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RNA 2000 6: 1585-1596

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Alternative designs for construction of the class II transfer RNA tertiary core

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ABSTRACT

The structural requirements for assembly of functional class II transfer RNA core regions have been examined by sequence analysis and tested by reconstruction of alternative folds into the tertiary domain of *Escherichia coli* tRNA^{Gln}. At least four distinct designs have been identified that permit stable folding and efficient synthetase recognition, as assessed by thermal melting profiles and glutaminylation kinetics. Although most large variable-arm tRNAs found in nature possess an enlarged D-loop, lack of this feature can be compensated for by insertion of nucleotides either 3' to the variable loop or within the short acceptor/D-stem connector region. Rare pyrimidines at nt 9 in the core region can be accommodated in the class II framework, but only if specific nucleotides are present either in the D-loop or 3' to the variable arm. Glutamyl-tRNA synthetase requires one or two unpaired uridines 3' to the variable arm to efficiently aminoacylate several of the class II frameworks. Because there are no specific enzyme contacts in the tRNA^{Gln} core region, these data suggest that tRNA discrimination by GlnRS depends in part on indirect readout of RNA sequence information.

Keywords: aminoacyl-tRNA synthetases; indirect readout; RNA engineering; RNA structure; tRNA identity

INTRODUCTION

Transfer RNAs possess remarkable structural similarity (Kim et al., 1974; Robertus et al., 1974; Moras et al., 1980; Woo et al., 1980; Rould et al., 1989; Basavappa & Sigler, 1991; Goldgur et al., 1997; Cusack et al., 1998; Nissen et al., 1999; Sankaranarayanan et al., 1999; Silvian et al., 1999), yet within the common L-shaped framework, each tRNA retains specific identity nucleotides that ensure correct aminoacylation by the cognate aminoacyl-tRNA synthetase (Giegé et al., 1998b). The identity elements are usually located in the spatially separated amino-acid acceptor and anticodon arms of the molecule. The bridging, core region of the transfer RNA molecule can often be viewed as a scaffold for presentation of these specific elements to the cognate synthetase. The scaffold is extraordinarily robust: a wide variety of sequence lengths and compositions are tolerated while retaining biological function in both aminoacylation and later stages of translation. This is demonstrated by extensive variations within a common design framework (e.g., the class I structure with a small variable arm), and by the existence of

alternative folds such as the class II large-variable arm tRNAs and the unusual mitochondrial species. There are also examples of RNAs that do not function conventionally in translation but are nevertheless capable of aminoacylation. These include tmRNA as well as tRNA-like pseudoknots in plant viral RNAs (Giegé et al., 1998a).

The tRNA core region consists of portions of the D, T, and variable arms, as well as the short connector bridging the acceptor and D-stems. Among these elements, the T-loop structure is by far the most highly conserved; it forms interdigitated base-stacking interactions with the conserved G18–G19 nucleotides in the D-loop to build the upper horizontal arm of the tRNA (domain II; Steinberg et al., 1997). The upper portion of the vertical arm (domain I) is constructed from the D-stem together with nucleotides from the D-loop, variable arm, and acceptor/D connector, and represents the most variable portion of the structure. It includes both base-triple interactions as well as noncanonical base pairings (Fig. 1).

The majority of tRNAs possess short variable loops of 4–5 nt (class I), whereas the isoacceptors for serine, leucine, tyrosine, and selenocysteine in many organisms have much longer insertions of 11 nt or more (class II; Sprinzl et al., 1998). Analysis of the tRNA sequence database shows that class II tRNAs typically

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FIGURE 1. Structural models of tRNA^{QSer}. Upper left: Presumed secondary structure of tRNA^{QSer}. In this tRNA, the 5-nt variable loop of *E. coli* tRNA^{Ser} is replaced with the 20-nt variable arm of *T. thermophilus* tRNA^{Ser} (GGA). tRNA^{QSer} also includes the mutations C9G and A13G, and the insertion of nucleotides U20a and G20b (Nissan et al., 1999; highlighted). Nucleotides in the core are color coded. Upper right: Tertiary structure model of tRNA^{QSer}, derived from replacing nucleotides in the crystal structure of tRNA^{Ser} followed by energy minimization. Bottom: Expanded view of boxed region at upper right. Nucleotide 20b is modified to dihydrouridine (D) in tRNA^{Ser}; because tRNAs in this study are derived from in vitro transcription, they possess instead a uracil at this position.

have larger D-loops than class I species; in *Thermus thermophilus* tRNA^{Ser} (GGA), one of these additional nucleotides stacks on the large variable arm, helping to define its orientation relative to the core region (Biou et al., 1994). Comparisons of structures and sequences suggests that other conserved differences between

class I and class II tRNAs may involve the precise positioning and identity of nt 9, which is found on different stack layers in the two structural types. Additionally, class I tRNAs use centrally located variable loop nucleotides to stack into and (presumably) help stabilize the core region, whereas in class II only the ex-

Class II transfer RNA folds

treme 5' and 3' distal nucleotides (positions 44 and 48) generally form tertiary pairing interactions with other bases.

Previously, we reengineered the core region of *Escherichia coli* tRNA^{Gln}₂ to incorporate the large class II extra arm of tRNA^{Ser} while retaining efficient recognition by glutamyl-tRNA synthetase (GlnRS) (Nissan et al., 1999). Thermal melting analysis showed that a 2-nt D-loop insertion accompanying the variable-arm exchange is essential for maintaining the class II structure, and a tRNA^{Ser}-like triple at position 9-13-22 is required for efficient glutamylation. Contrary to previous suggestions (Rogers & Söll, 1993; Frugier et al., 1994; Ibbi et al., 1996), the experiments indicated that GlnRS can be quite sensitive to alteration of tRNA tertiary structure.

These and other data (Ioudovitch & Steinberg, 1998), together with analysis of the extensive tRNA sequence database (Sprinzl et al., 1998), suggested the existence of alternative class II folding paradigms distinct from that observed in the crystal structure of tRNA^{Ser}. Class II tRNAs lacking D-loop insertions or possessing an unusual Py9 within the 9-13-22 triple are rare but do appear in the database, and these species appear to possess compensations elsewhere in the structure that may render them functional. To test the inferences made based on the sequence/structure analysis, we constructed a battery of additional mutants to attempt rescue of class II-like tRNA^{Gln} species lacking either a stable variable arm (due to a D-loop deletion) or the capacity for efficient glutamylation (due to a C9-A13-A22 triple). These data show that a class II-like thermal melting profile can be reconstituted in the D-loop deletion mutant by addition of nucleotides either 3' to the variable arm or within the acceptor/D-stem connector. Further, the ability of GlnRS to aminoacylate a class II framework containing C9 was recovered in two ways: by addition of two uracils 3' to the variable arm, and by replacement of the U20a/G20b D-loop insertion with C20a/A20b. Implications for class II tRNA sequence/structure relationships and for GlnRS recognition of the tRNA core region are discussed.

RESULTS

Experimental design

We wished to explore the possibilities for alternative functional folds among class II tRNA species. To accomplish this, advantage was taken of the extensive tRNA sequence compilation (Sprinzl et al., 1998), together with a previous RNA engineering experiment in which the class I *E. coli* tRNA^{Gln} was redesigned to class II structure while retaining efficient glutamylation properties (Nissan et al., 1999). The hybrid tRNA^{QSer} (Fig. 1) is a tRNA^{Gln} species possessing the 20-nt variable arm of *T. thermophilus* tRNA^{Ser} (GGA). This tRNA

is efficiently glutamylated, and possesses distinct structural features compared to class I tRNA^{Gln} as revealed by thermal melting analysis. The thermal melt of tRNA^{QSer} shows greater overall hyperchromicity and a much more cooperative transition, identifying the presence of an additional stable helical arm (Nissan et al., 1999).

tRNA^{QSer} possesses five other nucleotide alterations in addition to the exchange of the variable arm (Figs. 1 and 2). The importance of these was also previously tested by mutational analysis. Reversion of U44 to C44 produces a tRNA with slightly improved glutamylation properties (k_{cat}/K_m at 25% of wild-type tRNA^{Gln}), and which retains the class II thermal melting signature. However, removal of the 2-nt D-loop insertion produces a species (tRNA^{QSer-2D}) with a class I-like thermal melt despite the presence of the extra arm. This tRNA is efficiently glutamylated. Conversely, reversion of the presumed G9-G13-A22 base triple in

FIGURE 2. Secondary structures of species derived from tRNA^{QSer}, indicating mutations introduced in this study. Top: Secondary structure of tRNA^{QSer-2D}. Bottom: Secondary structure of tRNA^{QSer-C9/A13}.

tRNA^{QSer} to the tRNA^{Gln}-like C9-A13-A22 severely decreases function in glutaminylation, but the tRNA (tRNA^{QSer_C9/A13}) retains a class II melting profile (Nissan et al., 1999).

We searched the tRNA sequence database for examples of class II tRNAs that might possess a short class I-like D-loop or the presence of a pyrimidine at position 9, both of which apparently cause structural defects in the tRNA^{QSer} derivatives described above. Such a search can reveal examples in nature by which a functional class II core might be constructed according to different principles than exemplified by the crystal structure of tRNA^{Ser} (Biou et al., 1994). Indeed, of 388 class II sequences in the most recently updated database, there are 16 species possessing a short 2-nt D-loop β -region, and 13 with a pyrimidine at nt 9 in the core (Fig. 3). Guided by these data, reconstitution of the defective tRNA^{QSer} derivatives tRNA^{QSer_2D} and tRNA^{QSer_C9/A13} was attempted by directed mutagenesis.

Structural compensations for class II tRNAs lacking D-loop insertions

The tRNA D-loop is divided into two regions separated by the nearly invariant G18–G19 sequence; these guanine bases make key tertiary contacts with the T loop. Nucleotides 20, 20a, 20b, and 21 constitute the β -region of the D-loop (Fig. 1), with the 20a/20b insertion highly conserved among class II tRNAs. Most of the 16 class II species lacking this insertion possess instead one or more additional and apparently unpaired nucleotides (usually uridine or guanosine) between the variable and T-arms (Fig. 3). To test whether this alternate design indeed confers class II-like structure, each of the four naturally occurring ribonucleotides was separately inserted between the variable and T-arms of tRNA^{QSer_2D}, in the position immediately 5' to C48 of the 15–48 *trans* Levitt pair. The new tRNAs were produced by in vitro enzymatic synthesis using T7 RNA polymerase, purified, and subjected to thermal melting analysis (Fig. 4a). Each of these species reconstitutes the class II-like thermal melting profile characteristic of tRNA^{QSer} and lacking in tRNA^{QSer_2D}. The enhanced hyperchromicity and cooperativity of the transition is likely directly attributable to the additional melting of a well-ordered variable arm, in addition to the other four helical stems (Nissan et al., 1999). These data show that the tertiary structure of class II tRNAs can be stabilized by insertions either in the D-loop or at the variable arm/T-stem junction. The insertion of guanosine generates a tRNA with overall hyperchromicity significantly higher than the others. Because the variable arm itself is identical in all the mutants, this species may possess a particularly stable junction between this arm and the other nucleotides of the tRNA core.

The mutants were also tested for efficiency in aminoacylation by GlnRS (Table 1). Each of the four species

are aminoacylated at plateau levels within twofold of tRNA^{QSer} and tRNA^{QSer_2D}; although the proportion of functional constructs after refolding in the presence of Mg²⁺ cations is low, we have previously shown that the excess nonfunctional species do not inhibit the reaction (see Nissan et al., 1999, and Materials and Methods). The A47r, G47r, and C47r mutants exhibit similar kinetic parameters, with k_{cat}/K_m reduced approximately 20- to 25-fold (mostly at the level of k_{cat}) compared to tRNA^{QSer} and tRNA^{QSer_2D}. These data indicate significant sensitivity of GlnRS to tRNA core region structure. However, the U47r mutant is glutaminylated with the same efficiency as the parent species (Table 1). Thus, subtle and important effects on tRNA core structure and/or dynamics depend in part on the identity of the inserted nucleotide.

A single class II tRNA^{Ser} species from bacteriophage T5 appears to compensate for a short D-loop β -region by insertion of a purine in the short acceptor/D-stem connector region, lengthening this segment from 2 to 3 nt (Fig. 3). The unusual selenocysteine-encoding tRNA^{Sec} species also possess 1- to 2-nt insertions in this region. A model has been proposed for tRNA^{Sec} folding in which an acceptor/D-stem connector nucleotide stacks on the large variable arm in a manner analogous to D-loop nucleotide G20b of *T. thermophilus* tRNA^{Ser} (Ioudovitch & Steinberg, 1998). Thus, insertions in the acceptor/D-stem connector might represent a third design for the class II fold, specific to those species lacking the common D-loop insertion.

To test this idea we generated two further mutants via insertions of an adenosine residue either between U8 and G9 (A8a), or between G9 and C10 (A9a) of tRNA^{QSer_2D}. Thermal melting analysis of T7 RNA polymerase-generated transcripts of these tRNAs shows that both species indeed recover a class II-like thermal melting profile (Fig. 4b). The species containing the A9a insertion is efficiently glutaminylated, with an equivalent K_m and only twofold reduced k_{cat} compared with tRNA^{QSer} (Table 1). However, the A8a insertion instead produces a tRNA with 35-fold reduced k_{cat}/K_m relative to the parent tRNA^{QSer} and tRNA^{QSer_2D} molecules. These data provide a further example of the sensitivity of GlnRS to apparently small alterations in the tRNA core region.

Dissection of the 9-13-22 base triple

Most class II tRNAs possess purines at nt 9 and 13 and may form the 9-13-22 base-triple found in tRNA^{Ser} (Biou et al., 1994). With the exception of tRNA^{Cys}, which also possesses a 9-13-22 triple (Nissen et al., 1999), in class I tRNAs nt 9 lies instead on the same stack layer as D-stem base pair 12-23. Among non-selenocysteine-encoding tRNAs, only 13 class II species possess a pyrimidine at position 9, as in tRNA^{QSer_C9/A13} (Fig. 2). This tRNA is an extremely poor GlnRS substrate de-

A

Sequence alignment of class II tRNAs possessing short D-loops.

		Acceptor	D stem/loop	Anticodon	Variable Arm	TΨC stem/loop	Acceptor
DS0341	TGA	ARCHAEGLOBUS	FULG.	-GCCGGGGTTGCAGAGA--GGT--	ACTGCGCCAGCCTTGAGAGCTGGTGCCC ----	GTAA-----	GGG CTCCTGGGTTCAAATCCAGCCCGCGG ----
				*****	*****		
DS0343	GGA	ARCHAEGLOBUS	FULG.	-GCCGGGGTTGCCAGC--GGT--	AAGGCGCACGCTGGAGAGCGTGTGGGC ----	GTTC-----	GCC CGCTGGGTTCGAATCCACCCCGGCG ----
				*****	*****		
DS0651	GCT	METHANOCOCCUS	JAN.	-GCCGGGGTTGCCAGCCTGGT--	ACGGCGTGGGACTGCTAATCCCAT -TGGGC--	AAC----	GCC AG--CCCGGGTTCAAAGTCCCGGCCCGCG ----
				*****	*****		
DS0680	GCT	METHANOTHERM.	FER.	-GCCGGGGTTGCCAGCCTGGT--	AGGCGCTCGGCTGCTAAGCCGAT -GATCC--	GTTAA--	GGAT CG--CGCGGGTTCAAATCCCGTCCCGGCG ----
				*****	*****		
DS1500	CGA	LACTOBAC.	BULGARIC.	-GGAGAGTTGGCAGAGC--GGT--	AATGCAGCGGACTCGAAATCCGCC GAACCAATGTTGAATTGGT ---		CGCAGGTTCAAATCCTGACTCTCCT--
				*****	*****		
DK1280	TTT	BORRELIA	BURGDORF.	-GGGCTCATAGCTCAGGT--GGT--	AGAGCAGCGCCCTTTAAGGCGTTAAG ----	GCG-----	TTT GTCGTAGGTTGAGTCTCTACTGAGCTCA ---
				* *****			
DY0260	GTA	PHAGE	T5	-GGGGGAGTATTCTGTAA*GG--	TAG*GAGCTGACTGTAATTAGTT -GTC---	ATTGC----	GAC TCGGGTGGTTCGACTCCATCCTCCCCCT ---
				*****	*****		
RL2020	CAA	RHODOSPIRIL.	RUB.	-GCCUUUGUAGCGGAAD--GGD--	AACGCGCAGACUCAHAAPCUGCU -UUGG--	UAA--	CCAG G--UGGUAGTTCGACUCUCCCCAAGGACCA
				*****	*****		
RS0502	GGA	HALOFERAX	VOLCANII	-GCCAGGAUGGCCGA(C--GGU--	AAGGCGACGCCUGGAAGCGUGU -UCCC--	UCU-----	GGGA U--?GGGGG) PB0AAUCCUCCUUGGCGCCA
				*****	*****		
DS0260	TGA	PHAGE	T5	-GGAAGGTAG GGCGTAGT --GGT--	ACGCAACTAGTCTTGAAAAGTACG -CC*TGT-AGTG--	ATACGG--	TGATGGTTCGACTCCATTACCTTCTCCA
				*****	*****		
DS0340	GCT	ARCHAEGLOBUS	FULG.	-GCCGGGGTTGCCAGC--GGT--	AAGGCGACGCGCTGCTAAGCCGTTGGGC ----	GTTGC----	CCG--CGTGGGTT CGAATCCACCCCGGCG ----
				*****	*****		
DS1520	CGA	LACTOBAC.	DELBRUEC.	-GGAGAGTTGGCAGAGC--GGT--	AATGCAGCGGACTCGAAATCCCGGAGCCAATGTTGAATTGGGT ---		CGCAGGTTCAAATCCTGACTCTCCT--
				*****	*****		
DS9994	CGA	HUMAN		-GGAGATATGGCACAATCTGGT--	ACTGCAACGAGCTCGAAATCCGTCGAGCCG ---	CGTIT--	CGGGT TCGGGGTTCAAATCCGCTATCTCTCT ---
				*****	*****		
DW0010	CCA	HERPESVIRUS	68	-GCCAGGGTAGCTCAATT--GGT--	AGAGCGGCAGACCCACCTACGTGGTCT ---	AGTCT----	GTGGATCTCGGTTCAAGTCCGAGTCTTGCC--
				*****	*****		
DY0000	GTA	CHLORELLA	VIRUS K2	-CTCGCCATAGCTCAGTT--GGA--	AGAGCAGCGGACTGTAATGAAACACAAT ---	AATCC--	GTAAGTCCCGCTTCGAACCGGAGTGGCGAGA--
				*****	*****		
DY7520	GTA	TRYPANOSOMA	BRUCEI	-CCTTCTGTAGCTCAATT--GGT--	AGAGCATGTAGCTGTAGATATGC -GGGTATCACAGGT-----		CGTGGTTCGATTCCGCGCGAAGG--
				*****	*****		

B

Sequence alignment of non-selenocysteine class II tRNAs possessing a pyrimidine at position 9

		Acceptor	D stem/loop	Anticodon	Variable Arm	TΨC stem/loop	Acceptor
DL0650	TAG	METHANOCOCCUS JAN.	-GCAGGGG TCGCCAAGCTGGCCAAAGGCGCTGGGCTAGGACCCAGT -CCC----	GTAG----	GGG TTCCAGGGTTCAAATCCCTGCCCTGCA ---		
			=====	=====		=====	
DL0651	GAG	METHANOCOCCUS JAN.	-GCCGGGG TCGCCAAGCCAGGTCAAAGGCGCCAGATTGAGGGTCTGGT -CCC----	GTAG----	GGG TTCCGGGGTTCAAATCCCGTCCCCCGCA ---		
			=====	=====		=====	
DL0652	TAA	METHANOCOCCUS JAN.	-GCAGGGG TCGCCAAGCCTGGCCAAAGGCGCGGACTTAAGATCCGGT -CCC----	GTAG----	GGG TTCCGGGGTTCAAATCCCTGCCCTGCA ---		
			=====	=====		=====	
DS1350	GCT	STREPTOMYCES LIV.	-GGAGGGG TCGCCTAGTCCGGTCTATGGCGCCGCACTGCTAATGCGGTTTGGGT ---	CTTAA-AGCC AT--CGAGGGTTCAAATCCCTCCGCTCCG ---			
			=====	=====		=====	
DS0340	GCT	ARCHAEGLOBUS FULG.	-GCCGGGG TTGCCGAGC--GGT--AAGCGCAGCGCTGCTAAGCCGTTGGGC ---	GTTGC----	CC g -CGTGGG TTCGAATCCCACCCCGGCG ---		
			=====	=====		=====	
DS0341	TGA	ARCHAEGLOBUS FULG.	-GCCGGGG TTGCAGAGA--GGT--ACTGCGCCAGCCTTGAGAGCTGGTGCCC ---	GTAA----	GGG CTCCTGGGTTCAAATCCAGCCCGGCG ---		
			=====	=====		=====	
DS0342	CGA	ARCHAEGLOBUS FULG.	-GCCGGGG TTGCAGAGCCAGGCC--ACTGCGGAGACTCGAGATCTCCTGCC ---	GAAA----	GG CGCGTGGGTTCGAATCCCACCCCGGCG ---		
			=====	=====		=====	
DS0343	GGA	ARCHAEGLOBUS FULG.	-GCCGGGG TTGCCGAGC--GGT--AAGCGCGACGCTGGAGAGCGTGTGGGC ---	GTTT-----	GCC CGCGTGGGTTCGAATCCCACCCCGGCG ---		
			=====	=====		=====	
DL0660	TAG	METHANOCOC. VANI.	-GCAGGGG TTGTGCGAGCCTGGCCAAAGATGCAGGACTTAGAATCCTGT -CCA----	GTAG----	TGG TTCCAGGGTTCAAATCCCTGCCCTGCA ---		
			=====	=====		=====	
DY1820	GTA	PSEUDOMONAS AER.	-GGAGGGAT CCCTTAGT --GGCC--AAAGGATCAGACTGTAATCTGAC--GTC---	ATA-----	GAC TTTCGAAGGTTCGAATCCTTCTCCCTCCACCA		
			=====	=====		=====	
DY2000	GTA	HAEMOPHILUS INFLU.	-TGAGGGG TTCCCGAGC--GGCCAAAGGGGCGAGACTGTAATCTGTT--GGC---	TCA-----	GC TTTCGAAGGTTCGAATCCTTCTCCCTCCACCA		
			=====	=====		=====	
RY1660	QUA	E. COLI	-GGUGGGG 4UCCCGAGC--#GCCAAAGGGAGCAGACUQUA*APCUGCC--GUC---	AUC-----	GAC UUUGAAGGTPCGAAUCCUCCCGCCACCA		
			=====	=====		=====	
RY1661	QUA	E. COLI	-GGUGGGG 4UCCCGAGC--#GCCAAAGGGAGCAGACUQUA*APCUGCC--GUC---	ACA-----	GAC UUUGAAGGTPCGAAUCCUCCCGCCACCA		
			=====	=====		=====	

FIGURE 3. A: Sequence alignment of class II tRNAs possessing short D-loops, extracted from the updated tRNA sequence database (Sprinzl et al., 2000). β -regions following the conserved G18-G19 in the short D-loops are in bold. Additional unpaired nucleotides in the variable arm are in bold underline for those tRNAs that possess clear secondary structures in this region. The nucleotide at position 48 is considered to be paired, because it participates in the noncanonical Levitt pair 15-48. tRNAs DS0260, DS0340, DS1520, DS9994, DW0010, and DY0000 possess apparently mispaired bases in the region of the variable arm adjacent to the core, making the number and position of unpaired nucleotides uncertain. The *Thermus brucei* tRNA^{Tyr} (GTA) (DY7520) appears to have no regular base-paired secondary structure in its variable arm. An asterisk in the alignment indicates insertion of additional nucleotides at this position (not shown). **B:** Sequence alignment of non-selenocysteine class II tRNAs possessing a pyrimidine at position 9. The rare pyrimidines at position 9 in class II tRNAs are in bold. Extra nucleotides at the 3' end of the variable arm are in bold and underlined. The nucleotide at position 48 directly adjacent to the T-stem is paired in the noncanonical 15-48 Levitt pair interaction. The number of class II tRNA sequences analyzed is 388.

spite its class II-like thermal melting profile (Table 1; Fig. 4), suggesting a local structural rearrangement at the synthetase interface while retaining an ordered variable arm. To test whether the crucial lesion in this

tRNA is indeed the C9 nucleotide, each of the two single-substitutions tRNA^{QSer_C9} and tRNA^{QSer_A13} were constructed and synthesized. Both species retain class II-like thermal melting profiles (Table 2; data not

FIGURE 4. **a:** Left: Plot of the averaged absorbance at 260 nm as a function of temperature for tRNA^{QSer}_{2D} (red), tRNA^{QSer}_{2D}+G47r (blue), tRNA^{QSer}_{2D}+U47r (green), tRNA^{QSer}_{2D}+A47r (black), tRNA^{QSer}_{2D}+C47r (pink), and tRNA^{QSer}_{2D}+A47r (purple). These thermal melts were carried out after dialysis of the tRNAs into a buffer lacking Mg²⁺. Right: Plot of the thermal unfolding profile as the first derivative of absorbance with respect to temperature, revealing the transition at approximately 64 °C characteristic of tRNA^{QSer} and the derivatives with insertions at position 47r. Color scheme is the same as at left. **b:** Left: Plot of the averaged absorbance at 260 nm as a function of temperature for tRNA^{QSer}_{2D}+A8a (red), tRNA^{QSer}_{2D}+A9a (blue), tRNA^{QSer}_{2D}+A8a (green), tRNA^{QSer}_{2D}+A9a (black), and tRNA^{QSer}_{2D}+A9a (pink). Right: Plot of the thermal unfolding profile as the first derivative of absorbance with respect to temperature.

shown). As expected, the tRNA^{QSer}_{A13} mutant likely possessing a G9-A13-A22 triple is glutaminylated with an efficiency comparable to tRNA^{QSer} (Table 2). Surprisingly, however, k_{cat}/K_m for glutamylation of the tRNA^{QSer}_{C9} molecule is improved by 40-fold compared with tRNA^{QSer}_{C9/A13}, and is reduced only 14-fold with respect to tRNA^{QSer}. There are no examples of Py9 in class II tRNAs that lack a compensating insertion between the variable and T-arms (Fig. 3; see below). Such tRNAs may be rare in nature and not yet represented in

the database. One possibility for the large improvement in kinetic parameters could be that the poorly glutaminylated tRNA^{QSer}_{C9/A13} species is globally misfolded because of a dominating alternative non-tRNA-like secondary structure. Such a structure is not expected based on the tRNA^{QSer}-like thermal melting profile, or predicted by free-energy calculations for this sequence (data not shown). However, an alternative non-tRNA-like fold would be consistent with the very low level of functional molecules in the preparation (Table 2).

TABLE 1. Aminoacylation levels and steady-state kinetic parameters for variants based on tRNA^{QSer}_{2D}.

tRNA	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ M ⁻¹)	pmol/OD	% HC ^a
G1-tRNA ^{Gln} (b)	3.4 ± 0.16	0.30 ± 0.12	1.1 × 10 ⁷	267 ± 14	16.7
tRNA ^{QSer} (b)	0.81 ± 0.11	0.62 ± 0.12	1.3 × 10 ⁶	152 ± 5	21.5
tRNA ^{QSer} _{2D} (b)	0.70 ± 0.18	0.46 ± 0.12	1.5 × 10 ⁶	142 ± 14	16.8
tRNA ^{2D} +A8a	— ^c	—	(3.89 ± 0.17) × 10 ⁴	71.4 ± 0.4	20.8
tRNA ^{2D} +A9a	0.39 ± 0.07	0.64 ± 0.10	6.1 × 10 ⁵	133 ± 2	23.4
tRNA ^{2D} +A47r	0.031 ± 0.01	0.46 ± 0.15	6.7 × 10 ⁴	67 ± 8	19.3
tRNA ^{2D} +C47r	— ^c	—	(5.26 ± 0.50) × 10 ⁴	135 ± 14	19.5
tRNA ^{2D} +G47r	0.096 ± 0.002	1.19 ± 0.09	8.1 × 10 ⁴	150 ± 7	30.9
tRNA ^{2D} +U47r	0.18 ± 0.01	0.12 ± 0.02	1.5 × 10 ⁴	290 ± 39	21.8

^aThis parameter indicates the percentage increase in absorbance of the tRNA when measured at 25 °C versus 80 °C.

^bData for these tRNAs are taken from Nissan et al. (1999).

^cRates for these tRNAs are too slow for accurate estimation of the individual k_{cat} and K_m parameters.

All known class II tRNAs possessing Py9 conserve an insertion of one or (more commonly) two unpaired nucleotides 3' to the variable arm (Fig. 3). In 10 of the 13 species, the insertion contains at least one uridine nucleotide; seven of the tRNAs possess a U-U doublet at this position. We examined the consequences of additional uridines at this position by inserting either one or two such nucleotides into the tRNA^{QSer}_{C9/A13} background. Although the tRNA with a single uridine insertion (tRNA^{QSer}_{C9/U47}) was reduced in glutaminylation even below tRNA^{QSer}_{C9/A13}, the double-uridine insertion produced an efficient glutamine acceptor with k_{cat}/K_m within twofold of tRNA^{QSer} (tRNA^{9/13+UU47r}; Table 2). Each of these derivative species retains the enhanced hyperchromicity and cooperativity of the tRNA^{QSer} thermal melting profile (Table 2; data not shown).

Inspection of the Py9-containing class II tRNA sequences revealed a second correlation: 7 of the 13 sequences possess a CA doublet at positions 20ab in

the large β -region of the D-loop. By contrast, none of these 13 tRNAs contains the UG doublet present in tRNA^{QSer} and tRNA^{QSer}_{C9/A13} (in the tRNA^{QSer} species upon which the designs were originally based, this uridine is modified to dihydrouridine). The crystal structure of *T. thermophilus* tRNA^{QSer} shows that D20a is involved in a tertiary base-triple with the noncanonical Levitt pair at positions G15-C48, whereas G20b stacks on the proximal base pair of the variable arm (Biou et al., 1994). Thus, the identities of both inserted nucleotides are of potentially key significance in the class II architecture. A role for these nucleotides was explored by substituting U20a/G20b in tRNA^{QSer}_{C9/A13} with C20a/A20b. This tRNA is improved 100-fold in k_{cat}/K_m for glutaminylation, to within 10-fold of tRNA^{QSer} (tRNA^{9/13_CA20ab}; Table 2). In the tRNA^{QSer}_{C9/A13} background, combining C20a/A20b with the U-U doublet insertion 3' to the variable arm produced a class II tRNA (tRNA^{9/13_CA_UU}) with glutaminylation properties equivalent to tRNA^{QSer}. This is improved twofold com-

TABLE 2. Aminoacylation levels and steady-state kinetic parameters for variants based on tRNA^{QSer}_{C9/A13}.

tRNA	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ M ⁻¹)	pmol/OD	% HC ^a
tRNA ^{QSer} (b)	0.81 ± 0.11	0.62 ± 0.12	1.3 × 10 ⁶	152 ± 5	21.5
tRNA ^{QSer} _{C9/A13}	(1.96 ± 0.37) × 10 ⁻³	0.87 ± 0.09	2.3 × 10 ³	1.5 ± 0.3	20.2
tRNA ^{QSer} _{C9}	— ^c	—	(9.2 ± 1.9) × 10 ⁴	70.1 ± 4	22.6
tRNA ^{QSer} _{A13}	0.59 ± 0.05	0.33 ± 0.05	1.8 × 10 ⁶	211 ± 11	23.1
tRNA ^{9/13+U47r}	— ^c	—	<10 ³	8.6 ± 0.1	25.3
tRNA ^{9/13+UU47r}	0.22 ± 0.05	0.47 ± 0.12	4.7 × 10 ⁵	129 ± 11	24.1
tRNA ^{9/13_CA20ab}	0.13 ± 0.01	0.89 ± 0.02	1.5 × 10 ⁵	36 ± 5	22.7
tRNA ^{9/13_CA_UU}	0.30 ± 0.05	0.17 ± 0.07	1.8 × 10 ⁶	68 ± 3	21.1

^aThis parameter indicates the percentage increase in absorbance of the tRNA when measured at 25 °C versus 80 °C.

^bData for this tRNA is taken from Nissan et al. (1999).

^cRates for these tRNAs are too slow for accurate estimation of the individual k_{cat} and K_m parameters.

pared with the U-U insertion alone. As is the case for all the Py9-containing tRNAs, these mutants also retain a tRNA^{QSer}-like thermal melting profile (Table 2; data not shown).

DISCUSSION

Molecular design of class II tRNAs

Using a combined phylogenetic and biochemical analysis, we have identified structural elements essential to the construction of class II tRNA tertiary cores. Although the majority of class II molecules possess a 2-nt insertion in the β -region of the D-loop together with a purine at position 9, some exceptions to this design can be found in the tRNA sequence compilation (Sprinzl et al., 1998; Fig. 3). These unusual species were highlighted by the study of tRNA^{QSer}—a functional tRNA^{Gln} molecule engineered to contain the large variable arm from tRNA^{Ser} (Nissan et al., 1999; Fig. 2). Removal of the D-loop insertion in tRNA^{QSer} causes unraveling of structure in the extra arm (tRNA^{QSer_2D}), whereas introduction of tRNA^{Gln} nucleotides (including C9) at the 9-13-22 position results in a very small proportion of functional transcripts that are inefficiently glutaminylated (tRNA^{QSer_C9/A13}). Although the class II-like thermal melting profile is retained in this species, a different and more localized structural defect is likely responsible for the extreme effects on aminoacylation. The nucleotide compositions in the core regions of tRNA^{QSer_2D} and tRNA^{QSer_C9/A13} have so far not been found in naturally occurring tRNAs.

The thermal melting data presented here clearly indicate that a well-ordered variable arm can be generated with 1- or 2-nt insertions arising from either the β -region of the D-loop, an unpaired segment directly 3' to the variable stem, or the short connector between the acceptor and D-arms. All class II tRNAs found in Nature fall into one of these three structural categories. Inspection of the crystal structure of tRNA^{Ser}, which contains a D-loop insertion, shows that the three regions of the tRNA are in close proximity adjacent to the variable arm (Fig. 1). Thus, nucleotides from any segment could directly stabilize the crucial structure at the interface between the variable arm and the core of the molecule. This might be accomplished by base stacking on the proximal base pair of the variable arm, as observed for G20b from the D-loop insertion of *T. thermophilus* tRNA^{Ser} (Biou et al., 1994; Loudovitch & Steinberg, 1998). It is of interest to note that the position of adenosine insertion in the acceptor/D-stem connector has significant effects on glutaminylation efficiency, as does the identity of the nucleotide at position 47r (Table 1). A U47r insertion maintains efficient aminoacylation whereas the other base insertions at this site reduce k_{cat}/K_m by 20- to 30-fold. Thus, although these experiments immediately suggest three distinct folds

as defined by the arrangement of base-stacking interactions, additional arrangements may also be possible. Uridine is disfavored in base-stacking interactions compared to the other nucleotides (Burkard et al., 1999), and may promote yet another alternative structure that is in some way favorable to GlnRS recognition (see below).

We were able to reconstitute efficient glutaminylation in tRNA^{QSer_C9/A13} by introducing compensatory mutants based on sequence analysis of class II core regions, indicating that some of the same structural principles required for biological function of these class II tRNAs also allow for efficient GlnRS recognition. Both the U-U doublet inserted 3' to the variable arm, and introduction of the C20a/A20b double-mutant, were designed in this manner. Unlike the mutants designed to reconstitute tRNA^{QSer_2D}, tRNA^{9/13+UU47r} and tRNA^{9/13_CA_UU} contain insertions both in the D-loop and 3' to the variable arm. Thus, these species define a fourth distinct, functional class II fold within the class I tRNA^{Gln} framework. The four designs have the large variable arm in common, and differ with respect to the number and position of insertions in the D-loop, acceptor/D-stem connector region, and variable arm/T-stem junction.

The differences in the positions of insertions in these class II tRNAs very likely indicates distinct folds varying in the arrangement of base-stacking interactions in the core. However, a number of findings made here are not readily explainable on the basis of sequence alignments, suggesting the possibility that other stable packing arrangements of core nucleotides may also be possible. First, tRNA^{QSer_C9} is glutaminylated much more efficiently than is tRNA^{QSer_C9/A13}, despite possessing C9 while lacking inserted nucleotides 3' to the variable arm. Second, a single uridine insertion apparently suffices for the biological function of some class II tRNAs (Fig. 3), but in the tRNA^{QSer} context, two uridines are required to repair the structural defect in tRNA^{QSer_C9/A13}. Third, further sequence analysis shows that 21 of 22 class II tRNAs possessing UU insertions 3' to the variable arm (regardless of the nucleotide at position 9) are accompanied by Py-A at positions 20ab (data not shown). The remaining such tRNA has only a 1-nt pyrimidine insertion in the β -region of the D-loop. However, in tRNA^{Gln}, either C20a/A20b or U20a/G20b works in combination with the UU insertion to generate an efficiently glutaminylated species (Table 2). Interestingly, a single uridine inserted at the variable/T-arm junction does reconstitute a class II fold when working from the tRNA^{QSer_2D} framework (Table 1; Fig. 4), but two are required to restore efficient glutaminylation properties to tRNA^{QSer_C9/A13}, which already contains a D-loop insertion.

These experiments have resulted in the assembly of a variety of distinct class II core designs within the common architecture of a single tRNA^{Gln} species. We have shown that the analysis of compensatory substi-

tutions based on sequence alignments indeed allows prediction of nucleotides essential for alternative functional folds. We have also generated several examples of functional tRNA^{QSer} derivatives that have no known counterparts in Nature. Of course, the level of analysis presented here cannot yet provide a detailed explanation for why certain configurations of core regions allow extra-arm folding or efficient glutaminylation, whereas others do not (see also below). It is hoped that assembling a variety of class II designs within the same structural context will facilitate further thermodynamic and structural analysis of these tRNAs, with a view towards deeper understanding of function in aminoacylation and in ribosomal decoding/peptidyl transfer reactions.

tRNA core region recognition by glutaminyl-tRNA synthetase

The data presented here demonstrate that efficient recognition of tRNA by *E. coli* GlnRS can be quite sensitive to the architecture of the core region (Tables 1 and 2). Two of the mutants retaining C9 and A13 are apparently reduced by four orders of magnitude in k_{cat}/K_m . However, in both cases where steady-state aminoacylation is reduced to this extent, it is also found that less than 1% of transcripts are functional even under conditions of 10-fold enzyme excess. It is known from analysis of wild-type transcripts that a tRNA substrate pool containing 17% functional molecules can be aminoacylated with identical steady-state kinetic parameters to in vivo-derived tRNA^{Gln} (70–100% functional molecules). At this level the sixfold molar excess of nonfunctional tRNA in the assays clearly does not function as an inhibitor. tRNAs with plateau aminoacylation levels as low as 68 pmol/OD are efficiently aminoacylated as well (Table 2). However, the much greater molar excess of tRNA required for the kinetic analysis of tRNA^{QSer_C9/A13} and tRNA^{QSer_C9/A13/U47r} may render their k_{cat}/K_m values less reliable, because the nonfunctional species might function as inhibitors. Thus, the possibility that GlnRS shows a 10⁴-fold range of discrimination based on core region structure should be considered with caution. However, k_{cat}/K_m values vary over a 200-fold range among the other mutants, with the best class II framework tested capable of aminoacylation at 25% of k_{cat}/K_m compared to class I tRNA^{Gln} (Tables 1 and 2; Nissan et al., 1999). It is thus clear that the contribution of the core region is quite comparable to that of many previously identified identity elements in the acceptor stem and anticodon (Jahn et al., 1991; Hayase et al., 1992).

No systematic study of GlnRS recognition of altered core regions in the class I framework has as yet been performed. In the limited number of mutants examined thus far in vitro (Ibba et al., 1996; Sherlin et al., 2000), the effects found have been relatively small. However, the importance of backbone-mediated interactions at the inside corner of the L-shape is clearly demon-

strated from in vivo studies in the class I framework, which identify the noncanonical G15-C48 and A13-A22 pairs (among others) as determinants of GlnRS recognition (McClain et al., 1998). Given the large effects observed in our in vitro study, we think it likely that the core region also contributes importantly to GlnRS discrimination against class II tRNAs in vivo.

Because GlnRS interacts only with the inside of the tRNA L-shape, the mechanism by which noncognate tRNAs are rejected based on noncomplementary cores must arise from indirect propagation through the tRNA structure. The variable arm interacts with the D and augmented D-stems on the major groove side, thus helping to determine the detailed architecture of the helix and the consequent positioning of sugar-phosphate backbone moieties. By binding on the opposite minor groove side of the stem, it appears likely that GlnRS then discriminates against an altered core by making suboptimal contacts along this and adjacent portions of the interface. In the interaction with noncognate tRNA, we hypothesize that the mutual induced-fit conformational changes occurring in both partners are then in turn altered, leading to mispositioning of the terminal A76 nucleotide relative to the other two substrates in the active site (Fig. 5). This process may be considered as a form of indirect readout, analogous to the similar phenomenon in protein-DNA recognition (Rhodes et al., 1996). The tRNA core is much more complex than an isolated helix; thus, the structural variability of the sugar-phosphate backbone (within the confines of the global L-shaped fold) is likely to be greater. This could provide the basis for very substantial contributions to synthetase selectivity, although the details of this proposed process are so far poorly understood. Considerable further experimentation is needed to address both sequence/structure relationships within the tRNA (How might altered cores produce distinct D-stem geometries?), as well as the pathways by which D-stem binding interactions in turn affect molecular processes in the distant active site. Whether this structural coupling contains elements in common with proposed pathways for transmitting anticodon sequence binding to the GlnRS active site (Rould et al., 1989; Rogers et al., 1994; Sherman et al., 1996) is an interesting question for future research. Although there will be differences in detail, analogous pathways are likely present in other complexes of synthetases with class II tRNAs, where a strong influence of core region composition on aminoacylation has also been found (Soma et al., 1999; Tocchini-Valentini et al., 2000).

MATERIALS AND METHODS

Plasmid constructions

Oligonucleotides were purchased from IDT (Coralville, Iowa) and enzymes were purchased from New England Biolabs

FIGURE 5. Crystal structure of *E. coli* glutamyl-tRNA synthetase (ribbon) bound to tRNA^{Gln} and 5'-O[N-(L-Gln)sulfamoyl] adenosine (QSI; red) (Rath et al., 1998). Secondary structural elements in GlnRS providing a connection between the tRNA tertiary core and the active site are colored in green, yellow, and light blue.

except where noted. Transfer RNA genes tRNA^{Gln}, tRNA^{QSer}, tRNA^{QSer_2D}, and tRNA^{QSer_C9/A13} were originally constructed from four pairs of complementary synthetic oligonucleotides consisting of a 5' T7 RNA polymerase promoter followed by the tRNA gene (Nissan et al., 1999). All of the genes carried the U1G substitution required for efficient transcription, which is without effect on steady-state kinetic parameters (Jahn et al., 1991).

Mutagenesis was performed by QuickChange site-directed mutagenesis (Stratagene), in which highly efficient selection for mutants derives from cleavage of methylated parental plasmid strands by *DpnI*, leaving the newly synthesized and unmethylated mutant strand intact. The templates for mutagenesis were constructs encoding either tRNA^{QSer} lacking nucleotides 20a and 20b (tRNA^{QSer_2D}) or tRNA^{QSer} possessing a G9C/A13G reversion toward the tRNA^{Gln} sequence (tRNA^{QSer_C9/A13}), depending on the background desired (Nissan et al., 1999). All mutant tRNA plasmid constructs were verified by DNA sequencing.

Enzyme purifications

T7 RNA polymerase was purified as described (Grodberg & Dunn, 1988) from *E. coli* BL21 cells containing the plasmid

PAR1219. Purified enzyme was stored at -20°C in a buffer containing 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol, and 20 mM sodium phosphate (pH 7.8). Glutamyl-tRNA synthetase was purified by a two-column procedure (Hoben et al., 1982) from *E. coli* K12 Δ H1 Δ trp cells containing the overexpression vector pMN20 (Perona et al., 1988). The enzyme was stored at -20°C at 20 mg/mL in a buffer containing 50% glycerol, 50 mM KCl, 20 mM KH₂PO₄ (pH 7.2), and 20 mM β -mercaptoethanol.

In vitro transcription of RNA

Plasmid DNAs were used as templates for run-off transcription. Large quantities of plasmid were obtained from growth of 0.5–2 L of *E. coli*, followed by cell harvesting, lysis, and extraction of plasmid using Megaprep kits (Qiagen). Plasmid DNAs were subjected to three phenol/chloroform/isoamyl alcohol extractions followed by a chloroform/isoamyl alcohol extraction (Sambrook et al., 1989). The RNase-free template was linearized for transcription with *MvaI* (MBI Fermentas). Transcription reactions were performed with the following conditions: 40 mM Tris (pH 8.0), 24 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 0.01% Triton X100, 100 mM KCl, 2.5 mM ATP, 3.3 mM GTP, 3.3 mM CTP, 2.5 mM UTP, and 40 $\mu\text{g/mL}$

T7 RNA polymerase (Milligan et al., 1987). The transcription reactions were incubated for 8–12 h at 37 °C.

RNAs were purified following the general procedures described (Arnez & Steitz, 1994). Following transcription, RQ1 RNase-free DNase (Promega) was added (100 U/mg template) and the reactions incubated at 37 °C for 2 h. EDTA was then added to a final concentration of 20 mM to redissolve the Mg-PP_i precipitate. The RNA transcription reaction was then applied to a DEAE column, which was equilibrated with 2 mM PIPES (pH 7.5). The column was washed with 0.25 M NaCl and the RNA eluted with 1 M NaCl. Eluted RNA was ethanol precipitated and brought up in PMS buffer (5 mM PIPES (pH 7.5) and 10 mM MgSO₄). After a phenol/chloroform/isoamyl alcohol extraction, the RNA was dialyzed twice into PMS buffer. Dialyzed RNA was then refolded at 1 mg/mL in a solution of 10 mM MgSO₄ and 5 mM PIPES (pH 7.5), by heating to 80 °C for 10 min followed by slow cooling. Refolding was performed prior to aminoacylation. Gel excision of tRNA bands was not carried out because of the difficulty in separating, on a preparative scale, the large 94-nt tRNA^{G^{Ser}} and derivative species. Further, it has been shown for G1-tRNA^{G^{In}} that the presence of both 5'-end and 3'-end heterogeneity produces nonidentical transcripts of equivalent length, so that rigorous size purification of homogeneous RNA is not possible (Pleiss et al., 1998).

Aminoacylation assays

Aminoacylation was performed by filter binding assay (Hoben & Söll, 1985) to monitor incorporation of radiolabeled glutamine into bound tRNA. Conditions for aminoacylation assays were similar to those described (Sherman & Söll, 1996; Sherman et al., 1996; Nissan et al., 1999). The fraction of tRNA transcripts capable of being aminoacylated by GlnRS was determined by assays under conditions giving maximal incorporation (approximately 10-fold molar excess of enzyme over tRNA). Several tRNA concentrations (0.4–4.0 μM) were used for each substrate, and pmol/OD values were calculated separately for each concentration of tRNA. For all plateau-level charging determinations, the assay mix consisted of 50 mM Tris-HCl (pH 7.0), 4 mM ATP, 675 μM unlabeled glutamine, 5 μM [³H]-glutamine (Amersham), 10 mM magnesium acetate, 5 μM tRNA, and 30–50 μM GlnRS. Counting efficiencies for [³H]-glutamine bound to tRNA, relative to free [³H]-glutamine, were corrected by measuring aminoacylation levels for G1-tRNA^{G^{In}} with both [³H] and [¹⁴C] isotopes of glutamine, as described (Rogers & Söll, 1993; Sherman & Söll, 1996; Nissan et al., 1999). The low levels of plateau aminoacylation are mainly due to production of non-functional 3'-runover transcripts by T7 RNA polymerase. Transcription of wild-type tRNA^{G^{In}} from oligonucleotide templates containing 2'-O-methyl sugars at the 3' C75 and A76 positions (Kao et al., 1999) produces a tRNA pool in which the proportion of functional molecules increases from one-sixth (Table 1) to over two-thirds (1,100 pmol/OD; data not shown). Another possible contributing factor might be intermolecular association of the tRNA transcripts (Yang et al., 1972; Vogtherr et al., 1999).

For all steady-state kinetic determinations, the assay mix consisted of 50 mM Tris-HCl (pH 7.0), 4 mM ATP, 675 μM unlabeled glutamine, 5 μM [³H]-glutamine (Amersham), 10 mM magnesium acetate, and glutamyl-tRNA synthetase (1–

10 nM). tRNA at five concentrations bracketing the K_m was used for each assay. The tRNA:GlnRS ratio is 30:1 or greater in all assays, ensuring that steady-state conditions apply. The concentration of glutamine used represents the practical maximum and is three- to fourfold above the K_m for glutamine as determined in the presence of the cognate tRNA^{G^{In}}. Although this is likely to be near saturation for wild-type, it is known that the K_m for glutamine can be elevated for glutaminylation of modified complexes (Hong et al., 1996; Ibba et al., 1996; Kitabatake et al., 1996). The k_{cat} and K_m parameters should thus be understood as apparent values. The concentration of tRNAs used in the steady-state kinetic time courses represents the concentration of *functional* molecules as assessed by pmol/OD levels, and assuming 1,600 pmol/OD for fully functional tRNA. The tRNA concentrations used ranged between 0.05–2.5 μM. All data were taken at time points for which less than 20% of the tRNA substrate was converted to product. The data were analyzed by Eadie-Hofstee plots. For very weak substrates, the second-order parameter k_{cat}/K_m was determined directly by measurements at low substrate concentrations ($[S] \ll K_m$) using the relationship: $v = k_{cat}/K_m [E]_0[S]$ (Fersht, 1999).

Thermal melts

Melting curves were performed by refolding tRNA substrates at a concentration of 1 mg/mL in 10 mM MgSO₄ and 5 mM PIPES (pH 7.5). Refolded tRNA was then dialyzed into a buffer containing 50 mM PIPES (pH 7.5) and 30 mM (NH₄)₂SO₄. The change of absorbance at 260 nm was monitored on a UVIKON 9410 spectrophotometer (Kontron Instruments). The temperature was raised from 25 °C to 82 °C at a rate of 1 °C per minute with a NESLAB programmable temperature bath. Three data sets were collected for each substrate, each with one data point collected per degree of temperature rise. Data were normalized such that the A₂₆₀ at 82 °C corresponds to 1.0. Normalized values were averaged at each data point prior to plotting.

ACKNOWLEDGMENTS

This work was supported by Grant MCB-9600971 from the National Science Foundation (to J.J.P.).

Received June 14, 2000; returned for revision
July 10, 2000; revised manuscript received
August 8, 2000

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