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Accelerated Publications

RNA Tetraloops as Minimalist Substrates for Aminoacylation†

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ABSTRACT: Previous work established that seven-base-pair hairpin microhelices with sequences based on the acceptor stems of alanine, glycine, methionine, and histidine tRNAs can be aminoacylated specifically with their cognate amino acids. To obtain "minimalist" substrates with fewer base pairs, we took advantage of the high thermodynamic stability of RNA tetraloop motifs that are found in ribosomal RNAs. We show here that rationally designed RNA tetraloops with as few as four base pairs are substrates for aminoacylation. Major nucleotide determinants for recognition by the class II synthetases were incorporated into each of the respective tetraloop substrates, resulting in specific aminoacylation by the alanine, glycine, and histidine tRNA synthetases. An analysis of the kinetics of aminoacylation shows that, for the alanine system, the majority of the transition-state stabilization provided by the synthetase–tRNA interaction is reproduced by the interaction of the synthetase with nucleotides in its minimalist tetraloop substrate. In an extension of this work, we also observed specific aminoacylation with the class I methionine tRNA synthetase of RNA tetraloops based on sequences in the acceptor stem of methionine tRNA. Thus, the results demonstrate four different examples where specific aminoacylation is directed by sequences/structures contained in less than half of a turn of an RNA helix.

Nucleotide sequence elements in the acceptor stems of several tRNAs are recognition signals for aminoacylation by the cognate aminoacyl-tRNA synthetases (aaRSs)[1] (Normanly & Abelson, 1989; Pütz et al., 1991; Jahn et al., 1991; Rould et al., 1989, 1991; Ruff et al., 1991; Schimmel, 1989, 1991). These sequence elements have been incorporated into seven-base-pair hairpin helices that are joined at the 3′-end through the bridging "discriminator" base N73 to the single stranded CCA_{76} terminus that is common to all tRNAs[2] (Francklyn & Schimmel, 1989; 1990; Francklyn et al., 1992; Martinis & Schimmel, 1992). In this way, microhelices that are aminoacylated specifically by the alanine, histidine, or glycine tRNA synthetase have been obtained. For these three examples from the class II tRNA synthetase family, the nucleotide sequence elements that determine the specificity and efficiency of aminoacylation are concentrated within the span from N73 through the first three base pairs near the acceptor end of the helix. These nucleotide sequence determinants overlap, so that the introduction of a determinant for one aaRS simultaneously removes a determinant for another aaRS (Francklyn et al., 1992). Thus, for at least these three amino acids, there is a unique relationship between a specific sequence/structure in an acceptor helix and the particular amino acid which is directed to that helix.

In an effort to define the minimal RNA structure required for specific aminoacylation, we synthesized short complementary single strands based on the acceptor helix of $tRNA^{Ala}$ (Musier-Forsyth et al., 1991a). Although the single strands are not aminoacylated, when hybridized together they yield a duplex with a 3′-ACCA single-stranded tetranucleotide

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; HPLC, high-pressure liquid chromatography.
[2] The standard 76-nucleotide numbering system for a tRNA sequence has been used.

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segment that recreates the acceptor end of $tRNA^{Ala}$. We observed that duplexes of six to nine base pairs were efficiently aminoacylated, but when the structure was reduced to a four-base-pair duplex, the aminoacylation rate and extent were severely reduced to the point that it could not be quantitatively investigated. However, the melting temperature of the four-base-pair duplex was below the temperature of the charging assay (25 °C), indicating that little of the duplex had formed under the conditions of the experiment. Thus, whether a duplex shorter than six base pairs could serve as an efficient substrate for aminoacylation was unclear.

To circumvent this difficulty, we took advantage of the recent characterization of the highly stable RNA tetraloop motifs that are found in ribosomal RNAs (Woese et al., 1990) and in mRNAs of, for example, bacteriophage T4 (Tuerk et al., 1988). These motifs consist of hairpin helices that are capped by a sequence-specific four-base loop which is closed by a specific base pair at the top of the hairpin stem. An example is the UUCG tetraloop that is closed by C-G (Woese et al., 1990). Even with just four base pairs in the hairpin stem, these tetraloop structures have unusually high thermal stabilities (>65 °C, depending on the sequence of the stem) which are due to the participation of the first and last base of the loop in a novel pairing interaction that extends the stacking interactions of the stem (Cheong et al., 1990; Varani et al., 1991). Therefore, these tetraloop motifs afford an opportunity to determine the minimal sequence/structure required for specific aminoacylation.

Using recently described methods for chemical synthesis of RNA oligonucleotides (Musier-Forsyth et al., 1991a,b; Usman et al., 1987; Scaringe et al., 1990), we constructed small hairpin helices with as few as four base pairs and which incorporate the stabilizing features of RNA tetraloops (Woese et al., 1990). We confirmed the high thermal stability of these structures and introduced nucleotide sequence elements that confer aminoacylation in larger RNA substrates into the unconstrained positions of the short helical stems of the tetraloop structures. These "minimalist" substrates were then examined for aminoacylation with alanine, glycine, and histidine.

We also investigated minimalist tetraloop structures as substrates for one of the class I aminoacyl-tRNA synthetases, methionine tRNA synthetase. The class I enzymes have no apparent sequence, structural, or evolutionary relationship to the class II synthetases (Eriani et al., 1990; Cusack et al., 1990; Ruff et al., 1991; Nagel & Doolittle, 1991; Burbaum & Schimmel, 1991a). For the class I methionine tRNA synthetase, the anticodon is known to play a major role in determining aminoacylation efficiency (Schulman & Pelka, 1988; Pallanck & Schulman, 1991; Ghosh et al., 1990). However, recent experiments demonstrated a low, but consistent and specific aminoacylation with methionine of seven-to-nine-base-pair helical substrates that incorporate the acceptor stem base pairs and position 73 nucleotide that are held in common by the initiator and elongator species (Martinis & Schimmel, 1992). We designed tetraloop substrates based on the acceptor stem of $tRNA^{Met}$. Aminoacylation with methionine was achieved with a tetraloop substrate containing just four base pairs so that, in all four cases investigated, the tetraloop motif provided a route to the design of functional minimalist substrates.

Experimental Procedures

Chemical synthesis and purification of RNA substrates were carried out as outlined previously (Usman et al., 1987; Scaringe et al., 1990; Musier-Forsyth et al., 1991a). The RNA tetraloops were characterized using melting curves constructed from absorbance data at 260 nm determined on a Beckman DU-64 spectrophotometer (Puglisi & Tinoco, 1989). The experimental details are further described below and by Musier-Forsyth et al. (1991a).

The aminoacylation reactions for alanine (Hill & Schimmel, 1989), histidine (Francklyn & Schimmel, 1990), and glycine (Toth & Schimmel, 1990) were carried out in a reaction mixture containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 20 mM 2-mercaptoethanol, 2 mM ATP, 20 μM [^{3}H]amino acids, and a range of concentrations of RNAs and aminoacyl-tRNA synthetases. Because of the difference in the counting efficiency between [^{3}H]amino acid and [^{3}H]-aminoacyl-RNA on the filter pads, the specific radioactivities of [^{3}H]aminoacyl-RNAs were normalized by using [^{14}C]-aminoacyl-RNA as a control under the same experimental conditions.

The efficiency of trichloroacetic acid precipitation of RNA tetraloops was determined to be 85% using two different methods. In one method, gel-purified, ^{32}P-end-labeled RNA substrates (Sambrook et al., 1989) were added to an aminoacylation reaction mixture. Aliquots were spotted onto trichloroacetic acid-soaked filter pads and then washed as usual. The amounts of ^{32}P which remained on the pads were compared to those on control pads which were not washed.

The second method relied on an HPLC procedure for detecting aminoacyladenosine described by Francklyn et al. (1992). In brief, two sets of samples were taken from the aminoacylation mixture simultaneously. One set was precipitated onto the filter pad with trichloroacetic acid and washed; the other was treated with RNase A (in a molar ratio of 1:25 with respect to RNA) at 37 °C, pH 5, for 10 min and quenched with 3.5 N acetic acid. The precipitiated proteins of the reaction mixture were eliminated by centrifugation. The supernatant was diluted in 50 mM acetate buffer and loaded onto a cation-exchange column (Vydac 401 TP HPLC column) which was preequilibrated with 50 mM sodium acetate (pH 5). After being washed with the same buffer, a linear gradient from 50 mM to 1.0 M sodium acetate (pH 5) was used to elute the aminoacylated product. The peak of alanyladenosine on the elution profile was assigned by using tRNA as a substrate under the same experimental conditions. The amount of alanyladenosine derived from alanyl-RNA tetraloops was compared with that obtained from trichloroacetic acid precipitation.

The tetraloops which modeled $tRNA^{Met}$ and $tRNA^{fMet}$ were aminoacylated using the procedures outlined by Martinis and Schimmel (1992) with the following exceptions: the methionine tRNA synthetase (Burbaum & Schimmel, 1991b) and tetraloop substrate concentrations were 15 μM and 150–300 μM, respectively. The specific activity was increased to greater than 1.5 mCi/mL of [^{35}S]methionine. The aminoacylated products were separated on an acidic polyacrylamide gel and analyzed by a Molecular Dynamics phosphorimager interfaced with Image Quant Software.

Results

Design, Synthesis, and Thermal Stabilities of Tetraloop Substrates for Three Class II Enzymes. The previously described minihelix^{Ala} (mini^{Ala}) and microhelix^{Ala} (micro^{Ala}) hairpin duplexes are derived from the 12-base-pair acceptor-TΨC stem–loop and acceptor stem, respectively (Figure 1a; Francklyn & Schimmel, 1989). To reduce the size of these model substrates to fewer base pairs, four different tetraloop substrates based on the first three base pairs of $tRNA^{Ala}$ were synthesized. These tetraloops used the commonly occurring (in natural RNA sequences) UUCG sequence that is closed

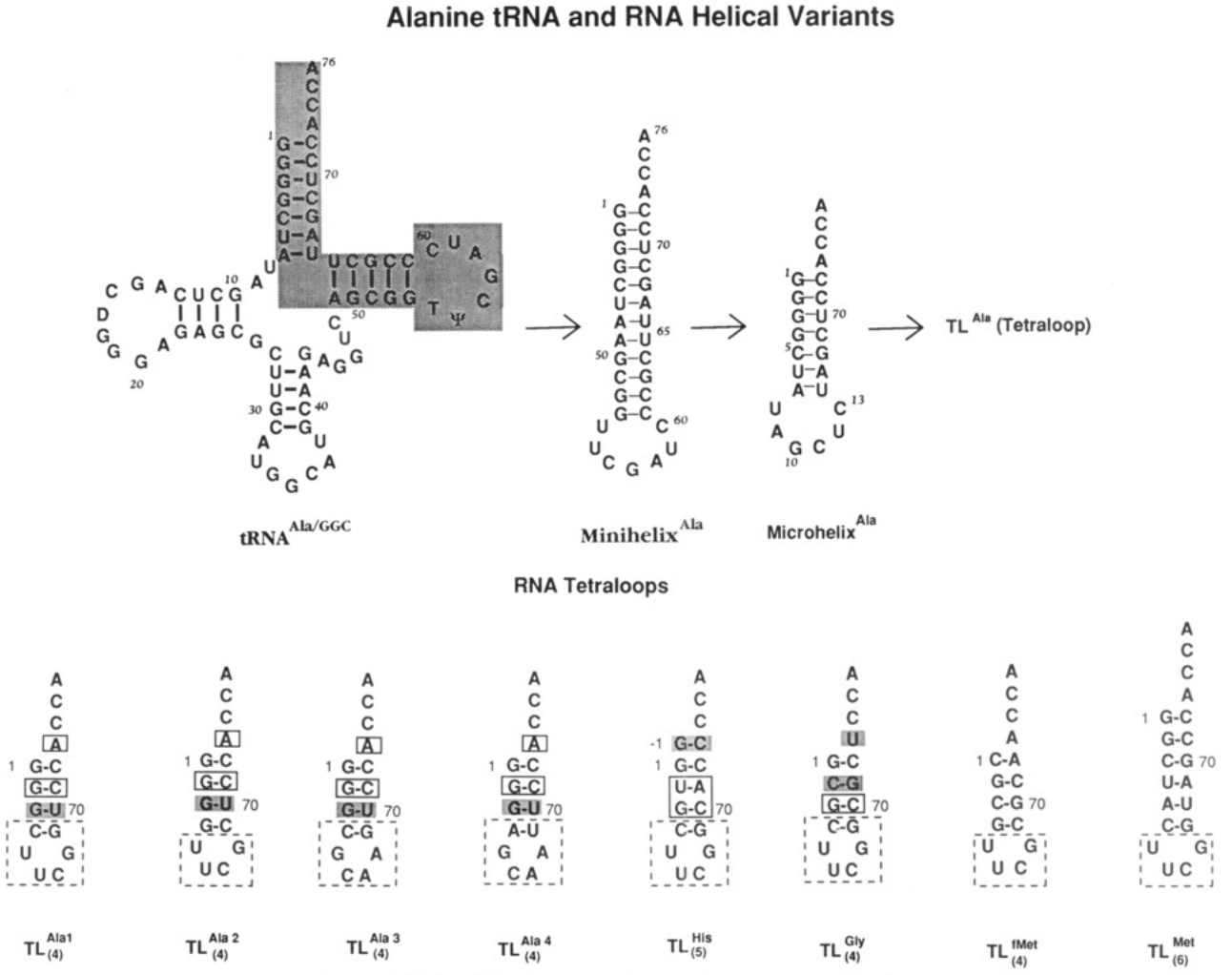

FIGURE 1: (a, top) Sequences of $tRNA^{Ala}$ and RNA model substrates. Numbering is based on that for intact tRNA. The acceptor-TΨC sequence of $tRNA^{Ala}$ is indicated by shading and comprises the nucleotides of $minihelix^{Ala}$. The seven-base-pair microhelix contains the acceptor helix portion only. The RNA tetraloop substrates have four to six base pairs, depending on the substrates. (b, bottom) Sequence variants of RNA tetraloops. Nucleotides essential for aminoacylation by the cognate enzyme are indicated by shaded boxes. Nonessential nucleotides that modulate activity are indicated by unshaded boxes. Dashed lines surround those nucleotides that are not natural to intact tRNA. In the case of $TL_{(4)}^{fMet}$ and $TL_{(6)}^{Met}$, the nucleotides essential for aminoacylation are yet to be determined [see Martinis and Schimmel (1992)].

by C-G or, less frequently, by G-C and GCCA closed by A-U and C-G (Tuerk et al., 1988; Woese et al., 1990) (Figure 1b). The essential (for aminoacylation with AlaRS) G3·U70 base pair (Hou & Schimmel, 1988) is shaded, and the G2–C71 and A73 nucleotides that influence the efficiency of aminoacylation of G3–U70 containing substrates are boxed (Shi et al., 1990, 1991; Francklyn et al., 1992). Dashed lines surround those nucleotides that are not natural to $tRNA^{Ala}$. These substrates are designated $TL_{(4)}^{Ala1}$, $TL_{(4)}^{Ala2}$, etc., where the subscript (4) designates four base pairs (Figure 1b). Similarly, tetraloop substrates for GlyRS, HisRS, and MetRS were synthesized on the basis of the UUCG tetraloop and the acceptor stems of the respective tRNAs. For HisRS, the tetraloop substrate has five base pairs, because of the inclusion of the extra nucleotide found at the 5′-end of all histidine tRNAs. This nucleotide, at the "−1" position, is essential for aminoacylation of an eight-base-pair $microhelix^{His}$ with histidine (Francklyn & Schimmel, 1990).

The thermal melting temperatures of these tetraloop substrates ranged from 61 °C to over 83 °C, depending on the sequence (Table I). The melting temperatures were concentration independent over a range of 3–60 μM, indicating that monomers and not dimers were responsible for the hypochromicity associated with RNA secondary structure (Puglisi & Tinoco, 1989). (This concentration range encompasses the concentrations at which aminoacylation assays were done.) For all of the tetraloop substrates, the amount of helix melting is negligible under the conditions of the aminoacylation reaction (37 °C).

Table I: Melting Temperature of RNA Tetraloops[a]

RNA	T_m (°C)	RNA	T_m (°C)
$TL_{(4)}^{Ala1}$	74.5[b]	$TL_{(4)}^{Ala4}$	61.7[b]
$TL_{(4)}^{Ala2}$	73.3[b]	$TL_{(5)}^{His}$	83.1[c]
$TL_{(4)}^{Ala3}$	72.6[b]	$TL_{(4)}^{Gly}$	81.3[d]

[a] It was technically difficult to obtain accurate melting temperatures above 85 °C. To reduce melting temperatures, the salt concentrations for different samples were adjusted. The resulting salt concentrations were lower than that of the standard assay conditions for aminoacylation (see Experimental Procedures). [b] 0.1 M NaCl, 10 mM sodium phosphate, pH 7, 0.1 mM EDTA. [c] 10 mM NaCl, 10 mM sodium phosphate, pH 7, 0.1 mM EDTA. [d] 10 mM sodium phosphate, pH 7, 0.1 mM EDTA.

Aminoacylation of Tetraloop Substrates for the Class II Enzymes. Each of the three class II enzymes aminoacylated its cognate RNA tetraloop substrate. Aminoacylation was specific, as no aminoacylation of noncognate substrates was detected (Figure 2). Thus, in addition to the five base pairs of the TΨC stem, three base pairs of the acceptor stem are dispensable for aminoacylation. In most cases, only the first

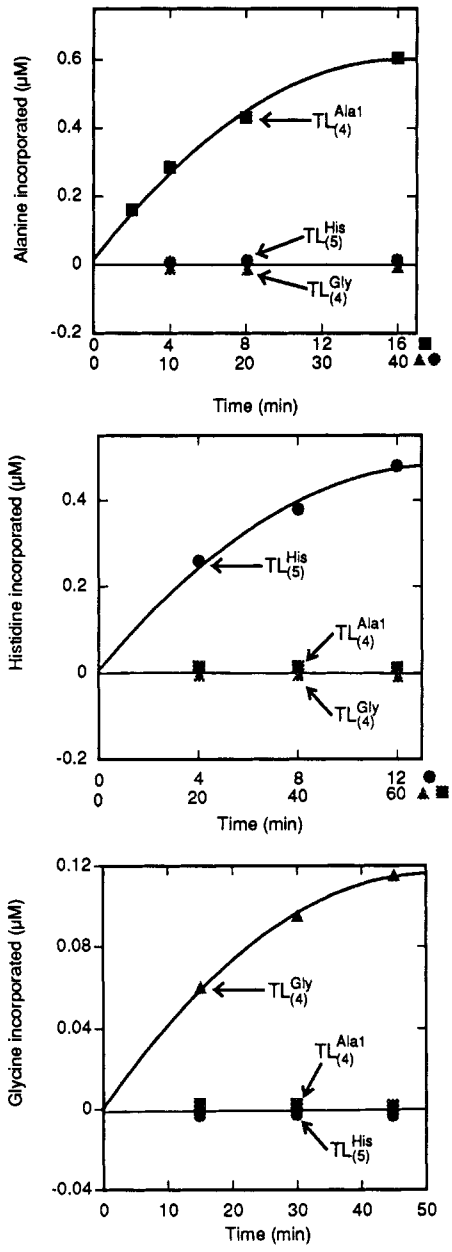

FIGURE 2: Specificity of aminoacylation of RNA tetraloops. (a, top) Aminoacylation with alanine using 41 μM $TL^{Ala1}_{(4)}$ and 27 nM AlaRS, 198 μM $TL^{His}_{(5)}$ and 1.6 μM AlaRS, or 119 μM $TL^{Gly}_{(4)}$ and 1.6 μM AlaRS. (b, middle) Aminoacylation with histidine using 49 μM $TL^{His}_{(5)}$ and 0.67 μM HisRS, 101 μM $TL^{Ala1}_{(4)}$ and 3.3 μM HisRS, or 108 μM $TL^{Gly}_{(4)}$ and 3.3 μM HisRS. (c, bottom) Aminoacylation with glycine using 96 μM $TL^{Gly}_{(4)}$ and 0.56 μM GlyRS, 101 μM $TL^{Ala1}_{(4)}$ and 1.2 μM GlyRS, or 101 μM $TL^{His}_{(5)}$ and 1.2 μM GlyRS, in the standard reaction mixture (see Experimental Procedures).

three base pairs of the tetraloop substrates were identical to those of the cognate tRNA, because the loop-closing base pair was not shared by the tRNA acceptor stem (Figure 1b). For example, only $TL^{Ala2}_{(4)}$ contains the first four base pairs of the acceptor stem of $tRNA^{Ala}$. In contrast, a loop-closing C-G base pair in $TL^{Gly}_{(4)}$ and $TL^{His}_{(5)}$ replaces the G4-C69 base pair that is present in $tRNA^{Gly}$ and $tRNA^{His}$. These results are consistent with the recent evidence that the first three base pairs of the acceptor helix are the most essential positions for determining enzymatic aminoacylations specific for alanine, glycine, and histidine (Francklyn et al., 1992). However, as shown below, the minimalist substrates are not necessarily as efficiently aminoacylated as the seven-base-pair microhelix counterparts, suggesting that the missing parts of the acceptor helix contribute to the efficiency and, additionally or alternatively, the loop nucleotides and particular RNA conformation of specific tetraloop substrates may interfere with enzyme recognition.

Table II: Relative Contribution to the Recognition Parameter k (V_{max}/K_m) of the Tetraloop Acceptor Stem versus the Rest of the tRNA Structure

		rel k	$-\Delta\Delta G^{\circ\ddagger}$ (kcal/mol)[a]
Ala	k_{tRNA}^{Ala}/k_{CCA}	$>8.6 \times 10^9$	>14
	k_{micro}^{Ala}/k_{CCA}	$>3.6 \times 10^7$	>10.7
	k_{TL}^{Ala1}/k_{CCA}	$>1.5 \times 10^7$	>10.2
	$k_{tRNA}^{Ala}/k_{micro}^{Ala}$	2.4×10^2	3.4
	$k_{tRNA}^{Ala}/k_{TL}^{Ala1}$	5.7×10^2	3.9
His	k_{tRNA}^{His}/k_{CCA}	$>1.6 \times 10^{10}$	>14.4[b]
	k_{micro}^{His}/k_{CCA}	$>8.6 \times 10^7$	>11.2[b]
	k_{TL}^{His}/k_{CCA}	$>1.1 \times 10^5$	>7.2
	$k_{tRNA}^{His}/k_{micro}^{His}$	1.9×10^2	3.2[b]
	$k_{tRNA}^{His}/k_{TL}^{His}$	1.5×10^5	7.3
Gly	k_{tRNA}^{Gly}/k_{CCA}	$>2.7 \times 10^{10}$	>14.7[b]
	k_{micro}^{Gly}/k_{CCA}	$>3.0 \times 10^5$	>7.7[b]
	k_{TL}^{Gly}/k_{CCA}	$>5.7 \times 10^3$	>5.3
	$k_{tRNA}^{Gly}/k_{micro}^{Gly}$	9.5×10^4	7.0[b]
	$k_{tRNA}^{Gly}/k_{TL}^{Gly}$	4.8×10^6	9.5

[a] $\Delta\Delta G^{\circ\ddagger}$ represents the incremental lowering of the free energy of activation for aminoacylation by recognition of nucleotide sequences in whole tRNA versus the CCA trinucleotide, in the RNA tetraloop (TL) versus CCA, or in tRNA versus the tetraloop etc. and is given by $\Delta\Delta G^{\circ\ddagger} = -RT \ln (k_{tRNA}/k_{CCA})$ etc. Free energy values pertain to 37 °C. [b] Data are from Francklyn et al. (1992).

Aminoacylation reactions for the class II tRNA synthetases were kinetically well-behaved so that kinetic parameters could be obtained. The results were cast into the analytical framework described by Francklyn et al. (1992), whereby contributions of acceptor stem components to the overall recognition of tRNA are determined. The basic idea is to compare the rate of aminoacylation of the trinucleotide CCA that is common to all tRNAs with that of microhelices, minihelices, and whole tRNA substrates that have CCA fused to specific structures/sequences. The recognition parameter k (V_{max}/K_m) is used to characterize the rate of aminoacylation of each substrate. The dynamic range of the system is established by $-RT \ln (k_{tRNA}/k_{CCA})$, which gives the lowering of the free energy of activation due to interactions with specific sequences in the whole tRNA. The incremental lowering of the free energy of activation $\Delta\Delta G^{\circ\ddagger}$ of aminoacylation of a tRNA versus a microhelix is given by $\Delta\Delta G^{\circ\ddagger} = -RT \ln (k_{tRNA}/k_{micro})$ and represents the contribution of the part of the tRNA outside of the microhelix to overall tRNA recognition. In an analogous way, contributions in the tetraloop structures can be analyzed.

None of the three class II enzymes appeared to aminoacylate the CCA trinucleotide, under conditions where 5 ppm of aminoacylated CCA could be detected (Francklyn et al., 1992). Thus, it is only possible to establish an upper bound for the rate of aminoacylation of CCA. With this limitation, rates of aminoacylation of the minimalist tetraloop substrates have been compared with the rates of charging of microhelix and whole tRNA substrates (Table II). The range of k_{tRNA}/k_{CCA} is greater than about 10^{10} in all three examples. For alanine, the contribution of the sequences in the tetraloop is greater than that of the rest of the tRNA. For glycine and histidine, while the microhelix contributes more than the rest of the tRNA, aminoacylation efficiency is lost when the substrate is reduced to the minimalist tetraloop motif, and because only the upper bound of the rate of aminoacylation of CCA could be established (Francklyn et al., 1992), it is not possible to conclude whether nucleotides in the tetraloop substrate also

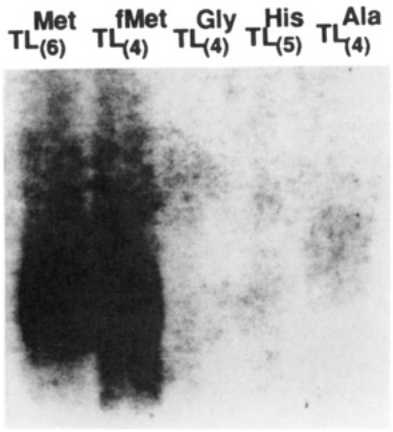

FIGURE 3: Aminoacylation of tetraloop substrates with [^{35}S]-methionine. The reaction mixture contained 300 μM each of tetraloop substrate except for $TL_{(6)}^{Met}$ (150 μM). Approximately 3.4 nmol of the RNA substrate was loaded into each gel lane. The aminoacylated products were analyzed by phosphorimaging.

contribute the major portion of the kinetic recognition.

Aminoacylation of Tetraloop Substrates by the Class I Methionine tRNA Synthetase. Although microhelices with sequences based on the acceptor stems of methionine tRNA are aminoacylated by MetRS (Martinis & Schimmel, 1992), less is known about the determinants essential for aminoacylation of model substrates by the class I MetRS. Aminoacylation efficiency of microhelix substrates is much lower than that for the three class II enzymes and their model substrates. This difference may be due to a much greater role for the anticodon in MetRS recognition than in the class II examples where, for example, with the alanine system there is no contact between the anticodon of $tRNA^{Ala}$ and AlaRS (Park & Schimmel, 1988).

Two tetraloop substrates were investigated for aminoacylation with MetRS (Figure 1b). The $TL_{(4)}^{fMet}$ hairpin incorporates the first four base pairs of the acceptor stem of $tRNA^{fMet}$, by virtue of closing the UUCG tetraloop with G-C. The $TL_{(6)}^{Met}$ substrate includes the native U4-A69 and A5-U68 base pairs of the $tRNA^{Met}$ acceptor stem. Thus, with the loop-closing C-G base pair, $TL_{(6)}^{Met}$ contains a perfect sequence match to the first six base pairs of $tRNA^{Met}$.

Because of the low efficiency of aminoacylation with methionine of truncated model substrates, and also of potential artifacts arising from an RNA-induced self-labeling of MetRS with methionine that can be mistaken for aminoacylation in the conventional trichloroacetic acid RNA precipitation assay, a previously described acidic gel electrophoresis system was used to provide direct visualization of aminoacyl-RNA species (Martinis & Schimmel, 1992). Aminoacylation of $TL_{(6)}^{Met}$ and $TL_{(4)}^{fMet}$ was observed (Figure 3). As was found with the microhelix RNA substrates, there appeared to be a slight aminoacylation of $TL_{(4)}^{Ala1}$, but there was no aminoacylation of $TL_{(4)}^{Gly}$ or of $TL_{(5)}^{His}$. Thus, sequence-specific tetraloop substrates are aminoacylated by the class I MetRS.

Discussion

This work establishes that less than a half of a turn of an RNA helix is sufficient for an aminoacyl-tRNA synthetase to produce an active Michaelis complex and transition state for specific aminoacylation. Although only the first three base pairs of each of the helices for alanine, glycine, and histidine aminoacylation are conserved with respect to the cognate tRNA acceptor stems from which they are derived, it is likely that the fourth base pair and the loop itself are affecting the efficiency of aminoacylation. This conclusion follows from the observation that, for example, the rate of aminoacylation (k) of $TL_{(4)}^{Ala4}$ is reduced relative to that of $TL_{(4)}^{Ala1}$ by over 20-fold (data not shown). These substrates have the same first three base pairs but differ in their tetraloop sequences and the loop-closing base pair (Figure 1b). Thus, the tetraloop and its adjacent base pair may be in direct steric contact with the enzyme and, additionally or alternatively, may indirectly affect the conformation of the first three base pairs and their interaction with the protein. However, in all of the examples studied here, these effects of the sequence of the loop and the closing base pair are not sufficient to obscure specific aminoacylation.

Remarkably, for $TL_{(4)}^{Ala1}$ the efficiency of aminoacylation is comparable to that for micro^{Ala} (Figure 1a). The slightly more than 2-fold lower k value for aminoacylation of $TL_{(4)}^{Ala1}$ (Table II) is almost entirely due to an increase in K_m (790 versus 340 μM). Moreover, the k_{cat} parameters for $TL_{(4)}^{Ala1}$, micro^{Ala}, and $tRNA^{Ala}$ are within a factor of 2 of each other (data not shown). This suggests that the interaction that are important for the transition state in the alanine system are concentrated within the first three base pairs and the ACCA acceptor end of the RNA substrates. Alanine may represent an extreme example, because neither the $TL_{(4)}^{Gly}$ nor $TL_{(5)}^{His}$ substrates are comparable to their microhelix counterparts in aminoacylation efficiency. However, unless more sequence variants of the tetraloop and closing base pair are made, we cannot be certain if a negative effect of these "foreign" sequence elements is responsible for the reduced aminoacylation efficiency of $TL_{(4)}^{Gly}$ and $TL_{(5)}^{His}$. Nonetheless, while the efficiency of aminoacylation is reduced, the specific aminoacylation of these substrates by their cognate enzymes (Figure 2) emphasizes that interactions with the first three base pairs are able to overcome whatever deleterious effects are introduced by the sequences/structures of the tetraloop motifs which are not native to the cognate tRNAs.

Because of the importance of the first three base pairs for synthetase interactions, and of the potentially negative effects of nonnatural sequences/structures that are proximal to the sites of recognition, the four-base-pair tetraloop structures are probably the limit to which the size of model helical substrates can be reduced. The relationship of these substrates to RNA components of early systems of protein synthesis is unknown. However, regardless of that relationship, the present results emphasize that, for several tRNA synthetases, specific RNA discrimination and aminoacylation can be achieved with a minimal amount of structure.

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