E. coli* (XL1-Blue), and isolated plasmids were sequenced to confirm the mutation.

Expression and Purification of Thg1 Variant Proteins. Thg1 alanine variant plasmids were transformed into *E. coli* BL21-DE3(pLysS) (Novagen). Cultures were grown at 30 °C in 1 L of LB media containing 100 μg/mL ampicillin until OD_{600} = 0.4, induced by addition of 1 mM isopropyl β-D-galactopyranoside, and shifted to 18 °C for approximately 20 h before harvest. Thg1 was purified by immobilized metal ion affinity chromatography, as previously described (*9*), dialyzed into buffer containing 20 mM Tris-HCl, 500 mM NaCl, 4 mM $MgCl_2$, 1 μM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 50% glycerol, pH 7.5, quantified by Bio-Rad protein assay using IgG as the protein standard, and stored at –20 °C. Typical concentrations of the resulting proteins ranged from 3 to 10 mg/mL, with the exception of Thg1-D153A (0.7–0.8 mg/mL). All proteins with the exception of Thg1-Y202A (which did not overexpress or purify to any significant degree) were >90% pure as judged by SDS−PAGE and migrated to the same position as wild-type Thg1.

Assay for G_{-1} Addition Activity Using ^{32}P-Labeled tRNA. Thg1 G_{-1} addition activity was determined using 5′-^{32}P-labeled $tRNA^{His}$ or $tRNA^{Phe}$ followed by treatment with RNase A and phosphatase, yielding $^{32}P_i$ from unreacted substrate and $G^{32}pGpC$ from G_{-1} addition products, as described previously (*12*). Reactions contained ~1 nM 5′-^{32}P-labeled tRNA (specific activity ~7000 Ci/mmol) in 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 3 mM DTT, 125 mM NaCl, 0.2 mg/mL BSA, 0.1 mM ATP, and 1 mM GTP and were initiated by addition of enzyme and carried out at room temperature for 4–5 h. Specific activity measurements were derived from 10-fold serial dilutions of enzyme (~1−0.001 μg/μL purified Thg1). Reaction products were resolved by silica thin-layer chromatography (TLC) in an 1-propanol−NH_4OH−H_2O (55:35:10) solvent system. Plates were visualized and quantified using a Storm imaging system and ImageQuant software.

Analysis of Thg1 Variant Function in Vivo. To analyze Thg1 function in vivo, Thg1 variants were inserted into a single copy (*CEN*) plasmid containing a *LEU2* selectable marker as well as the *THG1* gene and flanking regulatory sequences [*CEN LEU2* P_{THG1}-*THG1*]. Constructs were made by restriction cloning, replacing the wild-type *THG1* with the variant genes using a naturally occurring *Sna*BI site (at nucleotide +22) and an engineered *Mlu*I site (at nucleotide +645). The *Mlu*I site was created by QuikChange and does not affect the amino acid sequence at this position (T216/R217). Thg1 variant sequences were amplified by PCR from the *E. coli* expression plasmids using the following primer sequences: 5′CTAAATTTGGATACGTAAGGC3′ (containing the *Sna*BI site) and 5′ATTTCTCCTTTACGCGTTACTAGTGAACCCTTTT3′ (containing the *Mlu*I site). The resulting variant plasmids were verified by sequencing.

Testing Complementation of the thg1Δ Growth Phenotype. Single-copy yeast plasmids containing *thg1* variant genes (as described above) were transformed into yeast strain WG18 [relevant genotype: *MATα thg1Δ::kanMX his3-1 leu2Δ met15Δ ura3Δ* (*CEN URA3* P_{GAL}-*THG1*)]. Positive (Leu^+) transformants were selected at 25 °C on galactose-containing media to maintain expression of wild-type *THG1*. Then, strains were streaked onto media containing 5-fluoroorotic acid (5-FOA), to select against the wild-type *THG1 URA3* plasmid, and scored for their ability to grow at 25 °C. The 21 strains able to grow in the presence of 5-FOA (either ++ or +/− phenotype) were verified by testing all of the appropriate genetic markers, and then 19 of these that did not grow slowly were tested for temperature sensitivity. Serial dilutions (10-fold steps) of saturated overnight cultures (grown in rich media at room temperature) were spotted to plates containing either rich media containing dextrose (YPD) (*15*) or minimal synthetic complete media containing dextrose (SD) for incubation at multiple temperatures (18, 25, 30, 33, 35, and 37 °C).

Steady-State Kinetic Parameters for G_{-1} Addition to ppp-tRNA. Activity of wild-type and D68A Thg1 with ppp-$tRNA^{His}$, ppp-$tRNA^{Phe}$, and ppp-$tRNA^{Leu}$ was measured using

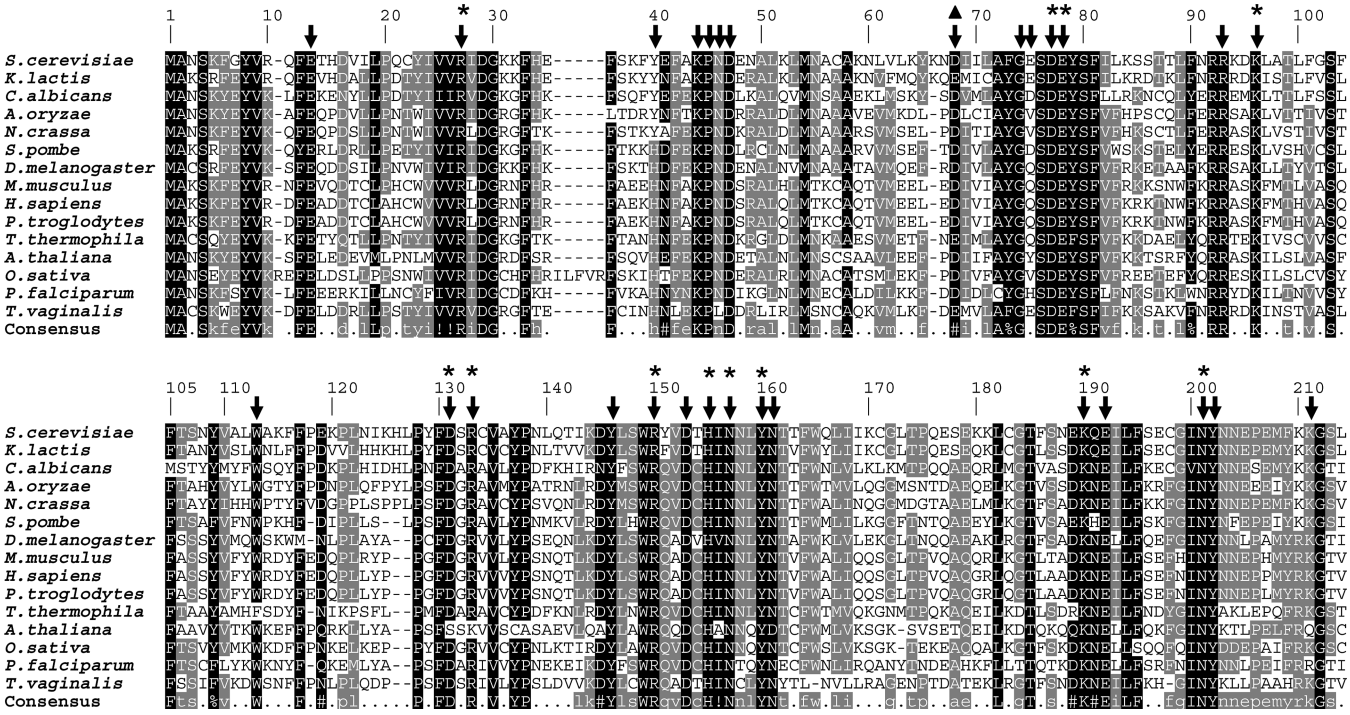

FIGURE 1: Alignment of eukaryotic Thg1 sequences. Eukaryotic Thg1 homologues were identified by BLAST search (*16*) against the yeast Thg1 sequence, and the resulting sequences were aligned using Multalin (*38*). Dark shading indicates >90% sequence identity; light shading indicates >50% sequence similarity. Positions chosen for alteration to alanine in this study are indicated by vertical arrows. The 12 positions identified as crucial for G_{-1} addition activity are indicated as starred residues, and D68 is indicated by the triangle.

a previously described assay with 5′-γ-^{32}P-labeled tRNA (*12*). Reactions were performed in the presence of saturating concentrations of GTP (1 mM) and varied concentrations of tRNA (50 nM to 50 μM for ppp-tRNA^{Phe} and ppp-tRNA^{Leu} and 50 nM to 4 μM for ppp-tRNA^{His}) in the same buffer described above for the p-tRNA assay. Reactions were initiated by the addition of enzyme under steady-state conditions (at least 5-fold excess of tRNA over Thg1), and time points were chosen to yield less than 10% formation of products. The resulting linear initial rates were plotted as a function of tRNA substrate concentration and fit to the Michaelis–Menten equation using the Kaleidagraph curve-fitting program (Synergy software) to determine k_{cat}, K_M, and k_{cat}/K_M with respect to tRNA for each enzyme and substrate tested. The estimate for the steady-state k_{cat}/K_M for wild-type Thg1 and ppp-tRNA^{Leu} was derived from the linear fit to the initial velocity vs substrate plot, which did not exhibit visible saturation, even at the highest concentrations of substrate achievable in the assay. This value was used to estimate a corresponding lower limit to k_{cat} and K_M for this enzyme–substrate pair.

RESULTS

Alanine-Scanning Mutagenesis of Highly Conserved Residues in Yeast Thg1. The overall level of sequence conservation between yeast Thg1 and its eukaryotic homologues is very high, based on BLAST analysis (*16*), reflecting the fact that Thg1 activity is likely to be required in all eukaryotes as it is in yeast, since eukaryotes universally lack a genomically encoded G_{-1} on their tRNA^{His} genes. Figure 1 shows an alignment of yeast Thg1 and homologous sequences from a variety of eukaryotic organisms representing a broad spectrum of species, including sequences from closely related fungi, distantly related fungi, insects, mammals (including humans), plants, and protozoa. Thg1 is much more highly conserved among eukaryotes than several other eukaryote-specific tRNA modification enzymes (*17, 18*). In total, 45 of the 238 residues in yeast Thg1 are absolutely conserved among these eukaryotic species, and an additional 66 residues are highly conserved (Figure 1).

To identify residues that may be important for G_{-1} addition activity, we focused on the highly conserved residues of Thg1, with a particular emphasis on amino acids with polar or charged side chains that could play an active role in catalysis. We also investigated three other highly conserved amino acids, P45, G74, and W113, and the less well-conserved E75, all of which are located within patches of highly conserved amino acids. As shown in Figure 1, these targeted residues (indicated by arrows) span nearly all of the highly conserved regions of Thg1. For each of the 29 targeted residues, we made alanine-substituted constructs and purified the resulting His_6-Thg1 variants after expression in *E. coli* for subsequent assay in vitro. The W113A variant also contained an I156V alteration that was revealed after sequencing; since I156 is also highly conserved, we included this double variant enzyme in our analysis.

Of the 29 variants, 27 are expressed at similar levels in *E. coli* based on SDS−PAGE of cell-free extracts (Figure 2, lanes a) and are each purified to near homogeneity with similar yields (Figure 2, lanes b). The similar yields and purity, combined with the stability of these variant enzymes during storage and repeated assay, suggest that the changes in activity reported here are more likely due to catalytically relevant alterations than to gross perturbations of protein structure.

Thg1-Y202A and Thg1-D153A were poorly expressed, but for different apparent reasons. The Y202A variant is present

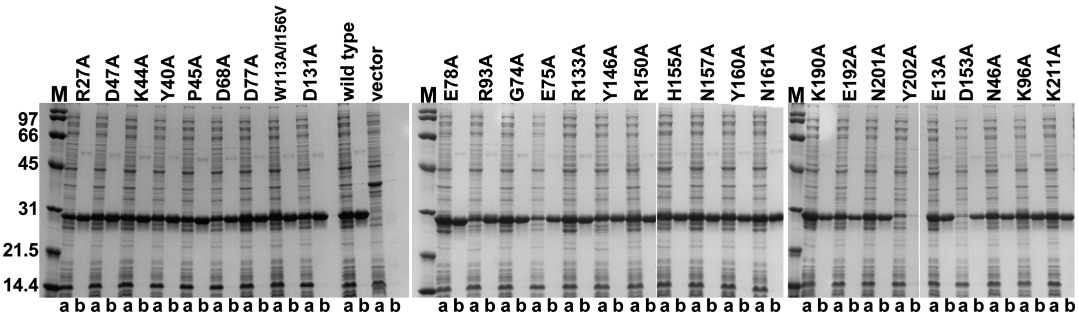

FIGURE 2: Thg1 variant protein expression and purification. Thg1 variants were analyzed by SDS−PAGE as indicated by the variant names at the top of each set of two lanes. Lanes a, a sample of the soluble crude extract from the overexpressing strain (15–20 μg of total protein each); lanes b, the resulting protein following affinity purification for each Thg1 variant (5 μg of total protein each). Crude extracts and purification from control strains overexpressing wild-type Thg1, or the empty vector only, are also shown.

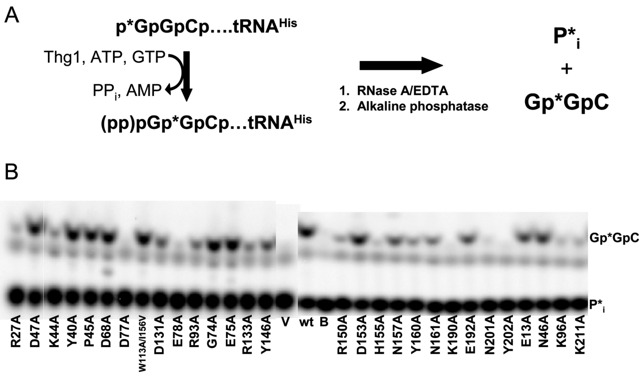

FIGURE 3: Assay for G_{-1} addition activity with p-tRNA^{His}. (A) Assay scheme using 5′-^{32}P-labeled p-tRNA^{His} as the substrate for G_{-1} addition in the presence of Thg1. Treatment of reactions with RNase A and EDTA, followed by phosphatase, results in the production of a labeled trimer (Gp*GpC) from the product species and inorganic phosphate (P*$_i$) from the remaining substrate. (B) Assay of purified Thg1 variants (1 μL each as indicated by the names below each lane) for G_{-1} addition activity with p-tRNA^{His}, performed as described above and in Materials and Methods. Lane V, mock-purified control from the strain containing the empty vector; lane wt, wild-type Thg1; lane B, buffer control.

at very low levels in soluble crude extracts and yields nearly undetectable levels of protein after purification (Figure 2), suggesting that this alteration leads to lack of overall stability. Thus, the G_{-1} addition activity of the resulting protein could not be determined. The D153A variant is also present at very low levels in the soluble crude extract (Figure 2), in large part because induction of its expression in *E. coli* is reproducibly accompanied by a marked decrease in cell growth. Nonetheless, the Thg1-D153A variant enzyme is readily purified, yielding an enzyme preparation with similar purity to the other variants, albeit with a reduced overall yield (5% of that obtained for other variants) reflecting the low levels of protein in the cell-free extracts (Figure 2). The facile purification of Thg1-D153A, coupled with the reproducible activity of the protein during assays, suggests that Thg1-D153A is stable, but its expression is somehow toxic to *E. coli*.

Assessment of in Vitro G_{-1} Addition Activity with Monophosphorylated tRNA^{His}. The purified Thg1 preparations were assayed for G_{-1} addition activity with 5′-^{32}P-labeled tRNA^{His}, in which the addition product appears as a G^{32}pGpC oligonucleotide, and unreacted substrate is converted into ^{32}P$_i$ (Figure 3A). The specific activity of each variant was quantified by titration of purified Thg1 variant enzymes and compared to the specific activity of wild-type Thg1 (Table 1).

Nineteen of the Thg1 variants exhibited 3% or less of wild-type G_{-1} addition activity. These can be further divided somewhat arbitrarily into three groups. The first group includes six variants (D77A, E78A, K96A, H155A, K190A,

Table 1: G_{-1} Addition Activity and in Vivo Complementation of Thg1 Variants

Thg1 variant	% wild-type specific activity (G_{-1} addition to p-tRNA^{His})	FOA phenotype in WG18
D153A	312	+/−[c]
wild type	112	++
E75A	105	++
P45A	62.3	++
D68A ▲	59.0	++
D47A	54.7	++
G74A	44.4	++ (ts)
Y40A	42.9	++
W113A/I156V	40.1	++ (ts)
N46A	22.6	++
E192A	3.0	++
N157A*	2.8	+/−
D131A*	2.4	−
Y146A	2.0	++ (ts)
E13A	1.0	++
N161A	0.9	++
R150A*	0.9	− −
R133A*	0.6	− −
R93A*	0.34	+/−
Y160A*	0.18	+/−[c]
R27A*	0.15	− −
K44A	0.14	++
K211A	0.10	++
K96A*	0.08	+/−
N201A	<0.05[a]	++
H155A*	0.04	+/−
K190A*	<0.04[a]	−
D77A*	<0.03[a]	− −
E78A*	<0.02[a]	− −
Y202A	ND[b]	− −
vector	<0.02[a]	− −

[a] Upper limit for G_{-1} addition activity, determined on the basis of the lack of detectable activity at the highest protein concentrations achievable in the assay. Activity results reported for the vector control are for a mock-purified preparation derived from cells containing an empty expression vector and assume an average amount of total purified protein per preparation. [b] ND = not determined for this variant. [c] Yeast strains containing these variants also exhibited a slow growth phenotype, in addition to variable colony size. Starred residues and the residue indicated with the triangle are the same as those indicated in Figure 1.

and N201A) with less than 0.1% of G_{-1} addition activity. The second group contains nine variants (E13A, R27A, K44A, R93A, R133A, R150A, Y160A, N161A, and K211A) that displayed between 0.1% and 1% of wild-type Thg1 specific activity. The third group contains the remaining four variants (D131A, Y146A, N157A, and E192A), which had only 2–3% of wild-type activity. This large number of variants with significant defects in the in vitro G_{-1} addition reaction is consistent with the highly conserved nature of the amino acids selected for this study and the critical function of Thg1 in eukaryotes.

A fourth group contained the remaining nine Thg1 variants, which had little or no defect in G_{-1} addition activity with tRNA^{His} (Table 1). Of these nine, three were the variants at positions without chemically active functional groups (P45, G74A, and W113A/I156V). Notably, the Thg1 D153A variant that resulted in toxic effects when overexpressed in *E. coli* is among this group, and in fact, this variant appears to be even more active than wild-type Thg1, displaying at least 3-fold greater G_{-1} addition activity under these conditions.

Assessment of the Ability of Thg1 Variants to Complement Growth Defects in a thg1Δ Mutant Strain. Since G_{-1} addition to tRNA^{His} is essential in yeast, the in vivo effects of these alterations could be assessed by determining whether expression of these Thg1 variants could support growth of a yeast strain. To this end, we transformed a collection of single copy *LEU2* plasmids encoding each Thg1 variant under control of the endogenous P_{THG1} promoter into a *thg1*Δ strain that also contains a *URA3* plasmid for galactose-inducible expression of wild-type *THG1* and then removed the wild-type *THG1* plasmid by selection on media containing 5-FOA. All of the Thg1 variants were successfully transformed under these conditions with no loss of viability, indicating no dominant negative effects caused by expression of the altered Thg1 proteins along with wild-type Thg1.

Of the 29 variant strains, six (*thg1-R27A*, *D77A*, *E78A*, *R133A*, *R150A*, and *Y202A*) were unable to grow at all on 5-FOA, appearing identical to a vector control strain lacking *THG1* (indicated by the "− −" phenotype in Table 1). The lack of growth of the *thg1-Y202A* mutant strain is consistent with the apparent folding defect of the corresponding enzyme that was observed in vitro. The lethality of the other five *thg1* mutant strains is consistent with the very low G_{-1} addition activity of the corresponding variant enzymes, two of which (D77A and E78A) are the two most inactive enzymes (Table 1). In addition, two other strains (*thg1-K190A* and *thg1-D131A*) are severely compromised for growth on 5-FOA (indicated by the "−" phenotype in Table 1), also consistent with their defective in vitro activity (Table 1). Thus an important role for these seven residues in Thg1 catalysis is corroborated by the lack of growth observed here.

Six mutant strains (*thg1-D153A*, *N157A*, *R93A*, *Y160A*, *K96A*, and *H155A*) formed single colonies on 5-FOA, but of variable size ("+/−" phenotype, Table 1). When the large and small colonies were subsequently streaked separately onto nonselective (YPD) media, the same mixed population of colonies was observed, indicating that the variable colony size is not a heritable property. Two of these strains (*thg1-D153A* and *thg1-Y160A*) also grew more slowly than the other strains, including the wild-type *THG1* strain. Five of these six strains contain mutations in Thg1 that cause defects in G_{-1} addition activity, but the sixth strain (*thg1-D153A*) contains a Thg1 variant that displays the highest level of G_{-1} addition activity in vitro (even greater than wild-type Thg1). Thus, the growth defect observed with the *thg1-D153A* strain may be due to some other function of this enzyme in vivo, perhaps unrelated to its function in G_{-1} addition to tRNA^{His}.

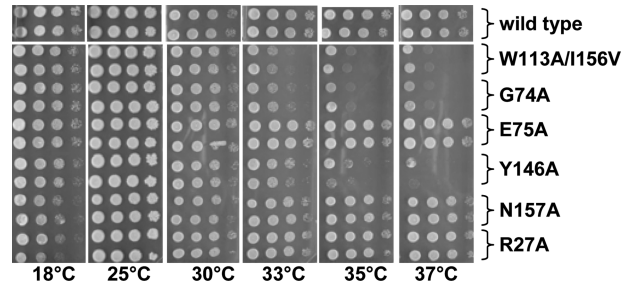

FIGURE 4: Temperature-sensitive growth phenotype of *thg1* variant strains. Viable strains containing Thg1 variant plasmids as the sole source of Thg1 activity (isolated after streaking onto 5-FOA at 25 °C) were grown overnight to saturation, plated on YPD media in serial 10-fold dilutions (starting from $OD_{600} \sim 2$), and allowed to grow at the indicated temperatures. The other 13 viable strains were also tested for temperature sensitivity but were omitted for clarity since they grew identically to the strain expressing wild-type Thg1.

Thus, of the 13 *thg1* strains with observable growth phenotypes on 5-FOA (five − −, not including *thg1-Y202A*, two −, and six +/−), 12 contain alterations in Thg1 that also significantly affect G_{-1} addition activity (indicated by starred positions on Figure 1 and Table 1). The agreement between the results of these two assays suggests that these 12 residues are catalytically important for G_{-1} addition to tRNA^{His}.

All viable *thg1* mutant strains that did not grow slowly (15 ++ and 4 +/−) were further tested for temperature sensitivity of growth, on both rich and minimal media. Most of the strains grew well at all temperatures tested (18, 25, 30, 33, 35, and 37 °C), compared to the control wild-type *THG1* strain (Figure 4 and data not shown). However, three of the strains (*thg1-G74A*, *W113A/I156V*, and *Y146A*) were strongly temperature sensitive, with little or no growth observed at 33 °C and higher (Figure 4). Although defects in G_{-1} addition are not observed for two of these temperature-sensitive variants, these residues may play more subtle roles in Thg1 function that are not immediately obvious from the limited number of assays employed here.

The remaining 12 Thg1 variants completely complement the *thg1* mutant strain, resulting in wild-type growth ("++" phenotype, Table 1). Half (6/12) of these strains (*thg1-Y40A*, *P45A*, *N46A*, *D47A*, *D68A*, and *E75A*) correspond to six of the nine Thg1 variants with near normal levels of G_{-1} addition activity (Table 1). Of the remaining six strains, three (*thg1-E13A*, *N161A*, and *E192A*) are altered at positions that resulted in moderate defects in G_{-1} addition activity in vitro (0.9−3% of wild type), perhaps indicating that moderate loss of Thg1 activity can be tolerated by the cell, although there may also be increased expression of these Thg1 variants due to their presence on extrachromosomal plasmids. Alternatively, the in vitro activity of these purified variant proteins may not accurately reflect their ability to catalyze G_{-1} addition in vivo. Somewhat surprisingly, the remaining three strains (*thg1-K44A*, *N201A*, and *K211A*) exhibited strong

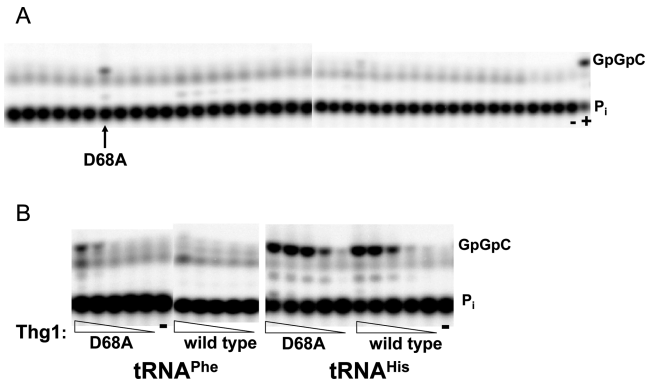

FIGURE 5: The Thg1-D68A variant catalyzes G_{-1} addition to p-tRNA^{Phe}. (A) Assay of all purified Thg1 variants (1 μL each) for G_{-1} addition activity with 5′-^{32}P-labeled tRNA^{Phe}. This assay yields the same Gp*GpC reaction products following G_{-1} addition as tRNA^{His}, due to the presence of identical 5′-nucleotide sequences in both tRNA species. The reaction containing D68A Thg1 is indicated by the arrow. Lane −, buffer only control reaction; lane +, positive control reaction for activity of wild-type Thg1 with p-tRNA^{His}. (B) Simultaneous assay of wild-type and D68A Thg1 for activity with 5′-^{32}P-labeled tRNA^{Phe} and tRNA^{His}. Enzyme assays with each tRNA substrate contained titrations of each enzyme as indicated (10-fold dilutions). Lanes −, buffer only control for each tRNA substrate.

complementation, yet the corresponding variant enzymes are extremely defective for G_{-1} addition activity with p-tRNA^{His} in vitro (≤0.1% of wild-type activity, Table 1). The possible significance of this result is discussed further below.

Alteration of D68 to Alanine Allows for Altered tRNA Substrate Recognition. Wild-type Thg1 is highly specific for monophosphorylated tRNA^{His} over other tRNA species, consistent with the crucial role of G_{-1} as the main determinant of tRNA^{His} identity in vivo. In fact, assay of 5′-^{32}P-labeled tRNA^{Phe} results in no detectable G_{-1} addition with wild-type Thg1 in vitro, even at very high protein concentrations and for long assay times (*12*). The vast majority of the assayable Thg1 variants (27/28) exhibit the same lack of activity with 5′-^{32}P-labeled tRNA^{Phe} as wild-type Thg1 (Figure 5A). However, a single variant enzyme, Thg1-D68A, catalyzes the addition of G_{-1} to p-tRNA^{Phe} at significant levels (Figure 5A). Indeed, Thg1-D68A is only about 100-fold less active with p-tRNA^{Phe} than with p-tRNA^{His} when both substrates were assayed simultaneously (Figure 5B). By comparison, wild-type Thg1 exhibits a >10000-fold difference in activity between the same substrates (*12*). Thus, the improvement of activity with p-tRNA^{Phe} is at least 100-fold for the Thg1-D68A variant.

To quantify the improvement of Thg1-D68A activity with tRNA^{Phe}, we used ppp-tRNA substrates instead of p-tRNA substrates, since wild-type Thg1 catalyzes G_{-1} addition to ppp-tRNA^{Phe} at measurable rates, albeit with approximately 1000-fold lower catalytic efficiency than for its cognate substrate, ppp-tRNA^{His} (Table 2). Therefore, using ppp-tRNA substrates allows for a quantitative comparison of the change in substrate specificity caused by the D68A alteration. The steady-state kinetic parameters for G_{-1} addition to ppp-tRNA species were measured using a previously described ppp-tRNA assay that follows release of the labeled pyrophosphate moiety upon G_{-1} addition (Figure 6, Table 2) (*12*). For this set of experiments, wild-type and Thg1-D68A enzymes were purified and assayed simultaneously to ensure that there were no preparation-dependent variations in activity; however, there was no evidence for any such variability, since the data described below agree well with previous Thg1 activity measurements (*12*).

As was observed with p-tRNA^{Phe}, Thg1-D68A is substantially more active than wild-type Thg1 with ppp-tRNA^{Phe}, exhibiting a k_{cat}/K_M for this substrate that is improved by nearly 100-fold, bringing the catalytic efficiency to within 10-fold of that exhibited by wild-type Thg1 with ppp-tRNA^{His}. This increase in activity is primarily driven by a substantial decrease in the K_M for ppp-tRNA^{Phe} compared to that of the wild-type enzyme. However, the steady-state kinetic parameters, including k_{cat}/K_M, measured for both enzymes with ppp-tRNA^{His} are nearly identical (Table 2), and moreover, these parameters are consistent with the previously published values for wild-type Thg1 (*12*).

The increased activity exhibited by Thg1-D68A is not restricted to ppp-tRNA^{Phe} but is also observed with ppp-tRNA^{Leu} (Table 2). Although the k_{cat} and K_M values for ppp-tRNA^{Leu} with wild-type Thg1 cannot be directly measured, due to the high tRNA concentrations that are necessary to saturate the observed initial rate, the estimate of k_{cat}/K_M obtained from the initial linear portion of the rate vs substrate plot indicates that the magnitude of the increased Thg1-D68A activity with ppp-tRNA^{Leu} is similar to that observed with ppp-tRNA^{Phe} (Table 2).

DISCUSSION

Thg1 is an essential eukaryotic enzyme that catalyzes a complicated set of reactions in order to add a G residue to the 5′ end of tRNA^{His}. Using the highly conserved amino acid sequence of Thg1 as a guide, we have altered 29 residues in yeast Thg1 to alanine in order to interfere with their function. To identify residues that are critical for this unusual activity, we determined the effects of these 29 alterations on in vitro G_{-1} addition activity, in vivo complementation of the *thg1*Δ mutant, and specificity for tRNA^{His}. Twelve Thg1 residues, indicated by starred positions on Figure 1 and Table 1 (R27, D77, E78, R93, K96, D131, R133, R150, H155, N157, Y160, and K190), are crucial for function, since the corresponding variants have defects in both G_{-1} addition and complementation. Only a single alteration (D153A) causes a significant growth defect that does not correlate with decreased G_{-1} addition activity, and the high level of activity (even greater than wild type) measured with this variant suggests that another Thg1 function may have been affected by this alteration (Table 1). An additional six positions (E13, K44, N161, E192, N201, and K211) may be important for catalysis, since the alanine variants have substantially reduced activity in vitro, yet there is no identifiable phenotype of the corresponding yeast *thg1* mutant strains (Table 1). Alterations at three other positions (G74, W113/I156, and Y146) result in a temperature-sensitive growth of the *thg1* mutant strains (Figure 4). Finally, a single variant (D68A, indicated with the triangle in Figure 1 and Table 1) demonstrated dramatically altered substrate specificity compared to the wild-type enzyme, which is rigorously specific for tRNA^{His} (Figures 5 and 6). Thus, of the 29 positions investigated, only five exhibited no effects on Thg1 function in any of the limited number of assays performed so far. These results are an important first step in the definition of

Table 2: Steady-State Kinetic Parameters for G_{-1} Addition to ppp-tRNA Species

substrate	enzyme	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	$(k_{cat}/K_M^{D68A})/(k_{cat}/K_M^{wild\ type})$
ppp-tRNA^{Phe}	D68A	0.62 ± 0.04	0.41 ± 0.10	417 ± 78	88
	wild type	$0.43^a \pm 0.06$	25 ± 8.0	4.7 ± 0.83	
ppp-tRNA^{His}	D68A	3.8 ± 0.29	0.24 ± 0.06	4360 ± 860	0.78
	wild type	$3.2^a \pm 0.35$	0.16 ± 0.06	5580 ± 1670	
ppp-tRNA^{Leu}	D68A	0.18 ± 0.02	1.3 ± 0.30	39 ± 5.8	38
	wild type	$>0.18^b$	$>50^b$	$1.0^b \pm 0.08$	

[a] Values for k_{cat} were determined using wild-type Thg1 purified at the same time as D68A Thg1 and are within 2-fold of previously published values determined with another preparation of the wild-type enzyme. [b] The k_{cat}/K_M value was measured from the slope of the linear initial rate vs substrate plot, due to the inability to reach saturating tRNA concentrations for ppp-tRNA^{Leu}, and was used to determine a lower limit to k_{cat} and K_M for the reaction with this substrate.

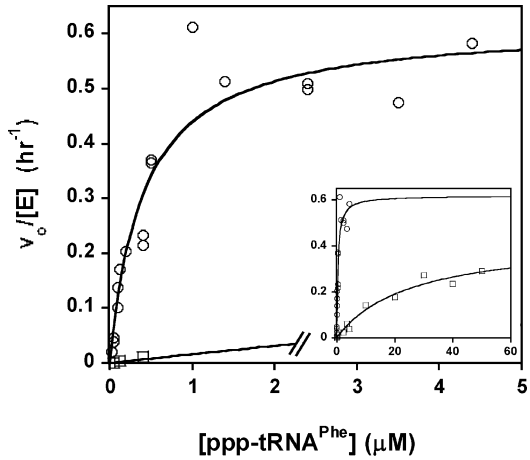

FIGURE 6: Steady-state kinetic parameters for G_{-1} addition to ppp-tRNA^{Phe}. G_{-1} addition activity was measured for wild-type (□) and D68A (○) Thg1 as a function of increasing ppp-tRNA^{Phe} substrate. The solid lines indicate the fit of the resulting initial rate vs substrate data to the Michaelis–Menten equation to yield kinetic parameters $k_{cat} = 0.62 \pm 0.04$ h^{-1}, $K_M = 0.41 \pm 0.10$ μM, and $k_{cat}/K_M = 417 \pm 78$ M^{-1} s^{-1} for D68A Thg1 and $k_{cat} = 0.43 \pm 0.06$ h^{-1}, $K_M = 25 \pm 8.0$ μM, and $k_{cat}/K_M = 4.7 \pm 0.83$ M^{-1} s^{-1} for wild-type Thg1. The inset contains the same data, plotted to show the complete ppp-tRNA^{Phe} concentration dependence of activity for wild-type Thg1.

individual amino acids that play important roles in the mechanism of this remarkable protein.

Although there is strong conservation throughout the entire Thg1 protein sequence in eukaryotes, the majority of the 12 residues that are important for the G_{-1} addition function of Thg1 are located in the C-terminal half of the enzyme (Figure 1). These residues are particularly concentrated in a patch of around 20 amino acids from position 143 to position 163 of the yeast Thg1 sequence, including an extremely well conserved patch that contains four of the 12 crucial residues (R150, H155, N157, and Y160). Therefore, this region seems to be centrally important for G_{-1} addition activity and may represent a "core" region of active site residues. However, it is notable that this region in particular is not well conserved in the amino acid sequences of the growing number of archaebacterial Thg1 homologues that have been identified (*9*). In fact, a protein residue that is analogous to the critical H155 residue in yeast Thg1 is completely absent from archaebacterial Thg1 sequences. Instead, a tryptophan residue is nearly universally found at this position in these species, and no histidine residues are present even at nearby positions. This may be indicative of significant mechanistic differences between the reaction mechanisms of eukaryotic and archaebacterial Thg1 that could potentially affect the properties of Thg1 enzymes from these two domains of life.

The 12 important residues that have been identified in this study are good candidates for residues with direct involvement in the catalysis of G_{-1} addition. However, the prediction of specific roles for these residues is difficult due to the lack of any identifiable homology between Thg1 and any other known enzyme family. Nonetheless, inspection of the chemistry of the Thg1 reaction in comparison to enzymes with known catalytic mechanisms can be used to suggest some possibilities. Activation of a nucleotide sugar hydroxyl group for nucleophilic attack by general base catalysis is a common mechanistic theme (*19–21*), and replacement of the general base residue with alanine in ribonuclease A, phosphatidylinositol phospholipase C, or adenylyl cyclase leads to substantial ($>10^4-10^5$-fold) inactivation of these enzymes (*21–23*), similar to the effects seen with several Thg1 variants (Table 1). Active site metal ions play significant roles in many reactions with nucleic acid substrates, including in the well-known two metal ion mechanism for catalysis of phosphoryl transfer reactions by polymerases and exonucleases (*24–26*). Removal of the protein ligands for these metal ions often results in significantly decreased catalytic activity (*27, 28*). Aminoacyl-tRNA synthetases share several features with Thg1, including strikingly similar chemical reactions and determinants for substrate recognition (*12*). These enzymes make use of active site strain and dynamics for proper positioning of substrates for catalysis and stabilization of the relevant transition states (*29–31*). In this respect, fully half of the residues that are important for Thg1 activity are either arginine or lysine which, due to their presumed positive charge, may be important for maintaining specific contacts with negatively charged tRNA and/or nucleotide substrates for Thg1 activity. Since Thg1 is not related to any enzyme family, further investigation of these 12 variants will reveal insights into the specific nature of the mechanism employed by this remarkable enzyme and possibly lead to the identification of unique mechanistic features that have not been previously seen with other enzymes.

Three of the residues investigated in this study (K44, K211, and N201) were exceptional because of the observation of extreme defects in G_{-1} addition activity with p-tRNA^{His} ($\leq 0.1\%$ of wild type) but wild-type growth of the corresponding *thg1* mutant strains. This discrepancy may have important implications for G_{-1} addition in vivo since G_{-1} is strictly required for growth as an identity element for HisRS, and therefore these Thg1 variants are presumably able to catalyze this reaction in vivo. While it is possible that the low in vitro activity of these variants does not accurately reflect activity in the context of an intact cell, this is less probable due to the extreme disparities between in vitro and in vivo activity observed here. Rather, these results

suggest another possibility, that the defective activity of these variants can be supplied by another in vivo function. The in vitro assay for G_{-1} addition used in this study measures two steps of Thg1 catalysis, adenylylation of the p-tRNA^{His} substrate and guanylyl transfer to add the G_{-1} nucleotide. Thus, variants that are only defective for the first step would still exhibit defects in overall G_{-1} addition activity. If additional cellular mechanisms exist to compensate for this adenylylation defect, perhaps involving another way to activate the 5′ end of the tRNA, these would allow the cell to bypass the need for Thg1 that is defective for this step of the reaction. Further investigation of the specific nature of the in vitro defect(s) of these variants is required to address this possibility.

The intriguing behavior of the D153A Thg1 variant raises the possibility that the observed growth defect of the *thg1-D153A* mutant strain is due to an inability of this variant to function in another cellular process, unrelated to tRNA^{His} G_{-1} addition. Although this investigation was focused primarily on tRNA^{His} G_{-1} addition activity, and identification of residues that are involved in this reaction, Thg1 also catalyzes a templated 3′−5′ polymerization reaction, resulting in the addition of multiple nucleotides to the 5′ end of certain tRNA substrates in vitro (*32*). Thg1 is the only gene product that is known to catalyze this type of reaction, although there are some similarities between 3′−5′ polymerization and a protozoan mitochondrial tRNA editing activity, for which the enzyme(s) that is (are) responsible is (are) unknown (*33, 34*). The discovery of Thg1 3′−5′ polymerase activity was unexpected, since this activity is quite distinct from G_{-1} addition, particularly with respect to the ability of Thg1 to recognize Watson–Crick G-C and C-G base pairs for polymerization while adding only a single G opposite an A residue for G_{-1} addition. In addition, unexplained severe cell-cycle progression defects have been associated with a *thg1*^{ts} yeast mutant, as well as with silencing of human Thg1 in a cell culture system (*35, 36*).

The distinct nature of G_{-1} addition and 3′−5′ polymerization, combined with the intriguing phenotypes described above, has led to the suggestion that there may be some alternative biological function for Thg1, beyond its known physiological role in G_{-1} addition to tRNA^{His}, which takes advantage of its ability to catalyze the 3′−5′ polymerization reaction. In this respect, nearly all of the Thg1 variant strains that are defective for growth have a corresponding defect in G_{-1} addition to tRNA^{His} of the purified variant enzyme, such that the effects on G_{-1} addition can explain the observed growth phenotypes. However, the *thg1-D153A* variant strain exhibits a defective growth phenotype that is characterized by variable colony size and slow growth, while the purified Thg1-D153A enzyme exhibits strong (even stronger than wild type) G_{-1} addition activity with tRNA^{His} (Table 1). Thus, this position may be important for cellular function(s) of Thg1 other than G_{-1} addition, and further investigation of its properties may provide insight into additional physiological role(s) for this enzyme.

The rigorous substrate specificity exhibited by yeast Thg1 for p-tRNA^{His} is consistent with the crucial nature of G_{-1} addition for recognition of the tRNA by the corresponding HisRS to avoid misacylation of this tRNA in vivo. The discovery of the single variation at D68 that dramatically alters this substrate specificity is intriguing, particularly since the presumed abrogation of functionality by replacement of the carboxyl group with a single methyl group results in a gain of function phenotype, i.e., the ability to recognize a previously unrecognized tRNA substrate. This is suggestive of a mechanism of tRNA substrate recognition that relies, at least in part, on prevention of interactions with inappropriate tRNA substrates in order to select for the correct substrate for G_{-1} addition. The molecular basis for this substrate recognition is unknown but may involve a direct interaction between D68 and the tRNA^{His} anticodon. In fact, alteration of several amino acids in HisRS that are implicated in anticodon recognition similarly affects K_M but not k_{cat} for aminoacylation of certain tRNA^{His} substrates (*37*).

The magnitude of the in vitro effect on substrate specificity exhibited by the Thg1-D68A variant is significant, with nearly 100-fold increased catalytic activity with ppp-tRNA^{Phe} compared with wild-type Thg1 and at least a similar effect with p-tRNA^{Phe}. However, since the k_{cat}/K_M for ppp-tRNA^{His} is unaffected by alteration of D68 to alanine, this variant enzyme still catalyzes G_{-1} addition more efficiently with ppp-tRNA^{His} than with ppp-tRNA^{Phe} ($k_{cat}/K_M = 4360\ M^{-1}\ s^{-1}$ vs $417\ M^{-1}\ s^{-1}$) (Table 2). Thus, tRNA^{His} is still likely the preferred substrate for G_{-1} addition by D68A Thg1 in vivo, which would account for the lack of a growth phenotype observed for the corresponding *thg1-D68A* strain. It is possible that other cellular conditions, such as decreased levels of tRNA^{His} expression or combination of this variation with other amino acid alterations to simultaneously decrease activity with tRNA^{His}, may be used to exacerbate this discrepancy, thus resulting in an observable growth phenotype due to promiscuous addition of G_{-1} to other tRNA species and resulting misacylation with histidine in those cells.

The work presented here provides a framework for more detailed investigation of the multifunctional Thg1 enzyme. The identification of 12 important amino acids for Thg1 activity will provide a starting point for the elucidation of the molecular mechanism of this unprecedented and unusual enzyme activity, and the identification of the single residue that plays an important role in tRNA^{His} substrate definition will be used to address questions regarding substrate selection and the definition of optimal substrates for each of the activities of Thg1.

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