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The effect of tRNA modification enzymes TrmA and TruB on aminoacylation of *in vitro* transcribed tRNA

MSc Thesis

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Tartu 2006

CONTENTS

LIST OF ABBREVIATIONS

aa	-	amino acid
aaRS	-	aminoacyl-tRNA synthetases in general; a given aminoacyl-tRNA synthetase is abbreviated by its cognate amino acid three letter code followed by RS; for example, AlaRS stands for alanyl-tRNA synthetase
aa-tRNA	-	aminoacyl-tRNA (prefix identifies the amino acid attached to tRNA and superscript determines the nature of tRNA)
DTE	-	dithioerythrol
EDTA	-	ethylenediaminetetraacetate
k_{cat}	-	catalytic constant
k_{cat}/K_M	-	specificity constant
K_M	-	Michaelis-Menten constant
m^5U	-	5-methyl uridine (also ribosylthymine, T)
mRNA	-	messenger RNA
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
PPase	-	pyrophosphatase
RNase	-	ribonuclease
RP-HPLC	-	reversed phase - high performance liquid chromatography
rRNA	-	ribosomal RNA
SAM	-	S-adenosyl-L-methionine
SDS	-	sodium dodecyl sulphate
TCA	-	trichloroacetic acid
Tm	-	melting point
TrmA	-	tRNA-(m^5U54) methyltransferase
$tRNA_{tot}$	-	total native tRNA of *Escherichia coli*
TruB	-	tRNA-(Ψ55) synthase
TSL	-	T stem-loop
V_{max}	-	maximal velocity
Ψ	-	pseudouridine

INTRODUCTION

Interpretation of the genetic message requires the coupling of nucleotide triplets to corresponding amino acids. Transfer RNA (tRNA) serves as the key to deciphering the nucleotide sequence in mRNA into the amino acid sequence of proteins. Amino acids are attached to the 3' end of tRNAs by appropriate aminoacyl-tRNA synthetases. The charged tRNAs then align on an mRNA template by complementary base pairing between codons in mRNA and anticodons in tRNA, finally, amino acids are joined to the growing chain of proteins, a reaction catalyzed by the ribosome.

From its biosynthesis to its function on the ribosome, tRNAs interact with many diverse proteins, including tRNA processing enzymes, aminoacyl-tRNA synthetases, and protein synthesis elongation and initiation factors. tRNAs thus provide excellent opportunities for studying various RNA-protein interactions and structure-function relationships.

tRNA molecules adopt a well-defined three-dimensional architecture that is crucial for protein synthesis. A large number of chemically diverse modified nucleosides have been described in all tRNAs studied so far. It is known that the modified nucleosides help to stabilize the functional structure of tRNA. However, little is known about the formation of the tertiary structure of tRNA. It is believed that folding of tRNA is facilitated by protein factors including tRNA modification enzymes.

tRNA modification enzymes TrmA and TruB are two potential "tRNA chaperones". Both of these proteins are involved in processing of vast majority of tRNAs in *Escherichia coli* and for both a second function distinct from tRNA modification has been proposed, but not yet identified.

The purpose of current investigation was to shed light on the role TrmA and TruB proteins play in the processing of tRNA. The effect of TrmA and TruB proteins on formation of correct tertiary structure of tRNA was monitored by its ability to be charged by aminoacyl-tRNA synthetases. Characterization of the mechanism by which TrmA promotes aminoacylation of *in vitro* transcribed tRNA by alanyl-tRNA synthetase was the central aim of current study.

I would like to gratefully acknowledge the always enthusiastic supervision and encouragement of Prof. Jaanus Remme. I would also like to thank Lauri Peil for help with all kinds of technical problems – at all times; Aivar Liiv and Kai Virumäe for teaching me the basics of experimental work; and all the other people in molecular biology and biochemistry labs for providing various support and creating a friendly atmosphere.

REVIEW OF LITERATURE

1. Structure and biosynthesis of tRNA

Transfer RNA (tRNA) was the first RNA molecule, whose primary sequence and secondary structure were determined *(Holley, 1965)* and tertiary structure described *(Kim et al., 1974; Robertus et al, 1974).* To date, thousands of primary sequences from various organisms are known. Irrespective of the primary sequence, all known tRNAs share similar overall structure, as might be expected given their common function in protein synthesis.

All tRNAs must be able to bind to the same sites on the ribosome and to participate in codon-anticodon interaction and peptide bond synthesis. tRNAs must also be recognized alike by elongation factor Tu that transports them to ribosomes. On the other hand, tRNAs must be different enough to guarantee their identity in the process of aminoacylation.

1.1. Primary and secondary structure of tRNA

Transfer RNAs are in general 74 to 92< (commonly 76) ribonucleotides long and numbered uniformly starting with the 5' terminus. Anticodon nucleotides are always numbered 34, 35 and 36. The 5' terminus is phosphorylated and the terminal residue is usually guanosine. All tRNAs have the sequence CCA (positions 74-76) at their 3' terminus (see Figure 1) where amino acids are covalently attached. Conserved, semi-conserved and variable positions are described in tRNA molecules *(for review Dirheimer et al., 1995).*

tRNA is the most extensively modified nucleic acid in the cell. tRNAs contain numerous modified bases, typically 7-15 per molecule (see Figure 1). All together, about 100 different modifications have been described. Although modified nucleosides are found at various positions in the tRNA, positions 34 and 37 contain the largest variety of rather complex modifications (hypermodified nucleosides). Some modifications (for example pseudouridine) are found in several positions of tRNA, others have unique location (see Figure 1) *(for review Björk, 1995).*

Transfer RNAs have secondary structure reminiscent of cloverleaf (see Figure 1) stabilized by intramolecular hydrogen bonding and extensive stacking interactions. On the secondary structure four arms are recognized: the acceptor arm is the one where the two ends of tRNA come together; the TΨC arm (also known as T arm, or TΨCG arm) is defined by this trinucleotide; the D arm is named after conserved dihydrouridine residues; and the anticodon arm

contains in the center of the loop the sequence of three nucleotides that base pair with a codon in mRNA. Some tRNAs have an "extra arm", which contains a variable number of residues (see Figure 1). Most of the invariant and semi-invariant bases are located in the loop regions *(for review Dirheimer et al., 1995).*

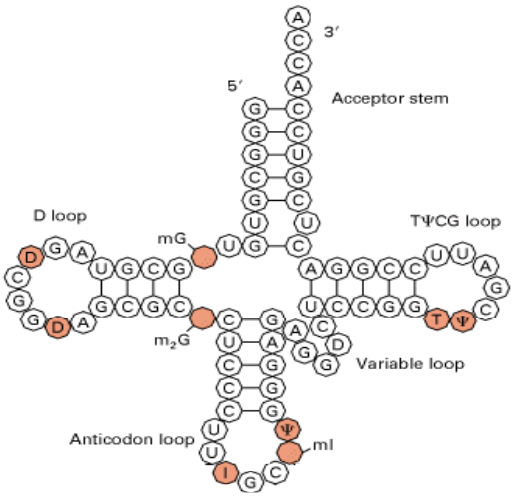

FIGURE 1. Primary sequence and secondary structure of yeast alanine tRNA. Modified nucleosides are shown: D – dihydrouridine, I – inosine, T – thymine, Ψ – pseudouridine, and m – methyl group. Although the primary sequence varies among tRNAs, they all fold into secondary structure reminiscent of cloverleaf. Dihydrouridine is nearly always present in the D loop of different tRNAs; likewise, thymine, pseudouridine, cytosine and guanidine are almost always present in the TΨCG loop *(adapted from Lodish et al., 2001).*

The structural diversity generated by this combination of helices and loops containing different bases and modifications ensure that the tRNAs can be uniquely distinguished, though structurally similar.

1.2. Tertiary structure of tRNA

X-ray crystallographic studies have shown that tRNAs fold into similar compact L shapes *(Kim et al., 1974; Robertus et al, 1974; Shi & Moore, 2000).* The helix containing the 5' and 3' ends stacks on top of the helix that ends in the TΨC loop to form one continuous arm; the remaining two helices stack to form the other continuous arm. The two RNA arms cross by 90° (see Figure 2). In all tRNAs the distance between the 3' end and the anticodon is about 70 Å *(for review Dirheimer et al, 1995).*

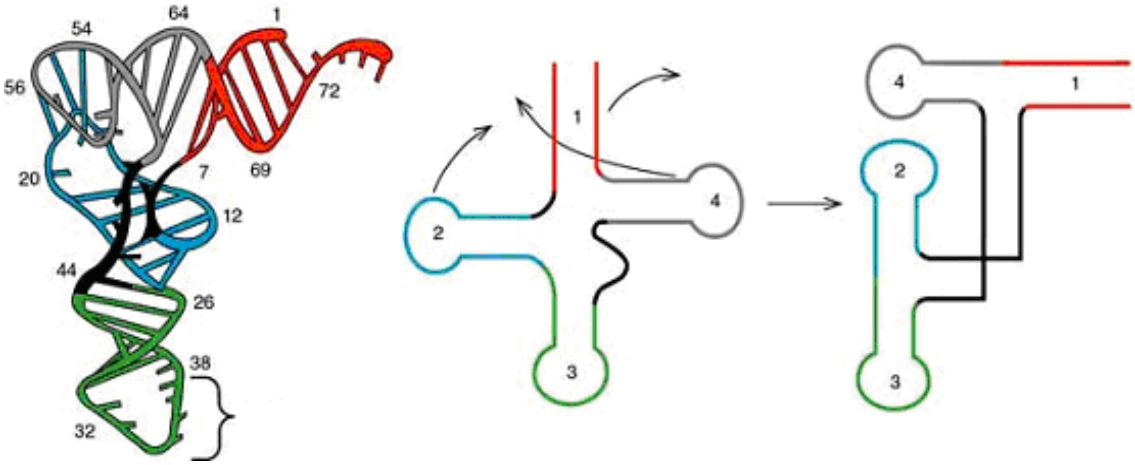

FIGURE 2. Tertiary structure of tRNA. Model of generalized backbone of tRNA. The folded tRNA molecule has a structure similar to the letter L. Different regions of tRNA molecule are colored accordingly to illustrate how the tRNA tertiary structure is related to the cloverleaf representation. However, a tRNA molecule does probably not attain its final structure by first base pairing and then folding into an L shape.

Tertiary structure of tRNA is stabilized by base stacking, base intercalation, and additional hydrogen bond formation between different regions of the molecule. Most of the bases, as well as the phosphate backbone, and the 2'-OH of the ribose of non-helical regions participate in tertiary hydrogen bonding interactions. For example, the conserved G19·C56 pair locks the nearby-situated D and TΨC loops. The U8·A14 reverse Hoogsteen pair on the other hand stabilizes the sharp turn in the D loop. Modified nucleosides are also important in stabilizing the tertiary structure of tRNA (see Chapter 2.2.1.), for example in the tertiary pairs of Gm18·Ψ55 and m^5U54·A58 *(for review Dirheimer et al, 1995; Shi & Moore, 2000).*

Magnesium stabilizes the tertiary structure of tRNA molecules. In the crystal structure of yeast $tRNA^{Phe}$, four strong Mg^{2+} binding sites are known: one is located in the pocket formed by a sharp bend of D loop; one in the anticodon loop; and two in the "elbow" of the L. These Mg^{2+} ions stabilize the interaction between the D and the TΨC loops. Mg^{2+} ions are coordinated by water molecules that in turn participate in hydrogen bonding interactions with nitrogens or oxygens of the bases, or phosphate oxygens *(Shi & Moore, 2000).* Polyamines (especially spermidine) also play an important role in the formation of the tertiary structure of tRNA *(for review Dirheimer et al, 1995).*

The tertiary structure of tRNA anticodon loop enables the anticodon to accurately interact with codons in mRNA. Conserved U33 residue situated in front of the anticodon is involved in the sudden turn of the tRNA backbone (U-turn). All three nucleotides of the anticodon are located on the same side of the anticodon loop in conformation suitable for interacting with

codons in mRNA *(for review Dirheimer et al, 1995).* Anticodon is followed by hypermodified nucleosides at position 37 (not capable to participate in base pairing interactions), that help to form the correct tertiary structure of anticodon loop thereby partaking in the accuracy of translation *(for review Yokoyama & Nishimura, 1995)*.

1.3. Biosynthesis of tRNA

tRNAs in both bacteria and eukaryotes are synthesized from long precursor molecules (pre-tRNAs), some of which contain several individual tRNA sequences *(for reviews Sprague, 1995 and Inokuchi & Yamao, 1995)*. In bacteria, some tRNAs are included in the pre-rRNA (ribosomal RNA precursors) transcripts. For example, in *Escherichia coli*, three types of rRNA molecules and a tRNA molecule are excised from a single primary RNA transcript that also contains spacer regions *(for review Deutscher, 1995).*

Pre-tRNAs are trimmed to produce the tRNA of mature size. Processing of the 5' end of pre-tRNAs involves cleavage by ribonuclease P (RNase P), which was one of the earliest catalytic RNA molecules to be recognized *(for review Altman et al., 1995).* The 3' end of tRNAs is generated by the action of numerous conventional protein RNases *(for reviews Deutscher; 1995 and Nakanishi & Nureki, 2005).*

The 3' terminal CCA nucleotide sequence of tRNA is essential for amino acid attachment and for interactions with the ribosome. The CCA sequence is often synthesized *de novo* by a template independent RNA polymerase, CCA-adding enzyme *(for reviews Deutscher, 1995; and Nakanishi & Nureki, 2005).*

Some pre-tRNAs from both bacteria and eukaryotes contain introns that are removed by splicing *(for review Westaway & Abelson, 1995).* Mutations that change the secondary structure of pre-tRNA prevent the splicing reaction, indicating that the secondary structure is important for intron excision to occur. Since introns are always located in the anticodon loop, pre-tRNAs are most likely folded similarly to mature tRNAs, thereby bringing the two intron-exon junctions into proximity *(for review Westaway & Abelson, 1995).* Trimming and splicing reactions are believed to act as quality control steps in generation of functional tRNAs, because misfolded tRNA precursors are not processed properly.

Another unusual aspect of tRNA processing is the extensive modification of bases *(for review Björk, 1995).* Synthesis and function of tRNA modifications are discussed in the next chapter.

During the formation of the tertiary structure of tRNA *in vivo,* various stable but biologically inactive conformations may arise (tRNA molecule becomes "kinetically trapped").

For example, several energetically stable alternative secondary structures of *E. coli* $tRNA^{Phe}$ have been described *(Weeks, 1997)*. Furthermore, different tRNA molecules may interact with each other to form stable but non-functional complexes. Even in the case of such a small molecule as tRNA, the transition to native conformation may take days *(Weeks, 1997)*.

tRNAs are generally associated with proteins and spend little time free in the cell. Protein factors may assist the formation of tRNA tertiary structure *in vivo*. Trbp111 is a small protein in *Aquifex aeolicus* that binds as a dimer to the "elbow" of the L structure of all tRNAs *(Morales et al., 1999)*. Trbp111 acts as a RNA chaperone by assisting the formation of the functional tertiary structure of tRNA, and by protecting the D and TΨC loops from nucleases. Trbp111 also delivers tRNA molecules to aminoacyl-tRNA synthetases and improves the overall rate of aminoacylation. Trbp111, tRNA and aminoacyl-tRNA synthetase form a tertiary complex, where tRNA is hidden between the two proteins *(Morales et al., 1999; Nomanbhoy et al., 2001; Kushiro & Schimmel, 2002)*. Yeast protein Arc1 (C terminal domain is homologous to C terminal domains in MetRS and TyrRS, and also to Trbp111 protein) promotes tRNA binding by MetRS and GlnRS. Association of aminoacyl-tRNA synthetases with Arc1 increases the affinity and selectivity for their cognate tRNAs. Arc1 may also participate in transport of tRNAs to cytosol *(Simos et al., 1998; Martinis et al., 1999)*.

Proteins involved in tRNA processing may also assist the formation of the functional tertiary structure of tRNAs or give a chance to misfolded molecules to obtain their correct structure. It has been proposed, that modification procedure itself may contribute to tRNA (re)-folding and the modification enzymes function as tRNA chaperones *(for review Nakanishi & Nureki, 2005)*. This subject is discussed in chapter 2.2.

Export of tRNAs from the nucleus is a critical step in eukaryotic cells. In eukaryotes the processing of tRNA, the synthesis of most modified nucleosides, and also the aminoacylation of tRNA takes place in the nucleus *(for review Wolin & Matera, 1999)*. Since aminoacyl-tRNA synthetases are sensitive to the correct tertiary structure (see Chapter 3.2.) and processing of the 3' and 5' ends of tRNA, aminoacylation reaction may act as the nuclear proofreading, ensuring that only correctly processed tRNAs leave the nucleus *(Lund & Dahlberg, 1998)*.

2. Modification of tRNA

Approximately 10% of the bases are altered during the post-transcriptional processing of tRNA to yield a variety of modified nucleosides at specific positions, mainly in the core of tRNA and in the anticodon loop. To date, about 100 different tRNA modifications have been described

(for abbreviations of modified nucleosides see Appendix). Several types of modifications occur: methylation of 2'-OH group of the ribose, isomerization of uridine to pseudouridine, and addition of various chemical groups to bases *(for review Björk, 1995)*.

Modified nucleosides have been described in all tRNAs from all organisms studied so far. A subset of these modified nucleosides is present in tRNA from all three phylogenetic domains, some modifications are even present at comparative positions *(Björk, 1984)*. In addition to the fact that tRNA modification is ubiquitous, both phylogenetic domain and species specifity has been described *(for review Björk, 1995)*.

The process of nucleoside modification must have evolved early in evolution, since it is likely that the tRNA progenitor already contained modified nucleosides. Apparently, these kinds of structural alterations of the tRNA were needed early in the development of the translational apparatus.

2.1. Function of modified nucleosides

The functions of tRNA modification are generally divided into two categories. Modifications located in the tRNA core region (D and TΨC loop) contribute mostly toward formation and stabilizing the L shaped tertiary structure. Whereas, modifications occurring within the anticodon loop are usually important for translation *(for review Björk, 1995)*.

Simple modifications like ψ, D and methylation of nucleosides may dramatically change the local structure and thereby affect the overall structure and stability of tRNA molecules *(for review Davis, 1998)*.

Methylation of 2'-OH group of the ribose serves to generally stabilize RNA structure. Methylation of nucleosides prevents the formation of usual base pairs, thereby rendering some of the bases in tRNA accessible for tertiary interactions. In addition, methylation imparts a hydrophobic character to some regions of tRNA, which may be important for their interaction with aminoacyl-tRNA synthetases, translation factors and ribosomal proteins *(for review Davis, 1998)*. Pseudouridine can form one additional hydrogen bond compared to uridine. Ψ can coordinate a water molecule between N1-H and the phosphate backbone and thereby decrease the conformational flexibility of pseudouridine itself and nearby situated structural elements *(for review Auffinger & Westhof, 1998)*. Dihydrouridine conserved in the loop regions of RNA molecules, is one of the few modifications that has a destabilizing effect on RNA structure, its function is to provide the necessary flexibility of the loops *(for review Davis, 1998)*.

Ψ and m^5U promote the *syn* conformation of the glycoside bond and the 3' endo conformation of the sugar, which may improve the stacking interactions between the bases *(for*

review Davis, 1998). Both Ψ55 and m^5U54 stabilize tRNA structure through improving the base stacking interactions. In addition, a bridge forms between Ψ55 N1-H and the neighboring anionic oxygen atom over a water molecule. Ψ55 also interacts with the Gm18 of the D loop. N3-H imino group of the m^5U54 interacts with the anionic oxygen atom of the nucleoside located at position 58 and gives TΨC loop motif similar to the U turn in anticodon loop *(Shi & Moore, 2000; for review Auffinger & Westhof, 1998).*

All tRNAs from *T. thermophilus* contain Gm18, s^2m^5U54 and m^1A58. It has been shown that these modifications are involved in reinforcing the thermal stability of tRNA *(Agris, 1996).* Melting temperature of un-fractionated tRNA from *Pyrococcus furiosus* (growth optimum 100°C) are approximately 20°C higher than predicted solely from G-C content and are attributed primarily to nucleoside modification *(Kowalak et al., 1994).* Addition of the m^5U modification into the 17 nucleotides long analog of the yeast $tRNA^{Phe}$ TΨC domain increased its melting point, whereas addition of Ψ did not *(Sengupta et al., 2000).*

When native $tRNA^{Phe}$ and *in vitro* transcript (lacks all modified nucleosides) were compared at low Mg^{2+} concentrations (0.1 mM), major structural differences were observed. When concentration of Mg^{2+} was increased (>1 mM) differences became less noticeable *(Serebrov et al., 1998).* Compared to the native fully modified tRNA, the *in vitro* transcribed tRNA adopts a less compact structure that can be at least partly compensated by Mg^{2+}.

It is known that compared to native tRNA, the *in vitro* transcript of *E. coli* $tRNA^{Val}$ has appreciably lower Tm, it is more sensitive to nuclease attack at low Mg^{2+} concentrations, interactions between the D and TΨC loops are disrupted, and the affinity of strong Mg-binding sites for Mg^{2+} has decreased *(Derric & Horowitz, 1993).* Unmodified yeast $tRNA^{Asp}$ also has an altered conformation disrupted in the D and TΨC loop interactions *(Perret et al., 1990, a).*

Hence, tRNA modifications stabilize the tertiary interactions and increase the overall structural stability of tRNA. In addition, tRNA modifications may prevent the formation of incorrect tertiary structures. The unmodified transcript of human mitochondrial $tRNA^{Lys}$ cannot adopt the functional cloverleaf structure. Addition of one methyl group (m^1A9) is necessary and sufficient for avoiding the formation of the non-functional extended hairpin structure and for ensuring the formation of the functional structure *(Helm et al., 1999; Helm & Attardi, 2004).* Thus, a post-transcriptional modification plays the role of a molecular internal chaperone. Modifications may stabilize intermediates and change local conformations, thereby making possible the formation of the overall tertiary structure of tRNA *(Nobles et al., 2002).*

Modifications occurring within the anticodon loop are generally important for accurate and effective synthesis of proteins, either by altering codon recognition properties of tRNAs,

affecting the efficiency of decoding and reading frame maintenance, or recognition of tRNAs by aminoacyl-tRNA synthetases *(for reviews Björk, 1995; Yokoyama & Nishimura, 1995; Curran 1998).*

In some cases, modifications occurring within the anticodon loop may have dual functions of precise codon pairing and accurate recognition by the cognate aminoacyl-tRNA synthetases. For example a mnm^5s^2U at the wobble position of $tRNA^{Lys}$ and $tRNA^{Glu}$ enables discriminate of purines and pyrimidines to prevent the decoding of the NNY codon. The mnm^5s^2U modification is also required for recognition by the cognate aminoacyl-tRNA synthetases (LysRS and GluRS respectively) *(for review Björk, 1995).* Similar case is described for the modification k^2C34, where decoding and aminoacylation specificities are both converted by the presence of this single nucleoside modification in *E. coli* $tRNA_2^{Ile}$ *(Muramatsu et al., 1988; Soma et al., 2003).* Function of modified nucleosides in tRNA aminoacylation is covered in more detail in chapter 3.3.

tRNA modifications have several other functions in various biological processes not discussed here. For many modifications, the biological relevance still remains unknown.

2.2. Synthesis of modified nucleosides

All of the modified nucleosides are formed by enzymatic modification of standard ribonucleotides in a tRNA precursor.

Since different enzymes may catalyze the formation of the same modified nucleoside (for example ψ) at different positions of the tRNA and since several enzymes are required to synthesize each hypermodified nucleoside, at least 45 different tRNA modification enzymes exist in the bacterial cell *(Buck et al, 1983).* The synthesis of these enzymes requires as much as 1% of the genome size present in bacteria, meaning that at least four times more genetic information is devoted to the synthesis of the tRNA modification enzymes, than to the synthesis of tRNA.

In both eukaryotes and bacteria, modifications occur at different stages during the processing of the precursor tRNA *(for reviews Wolin & Matera, 1999; and Ferre-D'Amare, 2003).* In bacteria, several modifications (m^5U, Ψ, D) occur in polycistronic precursors. However, no ribose methylation occurs in either dimeric or monomeric precursors, suggesting that such a methylation reaction requires an almost mature tRNA *(Sakano et al, 1974).* The formation of m^5U54 and Ψ55 was shown to precede the formation of Ψ39 and i^6A37, indicating stepwise modifications *(Ciampi et al, 1977).* Modified nucleosides inhibit the RNase P reaction, suggesting that this endonucleolytic cleavage occurs early in the tRNA maturation process and

precedes modifications *(Schaefer et al, 1973)*. In eukaryotes it was shown that all base modifications, except most of those present in the anticodon region, occur in the nucleus before splicing and in a sequential manner *(Nishikura & De Robertis, 1981; and Stange & Beier, 1987)*.

As modification is an integral part of tRNA processing, tRNA structure required for recognition by different modifying enzymes depends on the synthesis and processing kinetics of the primary transcript. Furthermore, modification enzymes must compete with other proteins such as aminoacyl-tRNA synthetases and elongation factor Tu that also use tRNA as substrate *(for review Björk, 1995)*. Therefore, tRNA modification is a function of the processing stage, the concentration of the substrate, and the amount and activity of the tRNA modification enzyme.

tRNA modification enzymes are site-specific and require recognition signals in tRNAs *(for review Björk, 1995)*. *In vitro* synthesis and mutation of unmodified tRNA molecules allows determination of tRNA sequences and structural elements required for recognition by different modification enzymes.

Most tRNA modification enzymes recognize several but not all tRNA species present in the cell. Some modifications are present in almost all tRNAs, other modifications are characteristic to only one or a few tRNAs. Enzymes that recognize elements common to all tRNAs synthesize first type of modifications, enzymes recognizing sequences and architectural motifs characteristic to specific tRNAs synthesize latter type of modifications. Synthesis of modifications present in the core of tRNA is often more sensitive to the overall structure of tRNA than is the synthesis of modifications in distal parts of tRNA *(Grosjean et al, 1996)*.

tRNA modification enzymes are divided into two large groups based on their sensitivity to the tertiary structure of tRNA. Enzymes sensitive to the overall structure of tRNA belong to the group I and less sensitive enzymes belong to the group II *(Grosjean et al, 1996)*. Hence, tRNA modification enzymes use different mechanisms to recognize their substrate tRNAs. The lesser sensitivity to the tRNA overall structure of some modification enzymes correlates to their function in early steps of the processing, when the correct tertiary structure may not be achieved yet *(Grosjean et al, 1996)*.

In vivo experiments have determined that the formation of modifications m^1G37, ψ40 and ψ13 is very sensitive to small perturbations in the tertiary structure of tRNA. Whereas the synthesis of m^2G26, Q34 and manQ34 is hindered only by drastic changes of tRNA tertiary structure. The synthesis of m^5U54, Ψ55, m^1A58, m^5C49 and m^2G26 depends largely on the elements in local regions of tRNA and not on the overall tertiary structure of tRNA *(Grosjean et al, 1996)*. For the latter modifications, some structural mutations turned out to be even better substrates than the native tRNA *(Grosjean et al, 1996)*. Disruption of tertiary interactions

between different regions of tRNA probably renders the target nucleosides more accessible to the enzymes. It is likely that these types of enzymes recognize and bind only to a small portion of tRNA. Alternative possibility is that these modifications enzymes are capable of changing the structure of tRNA and similarly to DNA modification enzymes turn the target nucleosides out of the tertiary structure (base-flipping) *(Huang et al, 2003).*

Enzymes that synthesize modifications in early stages of tRNA processing may "chaperone" (facilitate and/or stabilize the folding of tRNA precursors into correct tertiary structure, which is required for the synthesis of modifications in latter stages of processing or for aminoacylation of tRNAs *(Grosjean et al, 1996).*

It was shown that modifications $m^2{}_2G26$ and m^1A9 in human mithochondrial tRNAs affect the folding of tRNA molecules *(Steinberg & Cedergren, 1995).* Archaeosine (archaea specific modification) is introduced into G15 in the D loop by archaeosine tRNA guanine-transglycosidase (arcTGT) through a drastic conformational change of the tRNA from L to λ shape upon binding with the enzyme. Consequently, the enzyme precisely locates the exposed G15 in the active site. After the tRNA spontaneously refolds, it reinforces the canonical L shaped structure by intensive stacking interactions. Thus, arcTGT is thought to function as a kind of RNA chaperone *(Ishitani et al, 2003).*

Therefore, the pathway of tRNA modification may also be considered as the pathway of tRNA folding, where the modification enzymes act also as RNA chaperones, ensuring that only the correctly modified and folded tRNA molecules get to participate in the translation.

Two tRNA modification enzymes important for current study are described next.

2.2.1. TrmA

tRNA-(m^5U54) methyltransferase also known as tRNA-(T54) synthase (TrmA or RUMT) catalyzes the methylation of uracil at position 54 of all tRNAs in *E. coli* cells. Structural gene *trmA* is essential for viability, although the known catalytic activity of the TrmA protein is not *(Persson et al., 1992).* TrmA like nearly all other enzymes that methylate nucleic acids use S-adenosyl-L-methionine (AdoMet or SAM) as donor of the methyl group *(for review Björk, 1995).*

A mechanism that involves a covalent intermediate between the tRNA and a nucleophile in the TrmA was proposed *(Santi & Hardy, 1987).* The nucleophile was identified as the cysteine at position 324 *(Kealey & Santi, 1991).* The conserved Cys324 attacks C6 of U54, creating a nucleophilic center at C5, where the methyl group is added. The methyl group is directly

displaced from SAM by the C5 of U54 *(Kealey et al, 1991).* TrmA does not need the addition of cations in order to methylate the tRNA, but Mg^{2+} stimulates the reaction *(Ny et al., 1988).*

Since U54 is buried in tRNA through stacking between G53 and Ψ55, TrmA protein must either change the conformation of the TΨC loop to gain access to its substrate U54, or the methylation of U54 *in vivo* takes place before the final tertiary structure of the TΨC loop is formed *(Kealey & Santi, 1991; Yao et al., 1997).*

TΨC arm alone (positions 49-65) is a substrate for TrmA. However, k_{cat} is reduced to 36% and specificity constant (k_{cat}/K_M) to 6% of that of the complete tRNA *(Gu & Santi, 1991).* Moreover, an 11-mer oligonucleotide, with only 2 base pairs in the stem, is also a substrate for the enzyme, albeit at reduced efficiency. However, the 11-mer has the same K_M as the 17-mer. A consensus sequence for the methylation includes only 11 nucleotides of the TΨC arm: Pu52·Py62 and G53·C61 base pairs of the stem next to the loop, the conserved nucleosides U54, U55, C56 and A58 of the loop; and the semi-conserved bases Pu57 and Py60, also in the loop *(Gu & Santi, 1991).* Absolutely necessary is the 7 nucleotides long TΨC loop, whose nucleotide composition may vary, except for the positions 54 and 56 (absolutely required only U54 substrate nucleoside). At least 2 base pair long TΨC arm is also important. TrmA recognizes rather the local tertiary structure, than the exact nucleoside composition of the TΨC arm *(Kealey et al., 1994; Gu et al., 1996; Sengupta et al., 2000).* Structure of the rest of tRNA does not play an important role for TrmA *(Grosjean et al., 1996; Becker et al., 1997).* It was shown that TrmA can also methylate *E. coli* 16S rRNA position U788 *in vitro*, where the same consensus sequence is described *(Gu et al, 1994).*

TrmA from *E. coli* is a 42-kDa polypeptide *(Greenberg & Dudock, 1980).* This enzyme exists in several forms – the native form and a form covalently bound to RNA *(Gustafsson & Björk, 1993).* As much as 50% of the TrmA molecules present in the bacterial cell are covalently bound to rRNA (3'-end of 16S rRNA) and/or to a subset of undermodified tRNAs. In logarithmically growing cells, the enzyme is present in three forms: a 42 kDa native form, a 54 kDa TrmA-RNA complex, and a 62 kDa TrmA-RNA complex *(Gustafsson & Björk, 1993).* Although the reason for the presence of these RNA-TrmA complexes is not understood, it may be related to an unknown second function of the TrmA peptide that is essential for the cell *(Persson et al, 1992).*

Methylation of U54 of the TSL (T stem-loop) region of tRNA raised its melting temperature by 2 degrees and stabilized about 0.4 kcal/mol compared to unmodified TSL *(Garcia & Goodenough-Lashua, 1998).* m^5U54 in tRNA improves the accuracy of the decoding process in the A site of the ribosome, but does not affect the efficiency of formation of the

ternary complex between tRNAs and elongation factor Tu, binding of tRNAs to the P site, and the peptidyltransferase activity of the ribosome *(Kersten et al., 1981).*

Point mutation in the catalytic site of TrmA that abolishes the synthesis of modification m^5U54 completely reduces the growth rate of bacteria only by 4%. In mixed culture, however, the mutant cells have clear disadvantage compared to wild type cells *(Kealey et al., 1991; Persson et al., 1992; Urbonavicius et al., 2002).* Insertions and at the beginning of *trmA* gene that resulted in the lack of functional TrmA protein, were lethal to cells *(Persson et al., 1992).* Hence, TrmA protein itself is essential for cells and may have another rather important function. For example, TrmA may in addition to tRNAs other RNA molecules, synthesize different modification, take part in the assembly of ribosomes, or function as a chaperone in the processing of RNA *molecules (Persson et al., 1992; Johansson & Byström, 2002; Urbonavicius et al., 2002).* So far unknown function of TrmA protein may be related to the fact that TrmA binds covalently to a fragment of the 3' end of 16S rRNA molecules *(Ny et al., 1988).*

Yeast Trm2 protein (homologue of TrmA) was shown to be non-essential for viability. Nevertheless, it is essential for the stabilization of mutant $tRNA^{Ser}_{CGA}$ and may have a second function in assisting the folding of tRNA molecules *(Johansson & Byström, 2002).*

2.2.2. TruB

Pseudouridine (ψ) is the most common modified nucleoside in the cell. tRNAs from most organisms have Ψ at position 55. The tRNA-(Ψ55) synthase (TruB) is a 39.7 kDa protein that recognizes and modifies all the tRNAs in *E. coli* cells and does not require for synthesis of pseudouridine either cofactors, Mg^{2+}, or external input of energy *(Ivanetich & Santi, 1992; Nurse et al., 1995; for review Ferre-D'Amare, 2003).*

In contrast to uridine, the ribose of pseudouridine is linked to the C 5 not to the N1 of the base. The conserved Asp of TruB is involved in a nucleophilic attack to the C6 of the uracil at tRNA position 55. Glycoside bond between the ribose and the base is broken and due to conformational changes in the enzyme, the base is rotated and linked by C5 to ribose C1' *(Nurse et al., 1995).*

Crystal structure is known for TruB in complex with the TSL region of tRNA (see Figure 3, a) *(Hoang & Ferre-D'Amare, 2001)*. In the precursor tRNA structure the substrate for TruB U55 is hydrogen bonded to methylguanosine (Gm18) in the D loop and the pyrimidine base is unavailable for the enzyme (see Figure 3, b). The enzyme must disrupt at least partially the tertiary structure of tRNA precursor before the modification can be *synthesized (Hoang & Ferre-D'Amare, 2001).* TruB recognizes the A58·m^5U54 reverse Hoogsteen base pair and the

conserved histidine of the enzyme stacks with it, hence breaking the stacking interaction between the bases in the TΨC loop. Three bases (including the U at position 55) are flipped out of the stacked conformation of the loop and are positioned to the enzymatic cleft of TruB (see Figure 3, a and c) *(Ferre-D'Amare, 2003).* TruB becomes in contact with its substrate nucleoside, but during this process interactions between the D and TΨC loops are broken and reunited after the modification is made. Newly synthesized Ψ55 interacts with Gm18 of D loop leading to further structural rearrangements in tRNA molecules.

Recognition signals required by TruB are contained entirely in the TSL region of the tRNA, as the 17-mer of TSL region was as good a substrate for TruB as native tRNA. Important is the base pair 53:61 that ensures the 7 nucleotides long TΨC loop. The individual nucleotides in the T stem are not important as long as the correct base pairing is ensured. The function of the T stem is stabilization of the TΨC loop and presentation of the substrate U55 to TruB. Modified TSL substrate with TΨC loop one nucleotide longer or shorter was 30-fold less efficient substrate. Absolutely necessary for TruB were the nucleosides U54, U55, and A58, favorable was C56 *(Gu et al, 1996; Gu et al., 1998).* Mutations in tRNA that avoid the formation of tertiary interactions between D and TΨC loops, do not hinder the recognition of tRNA by TruB and the efficiency of Ψ55 synthesis. Hence, TruB does not need the tRNA to have a correct tertiary structure in order to be recognized *(Becker et al., 1997; Ferre-D'Amare, 2003).*

Comparison of TrmA and TruB enzymes showed that while both recognize the TSL region of tRNA, the 7 nucleotides long TΨC loop is a feature more strictly required by TrmA. TruB recognizes its consensus sequence also in TΨC loop with more variable length. TrmA, on the other hand, recognizes the tertiary structure of the 7 nucleotide long TΨC loop, with only one absolute requirement on sequence - substrate U54 *(Gu et al, 1996, Gu et al., 1998).* Both TrmA and TruB synthesize their modifications m^5U and ψ in TΨC loop even in the tRNA mutant where the anticodon and the TΨC loops are switched, showing that the recognition of these enzymes depends entirely on the sequence and/or conformation of the TΨC loop ant not on its location in the overall tRNA structure *(Becker et al., 1997).*

Biochemical studies have shown that Ψ55 is not required for tRNAs to be functional in protein synthesis. tRNA lacking Ψ55 is efficiently aminoacylated *(Pallanack et al., 1995),* forms a ternary complex with elongation factor Tu, and is capable to participate in poly-Phe synthesis *in vitro (Nazarenko et al., 1994).*

Deletion of *truB* gene in *E. coli* completely abolishes the formation of Ψ55. Lack of *truB* did not affect the rate of exponential growth of the cells. However when competing with wild

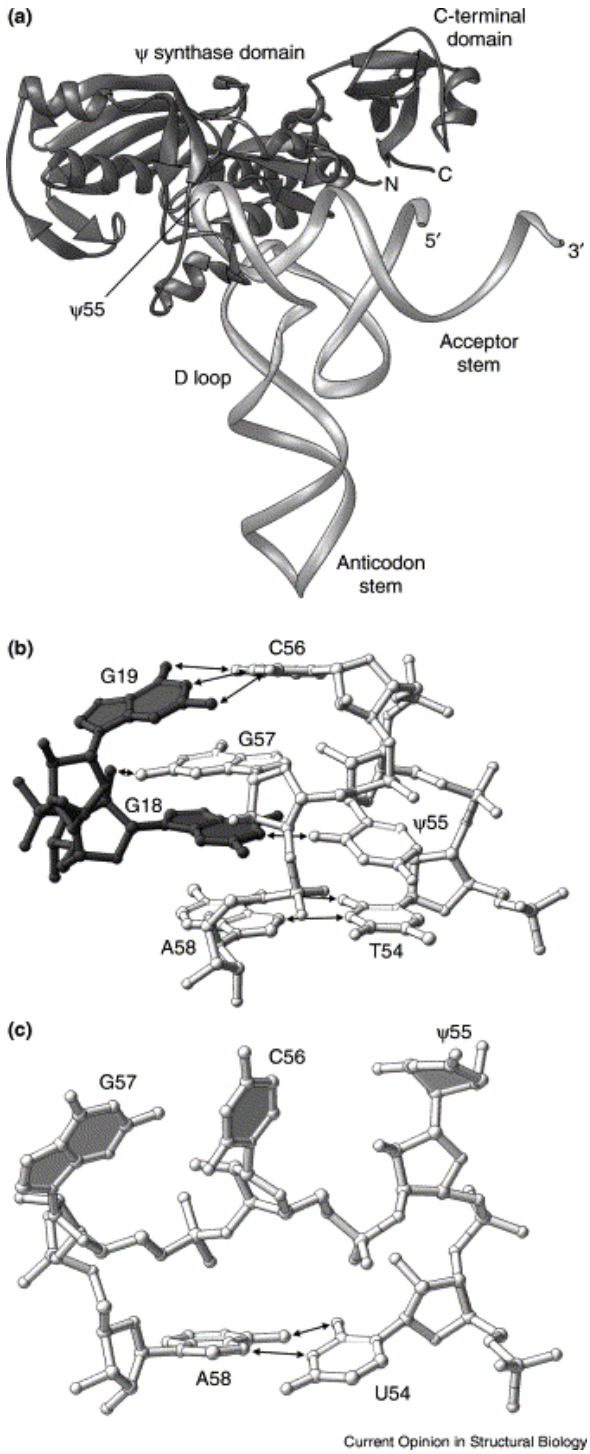

FIGURE 3. Binding of tRNA by pseudouridine synthase TruB. (a) Model of tRNA bound to TruB. TruB comprises a conserved Ψ synthase domain and a C-terminal PUA domain. The T loop is bound in a cleft that bisects the Ψ synthase domain. (b) Conformation of T loop nucleotides in intact, folded yeast $tRNA^{Phe}$. Two nucleotides from the T loop make tertiary contacts with nucleotides from the D loop. (c) Conformation of the T loop when bound to TruB. Ψ55, C56 and G57 have flipped out of the helical stack. This allows the enzyme to access the substrate base at position 55, but also disrupts the two base pairs formed between D and T loop nucleotides *(Ferre-D'Amare, 2003).*

type cells, latter had a strong selective advantage, especially under osmotic stress conditions and at elevated temperatures. The mutant phenotype could be complemented by cloned *truB* gene but not by D48C, catalytically inactive allele of *truB (Kinghorn et al., 2002)*. The *truB* deletion mutant also exhibited a defect in survival of rapid transfer from 37°C to 50°C. This mutant phenotype could be compensated by cloned *truB* gene but not by D48C mutant *truB*. The temperature sensitivity of *truB*$^-$ *E. coli* was even greater when TrmA activity was absent from the cells *(Kinghorn et al., 2002)*. Ψ55 is not essential but is believed to increase the stress tolerance of cells by stabilizing the tRNA population at higher temperatures, *(Gutgsell et al., 2000; Kinghorn et al., 2002)*.

The fact that transformation of *E. coli truB*$^-$ strain with plasmid containing D48C mutation in *truB* compensated the growth phenotypes *(Gutgesell et al., 2000)* refers to TruB protein exhibiting several functions in the cell. Since the change of tRNA conformation upon interaction with TruB has been described, it is possible that TruB has RNA chaperone activity and is actively involved in the process of tRNA folding *(Gutgsell et al., 2000; Ferre-D'Amare, 2003)*. The opening of tRNA structure during the process of pseudouridine synthesis may give to misfolded molecules a chance to acquire the structure needed to give correct tertiary interactions.

Hence, tRNA modification enzymes may act as RNA chaperones and assist the formation of the correct tertiary structure of tRNA, or give a possibility to misfolded molecules to obtain their functional structure.

It would be interesting to know whether the modification enzymes work individually, or form large multi-enzyme complexes. In other words, whether the modification enzymes interact with each other and/or other tRNA binding proteins, such as aminoacyl-tRNA synthetases, and work coordinately. Some evidence about the existence of high-molecular-weight complexes containing tRNA modification enzymes and aminoacyl-tRNA synthetases in mammals and bacteria has been reported *(Agris et al., 1976; Agris et al., 1983; Harris, 1990)*.

3. Aminoacylation of tRNA

Incorporation of correct amino acids into proteins depends on the specificity of codon-anticodon base pairing, as well as on the attachment of each amino acid to appropriate tRNAs.

The attachment of amino acids to specific tRNAs is mediated by a group of enzymes called aminoacyl-tRNA synthetases (aaRS). Each of these enzymes recognizes a single amino acid, cognate tRNA or tRNAs, and catalyzes a covalent bond formation *(for review Ibba & Söll, 2000)*.

The aminoacylation reaction proceeds in two steps and ATP energy is used to attach each amino acid to its tRNA molecule in a high-energy linkage between tRNA 3' terminal ribose and carboxyl group of amino acid *(Berg et al., 1961; for review Ibba & Söll, 2000)*. First, aaRS binds an amino acid and ATP. The amino acid is then activated by linkage of its α-carboxyl group directly to an AMP moiety forming a stable aminoacyl-AMP synthetase intermediate with the concomitant release of pyrophosphate.

(1) aaRS + ATP:Mg^{2+} + aa <=> aaRS:aa~AMP + PPi:Mg^{2+}

Without leaving the enzyme, the AMP linked α-carboxyl group of the amino acid is transferred to 2' or 3' O on the sugar at the 3' CCA terminus of the acceptor tRNA, and AMP is released. This transfer joins the amino acid by an activated ester linkage to the tRNA and forms the final aminoacyl-tRNA (aa-tRNA, also charged tRNA) *(reviews Ibba & Söll, 2000)*.

(2) aaRS:aa~AMP + tRNA <=> aaRS + aa-tRNA + AMP

The equilibrium of reaction is driven further toward aa-tRNA formation by converting pyrophosphate (PPi) released in the first step to inorganic phosphate (Pi) by pyrophosphatase. Certain aaRS (GluRS, GlnRS, ArgRS, and some LysRS) require prior binding of tRNA for amino acid activation. The binding of tRNA to aaRS occurs in the presence of a polyamine or a divalent cation, usually magnesium *(for reviews Meinnel et al., 1995; Arnez & Moras, 1997; Ibba & Söll, 2000)*.

Once a tRNA is loaded with an amino acid, codon-anticodon pairing directs its incorporation into proteins. Therefore, the accuracy of amino acid and tRNA recognition by aaRS is of first importance, governing to a large extent the fidelity of the translation *(for review Meinnel et al., 1995; and Ibba & Söll, 2000)*.

AaRSs utilize chemical properties, size, and shape of amino acids to prevent the attachment of an incorrect amino acid to a tRNA *(for reviews Meinnel et al., 1995; and Ibba & Söll, 2000)*. For HisRS, ProRS, and LysRS it has been shown that only the correct amino acid can promote the activation of the catalytic site *(for review Jakubowski, 2005)*. However, in certain cases, additional proofreading mechanisms of aaRS also contribute to the overall accuracy of the aminoacylation reaction by correction of errors *(for review Ibba & Söll, 2000)*. Such correction reactions may occur downstream from either aminoacyl-adenylate formation or tRNA esterification. Several aaRS (for example IleRS, ValRS, LeuRS, and ThrRS) contain editing sites in addition to acylation sites. These complementary sites function as a double sieve to ensure a very high fidelity *(for review Jakubowski, 2005)*.

Also the relative concentrations of amino acids, tRNAs and aaRSs may contribute to the accuracy of tRNA aminoacylation *in vivo*. The frequency of errors in amino acid synthesis is less than 10^{-5} *(for review Meinnel et al., 1995).*

3.1. Classes of aminoacyl-tRNA synthetases

There is a different aaRS for each amino acid in most cells that is 20 aaRS in all *(for review Ibba & Söll, 2000).* While all aaRS catalyze the same type of reaction, each is also specialized and has features that are unique. On the basis of aaRS sequences and structural studies, it has been concluded that aaRSs fall into two distinct classes, termed class I and class II, both classes are responsible for attachment of 10 out of the 20 amino acids to tRNA in *E. coli* (see Table 1) *(for review Delarue & Moras, 1993; and in Ibba & Söll, 2000).*

AaRSs may be monomers (α), homodimers (α_2), homotertramers (α_4), or heterotetramers ($\alpha_2\beta_2$), and may have one or two binding sites for tRNA (PheRS). Most class I aaRSs are monomers, whereas most class II aaRSs are dimers (see Table 1) *(for review Ibba & Söll, 2000).*

TABLE 1. Classification and subunit structure of aminoacyl-tRNA synthetases in *E. coli*

Class I	Class II
Arg (α)	Ala (α_4)
Cys (α)	Asn (α_2)
Gln (α)	Asp (α_2)
Glu (α)	Gly ($\alpha_2\beta_2$)
Ile (α)	His (α_2)
Leu (α)	Lys (α_2)
Met (α)	Phe ($\alpha_2\beta_2$)
Trp (α_2)	Ser (α_2)
Tyr (α_2)	Pro (α_2)
Val (α)	Thr (α_2)

Two classes of aaRS are defined by the characteristic sequence motifs and different structures of the activation domains *(for reviews Delarue & Moras, 1993; and in Ibba & Söll, 2000).* Class I aaRSs have an N-terminal catalytic domain for binding of amino acid and ATP. This domain contains conserved HIGH and KMSKS signature sequences. Class II aaRSs have

three conserved motifs: motif 1, 2 and 3. The activation domain of class I aaRS have a Rossmann fold (5 parallel β strands connected by α helices), whereas activation domain of class II aaRS consists largely of anti-parallel β strands *(for reviews Meinnel et al., 1995; Ibba & Söll, 2000)*. There is no sequence homology between the nucleotide binding sites of the aaRS from different classes and they bind ATP in different conformations *(for review Arnez & Moras, 1997)*.

Class I and class II aaRSs bind to the opposite faces of tRNA. Class I aaRSs bind to the minor groove side of the acceptor helix and the variable arm of tRNA is facing away from the enzyme. Class II aaRS bind to the major groove side of the acceptor stem and the variable region is facing the enzyme (see Figure 4) *(for review Arnez & Moras, 1997; and Ibba & Söll, 2000)*.

Class I aaRSs interact with the nucleotides of the 5' end of the acceptor stem and class II aaRS with the 3' end nucleotides. The CCA arm of tRNA adopts different conformations in complexes with the two classes of aaRS to accommodate these interactions; the arm is in the helical conformation observed in free tRNA for class II aaRS and in a hairpin conformation (U1-A72 base pair of the acceptor stem of tRNA is often disrupted) for class I aaRS *(for reviews Delarue & Moras, 1993; Ibba & Söll, 2000)*. Class II aaRS (except PheRS) link the amino acid

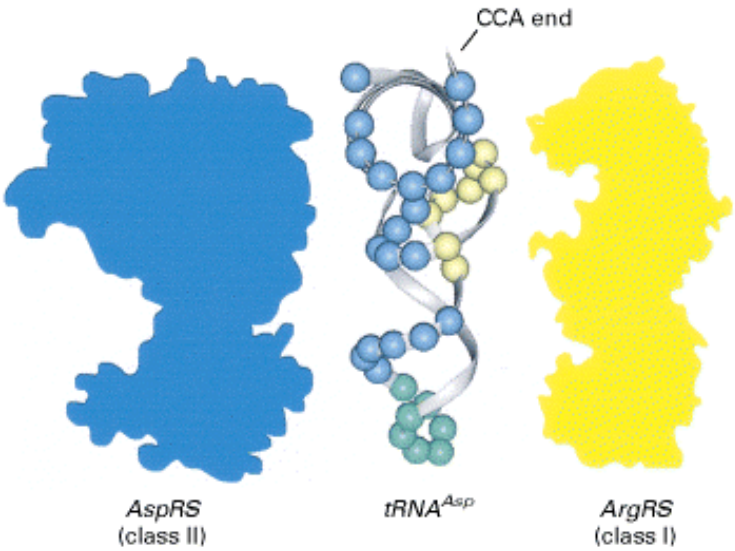

FIGURE 4. Binding of tRNA by aminoacyl-tRNA synthetases. Outlines of the tertiary structure of AspRS and ArgRS (class II and class I aaRS respectively), and a ribbon diagram of slightly modified version of $tRNA^{Asp}$ are shown. Sites on the opposite sides of tRNA make contact with the two aaRS: the blue circles show contacts with the AspRS; those that contact with ArgRS are indicated by yellow circles. The aaRS are shown positioned away from the tRNA for clarity, but the fit of the surfaces at close range is obvious *(adapted from Sissler et al., 1997)*.

directly to the 3'-OH group of the ribose, class I aaRS link it initially to the 2'-OH group and subsequent transesterification reaction shifts the amino acid to the 3' position *(for review Ibba & Söll, 2000).*

3.2. Recognition of tRNAs

The correlation between the amino acid and the nucleic acid world is established through the tRNA recognition by specific aaRS enzymes.

The selection of tRNAs by aaRS proceeds in two steps *(for review First, 2005).* First, aaRS binding is stable only to the correct tRNAs. Second, only the correct tRNAs are aminoacylated fast. Correct tRNAs induce conformational changes in aaRS that promote the aminoacylation reaction. Non-cognate tRNAs are not capable of causing conformational changes and are likely to dissociate from aaRS before the aminoacylation takes place *(for review First, 2005).* Selection based on the differences of the rates is known as kinetic proofreading.

Each aaRS can aminoacylate all the tRNAs (isoacceptor tRNAs) whose anticodons correspond to the same amino acid. The set of tRNA determinants that enable aaRS to discriminate among tRNAs are called identity elements and sometimes referred to as the "second genetic code" because of its central importance in flow of the genetic information *(for reviews Pallanack et al., 1995; Giege et al., 1998).* The identity of tRNA is determined by its primary, secondary, and tertiary structure. Major elements defining identity in all *E. coli* tRNAs have been deciphered and much is known about identity determinants of most yeast tRNAs and of a few tRNAs from other organisms.

Genetic, biochemical, and X-ray crystallographic evidence indicate that the specificity determinants of tRNAs are clustered at two distal extremities of the molecule: the acceptor stem and the anticodon loop (see Figure 5). These two regions of tRNA come in close contact with aaRSs (see Figure 4). Other regions of tRNA (like the variable loop and D stem) are only in few cases shown to interact with aaRS *(for review Giege et al., 1998).*

The acceptor arm is an especially important determinant for the specificity of tRNA recognition. For most tRNAs (except *E. coli* $tRNA^{Glu}$ and $tRNA^{Thr}$) the fourth nucleotide from the 3' end at the position 73 (discriminator base) is an identity determinant. First base pairs of acceptor stem (N1-N72, N2-N71) are also identity determinants of some tRNAs *(for review Giege et al., 1998).* In case of $tRNA^{Ala}$ a single base pair (G3-U70) in the right context of the acceptor stem is necessary and sufficient for recognition by AlaRS *(Hou & Schimmel, 1988; for review Ribas de Pouplana et al., 2005).* Minihelix (acceptor and TΨC stem of tRNA) is aminoacylated with kinetics only 5-fold below that of native $tRNA^{Ala}$. Even a microhelix

containing only 24 out of the 76 nucleotides (the acceptor stem and a hairpin loop) of the native $tRNA^{Ala}$ is specifically aminoacylated by AlaRS albeit 50-fold reduction in specificity *(Francklyn & Schimmel, 1989).* Thus the aminoacylation is possible for some aaRS even if the anticodon loop is completely lacking.

Nonetheless, the anticodon loop contributes frequently (except *E. coli* $tRNA^{Ala}$, $tRNA^{Leu}$, and $tRNA^{Ser}$) to discrimination as well. Some aaRS recognize their tRNA partners primarily on the basis of their anticodons (MetRS and ValRS) *(for review Giege et al., 1998).* N37 is an important identity determinant only for class I aaRS, however N35 is also important for class II aaRS that recognize anticodons of cognate tRNAs. N34 and N36 positions rarely determine the identity of tRNAs *(for review Giege et al., 1998).* X-ray crystallographic analysis of the $tRNA^{Gln}$/GlnRS complex revealed that each of the anticodon bases neatly fits into a separate pocket in the three-dimensional structure of GlnRS. In addition, contacts were made near the "elbow" of the tRNA molecule, particularly with the G10-C25 base pair *(Rould et al., 1989).* However, nucleotides in tRNA core are infrequently used as identity determinants. See Figure 5 about the distribution of identity elements in tRNA.

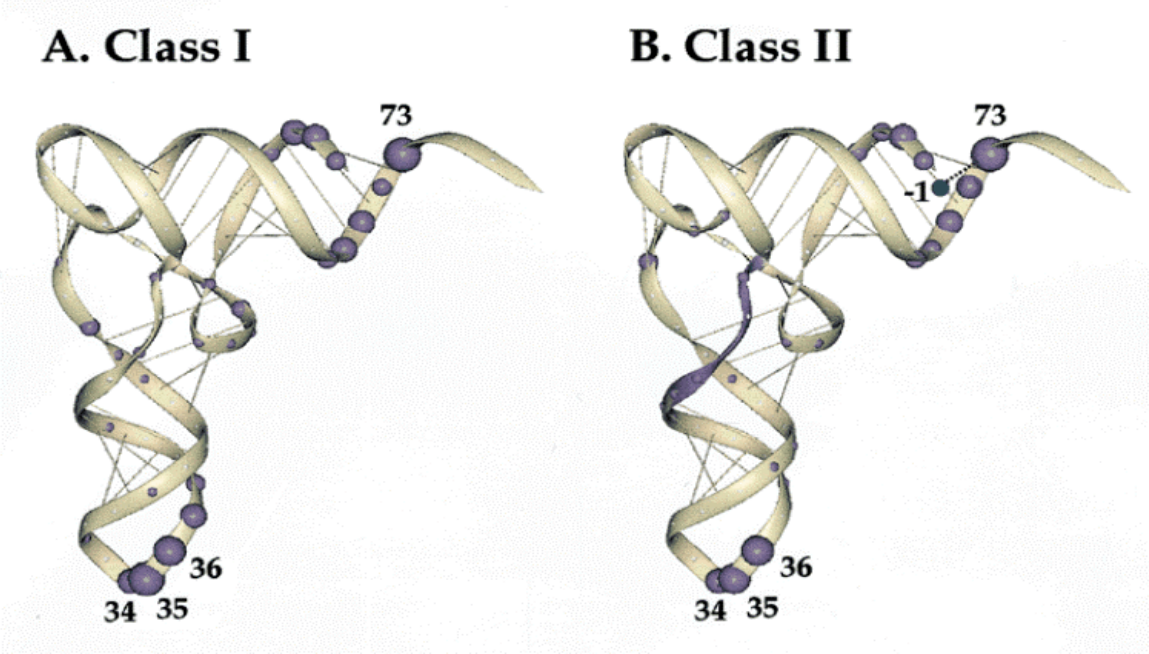

FIGURE 5. Distribution of identity elements in tRNA. Nucleotides that have been experimentally demonstrated to have a role in tRNA recognition by 10 class I aaRS (A), and by 10 class II aaRS (B) in *E. coli*. Increasing size of circles indicates the relative frequency that a given position acts as an identity element *(adapted from Giege et al., 1998).*

Strong identity determinants of tRNA interact with aaRS directly; hydrogen bonds are formed between identity nucleotides in tRNA and amino acids of aaRS *(for review Giege et al., 1998).* In many instances the chemical groups of identity nucleotides involved in the hydrogen

bonding interactions have been determined (for example N6 of nucleotide at position 73 in *E. coli* $tRNA^{Ala}$ and $tRNA^{Phe}$) *(for review Giege et al., 1998)*. Modified nucleosides may also be involved in formation of the hydrogen bonds with aaRS in few cases (see Chapter 3.3) *(for review Björk, 1995).*

Identity elements may also act indirectly through changing the conformation of tRNA recognized by aaRS *(for review Giege et al., 1998).* As noted already the G3-U70 wobble pair is the major identity determinant for AlaRS. However, whether AlaRS recognizes the chemical groups of the G3-U70 identity nucleotides or the conformation of acceptor stem caused by the wobble pair is debatable. In the absence of a crystallographic structure of a $tRNA^{Ala}$/AlaRS complex, only indirect evidence can be invoked. Experiments led to the conclusion that the exocyclic 2-NH_2 group of G3 is essential for alanylation *(Ramos & Variani, 1997; Beuning et al., 1997)*. Three 2'-OH groups located at positions 4, 70, 71 made contributions to the efficiency of aminoacylation *(Musier-Forsyth & Schimmel, 1992).* On the other hand, genetic investigations coupled with NMR analyses have shown that replacing the G3-U70 wobble pair with a C-C mispair preserves $tRNA^{Ala}$ aminoacylation *in vivo*. Likely, the C-C pair, as does a G-U pair, provides deformability in the acceptor stem that does not occur in a structurally more rigid stem with a G-C pair *(Chang et al., 1999).* Shifting the G-U base pair to an adjacent helical site preserves $tRNA^{Ala}$ identity *(for review Ribas de Pouplana et al., 2005).* These data are in line with an indirect recognition mechanism. Presumably, direct and indirect recognition mechanisms are not exclusive.

Conformational motifs of tRNA are important for efficient aminoacylation in several other cases. In contrast to AlaRS, *E. coli* ValRS prefers the regular A type geometry of the acceptor stem *(Liu et al., 1997).* The unusual G15·G45 Levitt pair is important for *E. coli* $tRNA^{Cys}$ and $tRNA^{Gln}$ giving more conformational flexibility to these tRNAs *(Sherlin et al., 2000).* The lengths of variable and D loops are important for *E. coli* $tRNA^{Gln}$, $tRNA^{Ala}$ and yeast $tRNA^{Phe}$. The long extra arm in $tRNA^{Ser}$ is also important for its recognition *(for review Giege et al., 1998).* Conformational motifs of tRNA thus act as additional identity determinants.

All identity determinants are not equally important. For *E. coli* $tRNA^{Ala}$ the G3-U70 is of most importance, for $tRNA^{Val}$ A35 is the strongest identity element *(for review Giege et al., 1998).* The effect of mutations of individual identity determinants on aminoacylation varies for different tRNAs in different organisms. Identity nucleotides that act directly by forming hydrogen bonds with aaRS affect the k_{cat} of the aminoacylation reaction. Determinants involved in formation of identity conformation change the K_M of the aminoacylation *(for review Giege et al., 1998).* Reaction conditions of *in vitro* experiments may affect the strength of the identity

determinants. Different identity elements may have additive, cooperative, anti-cooperative, or independent effects *(for review Giege et al., 1998).*

Anti-determinants are negative signals that hinder the recognition of tRNAs by non-cognate aaRS. For example, m^1G37 modification of yeast $tRNA^{Asp}$ hinders recognition by ArgRS *(for review Björk, 1995).* Structural motifs may also act as anti-determinants as is the case for G3-U70 wobble base, a determinant for *E. coli* AlaRS is an anti-determinant for ValRS *(Liu et al., 1997, Horowitz et al., 1999).* In addition to the identity determinants, aaRSs require the accommodation of tRNAs into active sites.

Permissive elements are not directly involved in tRNA identity, but they create the context for optimal recognition and aminoacylation of tRNAs *(Frugier et al., 1998).* Presence of permissive elements implies that no nucleotide within a tRNA is of random nature but has been selected by evolution so that tRNAs can fulfill their functions efficiently.

Isoacceptor tRNAs have mostly the same set of identity determinants. Alternative sets of identity elements sometimes exist in isoacceptor tRNAs. The identity determinants for the aaRS in different organisms may be different as the primary structure of tRNAs varies *(Giege et al., 1998).*

tRNAs with multiple identities exist in nature. Multi-identical tRNAs can be genetically engineered. Change of tRNA identity can be accomplished by transplantation of identity determinants. Aminoacylation efficiency of the transplanted tRNA, however, is often not optimal, once again indicating that sequence context and/or architectural features play a role in identity determination *(for review Giege et al., 1998).*

3.3. Role of tRNA modifications in aminoacylation

Modified nucleosides are usually not identity elements of tRNAs *(for review McClain, 1995; Giege et al., 1998).* This allows the use of *in vitro* transcribed and hence unmodified tRNA transcripts in aminoacylation reactions. Comparison of the kinetics of the aminoacylation reactions with cognate and non-cognate aaRS revealed that of the 14 different unmodified tRNAs, all except 3 *E. coli* tRNAs ($tRNA_1^{Ile}$, $tRNA^{Glu}$, $tRNA^{Lys}$) accept the cognate amino acid *(for review Björk, 1995).* However, in all cases but one (*E. coli* $tRNA^{Asp}$) the cognate interactions with the unmodified species have kinetic characteristics that are different from those of the fully modified species *(for review Björk, 1995).* This finding suggests that the modified nucleosides affect either directly or indirectly, the recognition of the tRNAs by cognate aaRSs.

Some aaRSs need specific interactions with modified nucleosides in the anticodon loop to recognize their cognate tRNAs *(for reviews Björk, 1995; and Ibba & Söll, 2000).* GluRS

forms a hydrogen bond with mnm^5s^2U34 in $tRNA_2^{Glu}$ *(Willick & Kay, 1976)*. A direct interaction with the mnm5 groups of mnm^5s^2U34 is also consistent with the x-ray analysis of the GlnRS-$tRNA^{Gln}_{CUG}$ complex *(Rould et al., 1989)*. Thus probably the mnm^5 group is a positive determinant for GluRS, GlnRS and also LysRS from *E. coli (for review Björk, 1995)*.

Even though the chemical structures of G34 (in $tRNA_1^{Ile}$) and k^2C34 (in $tRNA_2^{Ile}$) are quite different, *E. coli* IleRS recognizes them both. When k^2C34 in the anticodon of $tRNA_2^{Ile}$ is replaced with C34, the anticodon of $tRNA^{Met}$ (CAU) emerges. Such mutant $tRNA_2^{Ile}$ is efficiently misacylated with methionine as C34 (and also modification ac^4C34) is a positive identity determinant for the MetRS *(Stern & Schulman, 1977; Muramatsu et al., 1988)*. Thus, the modification of C34 to k^2C34 acts as a positive identity determinant for IleRS and an anti-determinant for MetRS *(for reviews Björk, 1995; and Yokoyama & Nishimura, 1995)*.

ψ34 and I34 are the identity elements of yeast $tRNA^{Ile}$ minor and major respectively. Also manQ34 in mammalian $tRNA^{Asp}$ and Q34 in *E. coli* $tRNA^{Tyr}$ are important for efficient aminoacylation *(for review Björk, 1995)*. Presence or absence of some modifications like I34 and perhaps mnm^5U34 in $tRNA_1^{Arg}$; ac^4C34 in $tRNA^{Met}$; and, Q34 in mammalian $tRNA^{His}$ do not affect the kinetic parameters of aminoacylation reaction, even though the wobble base may be a part of the major identity element for cognate aaRS *(for review Björk, 1995)*.

Native yeast $tRNA^{Tyr}$ has pseudouridine as the middle nucleoside of the anticodon. Specific hydrogen bonds may form between TyrRS and ψ35 of $tRNA^{Tyr}$ *(Bare & Uhlenbeck, 1986)*. Thus, ψ35 may be directly involved in the recognition process between the tRNA and TyrRS.

Modifications at position 37 may (t^6A37 in $tRNA_1^{Ile}$, m^1G37 in yeast $tRNA^{Asp}$) or may not (ms^2i^6A37 in *E. coli* $tRNA^{Phe}$, $tRNA^{Tyr}$, or $tRNA^{Sec}$; and yW37 in yeast $tRNA^{Phe}$, t^6A37 in $tRNA_3^{Thr}$) be involved as determinants or anti-determinants in the recognition process *(for review Björk, 1995)*. Hypermodified W37 is not an identity determinant for yeast $tRNA^{Phe}$, but without this modification the catalytic site of PheRS is not activated *(for review Giege et al., 1998)*.

The same modified nucleosides are often involved in codon recognition *(for reviews Björk, 1995; and Yokoyama & Nishimura, 1995)*. This means that the aminoacylation occurs only for the correctly modified tRNA species, which can take part in codon recognition. Such a link between the codon recognition and the tRNA identity is important for the correct translation.

Clearly, modified nucleosides in the anticodon loop may be important identity determinants for some aaRS. The lack of influence of modified nucleosides at other positions (such as 54 and 55) is reasonable, since these positions are not directly involved in tRNA

recognition by aaRS *(for review Björk, 1995)*. However, when some or all of the modified nucleosides are absent, conformation of tRNA may change thereby influencing the kinetic parameters of the aminoacylation.

Yeast PheRS aminoacylates *E. coli* $tRNA^{Phe}$ less efficiently than yeast $tRNA^{Phe}$ *(Roe et al., 1973)*. The *E. coli* $tRNA^{Phe}$ lacks modification m^2G10. When this modification was introduced to the *E. coli* $tRNA^{Phe}$, it became a much better substrate for the yeast PheRS (V_{max} increased 10-fold and was similar to V_{max} of yeast $tRNA^{Phe}$, K_M decreased slightly). At the same time, the efficiency of aminoacylation by *E. coli* PheRS was decreased. The same modification in different tRNAs can therefore either enhance or retard aminoacylation *(Roe et al., 1973)*. An introduction of a methyl group at position 10 of *E. coli* $tRNA^{Phe}$ changes its aminoacylation kinetics. However, position 10 is not one of the five important positions for recognition by yeast PheRS *(Sampson et al., 1989)*. The presence of modified nucleosides affect the structure of tRNA and the altered aminoacylation kinetics might merely reflect that fact.

Completely unmodified $tRNA^{Phe}$ is specifically aminoacylated by yeast PheRS and has a K_M only 4-fold higher than that of the native yeast $tRNA^{Phe}$. The V_{max} is dependent of Mg^{2+} concentration, whereas the K_M is not. Using high accuracy conditions (low Mg^{2+} and 1 mM spermidine), the $tRNA^{Phe}$ *in vitro* transcript shows a considerable decrease in the relative V_{max} of aminoacylation reaction, and consequently a decreased specificity constant (k_{cat}/K_M), the K_M is not altered, as compared to the native tRNA *(Sampson & Uhlenbeck, 1988)*. Under these conditions the $tRNA^{Phe}$ does not adopt native conformation, as shown by nuclear magnetic resonance and by analyses of chemical cleavage *(Chow et al., 1992; Hall et al., 1989)*. In aminoacylation of the unmodified tRNA the optimal Mg^{2+} concentration is much higher than for the native tRNA, in case of $tRNA^{Phe}$ 15 mM and 8 mM respectively *(Serebrov et al., 1998)*.

Unmodified yeast $tRNA^{Asp}$ has an altered conformation, interactions are disrupted between the D and TΨC loop *(Perret et al., 1990, a)*. Such unmodified tRNA is mischarged by ArgRS with considerable efficiency *(Perret et al., 1990, b)*. The major effect is in the rate of mischarging (k_{cat}), whereas the K_M for unmodified $tRNA^{Asp}$ in the cognate interaction increases only twofold. In the case of yeast $tRNA^{Asp}$ the modified nucleosides act as anti-determinants for non-cognate aaRSs *(Perret et al., 1990, b)*. However, the unmodified *E. coli* tRNAs specific for methionine or valine and the unmodified yeast $tRNA^{Phe}$ are not *mischarged (Schulman & Pelka, 1988; Sampson & Uhlenbeck, 1988)*.

In conclusion, modified nucleosides do not act as identity determinants or anti-determinants in most tRNAs. However, the absence of modified nucleosides influences kinetic parameters of the aminoacylation reaction, suggesting that the conformational changes caused by

the presence of modified nucleosides are indirectly involved in tRNA recognition. On the other hand, it is also possible that the presence of tRNA modification enzymes themselves rather than the modifications they synthesize is required for efficient aminoacylation of tRNAs.

EXPERIMENTAL WORK

1. Specific aims

tRNA modification enzymes TrmA and TruB are two potential "tRNA chaperones". Both of these proteins are involved in processing of vast majority of tRNAs in *Escherichia coli* and for both a second function in tRNA maturation distinct from tRNA modification has been proposed, but not yet identified.

The purpose of current investigations was to shed light on the role TrmA and TruB proteins play in tRNA maturation.

First, various tRNAs were *in vitro* transcribed and purified. The effect of purified TrmA and TruB proteins on the formation of correct tertiary structure of tRNA was tested by its ability to be charged by aminoacyl-tRNA synthetases.

Characterization of the mechanism by which TrmA promotes aminoacylation of *in vitro* transcribed tRNA by alanyl-tRNA synthetase was the central aim of this study. For that purpose kinetic parameters of tRNA aminoacylation by AlaRS in the presence and absence of TrmA protein were determined. Ternary complex formation between tRNA, TrmA and aaRS was tested by native gel mobility shift assay and by sedimentation in glycerol gradient.

Finally, effect of AlaRS on tRNA modification by TrmA was studied.

2. Materials and methods

Restriction endonucleases, T4 DNA ligase and RNase inhibitor (RNasine) were purchased from MBI Fermentas. Pwo polymerase was from Roche Applied Science, P1 nuclease from Boehringer and Mannheim Biochemicals, bacterial alkaline phosphatase (BAP) and pyrophosphatase (PPase) from Sigma. DNase was purchased from Amresco.

T7 RNA polymerase was purified in our laboratory by Kai Virumäe (as described in *Davanloo et al., 1984*) from *E. coli* protein purification strain BL21 (DE-3)/pAR1219 kindly provided by W. Studier (Brookhaven National Laboratory, Upton, USA).

Taq DNA polymerase was a kind gift from J. Sedman (Institute of Molecular and Cell Biology, University of Tartu, Estonia).

Radioactively labeled amino acids: L-[U-^{14}C]Ala (162 mCi/mmol), L-[U-^{14}C]Val (256 mCi/mmol), L-[2,3-^{3}H]Ala (48 Ci/mmol), L-[U-^{14}C]Phe (460 mCi/mmol), L-[3-^{3}H]Ser (26 Ci/mmol) and L-[3,4(n)-^{3}H]Val (37 Ci/mmol); [α-^{32}P]-UTP (3000 Ci/mmol) and S-adenosyl-L-[methyl-^{14}C] methionine (55.3 mCi/mmol) were purchased from Amersham Biosciences.

Total native tRNA ($tRNA_{tot}$) extracted from *E. coli* MRE600 strain was from Roche Molecular Biochemicals.

2.1. tRNA plasmid constructs

Plasmid constructs pCVX and pCAX containing *E. coli* $tRNA^{Val}$ and $tRNA^{Ala}$ encoding genes respectively, were a kind gift from A. Wolfson (Department of Chemistry and Biochemistry, University of Colorado at Boulder, USA). tRNA genes were modified so that *in vitro* transcribed tRNAs contain GAC anticodon and G3:U70 base pair in the acceptor stem. Plasmid construct containing *E. coli* wild type $tRNA^{Phe}_{AAG}$ encoding gene was a kind gift from O. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado at Boulder, USA). All tRNA genes were cloned into plasmid pUC19 under control of late promoter of T7 RNA polymerase.

E. coli wild type $tRNA^{Ser}_{UCG}$ encoding plasmid was constructed in our laboratory by Kai Virumäe and Pille-Riin Tamjärv. *serV* gene amplified from *E. coli* MG1655 strain was cloned into plasmid pUC18 between BamHI and HindIII restriction sites under control of late promoter of T7 RNA polymerase.

Plasmids were linearized by MvaI restrictase yielding *in vitro* transcribed tRNAs with correct 3' CCA end. Linearized DNA was extracted by phenol/chlorophorm, precipitated with ethanol, and dissolved in water.

2.2. tRNA transcription and purification

Reaction was performed in 200 µl transcription buffer (40 mM Tris/HCl, pH 8.0, 50 mM NH_4Cl, 20 mM $MgCl_2$, 2 mM spermidine and 1 mM DTE) containing 20 µg of linearized DNA, 5 mM of each NTP and 80 U RNasine. Transcription was initiated with the addition of 200 U of T7 RNA polymerase and performed at 37°C for 4 hours. For synthesis of radioactively labeled tRNA, 5 µCi [α-^{32}P]-UTP was added.

For extraction of *in vitro* transcribed tRNA, sodium acetate (pH 5.5) with final concentration (f.c.) 0.3 M was added and phenol/chlorophorm processing was performed. tRNA was precipitated with ethanol and dissolved in water. tRNA samples were heated for 2 minutes at 96°C in equal volume of formamide loading dye.

In vitro transcribed tRNA was purified by polyacrylamide gel electrophoresis (PAGE). After 30 minutes of pre-electrophoresis heated samples were loaded on 12% polyacrylamide gel containing 8 M urea, and electrophoresis was carried out for 3 hours (~200V).

tRNA transcripts were visualized by illuminating the gel in ultra violet light (256 nm) on Silufol plate. tRNA containing band was excised from the gel, sliced into small pieces and eluted in buffer (0.5 M NH_4OAc, pH 6, and 10 mM EDTA) overnight at 4°C on end-over-end mixer. Small particles of gel were removed by centrifugation. tRNA was ethanol precipitated and dissolved in water.

For renaturation, *in vitro* transcribed tRNA was incubated in water at 65°C for 3 minutes. $MgCl_2$ (f.c. 15 mM) was added, solution was slowly cooled to room temperature and set on ice.

Concentration of tRNA was determined spectrophotometrically. 1 µg of tRNA was taken equal to 40 pmol. tRNA solutions were stored at -20°C.

2.3. Aminoacyl-tRNA synthetases

Expression construct pET24A-VTA contains ValRS encoding gene from *Thermus aquaticus* fused with His_6 sequence, and kanamycin resistance gene. The plasmid was constructed in our laboratory and described in *Virumäe et al., 2002.*

Expression constructs AlaRS-pQE70 and pET21b-ecAlaRS-H6 were kindly provided by P. Schimmel (Scripps Institute, La Jolla, California, USA). AlaRS-pQE70 contains first 461 N-terminal codons of *E. coli* AlaRS (truncated AlaRS) encoding gene fused with His_6 sequence, and an ampicillin resistance gene. pET21b-ecAlaRS-H6 *(described in Beebe et al., 2003)* contains *E. coli* full-length AlaRS encoding gene fused with His_6 sequence, and an ampicillin resistance gene.

Expression constructs PheRS-pQE32 and PheRS-pQE60 were constructed in our laboratory by Tamara Aid. *pheS* and *pheT* genes of *E. coli* PheRS were cloned into plasmid pQE32 between XmaI and HindIII restriction sites, or into plasmid pQE60 between NcoI and HindIII restriction sites. PheRS-pQE32 and PheRS-pQE60 contain His_6 sequence in N terminus of S chain and C terminus of T chain of PheRS, respectively, and ampicillin resistance gene.

Expression construct pET19b-ecSerRS-H6 was a kind gift from T. Tenson (Institute of Technology, University of Tartu, Estonia) and contains *E. coli* SerRS encoding gene fused with His_6 sequence, and an ampicillin resistance gene.

pET24A-VTA, pET21b-ecAlaRS-H6 and pET19b-ecSerRS-H6 plasmids were transformed into *E. coli* protein expression strains BL21 (DE-3) (Stratagene). PheRS-pQE32, PheRS-pQE60 and AlaRS-pQE70 plasmids were transformed into *E. coli* protein expression strains M15 (pREP) (QIAgen). ValRS, PheRS, SerRS and N-terminal fragment of AlaRS were purified in our laboratory by Kai Virumäe; full-length AlaRS was purified by the author, using the standard protocol of protein purification under native conditions (QIA Expressionist).

2.4. TrmA, ΔTrmA, and TruB

Cloning procedures, plasmid preparations and bacterial transformations were performed according to standard methods (*Sambrook & Russell, 2001*). Expression constructs for C-terminally His_6-tagged TrmA and TruB proteins were made using vector pQE60 (QIAgen). *trmA* and *truB* genes were amplified by PCR from genomic DNA of *E. coli* MG1655 strain using Taq DNA polymerase and oligonucleotide primers flanked by Esp31 and BglII sites:

5’ GC**CGTCTC**CAATGACCCCCGAACACCTTC 3` (pQE60/*trmA* 5’ Esp31),

3’ CG**AGATC**TCTTCGCGGTCAGTAATACGC 5` (pQE60/*trmA* 3’ BglII),

5’ GC**CGTCTC**CAATGAGTCGTCCTCGTCGTC 3` (pQE60/*truB* 5’ Esp31), and

3’ CG**AGATCT**CGCCGGGTATTCAACCACC 5` (pQE60/*truB* 3’ BglII).

Mutant *trmA (ΔtrmA)* gene was produced by site-directed mutagenesis with two subsequent PCR reactions. Two point mutations were introduced into *trmA* during amplification using oligonucleotides:

5’ CATAACGTTTCCGGGTTA**GC**GGAGATGTACAAAATAC 3’

5’ GTATTTTGTACATCTCC**GC**TAACCCGGAAACGTTATG 3’

All oligonucleotides were purchased from DNA Technology A/S (Denmark).

PCR products were separated on 1% agarose gel in 1xTBE (Tris base, boric acid, EDTA) buffer, excised and purified from the gel by UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Inc.). Purified fragments were digested with Esp31/BglII and cloned into

BglII/NcoI treated expression vector pQE60 using T4 DNA ligase. pQE60 expression vector contains His_6 sequence and ampicillin resistance gene.

pQE60/*trmA*, pQE60/*ΔtrmA*, and pQE60/*truB* expression constructs isolated from XL1-Blue (Stratagene) transformants were confirmed by DNA sequencing (Uppsala Genome Center Sequencing Service, Sweden) using oligonucleotides Type III/IV and Reverse (QIAgen). The sequence obtained from *ΔtrmA* gene deviated from the *trmA* gene in that it contained the two desired point mutations T970G and G971C (catalytic cysteine-324 of TrmA is replaced by alanine in ΔTrmA).

Plasmid constructs pQE60/*trmA*, pQE60/*ΔtrmA* and pQE60/*truB* were transformed into *E. coli* M15 (pREP) protein expression strain (QIAgen). TrmA, ΔTrmA and TruB proteins were purified according to the standard protocol of protein purification by Ni-NTA (nickel-nitrilotriacetic acid) resin under native conditions (QIA Expressionist) using buffers (lysis buffer: 50 mM NaH_2PO_4, 1 M NaCl, 10% glycerol, 10 mM imidazole, pH 7; wash buffer: 50 mM NaH_2PO_4, 1 M NaCl, 20 mM imidazole, 10% glycerol, 0.5% Triton X-100, pH 7,0; and elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 6,0) modified by J. Parik (Institute of Molecular and Cell Biology, University of Tartu, Estonia).

Proteins were dialyzed for 8 hours against buffer: 20 mM Tris/HCl (pH 8.0), 100 mM NH_4Cl, 10% glycerol, 6 mM β-mercaptoethanol; and stored at -70°C in the same buffer containing 50% glycerol. TrmA(TEN) refers to TrmA protein that was dialyzed for 72 hours against buffer TEN (10 mM Tris/HCl, pH 7.6, 50 mM NH_4Cl, 6 mM β-mercaptoethanol and 1 mM EDTA) and stored in the same buffer containing 50% glycerol. Protein concentration was determined by Bradford method.

2.5. tRNA modification analysis

300 pmol of *in vitro* transcribed $tRNA^{Ala}$ was incubated with 70 µg of TrmA, ΔTrmA, or TruB enzymes in 50 µl modification buffer (50 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$ and 1 mM DTE) at 37°C for 3 hours. tRNA incubation with TrmA and ΔTrmA was performed with or without 50 µM S-adenosyl-L-methionine (SAM). After incubation, volume was enlarged with water 4 times and SDS (f.c. 1%) was added.

For purification of tRNA, sodium acetate (pH 5.5, f.c. 0.3 M) was added and phenol/chlorophorm extraction was performed. tRNA was ethanol precipitated and dissolved in water.

For enzymatic hydrolysis, 50 µl of tRNA solution was heated at 100°C for 2 minutes and rapidly cooled on ice water. $ZnSO_4$ (f.c. 1 mM) and 1 U of P1 nuclease were added and tRNA

was incubated at 37°C for 16 hours. Thereafter, Tris/HCl (pH 8.3, f.c. 0.1 M) and 20 mU of bacterial type III alkaline phosphatase (BAP) were added, and incubated at 37°C for additional 2 hours. Samples were clarified by centrifugation and filtered using Millex-LG filters (0.2 μm).

Ribonucleoside composition of tRNA hydrolysate was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) using Agilent 1100 series chromatographic system and 250 mm x 4.6 mm C_{18} nucleoside analysis column Supelcosil LC-18-S (Supelco).

Column was equilibrated with buffer A (2.5% methanol, 0.01 M $NH_4H_2PO_4$, pH 5.3) for 20 minutes. Sample was applied and the column was eluted with linear gradient of methanol and pH resulting from mixing buffer A and buffer B (20% methanol, 0.01 M $NH_4H_2PO_4$, pH 5.1) as described in Table 2 (modified from *Gehrke & Kuo, 1989*).

TABLE 2. HPLC eluation conditions:

Step	duration in minutes	Buffer composition %	
		Buffer A	Buffer B
1	12	100	0
2	8	90	10
3	5	75	25
4	7	40	60
5	4	38	62
6	9	0	100

Buffer B was changed to buffer A over time period of one minute and prior loading the next sample, column was equilibrated again with buffer A for 20 minutes. Flow rate was 1 ml per minute and the column was thermostatted to 26°C.

Column flow-through UV absorbance was analyzed continuously at 254 nm. Ribonucleoside composition of tRNA was determined and quantified. Retention times of standard and modified nucleosides important for current work are as follows: Ψ 4.6 min.; C 5.9 min.; U 8.4 min.; m^5U54 19.8 min.; G 20.0 min.; and A 33.5 min. *(Gehrke & Kuo, 1989).* Ribonucleoside composition of *in vitro* transcribed $tRNA^{Ala}$ analyzed is: 25 G (32.9%), 23 C (30.3%), 14U (18.4%), and 14 A (18.4%); as derived from the sequence of corresponding gene.

For radioactive methylation, 40 pmol of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Phe}$ were incubated with 0.03 μg of TrmA(TEN) and 50 μM S-adenosyl-L-[methyl-^{14}C] methionine (SAM) in 15 μl modification buffer at 37°C for 3 hours. Reactions were carried out also in the presence of $6.7x10^{-5}$-$6.7x10^{-2}$ μg/μl of PheRS or full-length AlaRS.

Reactions were stopped by adding ~1.5 ml of cold 5% trichloroacetic acid (TCA) and incubated on ice for 30 minutes. tRNA was collected on GF/A class fiber filters (Whatman). Filters were washed with ~10 ml 5% TCA and ~1 ml 70% ethanol. 5 ml of scintillator Optiphase "Hi Safe" 3 (Perkin Elmer Life Sciences) was added to dried filters. Radioactivity was determined by liquid scintillator counter Winspectral (Wallac).

2.6. tRNA aminoacylation assays

Aminoacylation reactions were performed in 15 µl aminoacylation buffer (same as modification buffer) containing 20 pmol of *in vitro* transcribed tRNA ($tRNA^{Ala}$, $tRNA^{Val}$, $tRNA^{Ser}$ or $tRNA^{Phe}$), or 400 pmol of native tRNA ($tRNA_{tot}$), radioactively labeled amino acid (20.5 µM L-[U-^{14}C]Ala, 13 µM L-[U-^{14}C]Val, 7 µM L-[U-^{14}C]Phe, 10 µM L-[3,4(n)-^{3}H]Val, 5 µM L-[3-^{3}H]Ser, or 3.3 µM L-[2,3-^{3}H]Ala), 5 mM ATP and 1.5-8.5 µg of aaRS (truncated AlaRS, ValRS, PheRS, or SerRS). When AlaRS was used 50 U of PPase and 20 U of RNasine was added. Reactions were carried out at 37°C for 30 minutes, if not otherwise indicated.

In experiments where tRNA was first incubated with TrmA, ΔTrmA, or TruB, a pre-incubation mixture was prepared containing 20 pmol of *in vitro* transcribed tRNA ($tRNA^{Ala}$, $tRNA^{Val}$, $tRNA^{Ser}$ or $tRNA^{Phe}$) or 400 pmol of native tRNA ($tRNA_{tot}$) and 0.07-1.35 µg/µl of modification enzymes TrmA, ΔTrmA, or TruB in 5 µl aminoacylation buffer. Pre-incubation was carried out at 37°C for 10-30 minutes and aminoacylation was performed as previously described. AaRS concentrations (0.06 µg/µl of truncated AlaRS, 0.01 µg/µl of ValRS, 0.03 µg/µl of PheRS, 0.07 µg/µl of SerRS and 3.3×10^{-4} µg/µl of full-length AlaRS,) and aminoacylation time (30-40 seconds) were suboptimal (based on previous experiments).

For determination of the kinetic parameters of aminoacylation reaction, *in vitro* transcribed $tRNA^{Ala}$ or $tRNA^{Phe}$ (at concentrations 0.016/0.032/0.08/0.16/0.32 µg/µl) were pre-incubated in the presence (0.01/0.02/0.05/0.1/0.2 µg/µl, respectively) or absence of TrmA protein in 10 µl aminoacylation buffer at 37°C for 10 minutes. Initial velocities for aminoacylation were obtained with full-length AlaRS or PheRS at final concentrations 0.006 µg/µl and 0.008 µg/µl, respectively. Aminoacylation of tRNA was performed as previously described in 25 µl aminoacylation buffer at 25°C for 10/20/30 seconds.

Reactions were stopped by adding ~1.5 ml of cold 5% trichloroacetic acid (TCA) and incubated on ice for 30 minutes. tRNA was collected on GF/A class fiber filters (Whatman). Filters were washed with ~10 ml 5% TCA and ~1 ml 70% ethanol. 5 ml of scintillator Optiphase "Hi Safe" 3 (Perkin Elmer Life Sciences) was added to dried filters. Radioactivity was determined by liquid scintillator counter Winspectral (Wallac).

Rates of aminoacylation, which are proportional to k_{cat}/K_M under the conditions used, were determined from the slope of a plot of pmol aminoacylated tRNA versus time. Kinetic parameters were determined by direct fitting of data points to the Michaelis-Menten equation.

2.7. Native gel electrophoresis analysis

20 pmol of *in vitro* transcribed and uniformly [^{32}P]-labeled tRNA^{Ala} was incubated with 2/4/8/12 μg of TrmA or TruB (or with TrmA, TruB, ValRS, and truncated AlaRS enzymes 4 μg each in various combinations) in 5 μl buffer (50 mM Tris/HCl, pH 8.0, 20 mM NH_4Cl, 10 mM $MgCl_2$ and 1 mM DTE) at 37°C for 20 minutes. Glycerol (f.c. 20%) and Triton X-100 (f.c. 0.5%) were added to the samples prior loading on a 12% or 6% native polyacrylamide gel (29:1 acrylamide:bisacrrylamide).

Gels were run at 4°C at ~100V (10-12mA) for 24 hours in native running buffer: 90 mM Tris-borate (pH 8.0), 10 mM $MgCl_2$ and 50 mM KCl. Buffer was re-circulated at rate >1 liter per hour. Gels were soaked in 5% acetic acid, 10% glycerol solution for 20 minutes, placed on Whatman 3MM paper, vacuum dried for 30 minutes at 70 °C and exposed to X-ray film (Fuji film).

tRNA* refers to tRNA that was prior to gel mobility shift analysis incubated with TrmA enzyme. In preparative TrmA treatment of tRNA, 800 pmol of *in vitro* transcribed and uniformly [^{32}P]-labeled tRNA^{Ala} was incubated with 60 μg of TrmA in 200 μl modification buffer at 37°C for 20 minutes. NaCl (f.c. 0.1 M) and SDS (f.c. 0.5%) were added, tRNA was extracted by phenol/chlorophorm, ethanol precipitated and dissolved in water. For renaturation, tRNA was incubated at 65°C for 3 minutes in the presence or absence of 20 mM $MgCl_2$, the solution was slowly cooled to room temperature and set on ice. Denaturated tRNA was incubated at 65°C for 3 minutes and immediately set on ice.

2.8. Glycerol gradient sedimentation analysis

30 μg of TrmA was incubated with 90 μg of PheRS or full-length AlaRS either in the presence or absence of tRNA (800 pmol *in vitro* transcribed tRNA^{Phe} or tRNA^{Ala} respectively) in 100 μl modification buffer at 37°C for 10 minutes. Samples were centrifuged at 13 000 rpm for 1 minute to discard the protein fallout.

Supernatant was loaded on a 5 ml 10–30% glycerol gradient in modification buffer and centrifuged in Sorwall SW55 rotor at 40 000 rpm for 12 h at 4°C. 11 fractions of 19 drops each (~460 μl) were collected from the bottom of the tube.

TCA (f.c. 10%) and 2 µg of $tRNA_{tot}$ (precipitation carrier) were added to the collected fractions and kept at 4°C for 2 hours. Samples were centrifuged at 13 000 rpm for 15 minutes at 4°C and the supernatant was carefully discarded. 1 ml of 80% acetone was added and centrifuged at 13 000 rpm for additional 10 minutes. Pellets (including the protein fallout from the incubation reaction) were dissolved in 20 µl SDS loading dye and analyzed by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue.

3. Results and discussion

The fidelity of protein synthesis depends on the specific tRNA aminoacylation by aminoacyl-tRNA synthetases. AaRS require identity elements (certain nucleosides at specific positions in tRNA) in order to recognize cognate and reject non-cognate tRNAs. The tertiary structure of tRNA is a critical requirement for efficient aminoacylation (see Chapters 3.2.). Aminoacylation reaction can therefore be used to estimate the correct structure (functionality) of tRNA.

In vitro transcription of a tRNA genes controlled by a phage T7 RNA polymerase specific promoter allows the synthesis of unmodified tRNAs, which are suitable for various physical and biochemical studies, including tRNA aminoacylation, since modified nucleosides are not required by aaRS used in this study (see Chapter 3.3.).

3.1. Aminoacylation of in vitro transcribed and native tRNA

For following experiments several tRNAs were *in vitro* transcribed and purified. To estimate the functionality, *in vitro* transcribed tRNAs were subjected to aminoacylation:

In vitro transcribed tRNA (*E. coli* $tRNA^{Ala}$, $tRNA^{Val}$, $tRNA^{Phe}$, or $tRNA^{Ser}$) was aminoacylated by purified aaRS (truncated AlaRS, ValRS, PheRS, or SerRS, respectively). Mutant $tRNA^{Ala}$ and $tRNA^{Val}$ contained identity determinants for both AlaRS and ValRS (G3:U70 bas pair in acceptor stem and GAC anticodon) and were aminoacylated accordingly. For comparison, *E. coli* native fully modified tRNA ($tRNA_{tot}$) was aminoacylated. Aminoacylation efficiencies of *in vitro* transcribed and native tRNA are presented in Table 3.

The efficiency of aminoacylation of *in vitro* transcribed tRNA varies between 25-65% depending on the tRNA type and preparation (when transcription yielded a large amount of tRNA it was relatively less active). Raising the concentration of aaRS and the aminoacylation time did not improve the yield of aminoacylated *in vitro* transcribed tRNAs (data not shown). Native tRNAs were aminoacylated with the efficiency of 90-100% (see Table 3).

Hence, *in vitro* transcribed tRNAs were significantly less efficiently aminoacylated by purified aminoacyl-tRNA synthetases than fully modified native tRNAs. This is in good agreement with previous aminoacylation experiments performed with *in vitro* transcribed tRNA *(for review, Björk, 1995).* Since the aminoacyl-tRNA synthetases used in current study do not require modified nucleosides as tRNA identity elements, the decreased yield of aminoacylation may be do to the fact that tRNA molecules do not adopt their correct tertiary structure when

synthesized *in vitro*. Various stable but biologically inactive conformations of tRNA may arise. Furthermore, different tRNA molecules may interact with each other to form stable non-functional complexes (see Chapter 1.3.).

TABLE 3. Aminoacylation of *in vitro* transcribed and native tRNA

tRNA	Ala-tRNA %	Val-tRNA %	Phe-tRNA %	Ser-tRNA %
$tRNA_{tot}$	**97±3**	**94±2**	**90±2**	**89±4**
$tRNA^{Ala}$	**47±9**	**49±6**	**—**	**—**
renaturated $tRNA^{Ala}$	**46±11**	**33±7**	**—**	**—**
$tRNA^{Val}$	**54±4**	**57±7**	**—**	**—**
renaturated $tRNA^{Val}$	**45±9**	**61±4**	**—**	**—**
$tRNA^{Phe}$	**—**	**—**	**35±7**	**—**
renaturated $tRNA^{Phe}$	**—**	**—**	**40±2**	**—**
$tRNA^{Ser}$	**—**	**—**	**—**	**33±10**
renaturated $tRNA^{Ser}$	**—**	**—**	**—**	**35±12**

Mutant $tRNA^{Ala}$ and $tRNA^{Val}$ were aminoacylated by both ValRS and truncated AlaRS.
aa-tRNA % - percent of aminoacylated tRNA out of the tRNA (specific tRNA in case of $tRNA_{tot}$) taken into experiment.
$tRNA_{tot}$ contains 6% $tRNA^{Ala}$, 7.9% $tRNA^{Val}$, 1.6% $tRNA^{Phe}$, and 5.9% $tRNA^{Ser}$ *(Dong et al., 1996).*

Magnesium is an important factor for the formation of correct tertiary structure of tRNA in the cell. However, incubation of *in vitro* transcribed tRNAs at 65°C for 3 minutes followed by slow cooling in the presence 15 mM $MgCl_2$ (tRNA renaturation) did not improve the efficiency of aminoacylation in current experiments (see Table 3).

It has been suggested that some protein factors are needed to ensure the correct folding of tRNA molecules in the cell (see Chapter 1.3.). This notion was further supported by the fact that aminoacylation of some *in vitro* transcribed tRNAs was more efficient by S100 (soluble protein fraction of *E. coli*) enriched with aaRS than by purified aaRS (data not shown). Some proteins present in S100 are likely to assist the formation of tertiary structure of tRNA (chaperones) and thereby improve the rate of aminoacylation. Proteins Arc1p and Trbp111 that bind to tRNA have been shown to increase the global aminoacylation efficiency of tRNA by chaperone-like effects in yeast and thermophilic bacteria (see Chapter 1.3.).

3.2. Modification analysis of tRNA incubated with TrmA and TruB

Proteins involved in tRNA modification may also be important for the formation of the functional structure of tRNA. It has been proposed, that the modification procedure itself contributes to tRNA (re)-folding where the modification enzymes function as tRNA chaperones (see Chapters 1.3.).

tRNA modification enzymes TrmA and TruB are two potential tRNA chaperones. Both of these proteins are involved in processing of vast majority of tRNAs in *Escherichia coli* and for both a second function distinct from tRNA modification has been proposed, but not yet identified. In case of TrmA, the protein itself but not the m^5U54 modification synthesized by it has shown to be essential (see Chapter 2.2.).

For the following experiments *E. coli* TrmA and TruB proteins fused to His_6-tag were cloned and purified using Ni-NTA resin. Functional activity of the isolated TrmA and TruB enzymes was tested according to the ability to catalyze m^5U and pseudouridine formation, respectively.

In vitro transcribed *E. coli* $tRNA^{Ala}$ was pre-incubated with TrmA (in the presence or absence of S-adenosyl-L-methionine) or TruB enzyme. For comparison, tRNA was pre-incubated under the same conditions without either enzyme. tRNA was then enzymatically digested to nucleosides and analyzed by HPLC. Results are presented in Table 4 and Figure 6.

Table 4. Analysis of ribonucleoside composition of *in vitro* transcribed $tRNA^{Ala}$ incubated with TrmA or TruB proteins.

	$tRNA^{Ala}$ +TruB		$tRNA^{Ala}$ +TrmA +SAM		$tRNA^{Ala}$ +TrmA -SAM		$tRNA^{Ala}$	
Ribonucleoside	**Ret. time**	**Area**	**Ret. time**	**Area**	**Ret. time**	**Area**	**Ret. time**	**Area**
Ψ	**4,9**	**113,8**	**4,9**	**67,0**	**4,8**	**52,7**	**–**	**–**
C	**6,2**	**2141,0**	**6,2**	**1228,1**	**6,2**	**1272,5**	**6,2**	**1753,5**
U	**8,8**	**2363,1**	**8,8**	**1286,8**	**8,8**	**1316,8**	**8,8**	**2047,8**
m^5U	**–**	**–**	**19,6**	**57,9**	**19,7**	**60,6**	**–**	**–**
G	**21,0**	**5255,0**	**20,9**	**2972,7**	**21,0**	**3097,7**	**21,0**	**4343,1**
A	**34,8**	**3827,8**	**34,8**	**2184,1**	**34,8**	**2257,8**	**34,9**	**3144,6**

Determined ribonucleosides with respective retention times and chromatogram peak areas are given. Incubation with TrmA enzyme was performed in the presence or absence of cofactor SAM.

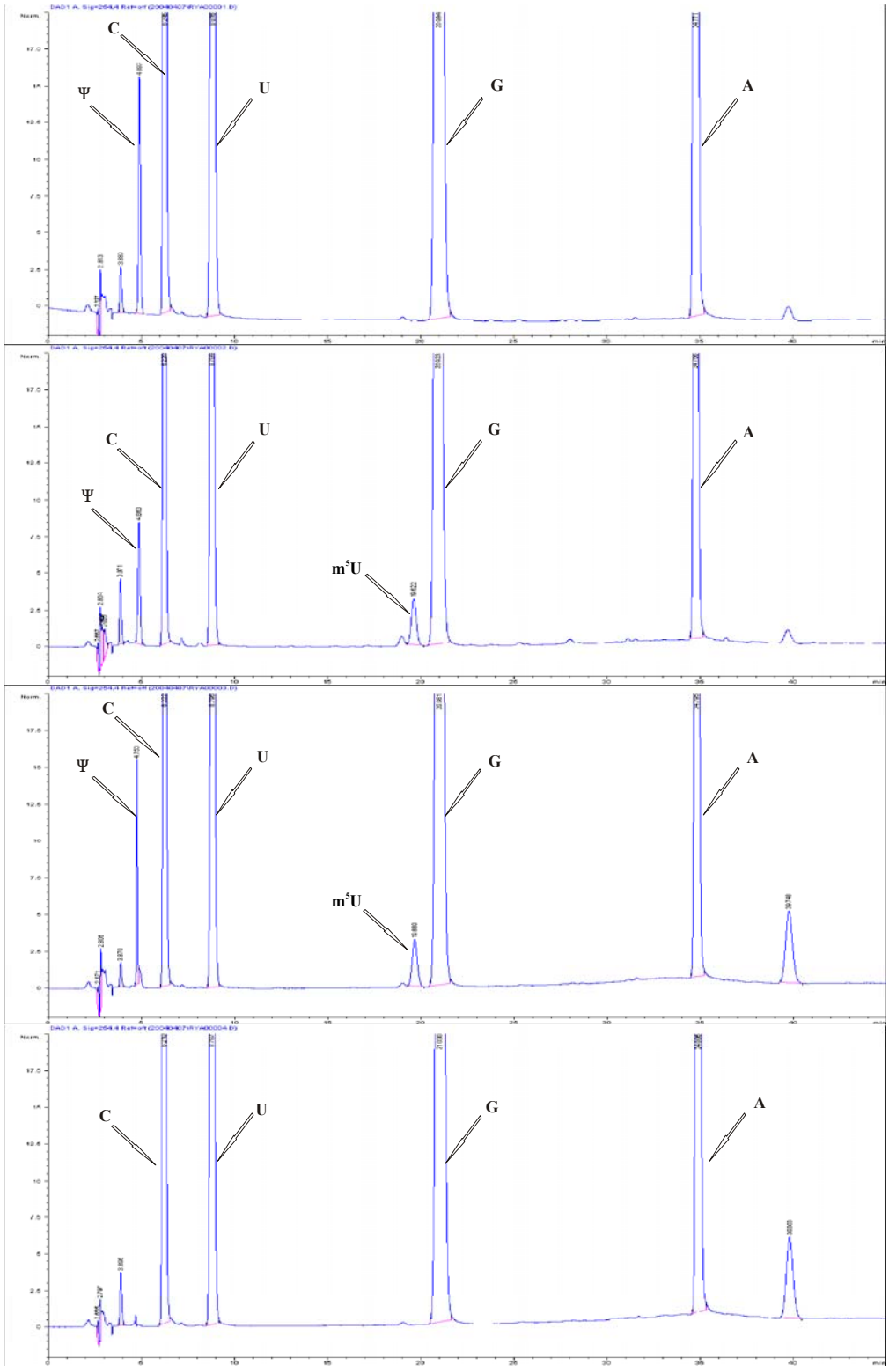

FIGURE 6. Analysis of ribonucleoside composition of tRNA pre-incubated with TrmA or TruB proteins. 300 pmol of *in vitro* transcribed *E. coli* $tRNA^{Ala}$ was pre-incubated with 75 µg of TrmA (in the presence or absence of 50 µM SAM) or 70 µg of TruB enzymes at 37°C for 3 hours. After incubation tRNA was deproteinized, digested to nucleosides by nuclease P1 and bacterial alkaline phosphatase, and analyzed by RP-HPLC (chromatograms at 254 nm are shown). Position of standard nucleosides, pseudouridine (Ψ) and m^5U are indicated.

Results of tRNA modification analysis show that the *in vitro* transcribed $tRNA^{Ala}$ contained the four standard ribonucleosides, as might be expected. tRNA pre-incubated with TruB enzyme contained in addition one modified nucleoside – pseudouridine, which was likely to be Ψ55, the product of TruB. tRNA pre-incubated with TrmA (both in the presence or absence of cofactor SAM), on the other hand, contained two modified nucleosides. One of the modified nucleosides was likely to be m^5U54 synthesized by TrmA and the other Ψ55, as the only pseudouridine present in $tRNA^{Ala}$ analyzed is located at the position 55 and synthesized by TruB.

Hence, purified TruB and TrmA proteins were functional with TruB synthesizing Ψ55 in approximately 65% and TrmA synthesizing m^5U54 in approximately 60% of tRNA molecules. The fact that TrmA synthesized m^5U even when cofactor SAM was not added to the reaction mixture, strongly suggest that TrmA protein co-purifies with endogenous SAM. Similar observations have been made for other methyltransferases as well *(Agris, 1996)*. Furthermore, formation of Ψ could be detected upon incubating tRNA with TrmA, showing that the purified protein fraction was not homogenous and very likely contained small amount of TruB. Co-purification of TruB with TrmA refers to the possibility that multi-enzyme complex containing several tRNA modification enzymes may facilitate tRNA processing *in vivo*.

3.3. Effect of TrmA, TruB, ΔTrmA on tRNA aminoacylation

To test TrmA and TruB as tRNA specific chaperones, the effect of these proteins on the functionality of tRNA transcripts was analyzed through the efficiency of aminoacylation:

In vitro transcribed tRNA (*E. coli* mutant $tRNA^{Ala}$, $tRNA^{Val}$) or native *E. coli* tRNA ($tRNA_{tot}$) were pre-incubated with TrmA or TruB enzymes. For comparison, tRNA was pre-incubated under the same conditions without either enzyme. Aminoacylation of tRNA was performed by truncated AlaRS or ValRS. AaRS concentrations and aminoacylation time were suboptimal (based on previous experiments, data not shown). Results are presented in Figure 7.

It was found that pre-incubation of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Val}$ with TruB enzyme inhibited aminoacylation by ValRS. Similar results were obtained using truncated AlaRS (see Figure 7, D and E). Likewise, pre-incubation of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Val}$ with TrmA enzyme inhibited aminoacylation by ValRS. In contrast, incubation with TrmA enzyme significantly increased the yield of aminoacylation by truncated AlaRS (see Figure 7, A and B). Similar results were obtained from experiments performed in the presence of 1mM, 10 mM or 40 mM $MgCl_2$ (data not shown). Aminoacylation of native tRNA was inhibited by both truncated AlaRS and ValRS (see Figure 7, C and F).

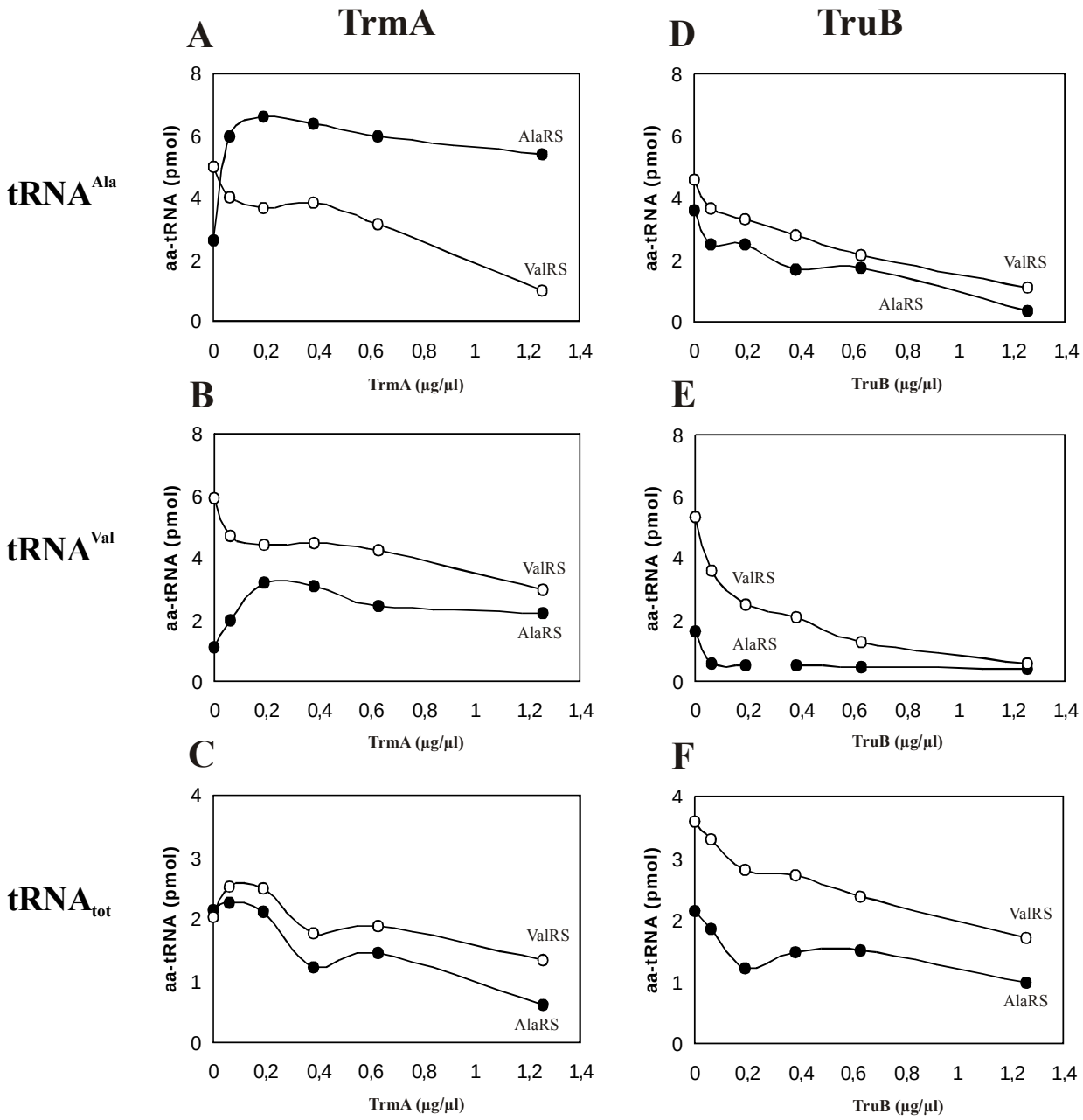

FIGURE 7. Aminoacylation of tRNAs pre-incubated with TrmA or TruB proteins. 20 pmol of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Val}$, or 400 pmol of native tRNA ($tRNA_{tot}$) were pre-incubated with 0.07-1.35 µg/µl of TrmA or TruB enzymes at 37°C for 30 minutes, and aminoacylated by 0.06 µg/µl of truncated AlaRS or 0.01 µg/µl of ValRS at 37°C for 30 seconds.

The inhibiting effect of pre-incubation with TruB protein on tRNA aminoacylation was not dependent on either tRNA type or aaRS class (ValRS and AlaRS are class I and class II aaRSs, respectively). TruB protein presumably competes with AlaRS and ValRS for binding to overlapping regions on tRNA. Though ValRS and AlaRS use different tRNA binding mechanisms, binding to tRNA acceptor stem is strictly required by all aaRS (see Chapter 3.2.).

When tRNA is bound to TruB, the C-terminal domain comes into close proximity of tRNA acceptor stem *(Ferre-D'Amare, 2003)* (see Figure 3 a) and may thereby hinder AlaRS and ValRS binding. The exact mechanism of TruB inhibition on tRNA aminoacylation remains unknown.

TrmA and ValRS enzymes as well may compete for overlapping binding sites on tRNA, since the C terminal coiled-coil domain of ValRS reaches the TΨC loop region of tRNA *(Nureki & Yokoyama, 2005)*, explaining the inhibiting effect of TrmA on the tRNA aminoacylation by ValRS.

AlaRS binds to the opposite face of tRNA as compared to ValRS (see Chapter 3.1.), it is possible that TrmA does not hinder AlaRS binding to tRNA and even facilitates it. The possibility of tRNA simultaneously binding to TrmA and AlaRS (formation of ternary complex with tRNA sandwiched between the two proteins) remains open. Though there is a design for tRNA acceptor stem recognition based on crystal structure of AlaRS, the three dimensional structure of TrmA is not determined yet. Alternatively, it is possible that TrmA protein could in fact act as a RNA chaperone and help *in vitro* transcribed tRNA to obtain its correct tertiary structure, whereby it becomes a better substrate for AlaRS. Yet another possibility is that the modification m^5U made by TrmA promotes the efficiency of aminoacylation by AlaRS indirectly, by stabilizing the tertiary structure of *in vitro* transcribed tRNA.

To find out if the effect of TrmA on aminoacylation of *in vitro* transcribed tRNA depends on the m^5U modification, mutant TrmA protein (catalytic cysteine at position 324 was replaced by alanine) was synthesized, purified and tested along with wild type TrmA. To analyze the effect of TrmA on aminoacylation of *in vitro* transcribed tRNA by other class II aaRSs, the following experiments were performed:

In vitro transcribed tRNA ($tRNA^{Ala}$, $tRNA^{Phe}$, $tRNA^{Ser}$ or $tRNA^{Val}$) was pre-incubated with TrmA or ΔTrmA enzymes. For comparison, tRNA was pre-incubated under the same conditions without either enzyme. Aminoacylation of tRNA was performed by truncated AlaRS (first 461 N-terminal codons of AlaRS), full-length AlaRS, PheRS, SerRS or ValRS. AaRS concentrations and aminoacylation time were suboptimal (based on previous experiments, data not shown). Results are presented in Figure 8.

It was found that pre-incubation with TrmA promoted aminoacylation of *in vitro* transcribed $tRNA^{Ala}$ by *E. coli* full-length AlaRS (up to three fold), as was the case with truncated AlaRS (see Figure 8 A and Figure 7 A). Pre-incubation with TrmA promoted aminoacylation of *in vitro* transcribed $tRNA^{Phe}$ by PheRS, but the effect was rather modest (see Figure 8, B). Low level of stimulation was observed by PheRS with His_6 sequences fused to

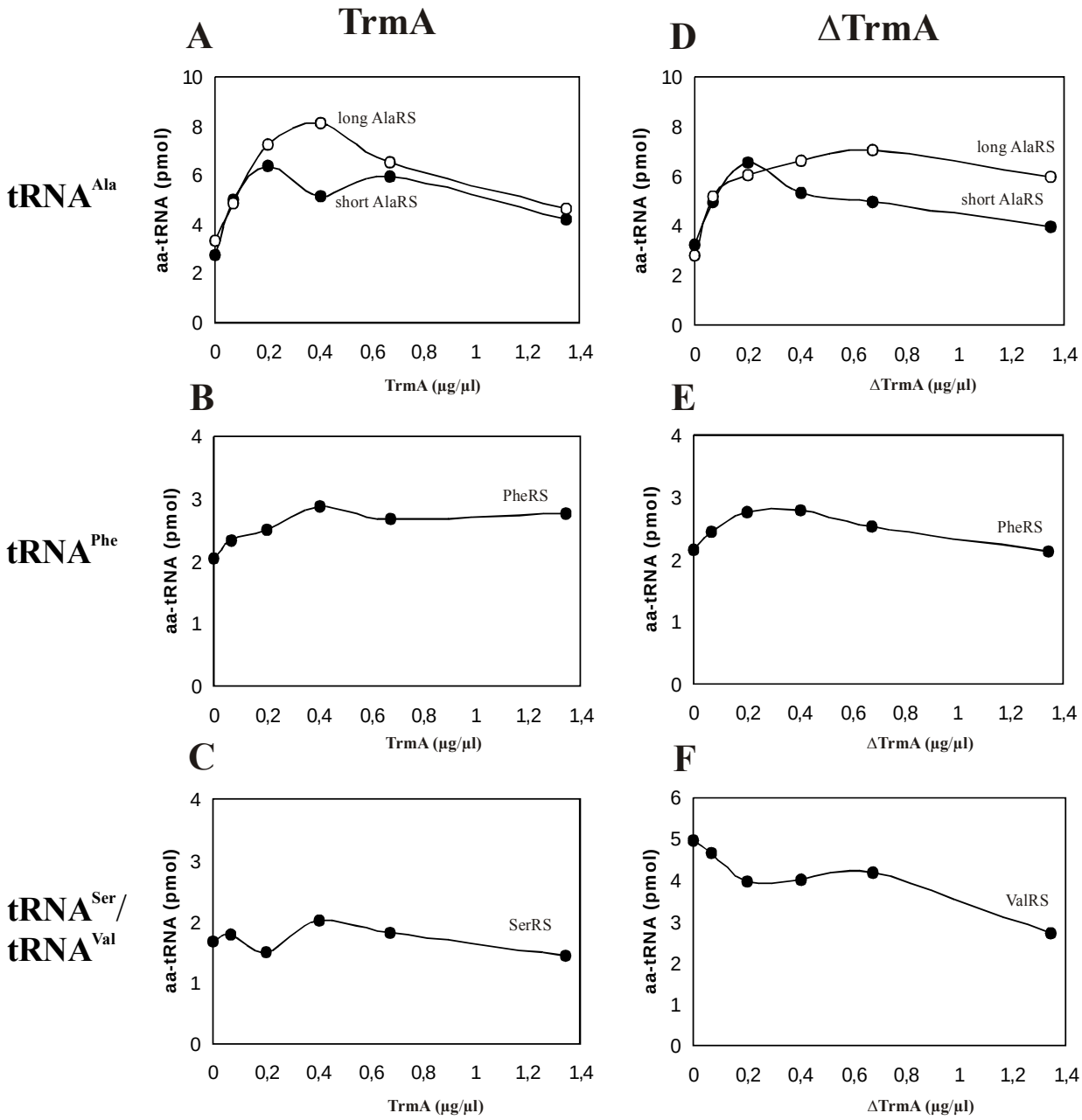

FIGURE 8. Aminoacylation of tRNAs pre-incubated with TrmA or ΔTrmA proteins. 20 pmol of *in vitro* transcribed $tRNA^{Ala}$, $tRNA^{Phe}$, $tRNA^{Ser}$, and $tRNA^{Val}$ were pre-incubated with 0.07-1.35 μg/μl of TrmA or ΔTrmA enzymes at 37°C for 30 minutes, and aminoacylated by 0.06 μg/μl of truncated AlaRS, $3.3x10^{-4}$ μg/μl of full-length AlaRS, 0.035 μg/μl of PheRS, 0.07 μg/μl of SerRS, or 0.01 μg/μl of ValRS at 37°C for 30-40 seconds.

either N terminus of S chain or C terminus of T chain (data not shown). Aminoacylation of *in vitro* transcribed $tRNA^{Ser}$ by SerRS was indifferent to tRNA pre-incubation with TrmA (see Figure 8, C).

The effect of mutant TrmA (ΔTrmA) on tRNA aminoacylation was similar to wild type TrmA, significantly promoting tRNA aminoacylation by truncated and full-length AlaRS, and

modestly by PheRS (see Figure 8, D and E). Similarly to wild type TrmA, the mutant TrmA inhibited aminoacylation of *in vitro* transcribed tRNA by ValRS (see Figure 8, F; and Figure 7, D and E). The lack of m^5U in tRNA incubated with ΔTrmA enzyme in the presence of cofactor SAM was confirmed by RP-HPLC (data not shown).

The fact that TrmA promoted aminoacylation of *in vitro* transcribed tRNA by full-length AlaRS shows that this phenomenon was not limited to truncated AlaRS, which was about 200-fold less active in tRNA alanylation. AlaRS, PheRS, and SerRS are all class II aaRS, but the effect of TrmA on aminoacylation of *in vitro* transcribed tRNAs by these enzymes differs. This indicates that the nature of TrmA effect on aminoacylation depends on the specific aaRS and is not tributed to class II aaRSs in general. It is known that AlaRS binds only to the acceptor arm of tRNA *(Swairjo et al., 2004)*, whereas the N-terminal coiled-coil domain of the PheRS α-subunit *(for review Safro et al., 2005)* and the helical arm of SerRS *(for review Weygand-Durasevic & Cusack, 2005)* come into close proximity of the TΨC loop region of tRNA, where TrmA protein is known to bind (see Chapter 2.2.1.). This can be the structural basis for the significant positive effect of TrmA only with AlaRS.

Effect of mutant TrmA on aminoacylation of *in vitro* transcribed tRNA was similar to wild type TrmA, indicating that neither promotion of tRNA aminoacylation by AlaRS and PheRS nor inhibition of tRNA aminoacylation by ValRS depends on m^5U modification. These effects are attributed to the presence of TrmA protein, supporting the notion that TrmA protein itself can facilitate tRNA aminoacylation by AlaRS and PheRS, but hinder aminoacylation by ValRS.

To determine whether TrmA causes an irreversible change in tRNA whereby it becomes a better substrate for AlaRS or the presence of TrmA protein is required for efficient aminoacylation of tRNA, the following experiment was performed:

In vitro transcribed $tRNA^{Ala}$ was first incubated with TrmA and subsequently deproteinized yielding tRNA*. Effect of pre-incubation with TrmA on the tRNA* aminoacylation was determined using truncated AlaRS. Results are presented in Figure 9.

Incubation with TrmA enzyme increased the yield of tRNA* aminoacylation by truncated AlaRS approximately three-fold (see Figure 9). The fact that *in vitro* transcribed $tRNA^{Ala}$ purified after incubation with TrmA behaved similarly to tRNA previously unprocessed with TrmA, supports the notion, that the presence of TrmA protein and not some irreversible change of tRNA structure caused by it (see Figure 7, A and Figure 8, A), was responsible for the promotion of aminoacylation by AlaRS.

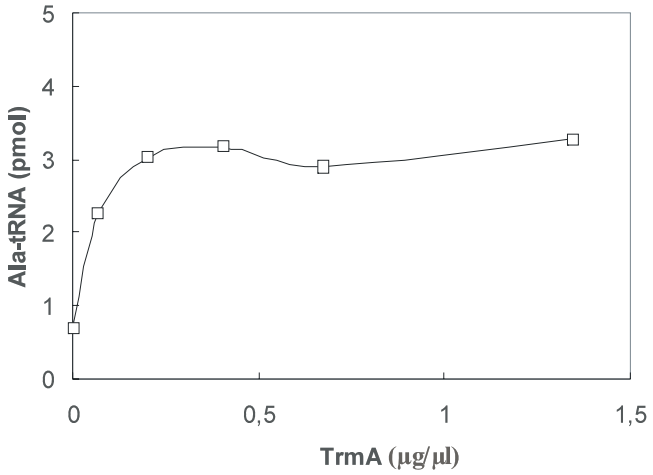

FIGURE 9. Aminoacylation of tRNA purified after incubation with TrmA. *In vitro* transcribed $tRNA^{Ala}$ was incubated with TrmA at 37°C for 20 minutes, phenol/chlorophorm extracted, ethanol precipitated and dissolved in water (tRNA*). 20 pmol of tRNA* was then pre-incubated with 0.07-1.35 µg/µl of TrmA at 37°C for 10 minutes, and aminoacylated by 0.06 µg/µl of truncated AlaRS at 37°C for 30 seconds.

To shed light on the mechanism by which TrmA promotes aminoacylation of *in vitro* transcribed tRNA, kinetic parameters of tRNA aminoacylation by AlaRS and PheRS were determined in the presence and absence of TrmA protein:

In vitro transcribed $tRNA^{Ala}$ and $tRNA^{Phe}$ were pre-incubated in the presence or absence of TrmA protein. Ratio of tRNA/TrmA was kept constant. tRNAs were then aminoacylated by full-length AlaRS or PheRS. Results are presented in Figure 10. Kinetic parameters were calculated based on Michaelis-Menten equation and are shown in Table 5.

TABLE 5. Kinetic parameters of aminoacylation

aaRS	Addition	K_M tRNA (µM)	k_{cat} (sec^{-1})	k_{cat}/K_M tRNA (sec^{-1} µM)
AlaRS	**none**	**2,4±0,3**	**0,067±0,007**	**0,028±0,008**
AlaRS	**TrmA**	**0,8±0,2**	**0,038±0,005**	**0,047±0,007**
PheRS	**none**	**3,2±0,6**	**0,61±0,08**	**0,192±0,03**
PheRS	**TrmA**	**2,7±0,5**	**0,61±0,1**	**0,225±0,08**

Kinetic parameters were determined over a tRNA range of 0.032-0.32 µg/µl

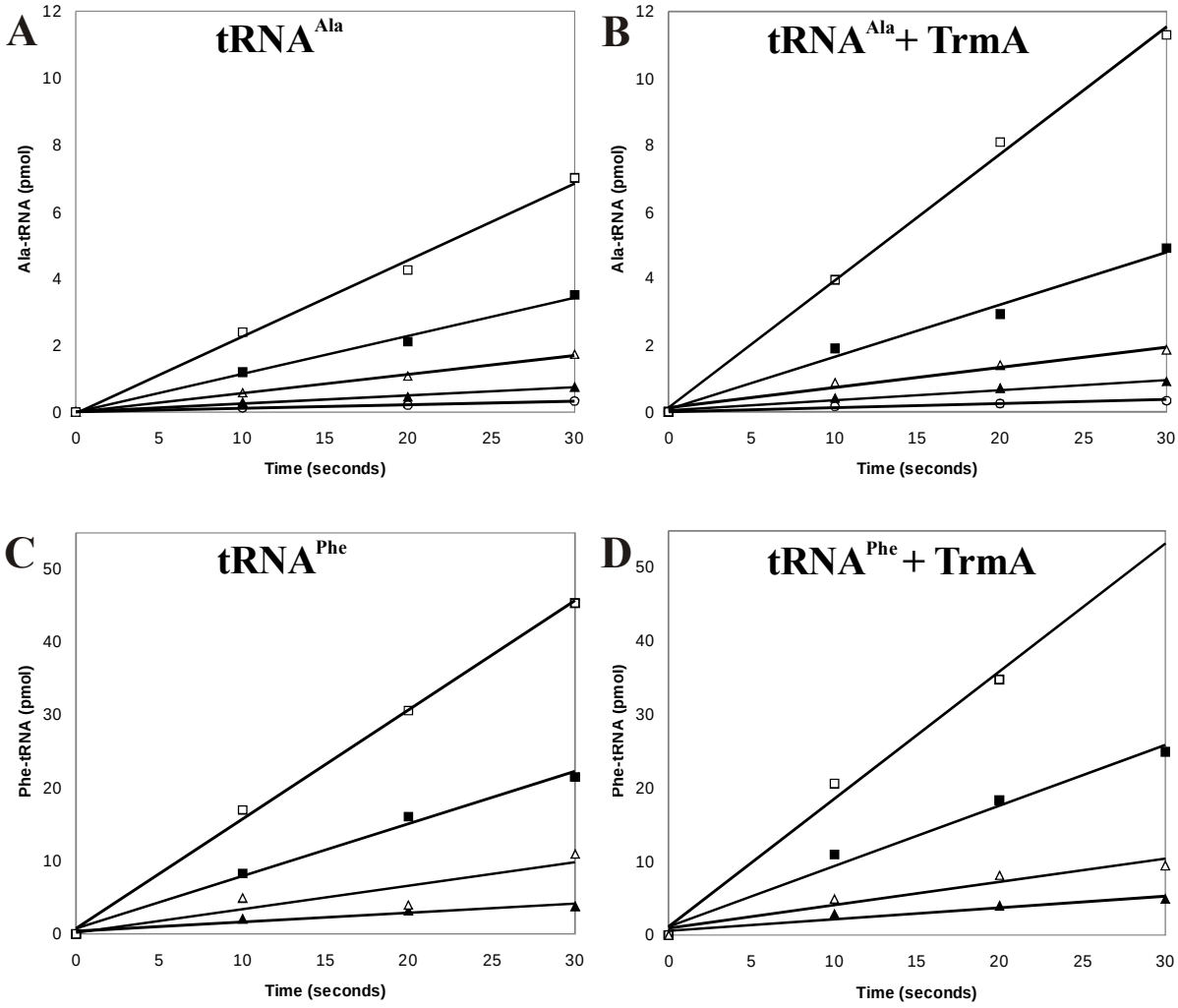

FIGURE 10. Effect of TrmA on the kinetics of tRNA aminoacylation. 0.016 μg/μl (open circles), 0.032 μg/μl (filled triangles), 0.08 μg/μl (open triangles), 0.16 μg/μl, (filled squares), 0.32 μg/μl (open squares) of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Phe}$ were pre-incubated in the presence (B,D) and absence of TrmA (0.01/0.02/0.05/0.1/0.2 μg/μl respectively) (A,C) at 37°C for 10 minutes, and aminoacylated by 0.006 μg/μl of full-length AlaRS or 0.008 μg/μl of PheRS at 25°C for 10/20/30 seconds.

Pre-incubation with TrmA lowered K_M of *in vitro* transcribed $tRNA^{Ala}$ three-fold and decreased k_{cat} of aminoacylation by AlaRS ca.1.5-fold (k_{cat}/K_M was increased approximately two-fold). In contrast, pre-incubation with TrmA did not significantly affect the kinetic parameters of *in vitro* transcribed $tRNA^{Phe}$ aminoacylation by PheRS (see Table 5).

Reduction of Michaelis-Menten constant in the presence of TrmA refers to increased affinity of AlaRS for tRNA. In other words, it is likely that TrmA, AlaRS, and tRNA form a ternary complex. Reduction of catalytic constant suggests that dissociation of tRNA from AlaRS is slower in the presence of TrmA.

3.4. Native gel electrophoresis analysis

tRNA binding to TrmA and TruB proteins was studied by analyzing tRNA mobility shift by native gel electrophoresis:

In vitro transcribed and radioactively labeled $tRNA^{Ala}$ was incubated with TrmA or TruB enzymes and analyzed for complex formation by 12 % native PAGE. Results are presented in Figure 11.

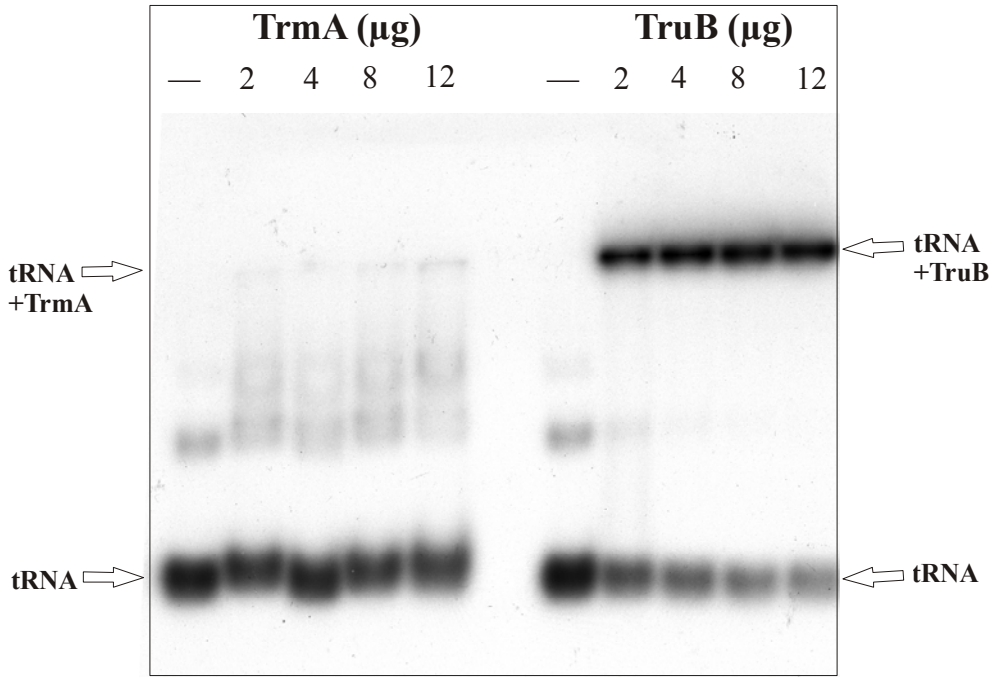

FIGURE 11. Gel mobility shift analysis of tRNA binding to TrmA and TruB proteins. 20 pmol of *in vitro* transcribed [^{32}P]-labeled $tRNA^{Ala}$ was incubated with 2/4/8/12 µg of TrmA or TruB proteins at 37°C for 20 minutes. Complex formation was detected by 12 % native PAGE. Gel was run at ~100V (10-12mA) for 24 hours at 4°C, vacuum dried on Whatman paper and exposed to X-ray film.

tRNA mobility shift characteristic to complex formation was detected with TruB protein. However, no clear tRNA mobility shift with TrmA protein could be detected (see Figure 11).

TrmA may bind tRNA very weakly under the conditions used. For binding to tRNA, TrmA may require AlaRS (formation of ternary complex).

Attempts to visualize ternary complex formation were done by incubating *in vitro* transcribed and radioactively labeled $tRNA^{Ala}$ with TrmA, TruB, ValRS, and truncated AlaRS enzymes in various combinations. tRNA was then analyzed for complex formation by 6 % native PAGE. Results are presented in Figure 12.

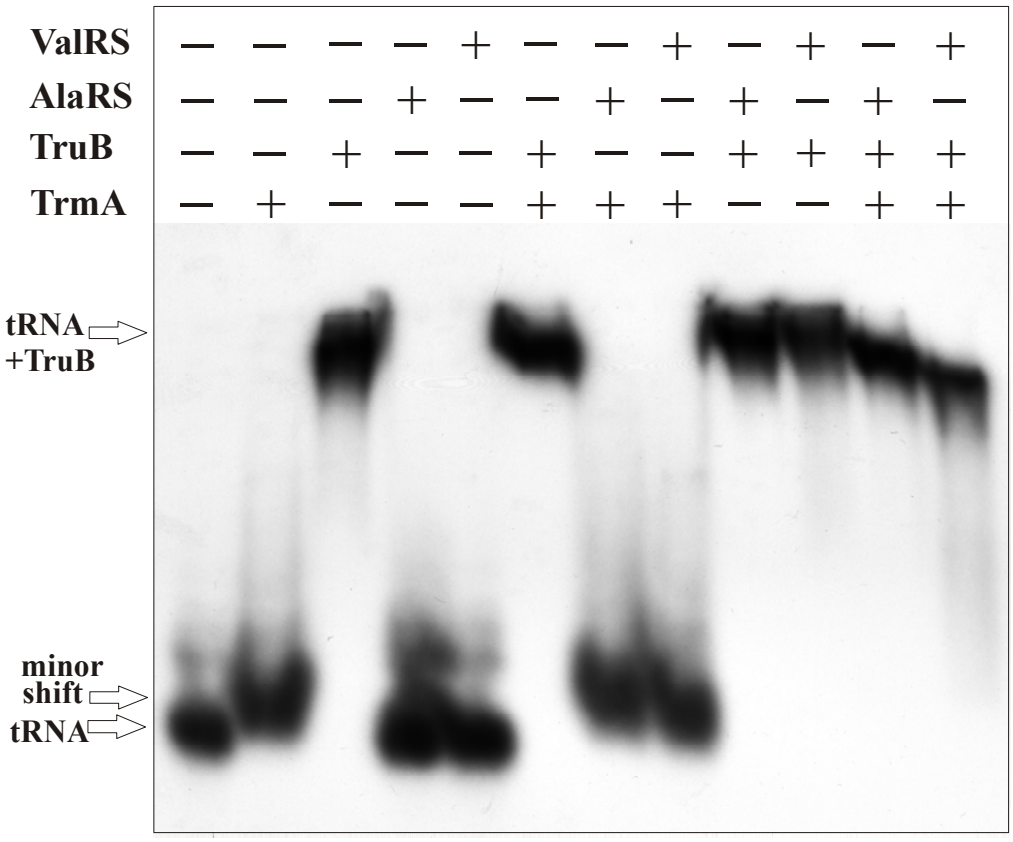

FIGURE 12. Gel mobility shift analysis of tRNA binding to TrmA, TruB, AlaRS and ValRS. 20 pmol of *in vitro* transcribed [^{32}P]-labeled tRNA^{Ala} was incubated with TrmA, TruB, ValRS, and truncated AlaRS enzymes 4 µg each in various combinations at 37°C for 20 minutes. Complex formation was detected by 6 % native PAGE. Gel was run at ~100V (10-12mA) for 24 hours at 4°C, vacuum dried on Whatman paper and exposed to X-ray film.

tRNA mobility shift was only detected with TruB protein. Neither tRNA binding to TrmA, truncated AlaRS and ValRS, nor tRNA mobility super-shift characteristic to ternary complex formation was detected under the conditions used (see Figure 12). tRNA binding to truncated AlaRS and ValRS was also analyzed in the presence of ATP, but no complex formation was detected (data not shown). tRNA mobility shift analysis by native gel electrophoresis may not be a suitable method for detection of ternary complex formation.

However, minor mobility shift of tRNA was detected upon incubation with TrmA protein (see Figure 12). This minor tRNA mobility shift was not caused by the m^{5}U modification synthesized by TrmA since the mutant TrmA (ΔTrmA) protein also inflicted the minor shift of tRNA mobility (data not shown). These results indicate that, either tRNA binding to TrmA protein is very weak and tRNA dissociates from TrmA rather early during the electrophoresis, or TrmA causes some kind of change in tRNA, making it to migrate somewhat slower.

To characterize the formation of minor band shift of tRNA incubated with TrmA protein, following experiment was performed:

In vitro transcribed $tRNA^{Ala}$ purified after previous incubation with TrmA protein (tRNA*) was analyzed along with *in vitro* transcribed $tRNA^{Ala}$ by 6% native PAGE for the formation of minor mobility shift of tRNA upon incubation with TrmA protein. Results are presented in Figure 13.

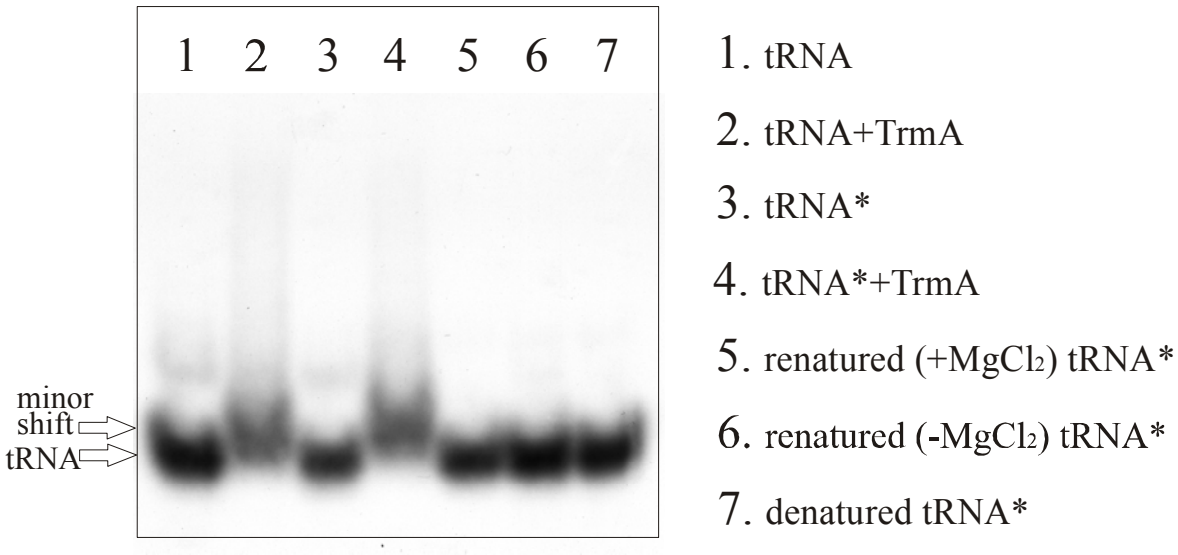

FIGURE 13. Analysis of tRNA mobility shift in the presence of TrmA. 20 pmol of *in vitro* transcribed [^{32}P]-labeled $tRNA^{Ala}$ was incubated with 4 μg of TrmA protein at 37°C for 20 minutes. tRNA* refers to *in vitro* transcribed $tRNA^{Ala}$ that was prior to gel mobility shift analysis incubated with TrmA enzyme at 37°C for 20 minutes. tRNA* was phenol/chlorophorm extracted, ethanol precipitated, dissolved in water and renaturated or denaturated (see Materials and methods). tRNA mobility shift was detected by 6% native PAGE. Gel was run at ~100V (10-12mA) for 24 hours at 4°C, vacuum dried on Whatman paper and exposed to X-ray film.

In vitro transcribed tRNA purified after previous incubation with TrmA protein (tRNA*) migrated similarly to *in vitro* transcribed tRNA previously unprocessed with TrmA (see Figure 13, lanes 1 and 3). Renaturation (in the presence or absence of magnesium) and denaturation of tRNA* did not affect its mobility (see Figure 13, lanes 5-7). Minor mobility shift of both unprocessed tRNA and tRNA* was detected only in the presence of TrmA protein (see Figure 13, lanes 2 and 4).

Results of native gel electrophoresis analysis confirm that TrmA does not lead to a stable change in the tRNA structure. The minor mobility shift of tRNA was dependent on the presence of TrmA protein. Ternary complex formation between TrmA, tRNA and AlaRS is highly likely, though it could not be detected by this method.

3.5. Glycerol gradient sedimentation analysis

Ternary complex formation between TrmA, tRNA, and AlaRS was followed by glycerol gradient sedimentation analysis:

TrmA was incubated with full-length AlaRS (α_4 homotetramer) or PheRS ($\alpha_2\beta_2$ heterotetramer) in the presence or absence of *in vitro* transcribed $tRNA^{Ala}$ or $tRNA^{Phe}$, respectively. Resulting complexes were separated by sedimentation in glycerol gradient. Results are presented in Figure 14.

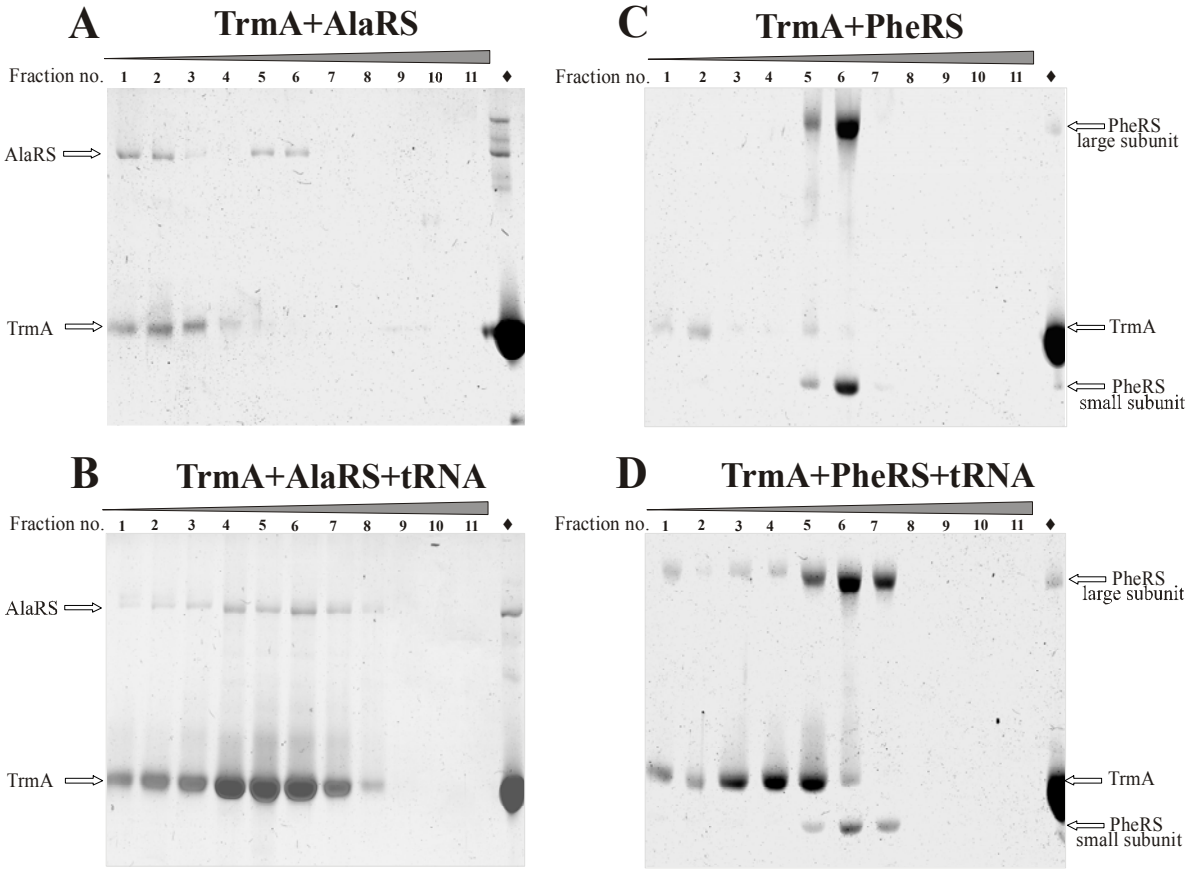

FIGURE 14. Glycerol gradient sedimentation analysis of ternary complex formation. 30 µg of TrmA enzyme was prior to centrifugation incubated with 90 µg of full-length AlaRS or PheRS either in the presence or absence of 800 pmol of tRNA (*in vitro* transcribed $tRNA^{Ala}$ or $tRNA^{Phe}$, respectively) at 37°C for 10 minutes. Samples were loaded on a 5 ml 10-30% glycerol gradient and centrifugation was carried out at 40 000 rpm for 12 h at 4°C. 11 fractions of 19 drops each were collected starting from the bottom of the tube (numbering of fractions starts from the top of the tube). Fractions were precipitated with TCA, dissolved in SDS loading dye and analyzed by 10% SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue. ♦ refers to protein fallout from the incubation reaction (see Materials and methods).

tRNA dependent co-sedimentation of TrmA was detected with AlaRS (see Figure 14, A and B) but not with PheRS (see Figure14, C and D).

tRNA dependent co-sedimentation of TrmA and AlaRS proteins clearly indicates the formation of ternary complex between tRNA, TrmA and AlaRS. Simultaneous binding of TrmA and AlaRS to tRNA is theoretically possible, since TrmA is predicted to bind only to the TΨC arm region of tRNA (see Chapter 2.2.1.) and AlaRS binds only to the acceptor arm of tRNA *(Swairjo et al., 2004)*. Determination of crystal structure of tRNA in complex with TrmA and in complex with AlaRS would be required for further characterization of the ternary complex.

3.6. Effect of AlaRS and PheRS on the m^5U formation in tRNA

Since it was determined that TrmA, tRNA and AlaRS form a ternary complex where the aminoacylation of tRNA was promoted, it was of great interest to study the effect of AlaRS on tRNA methylation by TrmA.

For this purpose TrmA protein was dialyzed for 72 hours against buffer TEN (see Materials and methods) in order to free it from the endogenous cofactor SAM. TrmA(TEN) was shown to be unable to synthesize m^5U when SAM was not added to the reaction (experiments performed in our laboratory by Kerli Piir, data not shown). Therefore it was possible to use the radioactive SAM as the sole source of methyl group during tRNA modification.

To analyze the effect of AlaRS and PheRS on tRNA methylation by TrmA, *in vitro* transcribed $tRNA^{Ala}$ or $tRNA^{Phe}$ were modified by TrmA(TEN) using radioactive SAM as substrate in the presence of full-length AlaRS or PheRS. Results are presented in Figure 15.

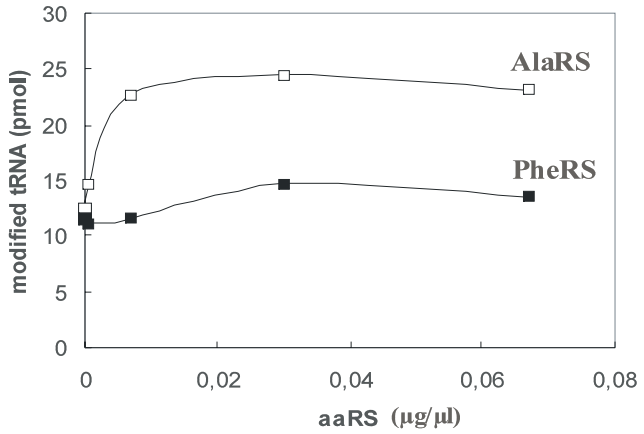

FIGURE 15. Modification of tRNA in the presence of AlaRS and PheRS. 40 pmol of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Phe}$ were modified by $2x10^{-3}$ µg/µl of TrmA(TEN) in the presence of $6.7x10^{-5}$- $6.7x10^{-2}$ µg/µl of PheRS or full-length AlaRS and 50 µM S-adenosyl-L-[methyl-^{14}C] methionine (SAM) at 37°C for 3 hours.

Modification of *in vitro* transcribed tRNA by TrmA was promoted about two-fold in the presence of AlaRS. PheRS did not have significant effect on the m^5U formation in tRNA (see Figure 15).

TrmA promotes the action of AlaRS and conversely, AlaRS promotes the action of TrmA, indicating that synergism occurs between TrmA and AlaRS in ternary complex. The exact mechanism as well as the biological relevance of synergism remains to be determined, but it refers to a link between tRNA processing and aminoacylation.

CONCLUSION

Transfer RNAs completely devoid of modified nucleosides were synthesized by *in vitro* transcription. Unmodified tRNAs were less efficiently aminoacylated by purified aminoacyl-tRNA synthetases than fully modified native tRNAs. Since the aminoacyl-tRNA synthetases used in this study do not require modified nucleosides, the decreased rate of aminoacylation may be do to the fact that tRNA molecules do not adopt their correct structure when synthesized *in vitro*.

It has been suggested that some protein factors may be needed to ensure the correct folding of tRNA molecules in the cell. tRNA modification enzymes TrmA and TruB are two potential "tRNA chaperones". Both of these proteins are involved in processing of vast majority of tRNAs in *Escherichia coli* and for both a second function in tRNA maturation distinct from tRNA modification have been proposed.

The purpose of current investigations was to shed light on the role TrmA and TruB proteins play in tRNA maturation. The effect of purified TrmA and TruB proteins on the formation of the correct structure of tRNA was tested through the efficiency of aminoacylation. It was discovered that incubation of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Val}$ with TruB protein inhibited aminoacylation of these tRNAs by both AlaRS and ValRS. TruB protein presumably competes with these aaRSs for binding to tRNA. Incubation of *in vitro* transcribed $tRNA^{Ala}$ and $tRNA^{Val}$ with TrmA enzyme inhibited aminoacylation by ValRS. TrmA and ValRS possibly compete for the same binding sites on tRNA. In contrast, pre-incubation with TrmA enzyme significantly increased the rate of $tRNA^{Ala}$ and $tRNA^{Val}$ aminoacylation by AlaRS.

Characterization of the mechanism by which TrmA promotes aminoacylation of *in vitro* transcribed tRNA by AlaRS was the central aim of this study. It was discovered that the stimulation of TrmA on aminoacylation of *in vitro* transcribed tRNA did not depend on the m^5U modification synthesized by TrmA. Neither did TrmA cause an irreversible change in tRNA structure, indicating that the presence of TrmA protein itself is required for efficient aminoacylation of *in vitro* transcribed tRNA. Kinetic parameters of tRNA aminoacylation by AlaRS in the presence and absence of TrmA protein were determined and referred to ternary complex formation between TrmA, tRNA, and AlaRS. Ternary complex formation was confirmed by glycerol gradient sedimentation analysis. Finally, it was determined that AlaRS promotes the methylation of *in vitro* transcribed $tRNA^{Ala}$ by TrmA.

The results of the experiments let us suppose that synergism occurs between TrmA and AlaRS proteins in the ternary complex with tRNA. The exact mechanism as well as the biological relevance of this synergism remains to be determined.

KOKKUVÕTE (Summary in Estonian)

tRNA modifikatsiooniensüümide TrmA ja TruB mõju *in vitro* transkribeeritud tRNA aminoatsüleerimisele

RYA ERO

Käesolevas töös sai *in vitro* transkriptsiooni teel sünteesitud täielikult modifitseerimata tRNA molekulid. Selgus, et modifitseerimata tRNA-d aminoatsüleeruvad puhastatud aminoatsüül-tRNA süntetaasidega vähem efektiivselt kui natiivsed modifitseeritud tRNA-d. Kuna antud töös kasutatud aminoatsüül-tRNA süntetaasid ei vaja modifitseeritud nukleosiide, võib vähene aminoatsüleerumise efektiivsus olla põhjustatud asjaolust, et tRNA-d ei omanda *in vitro* sünteesi käigus korrektset ruumilist struktuuri.

Arvatakse, et rakus osalevad tRNA-de korrektse ruumilise struktuuri moodustumisel valgulised faktorid. tRNA modifikatsiooniensüümid TrmA ja TruB on kaks potensiaalset „tRNA chaperoni". TrmA ja TruB osalevad valdava enamuse tRNA-de protsessimisel *Escherichia coli* rakus ning mõlemal valgul arvatakse olevat lisaks tRNA modifitseerimisele veel teinegi funktsioon tRNA-de biosünteesis.

Käesolevas töös sooviti teada saada, TrmA ja TruB valkude rolli tRNA molekulide korrektse ruumilise struktuuri moodustumisel. Puhastatud TrmA ja TruB valkude mõju tRNA funktsionaalsusele kontrolliti tRNA-de aminoatsüleerimise efektiivsuse kaudu. Katsetest selgus, et *in vitro* transkribeeritud $tRNA^{Ala}$ ja $tRNA^{Val}$ inkubeerimine TruB ensüümiga vähendas nende aminoatsüleerumise efektiivsust nii AlaRS kui ValRS poolt. TruB valk arvatavasti konkureerib nende aaRS-dega samadele seondumissaitidele tRNA-l. *In vitro* transkribeeritud $tRNA^{Ala}$ ja $tRNA^{Val}$ inkubeerimine TrmA ensüümiga pärssis aminoatsüleerimist ValRS poolt. Võimalik, et TrmA takistab aminoatsüleerimist seetõttu, et konkureerib ValRS-ga tRNA sidumisel. Vastupidiselt ValRS-le, TrmA ensüümiga preinkubeerimine tõstis oluliselt mõlema tRNA aminoatsüleerumise efektiivsust AlaRS poolt.

Järgnevalt sooviti kindlaks teha milles seisneb TrmA valgu positiivne efekt *in vitro* transkribeeritud tRNA aminoatsüleerimisele AlaRS poolt. Selgus, et stimuleeriv efekt tRNA aminoatsüleerimisele ei sõltunud TrmA poolt sünteesitud m^5U modifikatsioonist. TrmA ei põhjustanud ka pöördumatut tRNA struktuuri muutust, viidates, et TrmA valk is on vajalik *in vitro* transkribeeritud tRNA efektiivseks aminoatsüleerimiseks. Kineetilised parameetrid tRNA

aminoatsüleerimisel AlaRS-ga TrmA juuresolekul ja ilma TrmA valguta viitasid kolmikkompleksi tekkimisele TrmA, tRNA ja AlaRS vahel. Kolmikkompleksi teke leidis kinnitust sedimentatsiooni analüüsiga glütserooli gradiendis. Lõpuks tehti kindlaks, et AlaRS soodustab *in vitro* transkribeeritud $tRNA^{Ala}$ metüleerimisele TrmA poolt.

Katsete põhjal võib järeldada, et TrmA, tRNA ja AlaRS vahel moodustub kolmikkompleks, kus TrmA ja AlaRS valkude vahel valitseb sünergism. Sünergismi täpne mehhanism ja bioloogiline tähtsus vajavad edaspidist uurimist.

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APPENDIX

No.	Symbol	Common name
1	m^1A	1-methyladenosine
2	m^2A	2-methyladenosine
3	m^6A	N^6-methyladenosine
4	Am	2'-O-methyladenosine
5	ms^2m^6A	2-methylthio-N^6-methyladenosine
6	i^6A	N^6-isopentenyladenosine
7	ms^2i^6A	2-methylthio-N^6-isopentenyladenosine
8	io^6A	N^6-(cis-hydroxyisopentenyl)adenosine
9	ms^2io^6A	2-methylthio-N^6-(cis-hydroxyisopentenyl) adenosine
10	g^6A	N^6-glycinylcarbamoyladenosine
11	t^6A	N^6-threonylcarbamoyladenosine
12	ms^2t^6A	2-methylthio-N^6-threonyl carbamoyladenosine
13	m^6t^6A	N^6-methyl-N^6-threonylcarbamoyladenosine
14	hn^6A	N^6-hydroxynorvalylcarbamoyladenosine
15	ms^2hn^6A	2-methylthio-N^6-hydroxynorvalyl carbamoyladenosine
16	Ar(p)	2'-O-ribosyladenosine (phosphate)
17	I	inosine
18	m^1I	1-methylinosine
19	m^1Im	1,2'-O-dimethylinosine
20	m^3C	3-methylcytidine
21	m^5C	5-methylcytidine
22	Cm	2'-O-methylcytidine
23	s^2C	2-thiocytidine
24	ac^4C	N^4-acetylcytidine
25	f^5C	5-formylcytidine
26	m^5Cm	5,2'-O-dimethylcytidine
27	ac^4Cm	N^4-acetyl-2'-O-methylcytidine
28	k^2C	lysidine
29	m^1G	1-methylguanosine
30	m^2G	N^2-methylguanosine
31	m^7G	7-methylguanosine
32	Gm	2'-O-methylguanosine
33	$m^2{}_2G$	N^2,N^2-dimethylguanosine
34	m^2Gm	N^2,2'-O-dimethylguanosine
35	$m^2{}_2Gm$	N^2,N^2,2'-O-trimethylguanosine
36	Gr(p)	2'-O-ribosylguanosine (phosphate)
37	yW	wybutosine
38	o2yW	peroxywybutosine
39	OHyW	hydroxywybutosine
40	OHyW*	undermodified hydroxywybutosine

No.	Symbol	Common name
41	imG	wyosine
42	mimG	methylwyosine
43	Q	queuosine
44	oQ	epoxyqueuosine
45	galQ	galactosyl-queuosine
46	manQ	mannosyl-queuosine
47	$preQ_0$	7-cyano-7-deazaguanosine
48	$preQ_1$	7-aminomethyl-7-deazaguanosine
49	G+	archaeosine
50	Ψ	pseudouridine
51	D	dihydrouridine
52	m^5U	5-methyluridine
53	Um	2'-O-methyluridine
54	m^5Um	5,2'-O-dimethyluridine
55	$m^1Ψ$	1-methylpseudouridine
56	Ψm	2'-O-methylpseudouridine
57	s^2U	2-thiouridine
58	s^4U	4-thiouridine
59	m^5s^2U	5-methyl-2-thiouridine
60	s^2Um	2-thio-2'-O-methyluridine
61	acp^3U	3-(3-amino-3-carboxypropyl)uridine
62	ho^5U	5-hydroxyuridine
63	mo^5U	5-methoxyuridine
64	cmo^5U	uridine 5-oxyacetic acid
65	$mcmo^5U$	uridine 5-oxyacetic acid methyl ester
66	chm^5U	5-(carboxyhydroxymethyl)uridine
67	$mchm^5U$	5-(carboxyhydroxymethyl)uridine methyl ester
68	mcm^5U	5-methoxycarbonylmethyluridine
69	mcm^5Um	5-methoxycarbonylmethyl-2'-O-methyluridine
70	mcm^5s^2U	5-methoxycarbonylmethyl-2-thiouridine
71	nm^5s^2U	5-aminomethyl-2-thiouridine
72	mnm^5U	5-methylaminomethyluridine
73	mnm^5s^2U	5-methylaminomethyl-2-thiouridine
74	mnm^5se^2U	5-methylaminomethyl-2-selenouridine
75	ncm^5U	5-carbamoylmethyluridine
76	ncm^5Um	5-carbamoylmethyl-2'-O-methyluridine
77	$cmnm^5U$	5-carboxymethylaminomethyluridine
78	$cmnm^5Um$	5-carboxymethylaminomethyl- 2'-O-methyluridine
79	$cmnm^5s^2U$	5-carboxymethylaminomethyl-2-thiouridine
80	$m^6{}_2A$	N^6,N^6-dimethyladenosine

No.	Symbol	Common name
81	Im	2'-O-methylinosine
82	m^4C	N^4-methylcytidine
83	m^4Cm	N^4,2'-O-dimethylcytidine
84	hm^5C	5-hydroxymethylcytidine
85	m^3U	3-methyluridine
86	$m^1acp^3\Psi$	1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine
87	cm^5U	5-carboxymethyluridine
88	m^6Am	N^6,2'-O-dimethyladenosine
89	$m^6{}_2Am$	N^6,N^6,O-2'-trimethyladenosine
90	$m^{2,7}G$	N^2,7-dimethylguanosine
91	$m^{2,2,7}G$	N^2,N^2,7-trimethylguanosine
92	m^3Um	3,2'-O-dimethyluridine
93	m^5D	5-methyldihydrouridine
94	$m^3\Psi$	3-methylpseudouridine
95	f^5Cm	5-formyl-2'-O-methylcytidine
96	m^1Gm	1,2'-O-dimethylguanosine
97	m^1Am	1,2'-O-dimethyladenosine
98	tm^5U	5-taurinomethyluridine
99	tm^5s^2U	5-taurinomethyl-2-thiouridine
100	imG-14	4-demethylwyosine
101	imG2	isowyosine
102	ac^6A	N^6-acetyladenosine
103	inm^5U	5-(isopentenylaminomethyl)uridine
104	inm^5s^2U	5-(isopentenylaminomethyl)- 2-thiouridine
105	inm^5Um	5-(isopentenylaminomethyl)- 2'-O-methyluridine
106	$m^{2,7}Gm$	N^2,7,2'-O-trimethylguanosine
107	$m^4{}_2Cm$	$N^{4,4}$,2'-O-trimethylcytidine

Abbreviations and common names of modified nucleosides.

The RNA modification database: http://medlib.med.utah.edu/RNAmods/