tRNA^{His} guanylyltransferase adds G₋₁ to the 5' end of tRNA^{His} by recognition of the anticodon, one of several features unexpectedly shared with tRNA synthetases

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ABSTRACT

All eukaryotic tRNA^{His} molecules are unique among tRNA species because they require addition of a guanine nucleotide at the -1 position by tRNA^{His} guanylyltransferase, encoded in yeast by *THG1*. This G₋₁ residue is both necessary and sufficient for aminoacylation of tRNA by histidyl-tRNA synthetase in vitro and is required for aminoacylation in vivo. Although Thg1 is presumed to be highly specific for tRNA^{His} to prevent misacylation of tRNAs, the source of this specificity is unknown. We show here that Thg1 is >10,000-fold more selective for its cognate substrate tRNA^{His} than for the noncognate substrate tRNA^{Phe}. We also demonstrate that the GUG anticodon of tRNA^{His} is a crucial Thg1 identity element, since alteration of this anticodon in tRNA^{His} completely abrogates Thg1 activity, and the simple introduction of this GUG anticodon to any of three noncognate tRNAs results in significant Thg1 activity. For tRNA^{Phe}, k_{cat}/K_M is improved by at least 200-fold. Thg1 is the only protein other than aminoacyl-tRNA synthetases that is known to use the anticodon as an identity element to discriminate among tRNA species while acting at a remote site on the tRNA, an unexpected link given the lack of any identifiable sequence similarity between these two families of proteins. Moreover, Thg1 and tRNA synthetases share two other features: They act in close proximity to one another at the top of the tRNA aminoacyl-acceptor stem, and the chemistry of their respective reactions is strikingly similar.

Keywords: tRNA^{His}; tRNA^{His} guanylyltransferase; anticodon; tRNA synthetase; yeast

INTRODUCTION

Efficient translation by the ribosome requires two seemingly opposing evolutionary demands, requiring all tRNA species to be similar enough for accommodation at a single universal active site, while retaining enough unique features for each species to be specifically recognized by its own aminoacyl-tRNA synthetase and therefore maintain fidelity during translation. As a result, while all tRNA molecules share a basic secondary and tertiary structure, there is much diversity in both primary sequence and post-transcriptional modifications of the individual tRNA species that serve to differentiate them from one another (Sprinzl et al. 1998; Marck and Grosjean 2002).

One important way in which tRNA species differ is in their identity elements, the specific set of nucleotides that is

necessary and sufficient for aminoacylation of a tRNA species by its cognate tRNA synthetase. Although the identity elements vary substantially for individual tRNAs, the majority are located in either the aminoacyl-acceptor stem, with the discriminator nucleotide at position 73 often playing a substantial role, or in the anticodon, particularly at position 35 (Giege et al. 1998).

tRNA^{His} species contain a unique identity element, an additional universally conserved G residue at the -1 position. Whereas all tRNA^{His} species have G₋₁, only one other tRNA of the ~560 that have been characterized has *any* nucleotide at that position (Schnare et al. 1985; Sprinzl et al. 1998). In prokaryotes, G₋₁ is genome-encoded and retained during tRNA maturation because RNase P recognizes an additional G₋₁:C₇₃ base pair and cleaves the pre-tRNA at the -1 position (Orellana et al. 1986). In contrast, in eukaryotes G₋₁ is added post-transcriptionally across from A₇₃ by tRNA^{His} guanylyltransferase (Cooley et al. 1982), encoded in yeast by the essential *THG1* gene (Gu et al. 2003). G₋₁ addition by Thg1 is one of only two known examples of extension of a polynucleotide in the 3'-5' direction (Lonergan and Gray 1993a,b). G₋₁ is crucial for aminoacylation of tRNA^{His} in

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vivo, since conditional depletion of Thg1 causes accumulation of tRNA^{His} molecules lacking both G₋₁ and aminoacylation, although the tRNA is otherwise mature (Gu et al. 2005). Furthermore, the absence of G₋₁ in tRNA^{His} reduces charging in vitro by 740-fold, and its presence in other tRNAs can increase histidylolation ~230-fold (Rudinger et al. 1994; Nameki et al. 1995; Rudinger et al. 1997). Since G₋₁ addition is crucial for tRNA^{His} identity, Thg1 must be highly specific for tRNA^{His} to prevent misacylation of other tRNAs.

G₋₁ addition by Thg1 occurs by a three-step mechanism using the physiological monophosphorylated tRNA^{His} (p-tRNA) substrate that arises from RNase P cleavage of the precursor tRNA. First, p-tRNA is adenylylated at the 5' end with ATP, releasing pyrophosphate and generating an activated tRNA in which the AMP moiety is joined to the tRNA by a 5'-5' pyrophosphate (Ap-p-tRNA). Second, GTP is added to the 5' end of the tRNA by a guanylyltransfer step, in which the 3' OH of GTP attacks the pyrophosphate of the adenylylated tRNA, releasing AMP and yielding tRNA with the additional GTP residue on its 5' end in normal phosphodiester linkage (Fig. 1A; Jahn and Pande 1991; Gu et al. 2003). Finally, a Thg1-associated pyrophosphatase activity removes the pyrophosphate from G₋₁ yielding mature, monophosphorylated tRNA^{His}. Thg1 can also effect guanylyltransfer with triphosphorylated RNA species (ppp-tRNA), as occurs with synthetic transcripts (Gu et al. 2003); in this case the 5' triphosphate substitutes for the activated adenylylated intermediate, bypassing the requirement for ATP with monophosphorylated tRNA^{His} (Fig. 1B).

Here we investigate the source of Thg1 specificity for its tRNA^{His} substrate. We demonstrate that Thg1 specificity lies predominantly at the adenylylation step of the reaction, since Thg1 is >10,000-fold more selective for p-tRNA^{His} compared with p-tRNA^{Phe} but is only 600-fold more selective for ppp-tRNA^{His} over ppp-tRNA^{Phe}. We also show that the source of this selectivity is the tRNA^{His} anticodon, which is a very strong identity element that is both necessary and sufficient for Thg1 recognition of tRNA^{His} and G₋₁ addition activity. Recognition of the tRNA anticodon is one of several features that Thg1 and tRNA synthetases have in common, although the proteins are seemingly unrelated in overall sequence.

FIGURE 1. Thg1 reaction scheme with either monophosphorylated (A) or triphosphorylated (B) tRNA species.

RESULTS

Thg1 specificity for tRNA^{His} lies primarily at the adenylylation step

To measure the specificity of Thg1 for tRNA^{His}, we developed a sensitive in vitro assay for Thg1 activity using the physiologically relevant p-tRNA species, and compared the activity of Thg1 on p-tRNA^{His} with its activity on other p-tRNA species.

The p-tRNA assay exploits the phosphatase resistance that occurs upon addition of G₋₁ to 5'-[³²P]-tRNA species. After reaction of labeled p-tRNA with Thg1 in the presence of ATP and GTP, treatment with RNase A and phosphatase yields either [³²P]_i from unreacted substrate, or internally labeled G[³²P]GpC after G₋₁ addition, which are easily resolved by thin layer chromatography (TLC) (Fig. 2A). Formation of the GpGpC product is completely dependent on the addition of Thg1, GTP, and ATP to the assay and is time-dependent, with linear kinetics readily observed under steady-state reaction conditions (data not shown). Steady-state kinetic analysis of Thg1 activity with respect to p-tRNA^{His} (at saturating concentrations of ATP and GTP) yields a K_M of 1.7 μM and k_{cat} of 0.73 h⁻¹ for G₋₁ addition (Fig. 2B; Table 1). These parameters for Thg1 activity were determined using yeast Thg1 purified from *E. coli* rather than from yeast, since this preparation yields protein of similar specific activity but of higher concentration and slightly higher purity than the yeast-purified enzyme. Although the observed kinetic parameters are somewhat low, the specific activity of purified Thg1 is not further enhanced under multiple different reaction conditions, by removal or changing position of the His₆ affinity purification tag, or by the use of Thg1 protein purified from yeast instead of from *Escherichia coli*.

Examination of a series of p-tRNA substrates with this assay demonstrates that Thg1 is highly specific for p-tRNA^{His} relative to other p-tRNA species. In contrast to p-tRNA^{His}, no detectable Thg1 activity is observed with each of several other p-tRNA species, including p-tRNA^{Phe}, p-tRNA^{Leu}, p-tRNA^{Gly}, or p-tRNA^{Glu}, even at the highest enzyme concentration examined (Fig. 2C). Closer examination of Thg1 activity with p-tRNA^{Phe} under single turnover reaction conditions reveals no detectable products with 21 μM Thg1 and p-tRNA^{Phe} after 4 h, whereas 50 nM Thg1 produces quantifiable reaction products from p-tRNA^{His} within 10 min (data not shown). Based on these data, we estimate that Thg1 is >10,000-fold more selective for p-tRNA^{His} than for p-tRNA^{Phe} (Table 1).

Since Thg1 can bypass the adenylylation step for G₋₁ addition by using ppp-tRNA, we also wanted to determine if Thg1 exhibits a similar specificity for ppp-tRNA^{His} relative to other ppp-tRNAs. To measure Thg1 activity with ppp-tRNA, we incubated [γ-³²P]-labeled ppp-tRNA with Thg1 and GTP, and measured the release of [³²P]-labeled

FIGURE 2. Thg1 is specific for $p\text{-tRNA}^{\text{His}}$ over other $p\text{-tRNA}$ species. (A) Thg1 assay using 5' [³²P]-labeled $tRNA^{\text{His}}$. After treatment with RNase A and phosphatase (schematic) Thg1 reaction yields product trimer (Gp*GpC) and inorganic phosphate derived from unreacted substrate. The assay shown (for $p\text{-tRNA}^{\text{His}}$) contains fivefold serial dilutions of purified Thg1 (50–0.4 $\mu\text{g}/\text{mL}$; –, no Thg1 added) with products resolved using silica TLC. (B) Steady-state kinetic analysis of Thg1 reaction with $p\text{-tRNA}^{\text{His}}$. Thg1 was incubated with $p\text{-tRNA}^{\text{His}}$ at concentrations indicated, and the resulting initial velocity vs. [substrate] plot was used to determine steady-state kinetic parameters detailed in Table 1. (C) Examination of Thg1 activity with different $p\text{-tRNA}$ species. Thg1 was assayed using 5'-[³²P]-labeled $p\text{-tRNA}^{\text{Gly}}$, $p\text{-tRNA}^{\text{Glu}}$, $p\text{-tRNA}^{\text{Leu}}$, and $p\text{-tRNA}^{\text{Phe}}$, as indicated. Activity with $p\text{-tRNA}^{\text{His}}$ in the presence (+) or the absence (–) of Thg1.

pyrophosphate that occurs upon G_{-1} addition, using TLC. Pyrophosphate release measured by this assay is also dependent on the addition of Thg1 and GTP and yields linear initial rates under steady-state conditions.

In contrast to the results with $p\text{-tRNA}$ species, Thg1 has measurable activity with $ppp\text{-tRNA}^{\text{Phe}}$, as well as with $ppp\text{-tRNA}^{\text{His}}$. We could therefore directly compare the steady-state kinetic parameters for Thg1 with respect to both of these substrates (Table 1). We find that the k_{cat}/K_M for Thg1 activity with $ppp\text{-tRNA}^{\text{His}}$ (with saturating GTP only, since ATP is not required for this reaction) is 600-fold more than that observed with $ppp\text{-tRNA}^{\text{Phe}}$, due to a 62-fold increase in K_M from 0.42 μM to 26 μM , and a 90% reduction in k_{cat} from 8.4 h^{-1} to 0.84 h^{-1} (Table 1). However, this k_{cat}/K_M effect is relatively minor compared with the >10,000-fold specificity exhibited by Thg1 for $p\text{-tRNA}^{\text{His}}$ over $p\text{-tRNA}^{\text{Phe}}$. Thus, the major contribution to recognition of $tRNA^{\text{His}}$ occurs at the adenylation step of the Thg1 reaction. We note that the same overall patterns of specificity are observed with Thg1 purified from yeast (detection of activity with $p\text{-tRNA}^{\text{His}}$, $ppp\text{-tRNA}^{\text{His}}$, and $ppp\text{-tRNA}^{\text{Phe}}$, but not $p\text{-tRNA}^{\text{Phe}}$), although the lower concentration of protein in the yeast preparation limits our ability to define limits for the differences in selectivity

between $tRNA^{\text{His}}$ and other substrates. Because the physiological substrate is the $p\text{-tRNA}$ species that arises after RNase P cleavage, and because recognition of $tRNA^{\text{His}}$ appears to occur at this stage, we searched for determinants in $p\text{-tRNA}^{\text{His}}$ that were responsible for its recognition.

The determinants for Thg1 activity are not near its site of action

Although Thg1 acts at the 5' end of the tRNA, it does not appear to recognize residues in the acceptor stem. We constructed a series of $tRNA^{\text{His}}$ variants containing alterations in the aminoacyl-acceptor stem (Fig. 3) and assayed their ability to function as $p\text{-tRNA}$ substrates for Thg1. There is little, if any, change in observed Thg1 activity for any of the variant tRNA species, which include alterations in each of the first three base pairs (Fig. 3B–D, I), simultaneous alteration of either the fourth and fifth base pairs or the sixth and seventh base pairs (Fig. 3E,F), or alteration of four base pairs at once (Fig. 3G,H). Indeed, despite the fact that Thg1 has no detectable activity on $p\text{-tRNA}^{\text{Phe}}$, complete substitution of the entire acceptor stem of $tRNA^{\text{His}}$ with that of $tRNA^{\text{Phe}}$ results in no loss of activity (Fig. 3J).

Thg1 specifically recognizes the $tRNA^{\text{His}}$ anticodon

Since the aminoacyl-acceptor stem was not the source of $tRNA^{\text{His}}$ recognition by Thg1, we next examined the anticodon, a feature that is often used by aminoacyl-tRNA synthetases to distinguish tRNA species. We find that the anticodon has a dramatic effect on Thg1 activity. Alteration of the GUG anticodon of $tRNA^{\text{His}}$ to GAA (the anticodon of $tRNA^{\text{Phe}}$) results in no detectable Thg1 activity (Fig. 4A), exactly as is observed with $p\text{-tRNA}^{\text{Phe}}$ (Fig. 2C). This effect is not unique to the GAA anticodon sequence, since we also observe complete loss of Thg1 activity with $tRNA^{\text{His}}$ variants bearing UUA (amber) or UCA (opal) stop anticodon sequences (Fig. 4A). Thus, as for many tRNA synthetases, the anticodon plays a crucial role as a necessary determinant for G_{-1} addition to $p\text{-tRNA}^{\text{His}}$ by Thg1.

To determine if the $tRNA^{\text{His}}$ GUG anticodon is sufficient for Thg1 activity in the context of a foreign tRNA species, we introduced it into other tRNAs. Remarkably, a $p\text{-tRNA}^{\text{Phe}}$ variant with only the two nucleotide changes required to convert GAA to GUG at the anticodon position

TABLE 1. Steady-state kinetic parameters for Thg1 activity with respect to tRNA species				
Substrate tRNA	k_{cat} (hr^{-1})	K_M (μM)	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)	Loss of guanylyltransfer ^a
p-tRNA ^{His}	0.73 ± 0.09	1.7 ± 0.4	120 ± 19	
p-tRNA ^{Phe}		Undetectable activity ^b		>10,000
p-tRNA ^{Gly}		Undetectable activity ^b		N.D. ^c
ppp-tRNA ^{His}	8.4 ± 0.9	0.42 ± 0.13	5500 ± 1200	
ppp-tRNA ^{Phe}	0.84 ± 0.04	26 ± 2	9.2 ± 0.4	600
p-tRNA ^{Phe} GUG	$>0.070^d$	$>10^e$	1.9 ± 0.9^f	63
p-tRNA ^{Gly} GUG	$>0.096^d$	$>10^e$	2.7 ± 0.9^f	44
ppp-tRNA ^{Phe} GUG	8.4 ± 0.8	6.4 ± 1.5	350 ± 50	16

^aLoss of guanylyltransfer, reflecting the degree to which Thg1 discriminates between tRNA^{His} and other tRNA species, $= (k_{\text{cat}}/K_M)_{\text{tRNA}^{\text{His}}} / (k_{\text{cat}}/K_M)_{\text{tRNA}^{\text{X}}}$, in the case of substrates (X) with measurable activity. In the case of p-tRNA^{Phe}, the lower limit to the specificity ratio is determined from single turnover experiments in which Thg1 exhibits detectable activity with p-tRNA^{His} after 10 min with 50 nM Thg1, whereas there is still no detectable activity with p-tRNA^{Phe} after 4 h with 21 μM Thg1.

^bNo observable reaction products after up to 8-h reaction with the highest concentrations of Thg1 achievable in the assay, as shown in Figure 2C.

^cNot determined.

^dLower limit to k_{cat} was determined using the measured k_{cat}/K_M and the lower limit for K_M for each substrate.

^eLower limit to K_M is based on the highest concentration of substrate tested (10 μM tRNA) for which observed initial rates of Thg1 activity are still linearly dependent on tRNA concentration.

^f k_{cat}/K_M measured from the slope of the linear initial rate vs. substrate plot for each substrate, due to the inability to reach saturating tRNA concentrations for these substrates.

(p-tRNA^{Phe}_{GUG}) is ~15% as active as wild-type tRNA^{His}, converting the tRNA from one with no detectable activity as a substrate to an extremely efficient Thg1 substrate (Fig.

4B). Moreover, this effect is not specific for tRNA^{Phe}, since introduction of the GUG anticodon to either tRNA^{Gly} (Fig. 4B) or tRNA^{Leu} (data not shown) also converts the resulting tRNAs to effective Thg1 substrates. Thus, the GUG anticodon of tRNA^{His} is a potent identity element that is both necessary and sufficient to direct G₋₁ addition by Thg1 to a substrate tRNA.

To quantify the extent to which the tRNA^{His} anticodon confers recognition by Thg1 to a nonsubstrate tRNA, we determined the steady-state kinetic parameters for p-tRNA^{Phe}_{GUG} and p-tRNA^{Gly}_{GUG}, as well as for ppp-tRNA^{Phe}_{GUG}. We find that the major effect of introduction of the tRNA^{His} GUG anticodon is the significant increase in specificity for the p-tRNA species. Although the K_M values for p-tRNA^{Phe}_{GUG} and p-tRNA^{Gly}_{GUG} are higher than for p-tRNA^{His}, based on initial velocity plots that are still linear at the highest concentrations examined (10 μM), these substrates are recognized remarkably well by Thg1, with k_{cat}/K_M values that are only 40- to 60-fold lower than that observed with p-tRNA^{His} (Table 1). It is notable also that, despite the prominent role of the adenylation step in specificity for tRNA^{His}, introduction of the GUG tRNA^{His} anticodon into both p-tRNA^{Phe} and ppp-tRNA^{Phe} results in

FIGURE 3. The aminoacyl-acceptor stem of tRNA^{His} is not a determinant for Thg1 activity. 5'-[³²P]-labeled tRNA^{His} variants containing alterations in the aminoacyl acceptor stem, identified by the circled region on the diagram of tRNA^{His}, were assayed as described above. Variations from the wild-type tRNA^{His} sequence (shown in A) are indicated by lowercase letters; dashes indicate wild-type tRNA^{His} sequence. RNase A cleavage products GpGpC (A,B,D,E,F,G,J) and GpGpU (C,H) migrate identically in this TLC system, whereas the slower migrating GpGpGpC tetramer product results from RNase A cleavage of the variant shown in I.

FIGURE 4. The tRNA^{His} anticodon, GUG, is a major identity element for Thg1 activity. (A) The GUG anticodon is required for Thg1 activity with tRNA^{His}. Thg1 activity was examined with 5'-[³²P]-labeled tRNA^{His} variants containing alterations in the anticodon loop (circled on the tRNA diagram), as identified by lowercase letters in the boxed anticodon sequences. (B) The GUG anticodon is sufficient for Thg1 activity with noncognate tRNA species. Thg1 activity was examined with 5'-[³²P]-labeled wild-type p-tRNA^{His}, wild-type p-tRNA^{Phe}, and p-tRNA^{Phe}_{GUG} and p-tRNA^{Gly}_{GUG} variants, with alterations from the wild-type anticodon sequence indicated by lowercase letters.

increased activity relative to that observed with the normal GAA anticodon (Table 1).

DISCUSSION

We have demonstrated here that the tRNA^{His} guanylyltransferase Thg1 is highly specific for p-tRNA^{His} relative to other monophosphorylated tRNA species (at least 10,000-fold in one measured case), while exhibiting a substantial but comparatively moderate preference (600-fold) for ppp-tRNA^{His} relative to other triphosphorylated tRNA species. We have also demonstrated that although G₋₁ addition by Thg1 occurs at the top of the aminoacyl acceptor stem, the determinants for Thg1 specificity for tRNA^{His} are found at the opposite end of the tRNA, in the tRNA^{His} anticodon. Thus, alteration of the tRNA^{His} GUG anticodon completely abrogates Thg1 activity, and the mere introduction of the tRNA^{His} GUG anticodon into nonsubstrate tRNAs such as p-tRNA^{Phe} or p-tRNA^{Gly} results in recognition of these p-tRNA species as remarkably efficient Thg1 substrates, with only a slight decrease in k_{cat}/K_M relative to p-tRNA^{His}. Both the degree of improved selectivity and the magnitude of observed activity of Thg1 upon introduction of the GUG

identity element are comparable to those observed with tRNA synthetases when identity elements are introduced into a nonsubstrate tRNA (Nameki et al. 1995; Himeno et al. 1997). Thus, other than aminoacyl-tRNA synthetases, Thg1 is the only known example of a protein that uses the anticodon as a major identity element to distinguish tRNA species, while acting at a remote site in the tRNA. We note that two enzymes modify residues of the anticodon by aminoacylation; however, unlike Thg1, these enzymes are highly related to tRNA synthetases, do not act at a remote site from the anticodon, and catalyze aminoacylation and not nucleotide addition (Dubois et al. 2004; Salazar et al. 2004; Ikeuchi et al. 2005).

The large degree of specificity of Thg1 for p-tRNA^{His} relative to other p-tRNA species is consistent with the crucial role of G₋₁ addition for aminoacylation of tRNA^{His} in vivo, since the presumed physiological Thg1 substrate is the monophosphorylated tRNA species that arises after cleavage of the pre-tRNA^{His} by RNase P (Lee et al. 1991). Since there is no known physiological source of mature length ppp-tRNA species, the relatively weaker specificity of Thg1 for ppp-tRNA^{His} is not likely to result in G₋₁ addition to other tRNAs by Thg1 in vivo. However, the mild contribution to selectivity for tRNA^{His} at the level of ppp-tRNA may impart some added degree of specificity at the guanylyltransfer step for the correct activated tRNA^{His} species. It is also noteworthy that the overall catalytic efficiency of Thg1 with ppp-tRNA^{His} is greater than that observed with p-tRNA^{His} as indicated by the ≈ 10 -fold increase in the steady-state k_{cat} (Table 1). This may imply that adenylation is the rate-determining step for Thg1 under steady-state conditions with p-tRNA^{His} and that the second step of the Thg1 reaction, guanylyltransfer, occurs more rapidly. In this respect, it is possible that the ppp-tRNA species is a true mimic of the adenylylated intermediate tRNA species that arises during the physiological Thg1 reaction.

The catalytic strategy of ensuring correct tRNA specificity by recognition of the anticodon while acting at the opposite end of the tRNA is reminiscent of the strategy employed by aminoacyl-tRNA synthetases. Fidelity in protein synthesis is ensured by the correct selection of both the amino acid and tRNA species by synthetases, as well as, in some cases, by editing activities to correct any errors in recognition that may have occurred. For the vast majority of tRNA synthetases the nucleotides of the anticodon are quantitatively potent identity elements, although identity elements are also found throughout the tRNA, particularly in the aminoacyl-acceptor stem region, and the discriminator nucleotide at position 73 (Giege et al. 1998). Synthetases often recognize the anticodon by direct hydrogen bonding between amino acid residues in the enzyme and nucleotides of the anticodon (Cusack et al. 1998; Sekine et al. 2001). These interactions can be powerful enough for a single amino acid to determine specificity for

a given tRNA species (Sekine et al. 2001; Feng et al. 2003, 2005), although, in some cases, other regions of the tRNA can play an important role (Cusack et al. 1998; Martin et al. 2004). The mechanism by which Thg1 recognizes the anticodon of its tRNA^{His} substrate remains unclear, because Thg1 contains no identifiable similarity to tRNA synthetases, including in particular the known tRNA recognition motifs, and because important substrate recognition motifs of Thg1 have not yet been identified.

The relatively small size of Thg1 (28 kDa) relative to most synthetases poses two additional difficulties for anticodon recognition. First, the anticodon is found fully 70 Å away from the site of G₋₁ addition by Thg1, as it is for aminoacylation by synthetases. Synthetases usually bridge this distance in part because they are relatively larger enzymes, often >70 kDa, and, in addition, they frequently exist in dimeric forms, with both subunits of the enzyme making contacts with the tRNA species. In this respect Thg1 may function similarly to synthetases; it is likely that Thg1 is also active in some multimeric form since it interacts strongly with itself in both two-hybrid and direct protein interaction assays (Uetz et al. 2000; Hazbun et al. 2003). Second, recognition of the anticodon nucleotides by Thg1 requires yet another layer of complexity of this small enzyme, which already houses a number of nucleotide recognition features, including those for the 5' triphosphate of ATP, the 5' phosphate of tRNA, the 3' hydroxyl of GTP, and the pyrophosphate of the added G₋₁.

The unique presence of G₋₁ in all known tRNA^{His} species from all organisms allows this feature to serve as an important identity element of histidyl-tRNA synthetase (HisRS). Indeed, both *E. coli* and yeast HisRS rely on G₋₁ for determination of tRNA^{His} identity and maintenance of translational accuracy and are less dependent on the anticodon for tRNA recognition than most other synthetases (Francklyn and Schimmel 1990; Nameki et al. 1995; Giege et al. 1998). In a similar fashion, yeast seryl-tRNA synthetase relies on the unique longer variable loop region of tRNA^{Ser} (Himeno et al. 1997). For HisRS to rely on G₋₁ in eukaryotes, it requires the extraordinary specificity of Thg1 for its substrate tRNA, and the fact that specificity of Thg1 is conferred by the anticodon might suggest that it has somehow been transferred from the tRNA synthetase to Thg1 during evolution.

In addition to the similarity in terms of anticodon recognition, one of the most striking similarities between Thg1 and tRNA synthetases is that the chemical reactions catalyzed by these two enzymes are nearly identical, despite the fact that Thg1 catalyzes a nucleotide addition reaction and tRNA synthetases catalyze aminoacylation (Fig. 5). Both families of enzymes catalyze formation of an activated adenylated intermediate, by attack of the α-phosphate of ATP by an acid moiety, either the 5' phosphoric acid of tRNA^{His} in the case of Thg1 or the carboxylic acid of an amino acid in the case of a synthetase, to yield the cor-

FIGURE 5. Comparison of chemical reactions catalyzed by Thg1 and aminoacyl-tRNA synthetases.

responding anhydride. Also in both cases, the second step of the reaction involves attack of a 3' OH on the adenylated intermediate to yield the corresponding ester, either from GTP for Thg1 to add G₋₁ to tRNA^{His} in a phosphodiester linkage, or from A₇₆ of the tRNA for a synthetase to add the amino acid in an ester linkage. Since, in addition, the site of action of Thg1 on its tRNA substrate is very close in space to that of tRNA synthetases, it is tempting to speculate that these similarities underscore a shared evolution and/or mechanistic features, despite the lack of any obvious homology between the two enzyme families.

MATERIALS AND METHODS

Sources of Thg1

The source of Thg1p for steady-state kinetic analysis was a previously described N-terminally His₆-tagged Thg1p that was overexpressed in *E. coli* BL21(DE3)pLysS and purified to >90% homogeneity by immobilized metal ion affinity chromatography (IMAC) on TALON resin (Clontech) (Gu et al. 2003).

For purification of Thg1 from yeast, the *THG1* clone was obtained from the *Saccharomyces cerevisiae* Moveable Open Reading Frame (MORF) library (Gelperin et al. 2005), in which C-terminally tagged (His₆-3C-proteinA) Thg1 is expressed under control of the galactose promoter in yeast. Yeast cells bearing the *THG1* MORF plasmid were grown in -Ura synthetic media containing 2% raffinose, induced for expression of the MORF by addition of two volumes of media containing 3% yeast extract, 6% peptone, and 6% galactose and growth for 6 h, followed by harvest and lysis of the cells with glass beads as described (Gelperin et al. 2005). Thg1 was then purified by IMAC on TALON resin in buffer containing 20 mM Tris-HCl, 4 mM MgCl₂, 2 mM β-mercaptoethanol (BME), 0.5 M NaCl, and 5% glycerol (pH 7.5). After washing bound protein with buffer containing 10 mM imidazole, protein was eluted with buffer containing

250 mM imidazole. Protein-containing fractions were identified by A₂₈₀, pooled, and dialyzed using Slidealyzer 12–14 kDa MWCO cassettes (Pierce) against buffer containing 20 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5 M NaCl, and 50% glycerol (pH 7.5). The resultant protein was the major purifying polypeptide as determined by SDS-PAGE, was quantified using the BioRad protein assay, and stored at –20°C.

Thg1 activity assay with 5' [³²P]-tRNA

5'-[³²P]-tRNA species were prepared by phosphatase treatment of in vitro transcripts followed by labeling with [γ -³²P]-ATP using T4 polynucleotide kinase (PNK) (Roche) to a final specific activity of ~7000 Ci/mmol. Typically, the 5' [³²P]-tRNA was used as the substrate in a Thg1 assay containing 10 mM MgCl₂, 3 mM DTT, 125 mM NaCl, 0.2 mg/mL BSA, and 1 mM each of ATP and GTP in 25 mM HEPES (pH 7.5). Enzyme titration assays consisted of fivefold serial dilutions (~50–0.4 μ g/mL) of yeast-purified Thg1. Reactions were started by the addition of enzyme and carried out at room temperature. At indicated times, the reaction was stopped by removal of an aliquot (4 μ L) to a new tube containing 0.5 μ L of 0.5 M EDTA and 0.5 μ L of 10 mg/mL RNaseA, followed by digestion for 10–30 min at 50°C, and treatment with 0.5 U alkaline phosphatase (Roche) in 1 $\times$ phosphatase reaction buffer in a final volume of 6 μ L at 37°C for 30 min. Reactions were applied to silica-TLC plates (EM Science) and resolved in an *n*-propanol:NH₄OH:H₂O (55:35:10) solvent system. The plates were visualized and quantified using PhosphorImager and Imagequant software.

Thg1 activity assay with ppp-tRNA

[³²P]pp-tRNA species were prepared by in vitro transcription in the presence of [γ -³²P]-GTP. Reactions were carried out at room temperature in assay buffer containing 10 mM MgCl₂, 3 mM DTT, 125 mM NaCl, 0.2 mg/mL BSA, and 1 mM GTP in 25 mM HEPES (pH 7.5) and started by the addition of Thg1. At the indicated times, reactions were stopped by spotting 2 μ L to PEI-cellulose TLC plates, and the products were resolved in a saturated ammonium sulfate:isopropanol (80:20) TLC system. The plates were visualized and quantified using PhosphorImager and Imagequant software.

Variant tRNA species

All tRNA variants were cloned into a previously described pUC13-based plasmid under control of the T7 RNA polymerase promoter for purposes of in vitro transcription (Gu et al. 2003). Aminoacyl-acceptor stem tRNA^{His} variants were all created by mutagenic PCR; anticodon loop variants of tRNA^{His}, tRNA^{Phe}, tRNA^{Gly}, and tRNA^{Leu} were created using QuikChange site-directed mutagenesis (Stratagene).

Determination of steady-state kinetic parameters

Steady-state reactions were carried out using the above assays with 0.1–10 μ M tRNA (depending on K_M) with 10 nM–2 μ M Thg1 purified from *E. coli* (maintaining at least fivefold excess tRNA in the reactions) for time points ranging from 1 min to 8 h. All reactions were performed in the presence of saturating concen-

trations of nucleotide, either 1 mM each ATP and GTP for monophosphorylated substrates, or 1 mM GTP only for triphosphorylated tRNA. Timecourses were designed to yield <10% conversion to products, and rates were linear over the time courses studied. When possible, steady-state kinetic parameters k_{cat} and K_M for each substrate were determined by a fit of the initial velocities to the Michaelis-Menten equation, using the Kaleidagraph curve-fitting program (Synergy software). The steady-state k_{cat}/K_M values for p-tRNA^{Phe}_{GUG} and p-tRNA^{Gly}_{GUG} were measured from the linear portion (0.1–10 μ M) of the initial velocity versus substrate plot, and a corresponding lower limit to k_{cat} and K_M for each substrate was estimated from these data.

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