Use of semi-synthetic transfer RNAs to probe molecular recognition by *Escherichia coli* proline-tRNA synthetase

Li-Ping Yap, Catherine Stehlin and Karin Musier-Forsyth*

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Background: The attachment of specific amino acids to the 3'-end of cognate transfer RNAs (tRNAs) is catalyzed by a class of enzymes known as aminoacyl-tRNA synthetases (aaRS). We have previously demonstrated that *Escherichia coli* proline-tRNA synthetase (ProRS) can aminoacylate semi-synthetic tRNAs prepared by annealing two RNA oligonucleotides. We set out to examine the factors that are important in selective recognition of $tRNA^{Pro}$ by ProRS, using semi-synthetic tRNAs and full-length tRNA transcripts.

Results: Deletion of nucleotides A58, A59, and U60 in the TΨC-loop of semi-synthetic tRNAs has no adverse effect on aminoacylation. Nucleotide deletions that extend into the TΨC stem, particularly beyond C61, significantly reduce the efficiency of aminoacylation, however. Site-directed mutagenesis of full-length $tRNA^{Pro}$ transcripts shows that, although there is no strict sequence requirement at base pair 52•62 in the TΨC stem, helix destabilizing purine–purine mismatches at this position result in decreased aminoacylation activity. Moreover, aminoacylation is severely affected when a DNA–RNA hybrid helix is incorporated into the acceptor-TΨC stem domain.

Conclusions: At least three nucleotides in the TΨC-loop are dispensable for aminoacylation of *E. coli* $tRNA^{Pro}$. These results, combined with previous data, demonstrate that four out of five of the so-called 'variable pocket' nucleotides are not important for recognition of $tRNA^{Pro}$ by *E. coli* ProRS. ProRS is also sensitive to changes that are likely to alter the helical conformation in the TΨC stem.

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Introduction

In all living systems, 20 aminoacyl-tRNA synthetases (aaRS) catalyze the two-step attachment of specific amino acids onto the 3'-terminal adenosine of their cognate tRNAs. In the first step, the enzyme binds both the amino acid and ATP to form an activated enzyme-bound intermediate known as the aminoacyl-adenylate with release of pyrophosphate. The second step involves the transfer of the activated amino acid to the 2'- or 3'-hydroxyl of the terminal adenosine of the enzyme-bound tRNA. This step results in the release of AMP. The aaRSs have been divided into 10 class I and 10 class II synthetases based on conserved sequence motifs and structural data [1]. Transfer RNAs are generally 74–93 nucleotide residues in length [2] and form an L-shaped tertiary structure [3–6]. Most tRNAs can be divided into five basic domains known as the acceptor stem (the site of amino acid attachment), the D stem–loop (which typically contains one or more dihydrouridine (D) nucleotides), the anticodon stem–loop (which contains the tri-nucleotide sequence complementary to the mRNA codon), the variable loop (which can vary in size from 4 to 26 nucleotides), and the TΨC stem–loop (which typically contains the two modified nucleotides thymidine (T) and pseudouridine (Ψ)). Because of the structural similarity of the tRNAs and the chemical similarity of aminoacylation systems, discrimination by aaRSs remains a challenging and intriguing problem in molecular recognition [7]. Each tRNA is believed to contain within its sequence a set of 'recognition elements' that allows productive recognition and aminoacylation by cognate synthetases and another set of 'negative elements' that blocks non-cognate aminoacylation. Both components define the 'identity' of a tRNA [8,9]. In the known structure of yeast $tRNA^{Phe}$, nucleotides 16, 17, 20, 59, and 60 cluster in the hinge region of the L-shaped structure [4,6]. Due to the exposed and variable nature of this grouping of nucleotides, the so-called 'variable pocket', it was proposed that recognition of these nucleotides may be part of the discrimination mechanism used by aaRSs [6].

Recently, a great deal of progress has been made in locating the nucleotide determinants of particular tRNA molecules that specify aminoacylation identity [10–12]. Of relevance to this work, an *in vivo* study of *Escherichia coli* $tRNA^{Pro}$ has shown that important recognition elements can be found in the acceptor stem and anticodon loop [13]. Previous studies in our laboratory complemented this *in vivo* work and identified major determinants for the *in vitro* aminoacylation of $tRNA^{Pro}$ in at least three domains: the anticodon, the acceptor stem, and the D-loop/variable-loop domain [14,15]. Elucidating the importance of these elements in $tRNA^{Pro}$ structure, folding and aminoacylation has been facilitated by using semi-synthetic tRNAs [14,16]. Chemical RNA synthesis allows the specific incorporation of both base and backbone modifications [16]. A semi-synthetic approach to tRNA preparation also allows us to test the effect of nucleotide deletions on tRNA structure and function

*Corresponding author.

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[14]. We have previously shown that a functional full-length $tRNA^{Pro}$ molecule can be constructed by annealing a chemically synthesized 5'-derived oligonucleotide representing ~1/4 of the tRNA to a 3'-derived oligonucleotide representing the other 3/4 of the tRNA sequence, prepared by *in vitro* transcription [14]. Similarly, a 3'-derived oligonucleotide annealed to a 5'-3/4 tRNA is a substrate for ProRS [16]. Even though these annealed tRNA molecules contain a break in the phosphodiester backbone, they are efficiently aminoacylated [14,16].

In the present work, we prepared tRNAs with internal nucleotide deletions, multiple deoxynucleotide substitutions, and single base pair mutations in the TΨC stem–loop domain. Results of this analysis showed that while certain TΨC loop nucleotides, including those of the variable pocket, are dispensable for aminoacylation, maintenance of an A-form helix in the TΨC stem is required for optimal aminoacylation by ProRS.

Results

Deletion analysis using semi-synthetic tRNAs

In this study, we used a semi-synthetic $tRNA^{Pro}$ molecule containing a break in the TΨC loop after G57. Figure 1a shows the structure of this molecule and the scheme for producing it by annealing a 5'-ΔC1-57-mer to a chemically synthesized 3'-19-mer. The deletion of C1 facilitates *in vitro* transcription of the 5'-fragment and has previously been shown to have little effect on the aminoacylation of full-length $tRNA^{Pro}$ transcripts [14]. Figure 1b shows a space-filling model of *E. coli* $tRNA^{Pro}$ with the chemically synthesized 3'-19-mer (A58-A76) highlighted in gold. This semi-synthetic tRNA construct is an efficient substrate for ProRS despite the break in the TΨC loop [16]. The k_{cat}/K_M for the semi-synthetic tRNA was 0.027 $s^{-1}\mu M^{-1}$, which corresponds to only a ~2-fold reduction relative to a full-length ΔC1-$tRNA^{Pro}$ transcript [14]. To elucidate the importance of TΨC stem–loop nucleotides, seven truncated 3'-oligonucleotides were chemically synthesized (Fig. 2). Each 3'-fragment was annealed to an *in vitro* transcribed 5'-ΔC1-57-mer and the semi-synthetic tRNAs were tested for their aminoacylation activity. The difference in the free energy of activation ($\Delta\Delta G^{\circ\ddagger}$) between each mutant and the 'wild type' tRNA (3'-19-mer + 5'-ΔC1-57-mer) was then calculated from the experimentally determined values of k_{cat}/K_M. As shown in Figure 2 and Table 1, the deletion of three TΨC-loop nucleotides, A58, A59, and U60, did not affect aminoacylation by ProRS. However, as the deletions extended into the TΨC-stem region, a marked decrease in k_{cat}/K_M was observed (Fig. 2 and Table 1). Deletions beyond C61 were not well-tolerated and resulted in $-\Delta\Delta G^{\circ\ddagger}$ values greater than 2.6 kcal mol^{-1}.

To ascertain whether the observed reduction in aminoacylation activity was caused by incomplete annealing of the truncated fragments to the 3/4-tRNA molecule, the annealed tRNAs were visualized on a native polyacrylamide gel as previously described [14]. Figure 3 shows

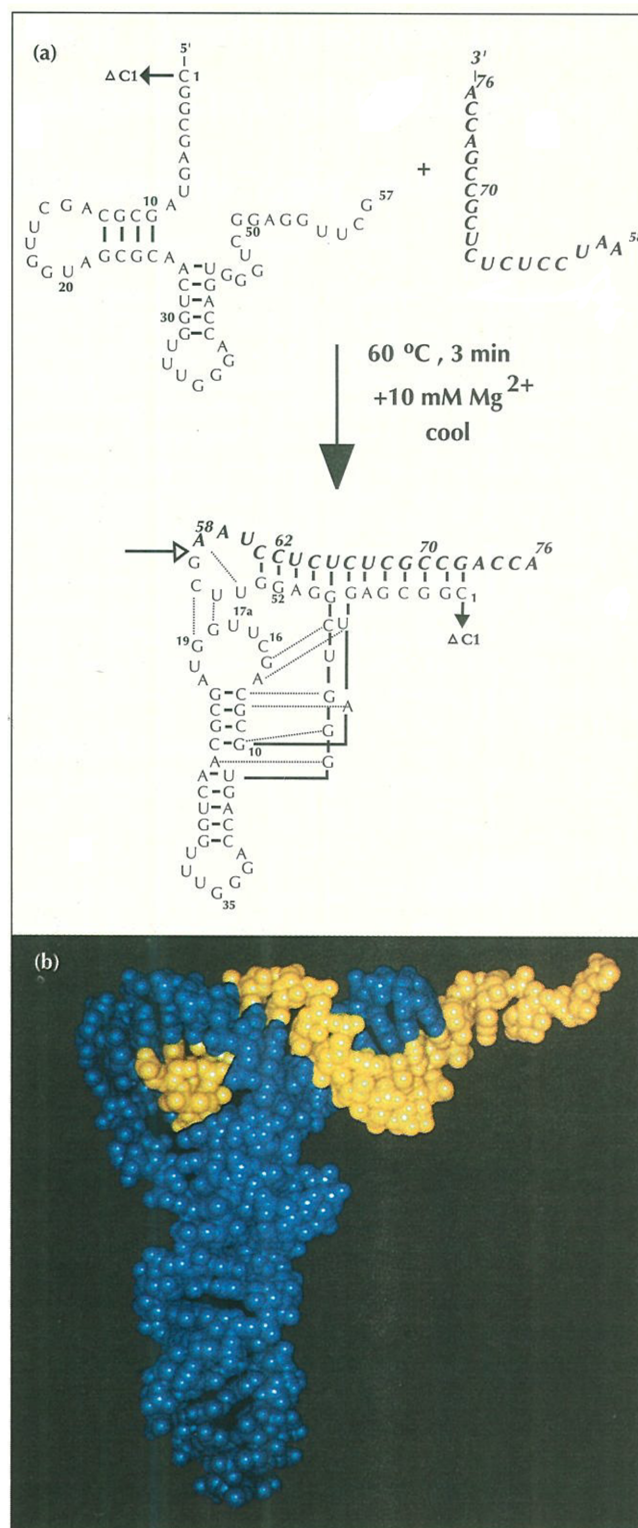

Fig. 1 Semi-synthetic tRNA analogs. **(a)** Scheme showing the preparation of semi-synthetic *E. coli* $tRNA^{Pro}$ containing a break in the phosphodiester backbone between nucleotides A58 and G57 (open arrow). A 3'-19-mer oligonucleotide (top right, bold, italics) was chemically synthesized and annealed to a 5'-ΔC1-57-mer prepared by *in vitro* transcription (top left). Dotted lines in the folded structure represent proposed tertiary interactions based on the known structure of yeast $tRNA^{Phe}$ [4]. **(b)** Space-filling model of $tRNA^{Pro}$ highlighting the chemically synthesized 3'-oligoribonucleotide (gold). Coordinates for $tRNA^{Phe}$ were used to generate this model using Insight II (Biosym Technologies) on an IRIS Indigo XS24 workstation (Silicon Graphics, Inc.).

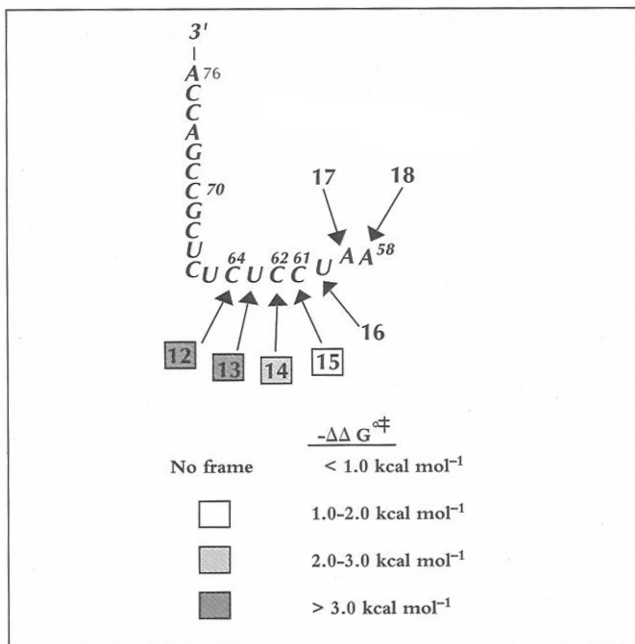

Fig. 2. Truncated 3'-oligoribonucleotides that were prepared synthetically, annealed to a 5'-ΔC1-57-mer, and assayed for aminoacylation activity. Numbered arrows indicate the length of each fragment from the 3'-end. $-\Delta\Delta G^{\circ\ddagger}$ values were calculated as described in Table 1.

that each of the annealed constructs (lanes 3–9) appeared as a single band and migrated to a similar distance on the native gel as the full-length ΔC1 tRNA^{Pro} (lanes 1 and 10). In contrast, the 5'-3/4-tRNA^{Pro} fragment alone ran as a series of smeared bands that were not easily detected upon visualization using ethidium-bromide staining (lane 2). These results suggest that when annealed to the 5'-3/4 tRNA^{Pro} molecule, the truncated fragments were able to fold into a global conformation resembling the functional full-length tRNA. The loss in activity observed upon deletion of C61 and beyond, therefore, is probably due to more subtle conformational changes or specific sequence requirements in the TΨC stem.

Table 1. The effect of nucleotide deletions in the TΨC stem-loop region on prolylation of tRNA^{Pro}.

3'-Synthetic oligonucleotide	k_{cat}/K_M (relative)	change in efficiency (fold)	$-\Delta\Delta G^{\circ}\ddagger$ (kcal mol^{-1})
all-RNA:			
19-mer	1.0	1.0	0
18-mer	0.87	–1.2	0.0086
17-mer	1.3	1.3	–0.15
16-mer	1.0	1.0	0
15-mer	0.17	–5.7	1.0
14-mer	0.013	–78	2.6
13-mer	0	>–600	>3.8
12-mer	0	>–600	>3.8

All 3'-fragments were chemically synthesized and annealed to a 5'-ΔC1-57-mer prepared by *in vitro* transcription. The k_{cat}/K_M of the annealed all-RNA 3'-19-mer + 5'-ΔC1-57-mer was assigned a value of 1.0, and all other values are reported relative to this. Under the experimental conditions employed, initial rates of aminoacylation were proportional to substrate concentration. The values reported are averages of at least three determinations, using annealed tRNA concentrations ranging from 1 to 12 μM, with an estimated error of ± 28 %. $\Delta\Delta G^{\circ\ddagger}$ is defined as RT $\ln(k_{cat}/K_M)^{\text{truncated variant}}/(k_{cat}/K_M)$wild-type, where 'wild-type' refers to the annealed 3'-19-mer + 5'-ΔC1-57-mer.

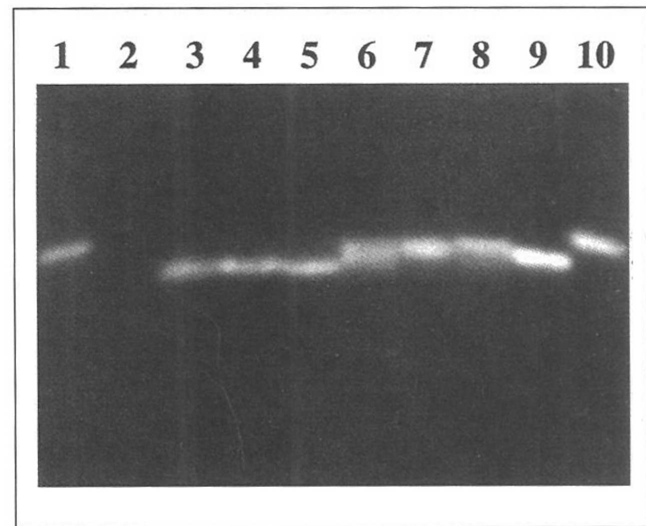

Fig. 3. Ethidium bromide-stained, native 12 % polyacrylamide gel showing full-length *in vitro* transcribed ΔC1-tRNA^{Pro} (lanes 1 and 10), 5'-ΔC1-57-mer alone (lane 2), annealed 5'-ΔC1-57-mer + 3'-19-mer (lane 3), + 3'-18-mer (lane 4), + 3'-17-mer (lane 5), + 3'-16-mer (lane 6), + 3'-15-mer (lane 7), + 3'-14-mer (lane 8), and + 3'-13-mer (lane 9).

Site-directed mutagenesis

To distinguish between the need for specific sequence elements versus conformational requirements, we first carried out site-directed mutagenesis of full-length tRNA transcripts. Because the aminoacylation activity was most severely affected when the nucleotides beyond C61 were deleted (Fig. 2 and Table 1), we were interested in probing the role of C62 and the G52•C62 base pair. This particular base pair is conserved in all tRNA^{Pro} isoacceptors from *E. coli*, phages T4 and T5, and *Salmonella typhimurium* [2,17]. Single and double mutations were introduced at position C62 and base pair G52•C62. The aminoacylation efficiency of full-length mutant transcripts was compared to that of wild-type ΔC1-tRNA^{Pro}. These results are shown in Table 2. Of the three single mutations made at position C62, changes to A62 and G62 resulted in substantial decreases in k_{cat}/K_M with a loss in activity of 6.6 and 14-fold, respectively (Table 2). Each of these changes introduced a purine–purine mismatch into the TΨC stem. A single mutation of C62 to U62 forming a G52•U62 wobble base pair resulted in only a minor (1.8-fold) decrease in activity. Four double mutants were also prepared at this site. The double mutation A52•G62 resulted in an 8.8-fold decrease in aminoacylation activity. This effect is very similar to the decrease observed upon introducing the other two purine–purine mismatches tested. Interestingly, the three double mutants that restored

Table 2. The effect of mutations at position G52•C62 on prolylation of $tRNA^{Pro}$.

$tRNA^{Pro}$ variant	k_{cat}/K_M (relative)*	Change in efficiency (fold)	$-\Delta\Delta G^{\circ\ddagger}$ (kcal mol^{-1})
ΔC1	1.0	1.0	0
ΔC1 + G52•C62→G52•**A**62	0.15	−6.6	1.1
ΔC1 + G52•C62→G52•**G**62	0.071	−14	1.6
ΔC1 + G52•C62→G52•**U**62	0.56	−1.8	0.34
ΔC1 + G52•C62→**A**52•**G**62	0.11	−8.8	1.3
ΔC1 + G52•C62→**C**52•**G**62	1.8	1.8	−0.35
ΔC1 + G52•C62→**U**52•**G**62	1.5	1.5	−0.24
ΔC1 + G52•C62→**A**52•**U**62	0.77	−1.3	0.15

*The k_{cat}/K_M of tRNA variants is reported relative to the full-length ΔC1-$tRNA^{Pro}$ which was assigned a value of 1.0. The values reported are an average of two or three determinations. The average estimated error of these measurements is ± 28 %. This set of assays was performed as described previously [15]. $\Delta\Delta G^{\circ\ddagger}$ is defined as $RTln(k_{cat}/K_M)^{mutant}/(k_{cat}/K_M)^{wild\text{-}type}$, where 'wild-type' refers to the ΔC1-$tRNA^{Pro}$ transcript.

standard Watson–Crick or wobble base-pairing at position 52•62 (C52•G62, A52•U62, and U52•G62) restored the activity to approximately wild-type levels (Table 2).

These results suggest that the large decreases in aminoacylation activity as a result of deletions in the TΨC stem of $tRNA^{Pro}$ beginning at position 62 (Fig. 2 and Table 1), are not due to the loss of a specific sequence requirement at this position. Our data show that as long as a standard Watson–Crick or a U•G or G•U wobble base pair is present at position 52•62 optimal aminoacylation activity is observed (Table 2). On the other hand, introduction of a helix-destabilizing purine–purine mismatch at this position results in a significant decrease in aminoacylation efficiency ($-\Delta\Delta G^{\circ\ddagger} > 1.0$ kcal mol^{-1}).

Effect of a DNA–RNA hybrid acceptor-TΨC stem

To examine the conformational requirements of the acceptor–TΨC–stem region of $tRNA^{Pro}$ further, we prepared a semi-synthetic tRNA with a 3'-16-mer containing deoxynucleotides at all positions except the 3'-terminal adenosine, the site of aminoacylation. This annealed hybrid tRNA was not a good substrate for ProRS (data not shown). The k_{cat}/K_M of the DNA-RNA substrate was reduced 68-fold ($-\Delta\Delta G^{\circ\ddagger}$ = 2.5 kcal mol^{-1}) relative to the all-RNA annealed substrate.

Discussion

Variable pocket nucleotides are dispensable

Using semi-synthetic tRNAs, we have shown that nucleotides A58–U60 in the TΨC-loop of *E. coli* $tRNA^{Pro}$ are dispensable for *in vitro* aminoacylation (Fig. 4, shaded oval). Previous studies from our lab showed that removal of C16, U17, and U17a from the D loop also had no effect on aminoacylation (Fig. 4, open oval) [14]. As mentioned earlier, the variable pocket nucleotides 16, 17, 20, 59, and 60 cluster in the hinge region of the known L-shaped structure of yeast $tRNA^{Phe}$ [4,6] and it has been proposed that their recognition forms part of the discrimination mechanism used by aaRSs [6]. The nucleotide at position 20 is a recognition element for a number of synthetases, including *E. coli*, yeast, and human $tRNA^{Phe}$ [18–20], *E. coli* $tRNA^{Ala}$ [21] and *E. coli* $tRNA^{Arg}$ [22,23]. Few studies to date, however, have addressed the role of the other four variable pocket bases. The replacement of the TΨC arm of *E. coli* $tRNA^{Ile}$ with a $(U)_5G$ sequence resulted in a 10-fold reduction in aminoacylation, while a similar replacement in *E. coli* $tRNA^{Phe}$ completely destroyed activity [24]. U59 and U60 have been shown to be important for *in vivo* $tRNA^{Phe}$ identity [18], while a U59→C change resulted in a 6.3-fold reduction in *in vitro* aminoacylation [20]. Our results with *E. coli* $tRNA^{Pro}$ show that four out of five of the variable pocket nucleotides are dispensable for aminoacylation. At least in the case of this *E. coli* tRNA, the variable pocket does not appear to form an important protein recognition site.

Importance of the TΨC-stem conformation

Deletion of C62 in the TΨC stem (Fig. 4, shaded square), on the other hand, has a dramatic effect on $tRNA^{Pro}$ aminoacylation efficiency ($-\Delta\Delta G^{\circ\ddagger}$ = 2.6 kcal mol^{-1}, Table 1). Furthermore, while introduction of alternative Watson–Crick and wobble base pairs at position 52•62 resulted in activities comparable to the wild-type tRNA, specific purine–purine mismatches that we have tested at this position resulted in significant decreases in aminoacylation efficiency ($-\Delta\Delta G^{\circ\ddagger}$ > 1.0 kcal mol^{-1}, Table 2). Relatively little experimental data is available regarding the effect of internal mismatches, such as those introduced into our $tRNA^{Pro}$ constructs, on the structure and stability of RNA helices. In most sequence contexts examined to date, however, the introduction of single purine–purine mismatches into RNA duplexes was destabilizing [25,26].

The sensitivity of ProRS to the helical conformation in the TΨC–stem is further supported by the inability of this enzyme to efficiently aminoacylate a semi-synthetic substrate with a DNA–RNA acceptor–TΨC–stem helix. It is known that DNA–RNA hybrid duplexes have a helical conformation that deviates from a standard A-form (RNA-like) or B-form (DNA-like) geometry [27,28]. Single deoxynucleotide substitutions were

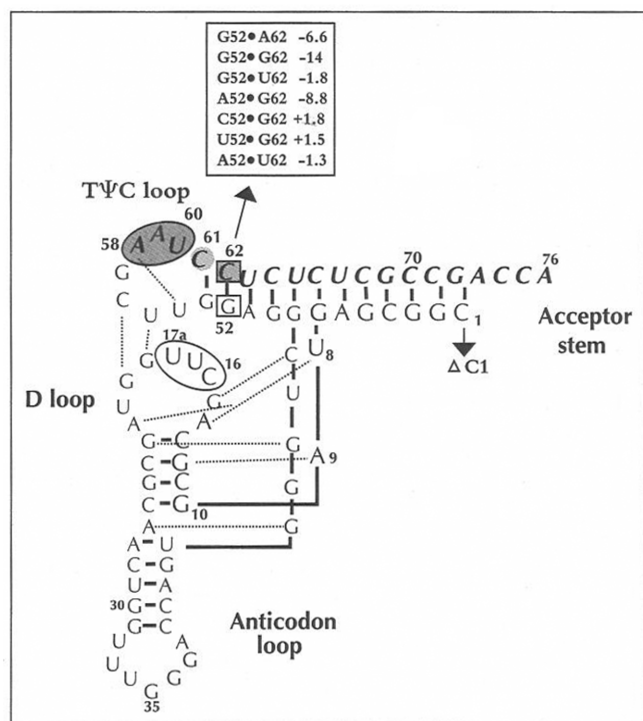

Fig. 4. L-shaped structure of *E. coli* tRNA^{Pro}. The open oval indicates nucleotides that were determined to be dispensable for aminoacylation in a previous study [14]. The shaded oval indicates nucleotides that were determined to be dispensable for aminoacylation in this study. Upon deletion of nucleotides beginning with C61 (shaded circle), a significant (5.7-fold) decrease in aminoacylation activity is observed. Deletion of C62 (shaded square) resulted in a 78-fold loss in activity. The results of introducing single and double mutations at position 52•62 are also shown. Numbers next to each mutation indicate the change in k_{cat}/K_M (x-fold) relative to wild type ΔC1-tRNA^{Pro}.

previously tested in nine positions of the 3'-16-mer and shown to have negligible effects [16]. The results presented here, therefore, support the hypothesis that ProRS is sensitive to changes in the helical conformation of the TΨC-stem domain. As only nine of the 2'-hydroxyl groups in the 3'-16-mer have been individually tested, however [16], we cannot rule out the possibility that the decrease in activity is due to a single 2'-hydroxyl contact that is missing in the DNA–RNA hybrid molecule examined in the present work.

We have previously determined that identity nucleotides for tRNA^{Pro} are located in the anticodon at positions 35 and 36, and at positions 72 and 73 of the acceptor stem [15]. Site-directed changes at these locations resulted in 9- to 185-fold decreases in aminoacylation efficiency. Our new results at position 52•62 illustrate the importance of the helical conformation, rather than specific sequence elements, in the TΨC stem. The subtle effects observed may be the result of conformational changes that affect the presentation of nearby (as of yet unidentified) recognition elements to the enzyme. On the other hand, the effects of a conformational change in the TΨC stem near the hinge region of the L-shape may be due to a global change in the tRNA structure, affecting distances between known recognition elements in the anticodon loop and the acceptor stem.

Significance

Due to the high degree of specificity between tRNAs and aaRSs, these biomolecules are ideal targets for studying the principles of protein–RNA interaction. The design and preparation of semi-synthetic tRNAs is a useful strategy for elucidating structure–function relationships that govern these key players in protein translation.

In the present work, using semi-synthetic tRNAs containing a break in the TΨC loop, we show that certain TΨC-loop nucleotides are dispensable for aminoacylation. These results, combined with previous data [14], demonstrate that four out of five of the so-called 'variable pocket' nucleotides are not important for recognition of tRNA^{Pro} by *E. coli* ProRS. On the other hand, we observe significant decreases in aminoacylation activity upon making changes that are likely to disrupt the helical conformation in the TΨC-stem. In particular, purine-purine mismatches at position 52•62 are not well-tolerated, while substitutions that recreate a Watson–Crick base-pairing or that result in wobble base pairings have little or no effect on aminoacylation activity. These results illustrate the subtle effects on aminoacylation of the helical conformation in a specific stem near the hinge region of the tRNA structure. The effects of helix conformation on recognition may be due to structural changes that alter critical distances between the amino acid acceptor terminus and important identity nucleotides.

Materials and methods

Enzyme preparation

ProRS was prepared from *E. coli* strain SY327 using a double plasmid expression system as described previously [14]. The adenylate burst assay was used to determine the active site concentration of ProRS [29]. T7 RNA polymerase was purified according to Grodberg and Dunn [30] from *E. coli* strain BL-21/pAR 1219, which was a gift of F.W. Studier.

Nucleic acids

A full-length ΔC1 tRNA^{Pro} transcript was prepared by *in vitro* transcription as described previously [14]. The deletion of C1 (ΔC1) facilitates *in vitro* transcription by T7 RNA polymerase and has no adverse effect on aminoacylation [14]. Plasmid DNA containing the gene for full-length ΔC1 tRNA^{Pro} in front of a T7 promoter was also used to prepare a 5'-ΔC1-57-mer tRNA^{Pro} fragment. This was accomplished by *Taq*I linearization of the plasmid, followed by *in vitro* transcription. RNA transcripts were purified using 12 % polyacrylamide gels, eluted, and stored in 10 mM Tris-HCl/1 mM EDTA (pH 8.0) at −20 °C. Oligoribonucleotides and deoxyribonucleotides were chemically synthesized on a Gene Assembler Plus (Pharmacia) using the phosphoramidite method [31]. The

oligomers were purified on 16 % polyacrylamide gels. To determine RNA concentrations, the following extinction coefficients were used: 57-mer, 41.8 x $10^4 M^{-1}$;19-mer, 15 x $10^4 M^{-1}$; 18-mer, 14 x $10^4 M^{-1}$; 17-mer, 13.5 x $10^4 M^{-1}$; 16-mer, 12.5 x $10^4 M^{-1}$; 15-mer, 12 x $10^4 M^{-1}$; 14-mer, 11.5 x $10^4 M^{-1}$; 13-mer, 10.7 x $10^4 M^{-1}$; 12-mer, 10.2 x $10^4 M^{-1}$. These values were determined as described previously [32].

Site-directed mutagenesis

Site-directed mutagenesis of full-length tRNAs was accomplished using the Kunkel method [33]. A mixture of oligonucleotides randomized at position 62 was used to construct the C62→G, C62→A, and C62→U mutants. Subsequent isolation of single-stranded DNA corresponding to mutant C62→G and C62→U permitted construction of double mutants G52→A:C62→G, G52→C:C62→G, G52→U:C62→G, and G52→A:C62→U. The existence of only the desired mutations was verified by sequencing the entire $tRNA^{Pro}$ coding region using Sanger's dideoxy chain-termination sequencing method [34].

Aminoacylation assays

The 5'-ΔC1-57-mer and 3'-variant oligonucleotides were annealed immediately prior to aminoacylation assays as follows: the oligonucleotides were mixed in a 1:1 ratio in 50 mM HEPES (pH 7.5) and heated at 60 °C for 3 min. $MgCl_2$ was then added to 10 mM and the mixture was cooled to room temperature for 5 min and placed on ice. All annealed tRNAs were visualized using native gel electrophoresis as previously described [14]. Aminoacylation assays of annealed tRNA fragments were carried out at 23 °C as described previously [14]. A factor of 1.3 was used to correct for incomplete precipitation of the charged 3'-oligonucleotide onto the Whatman 3MM filter pads employed in the assays. This factor was determined by using ^{32}P-end-labeled 3'-16-mer. Aminoacylation assays of full-length tRNAs were conducted essentially as described [15] using a final ProRS concentration of 0.1 μM and $tRNA^{Pro}$ concentrations ranging from 0.5 to 12 μM.

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