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REPORT

The relationship of thermodynamic stability at a G•U recognition site to tRNA aminoacylation specificity

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ABSTRACT

The G•U pair at the third position in the acceptor helix of *Escherichia coli* tRNA^{Ala} is critical for aminoacylation. The features that allow G•U recognition are likely to include direct interaction of alanyl-tRNA synthetase with distinctive atomic groups and indirect recognition of the structural and stability information encoded in the sequence of G•U and its immediate context. The present work investigates the thermodynamic stability and acceptor activity for a comprehensive set of variant RNAs with substitutions of the G•U pair of *E. coli* tRNA^{Ala}. The four RNAs with Watson–Crick substitutions had a lower acceptor activity and a higher stability relative to the G•U RNA. On the other hand, the RNAs with mispair substitutions had a lower stability, but either a higher or a lower acceptor activity. Thus, the entire set of variant RNAs does not exhibit a correlation between thermodynamic stability of the free, unbound tRNA and its acceptor activity. The substantial acceptor activity of tRNAs with particular mispair substitutions may be explained by their ability to assume the conformational preferences of alanyl-tRNA synthetase. Moreover, the G•U pair may provide a point of deformability for the substrate tRNA to adapt to the enzyme's active site.

Keywords: mid-point temperatures; protein synthesis; tRNA identity; wobble base pair

INTRODUCTION

The fidelity of protein synthesis depends on specific tRNA aminoacylation by aminoacyl-tRNA synthetase enzymes, which in turn depends on recognition of particular structural features in the substrate tRNA. The G•U wobble base pair at the third position in the acceptor helix of *Escherichia coli* tRNA^{Ala} is critical for aminoacylation by alanyl-tRNA synthetase (AlaRS) (Hou & Schimmel, 1988; McClain & Foss, 1988). The features that allow its recognition are likely to include direct protein interaction with the distinctive atomic groups and indirect recognition of the structural and stability information encoded in the sequence of G•U and its immediate context (McClain et al., 1988; Beuning et al., 1997; Chang et al., 1999). Biophysical analyses of a large array of mispair base appositions in RNA helices show that mispairs contribute to structural flex-

TABLE 1. Mid-point temperature (T_m) of RNAs.

Base pair	T_m (± 1.0 °C) ^a	
	22-mer	25-mer
G•C	100.0	85.4
C•G	96.9	84.0
A•U	91.6	76.3
U•A	90.7	77.5
G•U	87.6	73.0
G•U context	86.9	70.2
U•G	84.1	70.5
I•U	84.1	68.1
G•G	83.3	— ^b
U•I	83.3	—
G•A	81.5	64.5
C•A	80.8	63.0
A•C	80.5	63.7
C•U	80.5	61.4
A•G	79.9	—
U•C	78.9	—
C•C	78.5	59.5
U•U	77.7	60.3
A•A	77.3	—

^aThe standard error of the measurement of all RNAs was 1.0 °C, except it was 1.5 °C for G•C and C•G 22-mer RNAs.

^b—: Not determined.

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ibility, as they are often, but not systematically, less stable than Watson–Crick base pairs. To better characterize the parameters that influence G•U recognition, we have examined the effects of replacing it with other base combinations on RNA thermal stability and tRNA^{Ala} aminoacylation capacity.

RESULTS AND DISCUSSION

We first analyzed the stability of a 22-mer tetraloop hairpin RNA derived from the terminal 6 bp and single-stranded acceptor end of *E. coli* tRNA^{Ala} (Fig. 1). The RNA is a substrate for AlaRS (Francklyn & Schimmel, 1989) and its high-precision structure has been determined by nuclear magnetic resonance (Ramos & Varani, 1997). A comprehensive panel of variant tetraloop RNAs containing all fifteen natural base-pair substitutions of G•U was tested, as were the corresponding

RNAs with I•U and U•I analogue substitutions. We also analyzed the stability of a G•U context RNA where the base pairs flanking G•U were changed from G•C to C•G (Fig. 1).

Stability was assessed by collecting UV denaturing profiles between 20 and 102 °C. Figure 2 shows several denaturation profiles. All RNAs showed transition mid-point temperatures (T_m) between 77 and 100 °C (Table 1). The standard error of the measurements was 1.0 °C except that it was 1.5 °C for G•C and C•G 22-mer RNAs. Because the tetraloop may impose conformational and dynamic restrictions to the adjacent helical region, an additional series of 25-mer variant RNAs was prepared where the tetraloop was replaced by the less stable TΨ loop of tRNA^{Ala} (Fig. 1 and Table 1). These TΨ hairpins showed T_m values between 59 and 85 °C. Measurements of several 22-mers and 25-mers over an approximately 50-fold concentration range established that the profiles and T_m values of the main

FIGURE 1. Diagram of *E. coli* tRNA^{Ala} and the 22-mer tetraloop RNA and 25-mer TΨ loop RNA showing the location of the G•U wobble base pair and the substitutions studied here. Not shown is the G•U context RNA where the base pairs flanking G•U were changed from G•C to C•G. The RNAs were synthesized using Glen Research monomers on a model 392 Applied Biosystems DNA/RNA Synthesizer with several modifications to manufacturer's instructions: 5-ethylthiotetrazole activator, 5 min coupling time, cap-ox-cap procedure, 40% aqueous methylamine deprotection at room temperature for 2 h, Et₃N•3 HF desilylation at 55 °C for 1.5 h and *n*-butanol precipitation. The crude product was dissolved in 600 μL of 20 mM Na/K-phosphate, pH 7.0, heated in a closed Eppendorf vial for 1 min at 100 °C and injected into an HPLC system with high pressure gradient mixing. The column (25 × 8 mm, Nucleogen® DEAE 500-7, Macherey-Nagel) was eluted with buffer A (20 mM Na/K-phosphate, pH 7.0) and buffer B (0.6 M NaCl in buffer A) at a flow rate of 2 mL/min with a column oven temperature of 50 °C. The HPLC eluate was desalted by C₁₈ reversed-phase HPLC using a step gradient from nanopure water to 30% CH₃CN/H₂O. The fraction was subsequently concentrated in a SpeedVac to a total volume of 0.4–1.0 mL. All stock solutions contained 87–98% pure, full-length RNA as determined by several criteria, including anion exchange HPLC, capillary electrophoresis, and gel electrophoresis using both StainsAll® and 3'-pCp labeling. In addition, RNAs had the expected molecular mass (MALDI-TOF MS) and exhibited full integrity of the ACCA-3' terminus as monitored by a partial RNase U₂ digestion and gel electrophoresis of 3'-pCp-labeled RNA. The purified and completely desalted RNA stock solution could be stored at least 12 months in an RNase-free Eppendorf tube at 4 °C without notable degradation. Alternatively, after addition of 3 M NaOAc (final concentration 0.3 M Na⁺), RNA was stored as a wet pellet at –20 °C in 1–2 μL of 70% EtOH.

FIGURE 2. Denaturation profiles of several RNAs. The data for the 22-mer RNAs were scaled to the same hyperchromicity (100%), and the mid-point temperature was read at 50% hyperchromicity. The arrows in the graph represent the T_m value. The high- and low-temperature baselines of the 25-mer RNAs were connected with an orthogonal line and the T_m value read at the crossing point between the profile and the middle of the connecting line, as indicated. Each strand was measured four to six times in parallel, and the data was analyzed independently by two individuals. The standard error of the measurement of all RNAs was 1.0 °C, except it was 1.5 °C for G•C and C•G 22-mer RNAs. Within experimental error, the same T_m value was obtained by nonlinear-curve fitting. The 22-mers were more stable and showed nondrifting low-temperature baselines. The 25-mers were less stable and all showed drifting baselines, even at 285 nm (not shown). Some of the more stable 25-mers (e.g., C•G) produced a second minor transition at a lower temperature that presumably reflects competitive bimolecular formation of double strands. Data were collected using a Lambda 2 UV spectrometer/thermocontroller with a heating-cooling block for six 1-cm cuvettes/thermocontroller PTP-6 (Perkin Elmer). Data were handled with a Pentium PC computer using software UV WinLab® (for Lambda) and TempLab® (for PTP-6). The buffer contained 100 mM NaCl, 10 mM NaH₂PO₄, pH 7.5 (adjusted with NaOH). RNA samples (1.0 mL buffer, 4–20 µL RNA stock solution) in RNase ZAP® (Ambion)-treated quartz cuvettes were quickly preheated and after 1 min at 100 °C were cooled to 20 °C over ~60 min before beginning measurement. Each RNA was measured in one run of four parallel samples by recording the absorbency at 260 nm every 0.1 °C between 20 and 99 °C (or 20–102 °C for A•U, U•A, G•C, and C•G 22-mers). The initial RNA concentration at A₂₆₀²⁰ was ~0.04 (22-mers) or ~0.2 (25-mers) measured against buffer. The total run time was ~1.5 h per RNA. For stability reasons, we measured only in the “up” mode in one heating period. The baseline data points were subtracted from each set of raw data before further processing.

transitions were concentration independent, demonstrating that we were monitoring unimolecular hairpin-random coil transitions.

The substituted RNAs exhibit a gradation in stability relative to G•U (Table 1). The Watson–Crick G•C and C•G variant RNAs had T_m values 9–12 °C higher than the G•U RNA, whereas the increase was 3–5 °C for the A•U and U•A RNAs. The G•U context RNA and the U•G RNA were more similar to the G•U RNA, with T_m reductions of 2 °C. For the I•U RNA, the reduction was 4 °C, similar to that previously reported for the I•U-substituted duplex RNA (Limmer et al., 1993). Other variant RNAs had T_m values approximately 6–14 °C below the G•U RNA. The magnitude of the I•U reduction suggests that the abstraction of the unpaired 2-amino group of the guanine residue has a significant impact

on the structure of the RNA and may reflect disruption of the hydration network characteristic of G•U (Auffinger & Westhof, 1998; Mueller et al., 1999).

The steady-state level of alanyl-tRNA^{Ala} in *E. coli* has been determined for tRNA^{Ala} with G•U, the 15-bp variants, and the G•U context variant (Gabriel et al., 1996; McClain et al., 1999). Figure 3 plots the T_m of hairpin RNA versus the extent of aminoacylation of the corresponding tRNA^{Ala}. The plot shows that G•U confers distinctive properties on the molecule. The four variant RNAs where Watson–Crick pairs replace the G•U pair are more stable and also are poorer alanine acceptors. All other variant RNAs are less stable, but a clear-cut correlation with aminoacylation capacity is not evident. For example, it is striking that C•C and G•U tRNAs have high acceptor activity, but differ markedly in sta-

FIGURE 3. Plot of the deviation from G•U RNA of T_m versus steady-state level of alanyl-tRNA^{Ala}. The T_m data are from Table 1 and the alanyl-tRNA^{Ala} levels have been published (Gabriel et al., 1996; McClain et al., 1999). The fraction of wild-type G•U tRNA^{Ala} present as alanyl-tRNA^{Ala} was $82.1 \pm 4.8\%$. The in vivo analysis of alanyl-tRNA^{Ala} did not exclude the possibility that some variants have alternative conformations and/or defective termini that could potentially impair tRNA acceptor activity. The symbol G•U ctx represents the G•U context RNA. The pointers on the x axis indicate the relative T_m values for I•U and U•I RNAs.

bility, whereas pair-wise comparisons of tRNAs with either C•U and U•C or G•U and U•G reveal differences in acceptor activity, but similarity in stability. On the other hand, it is just as notable that the four RNAs with Watson–Crick substitutions have low acceptor activity and high stability, which by itself suggests that the thermodynamic stability at the third position of tRNA^{Ala} contributes to AlaRS recognition. A recent thermodynamic analysis of alanine acceptor helices corre-

sponding to a subset of G•U variants (Meroueh & Chow, 1999) gave results similar to those reported in Table 1. However, the subset did not include A•A-, A•G-, G•G-, or U•C-substituted RNAs that exhibit low stability and low activity, and thus provided an incomplete description of the relationship between stability and tRNA aminoacylation.

The recognition signature of the G•U pair in tRNA^{Ala} is likely to include direct and indirect readout features.

Therefore, it is not surprising that the entire set of variant RNAs does not exhibit a strong correlation between thermodynamic stability of the free, unbound RNA and tRNA acceptor capacity. Other aspects of recognition may obscure the correlation. For example, the RNAs exhibiting a lower stability and lower acceptor activity (U•G, A•A, A•G, G•G, and U•C) may be inactive due to tRNA conformational preferences that do not conform to those of AlaRS. For instance, the substitution of the nonisosteric U•G pair for the G•U pair reverses the helical twist values with the flanking base pairs so that deviations of up to 2 Å are observed 5 bp from the nonisosteric wobble pair (Masquida et al., 1999). Furthermore, the G•U backbone and the single-stranded acceptor end of tRNA^{Ala} adopt distinctive conformations in solution (Ramos & Varani, 1997). Thus, one role of the G•U pair that U•G and other inactive mispairs cannot mimic in promoting aminoacylation efficiency and specificity is the determination of a tRNA conformation that is most favorable for positioning the tRNA acceptor end in the enzyme catalytic site. In addition, after AlaRS binds the tRNA, its deformability may be important. In fact, the solution structures of wild-type and mutants of tRNA^{Ala} indicate that the G•U pair provides a point of deformability for an induced fit with the AlaRS enzyme (Chang et al., 1999). Furthermore, the acceptor capacity of the various tRNAs (Fig. 3) suggests that the pyrimidine•pyrimidine pairs are more easily deformed by the AlaRS protein than the purine•purine pairs. However, the deformability at the level of the G•U pair need not correlate with the thermodynamic stability of free, unbound tRNA^{Ala} because aminoacyl-tRNA synthetase recognition is driven by an ensemble of interactions that are spread over much of the substrate tRNA molecule (Cavarelli & Moras, 1993; Cusack, 1997).

The G•U wobble base pair provides specific recognition signals in numerous RNAs, ranging from ribosomal RNA and splicing regulatory signals (Li et al., 1996; Bénard et al., 1998) to RNA enzymes (Doudna et al., 1989). Because a detailed understanding of these systems is lacking, continued analysis of the G•U pair in tRNA^{Ala} can provide a paradigm to dissect the features that allow its widespread role as a distinctive recognition feature. Neither indirect recognition nor deformability of G•U in tRNA^{Ala} have been critically evaluated by the thermodynamic analysis reported here. At present, a determination of the three-dimensional structure of the AlaRS-tRNA^{Ala} complex corresponding to wild-type and several active tRNA mutants may be the best means of orchestrating the wealth of functional and biophysical data into a coherent picture of recognition.

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