Change of tRNA identity leads to a divergent orthogonal histidyl-tRNA synthetase/tRNA\textsubscript{His} pair

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ABSTRACT

Mature tRNA\textsubscript{His} has at its 5'-terminus an extra guanylate, designated as G\textsuperscript{-1}. This is the major recognition element for histidyl-tRNA synthetase (HisRS) to permit acylation of tRNA\textsubscript{His} with histidine. However, it was reported that tRNA\textsubscript{His} of a subgroup of \(\alpha\)-proteobacteria, including Caulobacter crescentus, lacks the critical G\textsuperscript{-1} residue. Here we show that recombinant C. crescentus HisRS allowed complete histidylation of a C. crescentus tRNA\textsubscript{His} transcript (lacking G\textsuperscript{-1}). The addition of G\textsuperscript{-1} did not improve aminoacylation by C. crescentus HisRS. However, mutations in the tRNA\textsubscript{His} anticodon caused a drastic loss of \textit{in vitro} histidylation, and mutations of bases A73 and U72 also reduced charging. Thus, the major recognition elements in C. crescentus tRNA\textsubscript{His} are the anticodon, the discriminator base and U72, which are recognized by the divergent (based on sequence similarity) C. crescentus HisRS. Transplantation of these recognition elements into an \textit{Escherichia coli} tRNA\textsubscript{His} template, together with addition of base U20a, created a competent substrate for C. crescentus HisRS. These results illustrate how a conserved tRNA recognition pattern changed during evolution. The data also uncovered a divergent orthogonal HisRS/tRNA\textsubscript{His} pair.

INTRODUCTION

Transfer RNAs (tRNAs) fold into a highly conserved and compact common structure, yet the necessity for accurate protein biosynthesis requires tRNA species-restricted recognition by their cognate aminoacyl-tRNA synthetases. This amino acid identity is largely determined by a set of tRNA features termed recognition elements. In the 1980's, the recognition elements for each single aminoacyl-tRNA synthetase class were unravelled; they showed a conservation of the major recognition elements for a particular amino acid among organisms in all three domains of life (1,2). Histidine tRNA (tRNA\textsubscript{His}) has an added guanylate at its 5' terminus and this was shown to be the major recognition element (3–5).

Maturation of the 5' terminus of precursor tRNA involves endonucleolytic cleavage by the ribonucleoprotein RNase P (6,7), which yields a tRNA molecule starting at position 1 [according to standard tRNA nomenclature (8)]. The nearly universal exception to this rule is the tRNA\textsubscript{His} that starts at the −1 position (9). The additional 5'-terminal nucleotide, usually a guanosine residue (G\textsuperscript{-1}), is required for HisRS to specifically recognize and aminoacylate its cognate tRNA (3–5). The extra base was shown to properly position the tRNA's 5'-monophosphate within an arginine cluster in the active site of \textit{Escherichia coli} HisRS (histidyl-tRNA synthetase) (10–12). The absence of G\textsuperscript{-1} causes a more than 100-fold reduction in the catalytic efficiency of both yeast and \textit{E. coli} HisRS (3,13–15). Other recognition elements include C73 in \textit{E. coli} tRNA\textsubscript{His}, as well as A73 and—to a lesser degree—the anticodon in yeast tRNA\textsubscript{His} (16).

Two pathways are known to guarantee that the G\textsuperscript{-1} residue is present in mature tRNA\textsubscript{His}. In the first pathway, which is found in many bacteria, RNase P exhibits altered cleavage specificity for the tRNA\textsubscript{His} precursor that maintains the genome encoded G\textsuperscript{-1} residue even though it cleaves the +1 position of all other tRNA precursors (9,17). It has been shown that the structure of the acceptor stem and primarily the pairing between the G\textsuperscript{-1} base and the C73 discriminator base are responsible for this unusual cleavage specificity (18–20). The second pathway is found in Eukaryotes (21,22) and some Archaea (23,24), which often do not encode a G\textsuperscript{-1} residue or remove it during RNase P cleavage. Rather, the G\textsuperscript{-1} is added post-translationally by a tRNA\textsubscript{His} guanylyltransferase (Thg1), which catalyzes the addition of a guanylate to the 5'-end of tRNA\textsubscript{His} as an unusual 3'-5' nucleic acid polymerase (25,26).
A few years ago, an exception to the assumed universal occurrence of G⁻¹ in tRNAHis was observed in some z-proteobacteria including members of the Caulobacterales, Parvularculales, Rhizobiales and Rhodobacterales (27,28). Surprisingly, these organisms are viable with a shortened tRNAHis (starting at position 1). Computational analyses suggested the co-evolution of a HisRS and analyze the orthogonality of this tRNAHis and its cognate proteins.

MATERIALS AND METHODS

Materials and reagents

Oligonucleotide synthesis and DNA sequencing were performed by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. [¹⁴C]Histidine (163 mCi/mmol) was obtained from Amersham Pharmacia Biotec and [¹³C]Histidine-5-methyl (163 mCi/mmol) was obtained from Amersham Pharmacia Biotec. [¹⁴C]-5-methyl-histidine, 5 mM DTT, 2.5 mM ATP and 0.1 mg/ml BSA. The concentrations of tRNA substrates range from 0.2 to 196 μM and the active fractions were estimated with reactions containing saturating amounts of enzyme. To calculate kinetic parameters, initial velocities of histidinylation reactions were obtained by monitoring [¹⁴C]-Hist-tRNAHis formation. In details, aliquots from the reaction mixtures were removed periodically and spotted on Whatman 3MM paper filter disks (Whatman). After washing in 10% trichloroacetic acid three times, the radioactivity was measured by liquid scintillation counter. Kinetic parameters for histidinylation of the tRNAs were obtained using KaleidaGraph to plot initial velocity versus tRNA substrate concentration followed by Michaelis–Menten curve fitting.

RESULTS

Histidinylation of tRNA

The histidinylation reaction was carried out with 3 nM purified recombinant C. crescentus HisRS at 30°C in buffer containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 μM [¹⁴C]-histidine, 5 mM DTT, 2.5 mM ATP and 0.1 mg/ml BSA. The concentrations of tRNA substrates range from 0.2 to 196 μM and the active fractions were estimated with reactions containing saturating amounts of enzyme. To calculate kinetic parameters, initial velocities of histidinylation reactions were obtained by monitoring [¹⁴C]-Hist-tRNAHis formation. In details, aliquots from the reaction mixtures were removed periodically and spotted on Whatman 3MM paper filter disks (Whatman). After washing in 10% trichloroacetic acid three times, the radioactivity was measured by liquid scintillation counter. Kinetic parameters for histidinylation of the tRNAs were obtained using KaleidaGraph to plot initial velocity versus tRNA substrate concentration followed by Michaelis–Menten curve fitting.

RESULTS

Analysis of tRNAHis nucleotide conservation

As the HisRS of nearly all organisms in all domains of life utilizes the unusual G⁻¹ base to select its cognate tRNA, we asked how the unusual tRNAHis without this extra base is recognized by the C. crescentus HisRS. Sequence alignments of tRNAHis genes reveal the different conservation of certain bases within the two groups of tRNAHis genes, particularly in the acceptor stem (27,28). The first group contains nearly all known tRNAHis genes, particularly in the acceptor stem (27,28). The first group contains nearly all known tRNAHis genes, particularly in the acceptor stem.

Cloning, in vitro transcription and purification of tRNA substrates

Caulobacter crescentus tRNAHis and E. coli tRNAHis variants were synthesized by in vitro T7 RNA polymerase run-off transcription as described (29). Two complementary oligonucleotides containing the respective tRNA gene downstream of a T7 RNA polymerase promoter were annealed and cloned into the vector pUC18. To generate tRNA transcripts with the proper 3'-CCA end, a BstNI restriction site was utilized at the 3'-end of each tRNA gene sequence. The in vitro transcription reaction was performed at 37°C for 3–5 h in a buffer containing 40 mM Tris–HCl (pH 8.1), 22 mM MgCl₂, 5 mM DTT, 1 mM spermidine, 16 mM guanosine monophosphate, 4 mM of each nucleoside triphosphate, BstNI-digested vector containing the template DNA (0.1 μg/μl) and 1 mM T7 RNAP. The tRNA transcripts were purified by electrophoresis on denaturing polyacrylamide gels with 8 M urea. Full-length tRNAs were eluted and desalted on Sephadex G25 Microspin columns (Amersham).
**Caulobacter crescentus** HisRS efficiently aminoacylates tRNA\(^{\text{His}}\) transcripts

*Caulobacter crescentus* HisRS protein (52.3 kDa each monomer) was produced and purified to enable histidylation efficiency analyses of a member of the /C1\-type HisRS group. The optimal histidylation reaction conditions for *C. crescentus* tRNA\(^{\text{His}}\) transcripts were determined by titrating the pH value and altering the temperature and salt concentrations. The enzyme showed optimal activity at pH 7.5, a reaction temperature of 30°C and in the absence of KCl. The optimal concentration of HisRS in the reaction was established as 3 nM, which ensures constant reaction velocity in the first 5 min during the histidylation of the wild-type tRNA\(^{\text{His}}\). *Caulobacter crescentus* tRNA\(^{\text{His}}\) transcripts showed a catalytic efficiency (Table 1) similar to histidylation of tRNA\(^{\text{His}}\) in *E. coli* (3).

*Caulobacter crescentus* HisRS recognizes the anticodon and the acceptor stem of tRNA\(^{\text{His}}\)

Several *C. crescentus* tRNA\(^{\text{His}}\) mutants were tested for their catalytic efficiency as HisRS substrates (Table 1, Figure 2A). The first set of mutants involved bases in the acceptor stem. It was determined that changing the discriminator base 73 to the standard C found in most bacterial tRNA\(^{\text{His}}\) species results in a slight increase in \(K_m\) value. However, mutation of base 72 decreased the \(k_{\text{cat}}/K_m\) value approximately 100-fold. Also, the addition of a G\(^{-1}\) residue did not improve the catalytic efficiency of the tRNA substrate, but rather increased the \(K_m\) value nearly 6-fold. These results indicate that the acceptor stem is still recognized by the G\(^{-1}\) type HisRS and substantiate that the absence of the G\(^{-1}\) in the *C. crescentus* cell does not negatively impact histidylation.

Next, we determined the location of new recognition elements (tRNA\(^{\text{His}}\) identity elements) that might have replaced the G\(^{-1}\) recognition mechanism *Caulobacter crescentus* tRNA\(^{\text{His}}\) variants with each of the three anticodon bases replaced with the remaining three bases were analyzed. All twelve possible anticodon mutants showed drastically reduced catalytic efficiency (Table 1) with losses of \(k_{\text{cat}}/K_m\) ranging between 91- and 1000-fold. Mutation of G37 only had a slight \(K_m\) effect on histidylation. In conclusion, the major new identity elements recognized by the G\(^{-1}\) type HisRS are the three anticodon bases and base U72 in the acceptor stem, highlighting a drastic deviation from the tRNA\(^{\text{His}}\) recognition mechanism of standard G\(^{-1}\) type HisRS.

**Escherichia coli** tRNA\(^{\text{His}}\) becomes a substrate for *C. crescentus* HisRS after identity element transplantation

Next, we tested whether the conventional G\(^{-1}\) type tRNA\(^{\text{His}}\) of *E. coli* can serve as a substrate for *C. crescentus* HisRS. Surprisingly, the catalytic efficiency was drastically reduced [over 250-fold loss of efficiency (Table 2)] even though this tRNA contains the same anticodon sequence (GUG) required for recognition by the G\(^{-1}\) type HisRS. Consequently, we decided to analyze the *E. coli* tRNA\(^{\text{His}}\) for the transplantation of *C. crescentus* recognition elements. Nevertheless, a transplant containing...
the terminal acceptor stem bases and the anticodon loop base G37 improved the charging efficiency of this hybrid tRNA (Trans 6) only approximately 19-fold. Even the transplantation of the entire *C. crescentus* tRNAHis acceptor stem or entire T-stem did not improve histidylation (data not shown). This suggested the presence of further negative determinants for *C. crescentus* HisRS in *E. coli* tRNAHis. A comparison of tRNAHis sequences from both HisRS groups (Figure 1) indicated that the D-loop of the ΔG−1 type tRNAHis always contains an extra residue, U20a, which is most often missing in other bacteria, including *E. coli*. The addition of this extra nucleotide to Trans 6 (Trans 7, Figure 2B) drastically increased histidylation efficiency, yielding a *Km* value similar to the *C. crescentus* wild-type tRNAHis transcript and only a slightly reduced *kcat* value (Table 2). Thus, the size of tRNAHis D-loop is important for the *C. crescentus* HisRS to eliminate a negative determinant in *E. coli* tRNAHis. A larger D-loop likely also influences the overall 3D structure of a tRNA molecule that forms 

### Table 1. Aminoacylation of *C. crescentus* tRNAHis variants by *C. crescentus* HisRS

| tRNA | *Km* (μM) | *kcat* (s−1) | *kcat*/Km | fractional *kcat*/Km | Loss of efficiency (x-fold) |
|------|-----------|-------------|-----------|---------------------|---------------------------|
| WT   | 3.25 ± 0.26 | 4.00 ± 0.11 | 1.231 ± 0.133 | 1                    | 1                         |
| ΔG−1 | 8.44 ± 0.39 | 4.21 ± 0.31 | 0.999 ± 0.096 | 0.405                | 2                         |
| G34A | Nd        | Nd          | 0.009 ± 0.003 | 0.007                | 143                       |
| G34C | Nd        | Nd          | 0.014 ± 0.004 | 0.011                | 91                        |
| G35A | Nd        | Nd          | 0.001 ± 0.001 | 0.001                | 1000                      |
| G35C | Nd        | Nd          | 0.007 ± 0.001 | 0.006                | 167                       |
| G35G | Nd        | Nd          | 0.010 ± 0.002 | 0.008                | 125                       |
| G36A | Nd        | Nd          | 0.006 ± 0.001 | 0.005                | 200                       |
| G36C | Nd        | Nd          | 0.005 ± 0.001 | 0.004                | 250                       |
| G36U | Nd        | Nd          | 0.010 ± 0.002 | 0.008                | 125                       |
| G37A | 8.86 ± 1.12 | 3.34 ± 0.41 | 0.377 ± 0.095 | 0.306                | 3                         |
| U32C | 13.74 ± 1.04 | 0.19 ± 0.01 | 0.014 ± 0.002 | 0.011                | 91                        |
| A32C | 9.93 ± 0.98 | 4.38 ± 0.16 | 0.441 ± 0.061 | 0.358                | 3                         |
| +G−1 | 19.02 ± 2.99 | 4.71 ± 0.28 | 0.248 ± 0.055 | 0.201                | 5                         |

Nd, Not determined. The rate of product formation was insufficient with respect to practical experimental time scales to allow determination of steady-state kinetic parameters. The *kcat*/Km value is estimated using subsaturating tRNA concentrations.

**Figure 2.** Recognition elements for *C. crescentus* HisRS. (A) The investigated mutants of the *C. crescentus* tRNAHis transcripts are indicated by arrows. (B) Transplantation of the *C. crescentus* HisRS recognition elements into *E. coli* tRNAHis (Trans7). Trans6 differs from Trans7 in the size of the D-loop due to the presence of base 20a.
tertiary interactions between the D- and T-loops. However, the reduction of the D-loop size in the *C. crescentus* tRNA\(^\text{His}\) backbone has only a small effect on histidylation efficiency (Table 1).

*Caulobacter crescentus* HisRS and tRNA\(^\text{His}\) form an orthogonal pair in *E. coli* background

Finally, we tested whether the *C. crescentus* HisRS is able to aminoacylate any *E. coli* tRNA or if the *C. crescentus* HisRS/tRNA\(^\text{His}\) pair would constitute a different orthogonal enzyme/substrate pair for histidylation. Indeed, *C. crescentus* HisRS was not able to aminoacylate total *E. coli* tRNA to a significant level above background (Figure 3). On the other hand, *E. coli* HisRS reached a 30-fold higher plateau level of total tRNA (Figure 3) containing 3.79% of tRNA\(^\text{His}\) (30).

### DISCUSSION

The divergence of tRNA\(^\text{His}\) recognition elements

The recognition of the tRNA\(^\text{His}\) acceptor stem and especially the presence of the G\(^-1\) base are believed to be necessitated by the low anticodon discrimination ability of HisRS (15,16). Studies focusing on the importance of the tRNA\(^\text{His}\) anticodon have generated conflicting results that might be the consequence of species-specific differences. It was recently shown that yeast can survive without a G\(^{-1}\)/C\(^0\)/1 containing tRNA\(^\text{His}\) as long as both tRNA and HisRS are overexpressed (31). It is also established that the C-terminal domain of *E. coli* HisRS forms binding interactions with the tRNA\(^\text{His}\) anticodon that are important for tRNA selection *in vivo* (15,18). Recent studies indicated that tRNA\(^\text{His}\) anticodon primarily affects the thermodynamics of initial HisRS/tRNA\(^\text{His}\) complex formation, while acceptor stem mutants force a specific kinetic block on aminoacyl transfer and decrease the tRNA-mediated kinetic control of amino acid activation (32). However, other studies concluded that the anticodon contribution for tRNA\(^\text{His}\) recognition is largely negligible (14,33). The importance of the acceptor stem, on the other hand, is unquestioned and evidenced by the histidylation capacity of tRNA\(^\text{His}\) suppressor tRNAs (34), tRNA\(^\text{His}\) minihelices (lacking the complete anticodon arm) (4) and viral tRNA-like structures (14).

The importance of the G\(^-1\) residue is also underscored by the evolution of two different mechanisms to ensure its presence, namely the unusual RNase P cleavage in Bacteria and guanylyltransferase activity by the essential Thgl enzyme in Eukaryotes and some Archaea. Therefore, it was initially puzzling to see that a few \(\alpha\)-proteobacteria are able to survive with a single tRNA\(^\text{His}\) species that does not contain a G\(^{-1}\)/C\(^0\)/1 residue (27,28). It has been suggested that such tRNA\(^\text{His}\) would be less distinctive among the set of tRNAs in the cell and that either the HisRS might compensate for the lost G\(^{-1}\) identity element or the organisms are able to tolerate a decreased tRNA\(^\text{His}\) discrimination (28).

Our results highlight that these \(\alpha\)-proteobacteria evolved a special HisRS, that emphasizes the recognition of the tRNA\(^\text{His}\) anticodon, as opposed to the G\(^-1\) recognition that is found in most organisms. Universal rules for tRNA identity have been described with major determinants that were conserved in evolution (1). Therefore, the described deviation serves as a fascinating example of
drastically altered tRNA species recognition that diverged from the universal tRNA discrimination established in the last universal common ancestor. Other unusual tRNA species recognition mechanisms have been described before, for example for certain mammalian mitochondrial aminoacylation systems (35), but we believe the described change of the tRNA\textsuperscript{His} recognition to be unique in its impact on RNase P, Thg1 and cytosolic HisRS evolution.

The loss of the standard identity elements G\textsubscript{−1} and C73 appears to be compensated by co-evolution with a HisRS that contains several inserts. HisRS alignments and modeling of the \textit{C. crescentus} HisRS-tRNA structure in comparison to the \textit{E. coli} HisRS indicated one insert that is unique among all anticodon-binding domains of class IIA aminoacyl-tRNA synthetases (28) and is a likely candidate for the observed anticodon discrimination of the ΔG\textsubscript{−1} type HisRS.

Where does this unusual bacterial tRNA\textsuperscript{His} come from? One striking feature is the difference in the discriminator base in tRNA\textsuperscript{His} as it is always an A73 in Eukaryotes and always a C73 in Bacteria with a G\textsubscript{−1} type HisRS. It is interesting to note, that of all its tRNAs, only tRNA\textsuperscript{His} has a C73 discriminator base in \textit{E. coli}. The unusual presence of A73 in the tRNAs of Bacteria with a ΔG\textsubscript{−1} type HisRS suggests that it might have been acquired by lateral transfer from a eukaryotic cell. However, recent phylogenetic analyses concluded that it diverged from an ancestral α-proteobacterial tRNA\textsuperscript{His} gene (27).

It has also been reported, that the discriminator base C73 plays a role in altered RNase P processing in the context of a stable G\textsubscript{−1}/C73 base pair (18). The presence of A73 in the ΔG\textsubscript{−1} type tRNA\textsuperscript{His} might decrease cleavage efficiency at the −1 position and might have encouraged evolution of the anticodon recognition mechanism.

It has been hypothesized that the G\textsuperscript{−1} might have a different important cellular function separate from its role in tRNA\textsuperscript{His} aminoacylation and that the standard HisRS may then have evolved to recognize this unique feature (31). While it is difficult to pinpoint such an alternate role for the G\textsuperscript{−1} residue, it should be noted that Thg1 (which guarantees the presence of G\textsuperscript{−1} in eukaryotic tRNA\textsuperscript{His}) has been associated with cell cycle control in yeast (36) and DNA replication (37). If this alternate role was not essential or lost in a group of organisms, they would be free to evolve a tRNA\textsuperscript{His} that does not require an elaborate G\textsuperscript{−1} addition mechanism such as either aberrant RNase P cleavage or Thg1 activity. Also, a tRNA\textsuperscript{His} with a conventional acceptor stem might be beneficial during translation in the ribosome as the base pairing between G\textsuperscript{−1} and C73 may decrease the flexibility of the 3′-terminal CCA end tail.

Two divergent HisRS/tRNA\textsuperscript{His} orthogonal pairs

The identification of the altered recognition elements of the ΔG\textsubscript{−1} type HisRS reveals the presence of two different orthogonal HisRS/tRNA\textsuperscript{His} pairs. Previous studies reported that conventional HisRS is not able to recognize a tRNA\textsuperscript{His} without the acceptor stem identity elements that are missing from the \textit{C. crescentus} tRNA\textsuperscript{His} (Figure 4) (10,18). Similarly, we found that the \textit{C. crescentus} HisRS is also not able to recognize the tRNA\textsuperscript{His} of \textit{E. coli} even though the necessary anticodon is present (Figure 4). Instead, the acceptor stem and especially the D-loop size are antideterminants in \textit{E. coli} tRNA\textsuperscript{His}. These observations indicate that it should be possible to create a cell that contains both of these two different orthogonal HisRS/tRNA\textsuperscript{His} pairs. As the anticodon of the tRNA\textsuperscript{His} with G\textsuperscript{−1} is not recognized by the G\textsuperscript{−1} type HisRS, its backbone can be utilized to construct an amber suppressor tRNA with an 8–20-fold $K_m$ increase (15,34). Thus, a HisRS/tRNA\textsuperscript{His}_\text{CUA} could be employed in a scenario where the normal histidine codons are accounted for by the ΔG\textsubscript{−1} type HisRS/tRNA\textsuperscript{His} system. This enables

![Figure 4. Two divergent HisRS/tRNA\textsuperscript{His} pairs. Two different HisRS/tRNA\textsuperscript{His} pairs are found in Bacteria, the G\textsuperscript{−1} type HisRS/tRNA\textsuperscript{His} pair present in most bacteria (\textit{E. coli} HisRS structure, pdb id 2EL9) and the ΔG\textsubscript{−1} type HisRS/tRNA\textsuperscript{His} pair found in a few α-proteobacteria. Recognition elements are indicated in red on the schematic tRNA depiction and the elements that prevent charging of the contrary tRNA\textsuperscript{His} are indicated by red arrows. A ΔG\textsubscript{−1} type HisRS crystal structure is not available.](image-url)
the engineering of HisRS variants for the targeted incorporation of histidine analogs into proteins. Such analogs would be useful for various studies involving enzyme activity, protein engineering and metal binding site studies among others (38–40).

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