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MARGUS LEPPIK

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Substrate specificity of the multisite specific pseudouridine synthase RluD

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Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

Current dissertation is based on the following publications which will be referred to in the text by their Roman numerals:

I **Leppik, M; Peil, L; Kipper, K; Liiv, A; Remme, J:** Substrate specificity of the pseudouridine synthase RluD in Escherichia coli. FEBS J 2007, 21:5759–66.

II **Ero, R; Leppik, M; Liiv, A; Remme, J:** Specificity and kinetics of 23S rRNA modification enzymes RlmH and RluD. RNA 2010, 11:2075–84.

III **Leppik, M; Ero, R; Liiv, A; Kipper, K; Remme, J:** Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in Escherichia coli 23S rRNA. Biochimie 2012, 94(5):1080–9.

My contribution to the publications is as follows:

Ref I I introduced single point mutations A1916U and A1916C into plasmid, containing rrnB operon. I purified most of the ribosomes and performed most of the CMCT/alkali and primer extension analysis. I prepared Figures 1–4 and participated in manuscript preparation.

Ref II I determined the time course for RluD dependent isomerization of positions 1911, 1915 and 1917. I prepared Figure 5 and participated in manuscript preparation.

Ref III I share the first authorship. I did all the RluD experiments. I prepared Figures 1–3 and 5. I participated in writing of the manuscript.

LIST OF ABBREVIATIONS

A-site	acceptor site for aminoacyl tRNA on the ribosome
DC	decoding center
DMS	dimethylsulfide
h44	stem-loop 44 of the ribosomal small subunit RNA
H69	stem-loop 69 of the ribosomal large subunit RNA
IF3	ribosome initiation factor 3
L-proteins	ribosomal large subunit proteins
LSU	ribosomal large subunit
mRNA	messenger RNA
MS	mass spectrometry
NMR	nuclear magnetic resonance
nt	nucleotides
P- site	acceptor site for peptidyl tRNA on the ribosome
PKR	RNA-dependent protein kinase
PS	pseudouridine synthase
PTC	peptidyl transferase center
RF1 and RF2	ribosomal release factors 1 and 2
r-proteins	ribosomal proteins
RRF	ribosomal recycling factor
rRNA	ribosomal RNA
S4-like domain	protein domain that resembles ribosomal small subunit protein S4
snoRNA	small nucleolar RNA
s-proteins	ribosomal small subunit proteins
SSU	ribosomal small subunit
tRNA	transfer RNA
Ψ	pseudouridine

INTRODUCTION

Protein synthesis is one of the fundamental processes in every cell and it is carried out by ribonucleoparticles called ribosomes. Ribosomes translate the information encoded in the nucleotide sequence of mRNA into amino acid sequence of proteins. Functional ribosome is composed of two unequal subunits which contribute differently for translation (Tissieres & Watson 1958). Smaller particle is responsible for accuracy and larger particle is responsible for catalysis. To achieve fast and accurate translation, binding of extra-ribosomal factors and communication between the two particles are required.

Ribosomal RNA (rRNA) is highly structured and modified molecule. Its structure and many modifications are well conserved (Ofengand & Del Campo 2004). Although, most of the modifications are not essential for ribosome functioning, their deletion leads to reduced fitness of the cells, indication for small but significant contribution into rRNA functionality (Sergiev *et al.* 2006; Purta *et al.* 2008b).

The most abundant modification in rRNA is pseudouridine (Ψ). *E. coli* ribosomal large subunit contains ten and small subunit one Ψ (Ofengand & Del Campo 2004). Despite being discovered over 50 years ago (Davis & Allen 1957), little is known about the function of Ψ in RNA sequence. In bacteria, substrate recognition of pseudouridine synthases is done in protein level and the exact mechanism how the proteins recognize their substrate is mostly unknown. Most pseudouridine synthases isomerize only one specific uridine residue in rRNA to Ψ. However, in *E. coli* there are three rRNA pseudouridine synthases that exhibit multisite specificities (RluA, RluC and RluD) (Koonin 1996). The study of substrate recognition mechanism of these enzymes is very challenging task. Understanding the substrate recognition and catalytic mechanism of rRNA modification enzymes are needed to completely understand the ribosome biogenesis process. Notably, defects in ribosomal biogenesis can cause rare genetic diseases (Freed *et al.* 2010).

First part of current thesis is focused on most abundant modification, pseudouridylation, and rRNA pseudouridine synthases. Structure of ribosome and rRNA are also discussed.

The experimental part is focused on RluD, the multisite specific pseudouridine synthase, which is responsible for synthesizing three pseudouridines into highly conserved rRNA structural element the 23S rRNA helix-loop 69 (H69). We have revealed a nucleotide (A1916) in H69 loop region that affects the substrate specificity of the RluD. We also propose that S4-like domain is necessary for initial binding of the RluD to its substrate. Although RluD exhibits highly specific nature *in vivo*, its *in vitro* specificity is significantly reduced.

I. REVIEW OF LITERATURE

I.1. Structure of the bacterial ribosome

All organisms, known so far, use ribosomes to convert nucleic acid sequence in mRNA codons to proper amino acid sequence of proteins. Bacterial ribosome is a ribonucleoprotein that has molecular weight of about 2,3 MDa and it is composed of two unequal subunits. Ribosomal proteins (r-proteins) form about 1/3 and ribosomal RNA (rRNA) about 2/3 of total mass of bacterial ribosome (Tissieres & Watson 1958). Small subunit (SSU) has molecular weight about 0,8 MDa and it contains the decoding center (DC) where correct aminoacylated tRNA is chosen based on mRNA codon sequence. Large subunit (LSU) has molecular weight about 1,5 MDa and it contains the peptidyl transferase center (PTC) which catalyzes addition of a new amino acid to the C-terminal end of the growing polypeptide chain. Particle, containing one SSU and one LSU bound to each other, is responsible for protein synthesis in cells (Tissieres & Watson 1958; McQuillen *et al.* 1959). Instead of just molecular weight, size of the ribosome is more often characterized by the sedimentation coefficient ($1S=10^{-13}$ sec), which is complex function of molecular mass, density, and shape. Functional bacterial ribosome sediments as 70S, SSU and LSU sediment as 30S and 50S particle, respectively (Tissieres & Watson 1958).

Catalytic sites of both ribosomal subunits are entirely composed of rRNA. Ribosomal proteins are located more distal from the catalytic sites of the ribosome. Thus localization of rRNA and r-proteins indicates unambiguously that rRNA has the catalytic role in protein synthesis, which makes the ribosome a ribozyme (Picking *et al.* 1992; Ban *et al.* 2000; Nissen *et al.* 2000; Wimberly *et al.* 2000; Harms *et al.* 2001; Yusupov *et al.* 2001). 50S subunit of thermophilic bacterium *T. aquaticus* maintains its peptidyl transferase activity even after removal of most of the r-proteins from the subunit core, confirming the catalytic role of the rRNA (Noller *et al.* 1992). In contrast, *E. coli* 50S subunit loses its catalytic activity after protein removal. rRNA structure of *T. aquaticus* is probably inherently more robust than structure of *E. coli* rRNA indicating that proteins are required for stabilization of rRNA structures in ribosome (Noller *et al.* 1992).

Prokaryotic ribosomes are composed of three rRNA molecules (5S; 16S and 23S) and around 50 proteins. *E. coli* SSU is composed of the 16S rRNA (1542 nt) molecule and 21 proteins (s-proteins) (Kaltschmidt & Wittmann 1970). Six morphological features can be distinguished from the tertiary structure of the 30S subunit: head, neck, body, shoulder, platform and spur (Figure 1.D). Such structural arrangement indicates the need for extensive movement within the 30S subunit during the translational process, an assumption confirmed by cryo-electron microscopic studies. Head and spur regions of the SSU are known to undergo the largest movement during the translation (Gao *et al.* 2003). 30S subunit rotates with respect to the 50S subunit about 6 degrees during translocation which accompanies the tRNA movement through the ribosome. Extensive movement of head and spur is partly due to their location in periphery

of 30S subunit and the large scale movement can be explained with the ratchet-like movement of the subunits (Frank & Agrawal 2000). Pivotal point of ratchet-like movement is the center of both ribosomal subunits (Dunkle *et al.* 2011). SSU head domain also moves 15º–18º toward the E site (away from the shoulder) in respect to the body domain of the 30S subunit, called head swiveling. Swiveling is coupled with ratcheting and tRNA translocation. Head swiveling causes opening of the so-called latch of the mRNA opening channel which facilitates translocation of the mRNA/tRNA complex. Therefore head swiveling, coupled with partial unratcheting event, leads to translocation of the tRNA/mRNA complex (Ben-Shem *et al.* 2010; Ratje *et al.* 2010).

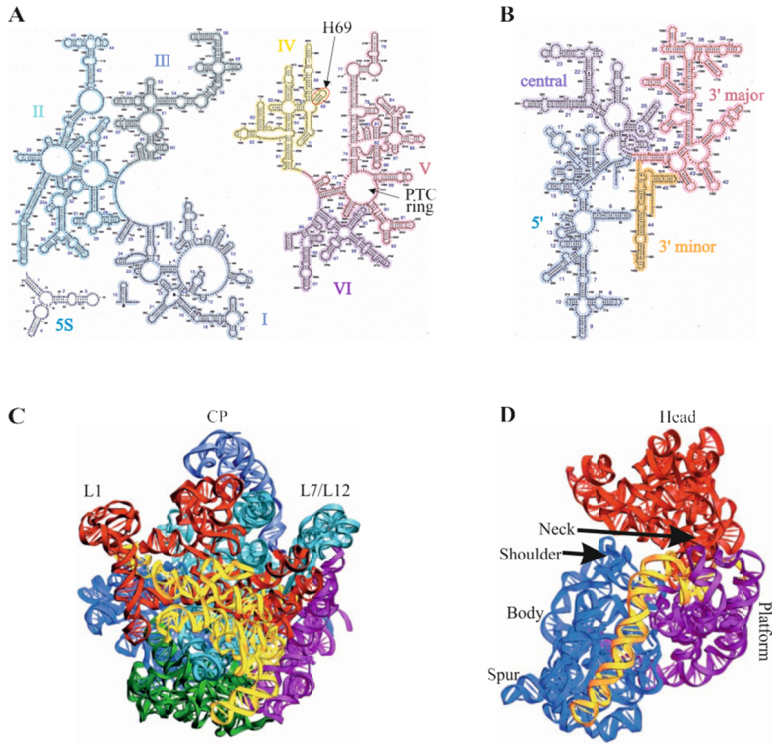

Figure 1. Secondary and tertiary structures of rRNAs. **A and B.** Secondary structure of 5S, 23S and 16S rRNA, respectively. **C and D.** Three dimensional structures of 50S and 30S subunits of 70S ribosome, respectively. **A.** Distinguishable domains of 23S rRNA secondary structure I (blue), II (cyan), III (green), IV (yellow), V (red), VI (magenta) and 5S rRNA (cyan) are indicated. Arrows are pointing to the universally conserved stem-loop H69 in domain IV and peptidyl transferase ring in domain V. **B.** 5' (cyan), central (magenta), 3' minor (yellow) and 3' major (red) domains for 16S rRNA secondary structure. **C.** Three dimensional structure of 23S and 5S rRNA (located in central protuberance) colored as in A. Morphological features as L1 stalk, L7/L12 stalk and central protuberance (CP) are indicated. **D.** Three dimensional structure of 16S rRNA colored as in B. Head, neck, body, shoulder, platform and spur are indicated. Illustration adapted from (Yusupov *et al.* 2001).

LSU is composed of 23S rRNA (2904 nt), 5S rRNA (120 nt) and 34 proteins (L-proteins) (Kaltschmidt & Wittmann 1970). In contrast to the SSU, tertiary structure of the LSU cannot be divided into distinct morphological features (Mueller *et al.* 2000) (Figure 1.C). LSU resembles a crown like structure consisting of a compact rounded base with three protuberances, L1 stalk, central protuberance and, L7/L12 stalk on top of the subunit (Figure 1.C). L1 stalk consists of protein L1 and its rRNA binding site. Central protuberance consists mostly of the 5S rRNA and proteins L5, L18, and L25 (Yusupov *et al.* 2001). L7/L12 stalk consists of 4–6 copies of L7 and L12 proteins connected to N-terminal domain of the L10 (Diaconu *et al.* 2005; Mandava *et al.* 2012) whereas L7 is N-acetylated form of the L12 (Wilson & Nierhaus 2005). L7/L12 proteins are necessary for binding of extra-ribosomal GTPases to the ribosome (Diaconu *et al.* 2005). In contrast to the SSU, majority of the structural elements of the LSU are not very mobile during the translation process, moving generally less than 3Å. Only three protuberances are exhibiting movement around 3Å (Gao *et al.* 2003).

Ribosome assembly is a fast and highly precise process including coordinated synthesis, proper folding, and modification of rRNA and r-proteins (Lewicki *et al.* 1993). Assembly process from the start of rRNA transcription to the formation of fully active 70S ribosome takes only about 2–3 minutes at 37ºC (Lindahl 1975) and 5–10 minutes at 25ºC (Peil *et al.* 2008) *in vivo*. Functional ribosomal particles can also be reconstituted *in vitro* from purified ribosomal components indicating that most of the information needed for proper assembly of a ribosome is encoded in rRNA and r-protein sequences. However, higher temperatures, high Mg^{2+} concentration, and considerably more time are necessary to assemble the ribosomal subunits *in vitro* (Traub & Nomura 1968; Nomura & Erdmann 1970; Dohme & Nierhaus 1976; Green & Noller 1999). It has to be noted that assembly intermediates of *in vivo* and *in vitro* assembly are very similar. Thus the *in vivo* and *in vitro* assembly seems to follow the same path (Lindahl 1975; Dohme & Nierhaus 1976). Assembly of LSU proceeds via three precursors and SSU via two precursors *in vivo* (Lindahl 1975) and *in vitro* (Traub & Nomura 1969; Dohme & Nierhaus 1976; Talkington *et al.* 2005). *In vitro* reconstitution studies have revealed that rRNA post-transcriptional modifications are not essential for basic functions of 23S rRNA. Although *in vitro* synthesized 23S rRNA, lacking all post-transcriptional modifications, can be assembled into 50S subunit *in vitro*, the latter exhibits reduced activity in peptidyl transferase and translational activity compared to 50S ribosomes reconstituted from fully modified 23S rRNA (Khaitovich *et al.* 1999). *In vivo* ribosomal assembly begins with rRNA synthesis and processing. *In vivo* rRNA synthesis induces translation from r-protein mRNAs (Ecker 1965) by competing for r-proteins which would otherwise bind to their own mRNAs and thereby repressing their translation (Fallon *et al.* 1979; Nomura *et al.* 1980).

Translation is highly complex sequence of events that requires the assistance of many extra-ribosomal factors, and fine communication between the two ribosomal subunits. SSU and LSU are joined together by a number of connections

called the intersubunit bridges. Intersubunit bridges are, at least partly, responsible for the communication between two ribosomal subunits. Cognate tRNA in ribosomal A-site is likely also responsible for transfering of the signal from the decoding site to the PTC (Stark *et al.* 2002; Daviter *et al.* 2005). All the intersubunit bridges are not formed concurrently upon the association of subunits during initiation of translation. At first, formation of a few connections between LSU and SSU take place which leads to structural rearrangements at the interface between the subunits. Early formed intersubunit bridges are speculated to be necessary for the formation of later ones (Hennelly *et al.* 2005). One of the connections that is formed early during ribosome subunit association is the intersubunit bridge B2a (Hennelly *et al.* 2005). Most of the intersubunit bridges are RNA-RNA bridges but in the periphery, bridges where r-proteins contribute into bridging, also occur (Cate *et al.* 1999; Gabashvili *et al.* 2000; Yusupov *et al.* 2001; Gao *et al.* 2003). rRNA elements, forming the intersubunit bridges, fall into domains II and IV of the 23S rRNA and 3'-minor, central and 5' domains of the 16S rRNA (Figure 1. A and B) (rRNA domains are discussed in Chapter 1.2.1) (Merryman *et al.* 1999a; Merryman *et al.* 1999b; Yusupov *et al.* 2001). Ribosomal subunits from two distinct organism, 40S from eukaryote *A. salina* and 50S from prokaryote *E. coli*, can be joined together to form a hybrid ribosome. The resultant hybrid ribosome is able to carry out *in vitro* protein synthesis (Klein & Ochoa 1972). Latter fact that elements for intersubunit bridges have remained intact during evolution indicates that the location and nature of basic intersubunit bridges are vital for the ribosome function. Although intersubunit bridges hold two subunits together, they also exhibit high conformational dynamics due to the ratchet-like movement of subunits during translation. B3 is the only bridge that maintains its conformation during the intersubunit movement and can therefore be considered as pivot point of the movement (Dunkle *et al.* 2011). All other bridges change their conformation during the translation. The extent of conformational change depends on the distance from pivot point of the intersubunit movement. Some bridges, located at periphery, are even disrupted and formed again during translation (Spahn *et al.* 2004; Ben-Shem *et al.* 2010; Dunkle *et al.* 2011).

1.2. Maturation of ribosome and rRNA

1.2.1. Ribosomal RNA

About 2/3 of bacterial ribosome is made up of rRNA. Secondary and tertiary structures of the rRNA are highly conserved. In bacteria 5S, 16S, and 23S rRNA genes are typically organized into an operon and are transcribed as a single transcript. Different bacterial species carry different number of rRNA operons varying from one in *Ricettsia prowazekii* (Pang & Winkler 1993) to 15 in *Clostridum paradoxum* and not all copies are always identical, at least as far as the rDNA sequence is considered (Rainey *et al.* 1996; Klappenbach *et al.* 2001). The different number of rRNA operons between species seems to be

evolved because of adaptation to certain growth conditions. The number of rRNA operons has a mild effect on the maximal growth rate of bacteria (Condon *et al.* 1995a). More rRNA operons allow cells to start transcription from multiple loci, permitting rapid increase of intracellular rRNA levels thereby reducing the lag phase. Latter gives an advantage under the conditions where environment becomes rapidly more favorable, for example when amount of nutrients is constantly fluctuating or cells experience rapid temperature changes (Condon *et al.* 1995a; Condon *et al.* 1995b). Higher number of rRNA operons has a disadvantage due to the metabolic burden, when nutrients are constantly poor supply (Stevenson & Schmidt 1998; Klappenbach *et al.* 2000). It has been speculated that different rRNA operons are expressed under specific physiological conditions as has been described in *Plasmodium* (Gunderson *et al.* 1987; Zhu *et al.* 1990). Genome of malaria parasite *P. berghei* contains four rRNA operons and the operons appear to exhibit microheterogeneity (Dame & McCutchan 1983). Different rRNA genes are expressed during life cycle of *P. berghei*. Transcripts of one gene predominate in the parasite, developing in mosquito and transcripts of another gene predominate in the parasite, entered into bloodstream of the host (Gunderson *et al.* 1987; Zhu *et al.* 1990). However, no evidence of such regulation has yet been reported in *E. coli*. Yet, it has to be noted that the rRNA is not always transcribed as one covalently continuous polyribonucleotide chain as in *E. coli*. In some organisms the rRNA is fragmented into coding modules that can be interspersed with other genes (Heinonen *et al.* 1987; Nedelcu 1997). Moreover, the coding sequences of rRNA gene can deviate from conventional, highly conserved, 5'-3' order of sequence domains as has been described in mitochondria (Heinonen *et al.* 1987).

Ribosomal RNAs are highly structured and modified molecules. According to the secondary structure, 16S rRNA is divided into four domains (5', central, 3'-major and 3'-minor) (Woese *et al.* 1980; Yusupov *et al.* 2001) (Figure 1.B). Different secondary structure domains of the 16S rRNA correspond to nearly structurally autonomous three-dimensional domains in SSU (Wimberly *et al.* 2000; Ramakrishnan & Moore 2001; Yusupov *et al.* 2001) (Figure 1.B and D). 23S rRNA is divided into six domains (I–VI) (Noller *et al.* 1981; Yusupov *et al.* 2001) (Figure 1.A). Unlike 16S rRNA, 23S rRNA domains are extensively intertwined with each other in LSU, forming a single large hemispherical structure (Harms *et al.* 2001; Ramakrishnan & Moore 2001; Yusupov *et al.* 2001) (Figure 1.A and C). 5S rRNA being a part of the LSU virtually forms the seventh independent domain of the LSU (Yusupov *et al.* 2001) (Figure 1.A and C).

Logical assumption would be that regions of rRNA that are absolutely essential for ribosomal function are very highly conserved in nature and less important regions can be varied or even be absent. Interestingly nearly all of the conserved helixes of 23S rRNA are located in domains II, IV, and V, furthermore, domains IV and V also exhibit the highest percentage of universally conserved residues of rRNA (29% and 28% respectively) (Mueller *et al.* 2000; Mears *et al.* 2002). Moreover, nearly all of the post-transcriptionally modified

nucleotides are also located in 23S rRNA domains II, IV, and V (Noller *et al.* 1981; Ofengand & Bakin 1997). The modified nucleotides tend to cluster around CCA end of A and P site tRNAs in tertiary structure of LSU (Mueller *et al.* 2000) (Figure 2), hypothetically linking post-transcriptional modifications to ribosomal functioning. All highly conserved domains (II; IV; V) of 23S rRNA have been linked to different ribosomal functions. Domain V is responsible for carrying out peptidyl transferase reaction. Peptidyl transferase cleft is formed of helices surrounding PTC-ring of 23S rRNA (Figure 1.A) (Mueller *et al.* 2000). Universally conserved G2553, located in domain V of the 23S rRNA, has been shown to crosslink with aminoacyl-tRNA analog 4-thio-dT-p-C-p-puromycin (Green *et al.* 1998). The crosslink is dependent on occupancy of the P-site with deacylated tRNA and is inhibited with peptidyl transferase specific antibiotics. Thus this specific crosslink appears only in biologically active conformation of ribosomes. The G2553 has also been shown to involve in base-pair interaction with C75 of the A-site tRNA in crystal structure (Nissen *et al.* 2000). Hence the G2553 seems to play a vital part in the peptidyl transferase reaction. Domain IV of the 23S rRNA is responsible for correct binging of ribosomal subunits (Leviev *et al.* 1995). Nearly all RNA-RNA bridges between ribosomal subunits are formed between domain IV of the 23S rRNA and 3' minor and central domains of the 16S rRNA (Yusupov *et al.* 2001). Moreover, mutations in 23S rRNA domain IV cause misreading of tRNAs by the SSU (O'Connor & Dahlberg 1995). This leads to a speculation that domain IV is also responsible for transmission of signals from the decoding center of the SSU to the peptidyl-transferase center of the LSU. Antibiotic thiostrepton binds to domain II of the 23S rRNA and causes loss of the GTPase activity of EF-G, thus domain II is part of the ribosomal GTPase center (Rodnina *et al.* 1999).

Structure and location of the 5S rRNA, in central protuberance of the LSU, is highly conserved. The 5S rRNA is located in the vicinity of the PTC in LSU (Dontsova *et al.* 1994), it makes several contacts with 23S rRNA and L-proteins. Lack of 5S rRNA during LSU assembly causes drastic reduction in LSU function (Barciszewska *et al.* 2001). 5S rRNA contacts with the domains II and V of 23S rRNA and participates in proper folding of PTC during assembly of the LSU. Lack of 5S rRNA can be compensated with aminoglycoside antibiotic that binds simultaneously to domains II and V, stabilizing 23S rRNA structure during *in vitro* assembly (Khaitovich & Mankin 1999). U89 of 5S rRNA has been cross-linked with highly conserved residues A960 of domain II and C2475 of domain V. Mutations at position A960 cause structural rearrangements in D loop of the 5S rRNA and also in domain V of 23S rRNA, thus 5S rRNA has been proposed to be necessary for signal transmission between ribosomal PTC and GTPase centers during translation (Sergiev *et al.* 2000).

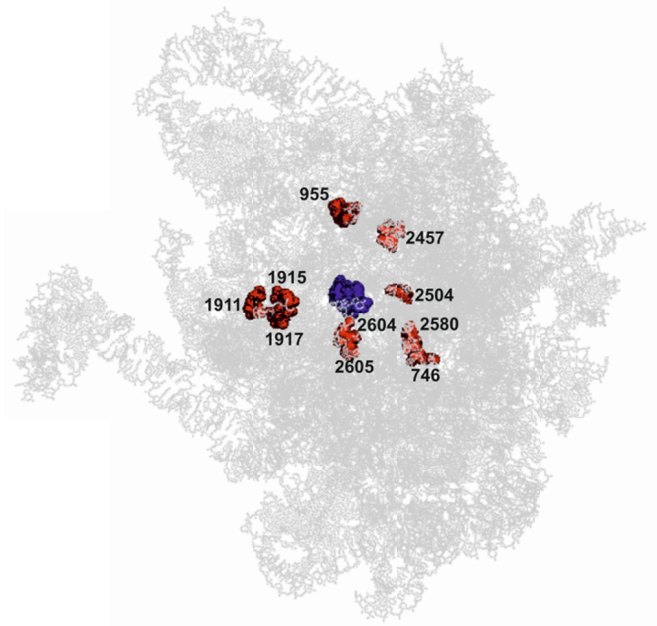

Figure 2. Pseudouridines are clustered around tRNA CCA end in LSU tertiary structure. 23S rRNA is colored grey, pseudouridines are marked as red dots and P-site tRNA CCA end is colored blue. Location of pseudouridines are indicated according to *E. coli* 23S rRNA sequence. Illustration was created with PyMOL DeLano Scientific (PDB ID: 2WWQ).

1.2.2. rRNA maturation

Maturation of rRNA is taking place during the ribosomal assembly process and comprises of proper folding, enzymatic cleavage, and modification of rRNA. rRNA maturation depends on several factors, including nucleases, helicases, and modification enzymes. Maturation of rRNA begins when the polymerase is still synthesizing the rRNA sequence and the last maturation steps take place after the newly assembled ribosome has reached the cells polysome pool (Srivastava & Schlessinger 1990; Ero *et al.* 2008). RNase III cleavage of the rRNA releases pre23S, pre16S, and pre5S rRNAs from the primary rRNA transcript and is likely the first step in rRNA maturation. The cleavage occurs before RNA polymerase has finished the transcription of the whole RNA operon and it has been suggested that RNase III cleaves 23S rRNA 5' end before the 3' end has been synthesized (King & Schlessinger 1983).

All available data indicate that the maturation of the rRNA is highly ordered process. Data from assembly mapping experiments shows that the newly transcribed rRNA binds r-proteins in a specific order (Mizushima & Nomura 1970; Dohme & Nierhaus 1976; Roth & Nierhaus 1980). This observation suggests that binding of early proteins leads to a conformational change in rRNA

necessary for binding of the following r-proteins. It is noteworthy that expressing of rRNA genes with scrambled rRNA secondary structural domain elements, leads to assembly of functional ribosomes, however, slightly reduced growth rate and high dependence on rRNA specific helicases still refers to reduced efficiency of the assembly. Thus, the ordered transcription of rRNA and the hierarchical incorporation of r-proteins from the 5'-terminal domain are not essential. However, transcription of 23S rRNA domains IV and V cannot be separated without the loss of functional ribosomes (Kitahara & Suzuki 2009).

Enzymatic cleavage of the freshly synthesized rRNA also follows specific ordered pathway. As already mentioned, RNase III is the first nuclease to cleave the primary transcript. RNase III cleavage leaves relatively long 3' and 5' ends for rRNAs which require further maturation. It must be noted that initial cleavage of RNase III is absolutely essential for maturation of the 23S rRNA but 16S rRNA can be matured also without this initial cleavage (King *et al.* 1984). In *E. coli*, the nuclease responsible for the final maturation of the 3' ends of 23S and 5S rRNA is RNase T, an exoribonuclease that has previously been identified as the enzyme responsible for the removal of nucleotides in tRNA 3'-end turnover (Deutscher *et al.* 1985). RNase T cleavage occurs preferably after the rRNA is completely associated with r-proteins. Maturation of 23S and 5S rRNA 5' end is independent from 3' end but the nuclease(s) responsible for final maturation of 5' ends are not clear yet (Li & Deutscher 1995; Li *et al.* 1999a). *In vitro* studies have shown that RNase M5 is responsible for final maturation of 5S rRNA 3' and 5' ends in *Bacillus subtilis* (Sogin *et al.* 1977) and tRNase Z is responsible for the maturation of 5' end of 5S rRNA in archaeon *Haloferax volcanii* (Holzle et al. 2008). tRNase Z is an endonuclease that has shown to generate mature 3' end of tRNA. Interestingly, structural modeling reveals that in *H. volcanii* the 40 nt upstream sequence of 5S rRNA can be folded into mini-tRNA structure and the nuclease recognizes this formed structure rather than the actual sequence of the 5S rRNA. Unfortunately, tRNase Z is an essential gene and its actual *in vivo* function is not known (Holzle *et al.* 2008).

Unlike with the 23S rRNA, RNase III cleavage is not essential for the final maturation of 16S rRNA (Srivastava & Schlessinger 1989). Maturation of the 16S rRNA 5' end occurs in two steps: first, RNase E cleavage leaves 66 nucleotides longer end, followed by final maturation with RNase G. RNase G is able to cleave 16S 5' end even without the previous RNase E cleavage step but exhibits, in this case, significantly reduced efficiency. Nuclease(s) responsible for the maturation of 3' end of the 16S rRNA is (are) still unknown (Li *et al.* 1999b).

It is known that immature 23S can be assembled into functional 70S ribosomes (King *et al.* 1984), however, the maturation of 16S rRNA is essential for the assembly of functional 30S subunit. Yet, 16S rRNAs isolated from *E. coli* BUMMER strain polysomes contains 66 additional nucleotides at 5' end. The fact that such 16S is included into polysomes indicate at least some activity for the 30S containing partly unprocessed 16S rRNA (Dahlberg *et al.* 1978). SSU, containing immature 16S rRNA exhibits reduced translational fidelity (Roy-

Chaudhuri *et al.* 2010). Immature 5' end interferes with the formation of helix 1 of the 16S rRNA. Formation of the helix 1 in biogenesis of SSU is essential for formation of accurate ribosomes (Roy-Chaudhuri *et al.* 2010).

Escherichia coli mature 16S and 23S rRNAs contain 36 modified nucleotides in total and the modifications can be divided into three major groups: pseudouridines, base methylated nucleotides, and ribose methylated nucleotides. Although distribution of modifications in rRNA secondary structure is seemingly dispersed, the modifications are becoming clustered at functionally important regions in tertiary structures. Moreover, during association of the functional 70S ribosome, modifications of 16S and 23S rRNAs become into close proximity (Merryman *et al.* 1999a). Although rRNA modifications have been shown not to be essential for either ribosome assembly or functioning (Green & Noller 1999), these observations are mostly based on *in vitro* studies and are not necessarily reflecting the *in vivo* conditions. High conservation of rRNA modifications throughout all kingdoms of life and the fact that peptidyl transferase reaction, catalyzed by unmodified rRNA is inefficient, firmly argues against this proposal. Synthesis of modifications causes a metabolic burden for the cell and the fact that the enzymes, catalyzing modifications, have not faded away during evolution is strong evidence that modified nucleotides are necessary (Ofengand & Del Campo 2004). No *in vivo* assembly or activity experiments have been carried out with rRNA without any modifications. It is known that three methylase genes can be knocked out simultaneously without major effect on cells viability (Sergiev *et al.* 2008). Only the deletion of one rRNA pseudouridine synthase, RluD, was shown to have a significant effect on cells viability (Huang *et al.* 1998a; Gutgsell *et al.* 2005). However, in this particular case, the growth rate defect turned out to be combination of several different factors (Ejby *et al.* 2007; O'Connor & Gregory 2011; Schaub & Hayes 2011) which will be discussed in Chapter 1.3.3. While not essential for cells viability, many of the post-transcriptional modifications have shown to affect bacterial fitness. Although most of the rRNA modification enzyme deletion strains exhibit normal ribosomal assembly and cell growth rate, they often fail to compete with wild type cells in growth competition experiments (Andersen & Douthwaite 2006; Lesnyak *et al.* 2006; Sergiev *et al.* 2006; Purta *et al.* 2008b; Sergiev *et al.* 2008) indicating that modifications still play an important role in rRNA maturation and/or ribosome functioning. Notably, RrmJ (RlmE) responsible for the 2'-O methylation at position 2552 of 23S rRNA is regulated by a heat shock promoter indicating the involvement in cellular stress response mechanism (Caldas *et al.* 2000).

Remme and coworkers have divided rRNA modifications into three groups based on their time of occurrence *in vivo*: early, intermediate, and late modifications (Siibak & Remme 2010). In this work ribosomal precursor particles induced by chloramphenicol or erythromycin, were studied to reveal at which stage the different modifications are introduced into rRNA during the maturation and assembly. This gives a clue at which time different modification enzymes modify rRNA. Until then, most of the knowledge about rRNA modi-

fications was collected from *in vitro* studies. The function and the exact order the modification enzymes follow during the maturation remains to be determined.

1.2.3. RNA helicases

Several extra-ribosomal factors, including RNA helicases, are required to achieve fast and proper assembly of a ribosome, needed to support bacterial exponential growth rate (Strunk & Karbstein 2009). *In vitro* reconstitution of ribosomal particles requires a step where increased temperature is used (Traub & Nomura 1969; Nomura & Erdmann 1970; Dohme & Nierhaus 1976; Green & Noller 1999). The heating step is probably necessary for breaking number of non-covalent bonds required for loosening the rRNA structure for binding of final r-proteins and final maturation of the ribosome. RNA helicases are proteins, able to unwind double stranded RNA helices in an ATP dependent manner. Helicase activity, of the DEAD-box helicases, depends on presence of ATP and ATPase activity depends on presence of RNA (Fuller-Pace 1994; Bizebard *et al.* 2004). Partial unwinding, induced by helicases, can promote proper folding of the rRNA and/or interaction with r-proteins *in vivo*.

Five DEAD-box helicases have been identified in *E. coli.* Four DEAD-box family helicases (CsdA/DeaD, DbpA, RhlE and SrmB) have been implicated in ribosome biogenesis (Charollais *et al.* 2003; Charollais *et al.* 2004; Jain 2008; Peil *et al.* 2008; Sharpe Elles *et al.* 2009) and one (RhlB) has been found as component of the RNA degradosome (Liou *et al.* 2002). Although most of the DEAD-box helicases have been determined as essential in *S. cerevisiae* (de la Cruz *et al.* 1999), the knockout studies have revealed that none of the helicases are essential for cell's viability in *E. coli*. Moreover, all the genes encoding DEAD-box helicases can be removed from the cell (Jagessar & Jain 2010).

Deletion of *csdA/deaD* and *srmB* genes leads to growth defect of *E. coli*. Moreover, sucrose density gradient profiles show reduction in polysomes and 70S ribosomes, an increase in SSU and occurrence of a pre-LSU particle, containing pre-23S rRNA and reduced amount of r-proteins. The phenotypic effect is more pronounced at lower temperatures (Charollais *et al.* 2003; Charollais *et al.* 2004; Peil *et al.* 2008). Latter can be explained with increased stability of misfolded rRNA structures, requiring assistance of helicases to achieve correct structure. Furthermore, expression of *srmB* gene has been shown to be specifically induced after temperature shift from 37ºC to 15ºC (Jones *et al.* 1996). Notably, DEAD-box helicases bind specifically to pre-50S particles and not to fully assembled 50S ribosomes (Charollais *et al.* 2003; Charollais *et al.* 2004). How the proteins distinguish between pre-50S and 50S particles, has remained unclear. The most probable substrate for the helicases is misfolded structure of rRNA.

In contrast to helicases CsdA/DeaD and SrmB, deletion of the DEAD-box helicase DbpA does not affect ribosome assembly. However, overexpression of

DbpA active site mutant R331A, causes similar phenotypic effects as deletion of CsdA/DeaD and SrmB (Sharpe Elles *et al.* 2009). DbpA, containing a mutation R331A, exhibits reduced ATPase and RNA unwinding ability but has minimal effect on RNA binding, compared to wild type enzyme (Elles & Uhlenbeck 2008). Latter leads to a speculation that accumulation of pre-LSU is caused by binding of the DbpA that is unable to catalyze rRNA conformational change (Sharpe Elles *et al.* 2009). DbpA is the only DEAD-box helicase that has been shown to require specific rRNA element, hairpin 92 of the 23S rRNA, for its activity (Diges & Uhlenbeck 2001).

The biological significance of DEAD-box helicase RhlE is interesting. RhlE has been found to be primarily associated with ribosomes but deletion of the *rhlE* gene leads to modest (Jain 2008) or no growth defect of *E. coli* cells (Jagessar & Jain 2010). Interestingly gene deletion and overexpression studies have revealed that RhlE has an opposite effect on csdA/*deaD* and *srmB* gene knockouts, indicating for regulatory role of the RhlE. Overexpression of RhlE dramatically reduces the growth rate of *ΔsrmB* strain and enhances the growth rate of *ΔcsdA/ΔdeaD* strain compared to isogenic *ΔsrmB* and *ΔcsdA/ΔdeaD* strains respectively. The opposite effect was seen in RhlE knockout experiments (Jain 2008). These results indicate that CsdA/DeaD and SrmB act on a non-overlapping intermediates in LSU maturation pathway and RhlE acts as switch between the intermediates (Jain 2008).

It is notable that deletion of a DEAD-box helicase cause only LSU assembly defects, no SSU assembly intermediates has been observed (Charollais *et al.* 2003; Charollais *et al.* 2004; Jain 2008; Sharpe Elles *et al.* 2009; Jagessar & Jain 2010). Notably, SSU containing pre-16S rRNA (17S rRNA), sendiment as mature 30S particle (Lindahl 1975). Although, SSU particles containing pre-16S rRNA has been found from DEAD-box helicase deletion strains (Charollais *et al.* 2003; Sharpe Elles *et al.* 2009), this effect can be due to misassembly of the LSU because final maturation of rRNA takes place after LSU and SSU have been associated (Udem & Warner 1973; Srivastava & Schlessinger 1988, 1989). Accumulation of pre-LSU reduces the amount of functional LSU needed for final maturation of 16S rRNA.

1.2.4. Helix-loop 69

One of the most intriguing structure in the 23S rRNA is the stem-loop 69 (H69) (Figure 3). Its location in free 50S as well as in 70S ribosomes during translation, and also high degree of modification has made the H69 one of the most extensively studied structures in the ribosomal RNA. H69 has been indicated to be important for many translational events including ribosomal assembly (Liiv *et al.* 2005), subunit association (Maivali & Remme 2004; Kipper *et al.* 2009), translational accuracy (O'Connor & Dahlberg 1995), initiation (Kipper *et al.* 2009), elongation (Kipper *et al.* 2009), termination (Klaholz *et al.* 2004; Ejby *et*

al. 2007; O'Connor & Gregory 2011) and recycling (Wilson *et al.* 2005; Borovinskaya *et al.* 2007).

H69, consisting of 23S rRNA nucleotides 1906–1924 in *E. coli,* exhibiting a 7 nucleotide loop flanked by 6 base pair helical region (Figure 3), is a relatively small structural element. H69 is located in domain IV of 23S rRNA (Figure 1.A) and it contacts with helix 44 of 16S rRNA (h44) in 70S ribosome (Mueller *et al.* 2000; Schuwirth *et al.* 2005), forming the intersubunit bridge B2a. H69 and intersubunit bridge B2a are both highly conserved structures in all three kingdoms of life and also in organellar rRNA (Mears *et al.* 2002). Nucleotides in H69 loop region are extremely conserved, exhibiting about 99% of conservation if nucleotide 1918 is excluded. Adenine or guanosine has been almost equally found at position 1918 (Cannone *et al.* 2002). Adenine is highly conserved in bacteria and guanosine is >98% conserved in eukaryotes (Cannone *et al.* 2002; Sumita *et al.* 2005).

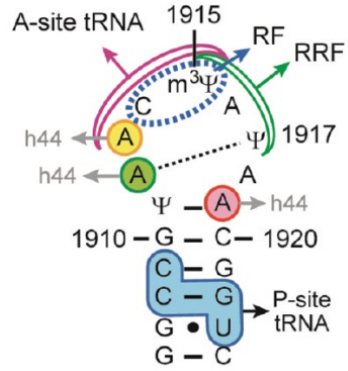

Figure 3. Sequence and secondary structure of 23S rRNA stem-loop 69 (H69). H69 contains two pseudouridines (Ψ) (at positions 1911, and 1917) and one N3 methylpseudouridine ($m^3\Psi$) (at position 1915), in the loop region. Key interactions with A and P site tRNAs, 16S rRNA h44 and ribosomal factors RF and RRF are shown. Illustration adapted from (Sakakibara & Chow 2012).

H69 is also one of the most extensively modified elements in *E. coli* ribosome, containing three out of ten pseudouridines of 23S rRNA at positions 1911, 1915 and 1917 (Figure 3). The pseudouridine at position 1915 also has a methyl group incorporated into the third position of the base, yielding $m^3\Psi$ (Kowalak *et al.* 1996). It is interesting to note that human 28S contains five pseudouridines in its H69 region, hence all H69 uridines are converted to pseudouridines (Sumita *et al.* 2005). X-ray crystallographic and cryo-electron microscopic studies have placed H69 to the heart of ribosome, in the vicinity of peptidyltransferase and decoding centers, between A and P site tRNAs (Cate *et al.* 1999; Yusupov *et al.* 2001). H69 is located at the intersubunit interface and its orien-

tation is considerably different in the free 50S subunit and in the 70S ribosome (Harms *et al.* 2001; Schuwirth *et al.* 2005). In 70S the tip of H69 is stretched toward the 30S subunit. In free 50S subunit, the location of H69 differs by 13,5Å and makes contacts with 23S rRNA H71 (Harms *et al.* 2001). H69 exhibits mobile nature not only upon subunit association, but also during subunit ratcheting in translation. In fully ratcheted state of the ribosome, the H69 is compressed about 5Å to maintain the bridge B2a. Base pair C1925-G1929 of the 23S rRNA is disrupted to enable sufficient compression of the H69 (Dunkle *et al.* 2011).

During accommodation of cognate tRNA into ribosomal A-site, nucleotides A1492 and A1493 flip out from h44 of the 16S rRNA toward the minor groove of first two codon-anticodon base pairs (Figure 4.A). In crystal structure A1913, located at the loop region of H69, is flipped out from the H69, upon tRNA binding, to give hydrogen bond contact with A37 of the A-site tRNA (Figure 4.A). Base of the A1913 also forms Mg^{2+} mediated contacts with nucleotide at position 38 of the A-site tRNA and with A1493, G1494 of the 16S rRNA (Selmer *et al.* 2006). Contact between A1913 and the A-site tRNA seems to be necessary for accommodation of near cognate tRNA, containing mismatch at third codon-anticodon base pair (wobble position). The A1913 is probably necessary for stabilization of more compromised interactions between tRNA, mRNA, and the ribosome (Ortiz-Meoz & Green 2011). Moreover, binding of class 1 release factor (RF1 or RF2) causes H69 to change its conformation. A1913 is displaced about 6Å to make stacking interactions with A1493 of h44 (Figure 4.B). Unlike binding of tRNA, binding of calss 1 release factor induces only A1492 to flip out from the h44 and vacated space is filled by A1913 (Figure 4.B). Latter probably plays a role in transmission of the termination signal from the decoding center to the peptidyl-transferase center, and directing the universally conserved GGQ motif of class 1 release factors to the PTC (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). Contacts between nucleotide A1913 and the h44 of 16S rRNA change noticeably during intersubunit rotation. Antibiotics viomycin and caperomycin bind to the vicinity of A1913 and may stabilize the compressed state of H69 favoring fully ratcheted state of the ribosome, which causes inhibition of the protein synthesis (Stanley *et al.* 2010; Dunkle *et al.* 2011). Ribosomes, containing 23S rRNA mutation A1913G, exhibit slightly reduced poly-U dependent translation *in vitro* and modest counter-selection of mutant ribosomes in polysome fraction *in vivo,* suggesting that A1913 has an important role in translation (Liiv *et al.* 2005).

Aminoglycoside antibiotics bind to the ribosome and cause errors in translational accuracy, termination, and recycling. Several aminoglycosides like neomycin, gentamycin, tobramycin, paromomycin, streptomycin, etc. have been shown to bind H69 and seem to inflict stability of the H69 (Borovinskaya *et al.* 2007; Scheunemann *et al.* 2010). Aminoglycosides gentamycin and neomycin bind to the major groove of the H69 at the base of its stem (nucleotides 1920–1925 and 1906) which would contact to P-site tRNA (Figure 3). Binding of the

drug stabilizes the H69 in its conformation that is necessary for maintaining intersubunit bridge B2a. During ribosomal recycling, binding of RRF induces H69 to swing away from the subunit interface and cause disruption of bridge B2a which in turn causes dissociation of subunits. Stabilization of the H69 by aminoglycosides leads to maintenance of the B2a even after binding of the RRF, leading to defects in ribosome recycling (Borovinskaya *et al.* 2007).

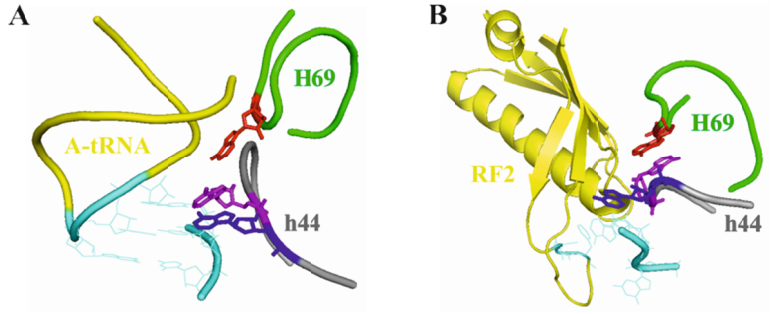

Figure 4. Conformational states of the H69 and h44 upon binding of tRNA (**A**) and RF2 (**B**) to the ribosome. H69 is colored green, h44 is grey, A1913 of the 23S rRNA is red, A1492 and A1493 of 16S rRNA are blue and magenta respectively, A-site tRNA and RF2 are colored yellow. Sense and nonsense codon interactions with anticodon of the tRNA or conserved SPF motif of the RF2 are cyan. **A.** Upon tRNA binding, A1492 and A1493 flip out from the h44 to form A-minor interactions with codon-anticodon base pairs. A1913 is also flipped out and contacts the A-tRNA, stabilizing tRNA binding to the ribosome (PDB ID:2J00 and 2J01). **B.** Only A1492 is flipped out upon RF2 binding and A1913 replaces the A1492, making stacking interactions with A1493 (PDB ID: 3F1E and 3F1F). Illustration was created with PyMOL DeLano Scientific.

Nucleotides A1912 and A1919 of the H69 make hydrogen bonding network with nucleotides G1494 and U1495 of 16S rRNA H44 (Schuwirth *et al.* 2005; Selmer *et al.* 2006). Disruption of this intersubunit bridge strongly affects association of ribosomal subunits. Modification of positions A1912 and A1918 by DMS reduces the association of ribosomal subunits 20%–50% (Maivali & Remme 2004). A1918 is not directly participating in formation of the B2a, but is a part of the hydrogen bonding network (Schuwirth *et al.* 2005) and DMS modification may indirectly impede the bridge from forming.

IF3 binds to the 30S subunit (Pon & Gualerzi 1974) and dissociates only after proper translational initiation complex is formed (Vermeer *et al.* 1973). Binding site of the IF3 is overlapping with H69 binding site on the 30S subunit, masking the binding site of the H69, preventing formation of the bridge B2a, and association of subunits (Dallas & Noller 2001). Mutational analysis has revealed that mutations at positions 1912 and 1919 of 23S rRNA cause defects in subunit association and *in vitro* protein synthesis, whereas not affecting 50S peptidyl-transferase activity (Kipper *et al.* 2009). These mutations also lead to

depletion of mutant 50S subunits, in polysome pool, indicating translational defects *in vivo* and *in vitro*. Molecular dynamic studies revealed that ribosomes, containing mutations at positions 1912 or 1919, exhibit higher amplitude movement of the H69 in respect to the h44 of 16S rRNA, destroying the B2a core part. Translational defects, caused by mutations at positions 1912 and 1919 of the H69, are caused by inefficient subunit association due to the disruption of bridge B2a (Kipper *et al.* 2009). Interestingly, deletion of entire H69 (ΔH69) has milder effect on *in vitro* translation than single point mutation at position 1912 or 1919 (Ali *et al.* 2006). Milder defects of ΔH69 can be explained with steric clash between H69 and A site tRNA. Mutations at positions 1912 and 1919 cause conformational change of the H69 in a way that H69 moves toward the A site and impedes binding of tRNA to the A-site. Deletion of H69 abolishes this steric clash (Kipper *et al.* 2009). However, *in vivo* expression of 23S rRNA variant, containing deletion of entire H69 from 23S rRNA, is lethal for bacterial cells, even in presence of wild type 23S rRNA. 50S subunits, containing ΔH69, are not able to associate with 30S subunits without presence of tRNA and exhibit RRF independent ribosomal recycling *in vitro* (Ali *et al.* 2006). It is noteworthy that poor association itself shouldn't be toxic for the cells and the exact lethal effect of the ΔH69 ribosomes remains to be studied.

Helix 69 has also been shown to affect a selection of correct tRNA by ribosomes, a function that is usually attributed to the 30S subunit and 16S rRNA. Base alternation C1914U, and deletion of A1916 leads to reduction of cell growth, frameshifting, and stop codon readthrough caused by defects in tRNA selection (O'Connor & Dahlberg 1995). 50S ribosomes containing mutation A1916G, exhibit defects in translation *in vitro*, and assembly defects of the 50S subunit are seen in sucrose density gradients. Base substitution C1914A cause no similar defects (Liiv *et al.* 2005). Why single mutation at position 1916 causes assembly defect remains to be studied.

1.3. rRNA pseudouridines and pseudouridine synthases

1.3.1. Pseudouridines in rRNA

Pseudouridines (Ψ) were the first modified ribonucleotides discovered (Davis & Allen 1957) and they have been found from many structured RNAs including rRNA, tRNA and snoRNA (Charette & Gray 2000). Pseudouridine is also the most abundant modification of a specific nucleotide in RNA. It must be noted that the sum of methylated nucleotides is higher in rRNA but methyl groups are divided between four canonical nucleotides (Ofengand & Del Campo 2004). Notably, pseudouridines have never found from naturally occurring mRNAs. When *in vitro* transcribed mRNAs are inserted into mammalian cells, mRNA, containing Ψ-s, yield increased translational capacity and stability compared to mRNA, containing only canonical U-s (Kariko *et al.* 2008; Anderson *et al.* 2010). It seems that mRNAs containing Ψ does not activate cellular defense system upon insertion, making such mRNAs potentially useful for therapeutic

applications (Kariko *et al.* 2008). *In vitro* synthesized mRNA, containing canonical U, activates cellular PKR system that phosphorylates translation initiation factor aIF2α resulting in repression of translation. However, PKR is not activated if mRNAs, containing Ψ, are inserted into the cells (Anderson *et al.* 2010). It was recently reported that pseudouridylation of stop codon leads to suppression of the stop codon both *in vivo* and *in vitro* (Karijolich & Yu 2011). Serine or threonine is incorporated into polypeptide chain when ribosome reaches the ΨAG or ΨAA codon and phenylalanine or tyrosine is incorporated when ΨGA codon is reached (Karijolich & Yu 2011). Yeast contains H/ACA RNA genes that can guide pseudouridine synthases to its substrate. It has been speculated that in certain conditions mRNA pseudouridylation can occur to alter mRNA properties or expand the genetic code (Karijolich & Yu 2011; Ge & Yu 2013). However, there is no experimental evidence to prove this speculation.

It is noteworthy that higher organisms contain more pseudouridines in rRNAs than prokaryotes. The proportion of Ψ is 0,9%–1,4% in eukaryote 28S rRNA, and about 0,03%–0,4% in bacterial or organellar counterpart 23S rRNAs (Ofengand & Bakin 1997). Although all Ψ-s are mapped in nucleotide resolution in several species, and pseudouridylated positions seem to be well conserved, their function has still remained enigmatic. As all other modified nucleotides in rRNA, pseudouridines are also clustered around functionally important regions of the ribosome and are therefore considered to be important for ribosomal functioning (Brimacombe *et al.* 1993; Bakin *et al.* 1994) (Figure 2). Pseudouridine at position 1917 in *E. coli* has been found in every cytoplasmic ribosome studied. Thus Ψ1917 is absolutely conserved and probably important for ribosomal functioning (Ofengand 2002).

Formation of pseudouridine introduces an additional imino group to the pyrimidine ring as an extra H-bond donor (Figure 5), making the nucleotide more hydrophilic. Compared to uridine, Ψ exhibits more stable stacking interaction and is capable of contributing to stabilization of rRNA local structure (Davis 1995; Desaulniers *et al.* 2008). N1 imino group is also able to give an extra H-bond to stabilize RNA structure by forming water mediated bridge between the base and RNA backbone, reducing structural mobility close to the Ψ (Auffinger & Westhof 1997) (Figure 6). Because of the unusual C-C glycosidic bond (Figure 5), Ψ is anticipated to exhibit greater conformational flexibility due to the enhanced rotational freedom. Latter is the reason for speculations that Ψ can act as a molecular switch in RNA molecule (Charette & Gray 2000). U and Ψ tend to prefer different conformation in solution. Ψ tends to be in *syn*- and U in an *anti*-conformation, but the conformations can vary when the nucleotides are inserted into the polynucleotide chain (Neumann *et al.* 1980).

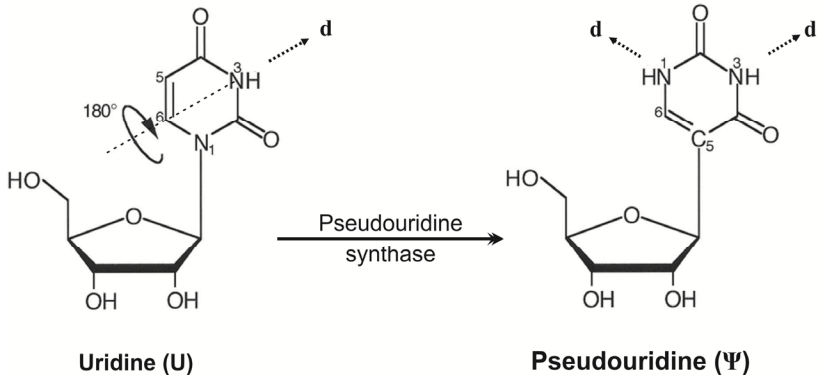

Figure 5. Chemical differences between uridine and pseudouridine. Pseudouridine synthase hydrolyses N1-C1' glycoside bond of uridine, rotates nitrogen base around C6-N3 axel and forms new C5-C1' glycoside bond. No external energy or factors are used. Pseudouridine contains one extra hydrogen bond donor and new C-C glycoside bond exhibiting higher conformational flexibility. Illustration adapted from (Charette & Gray 2000).

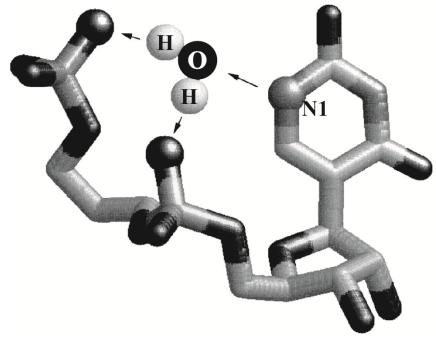

Figure 6. Water molecule mediated bridge between pseudouridine and phosphate backbone. Nitrogen base interaction with phosphate backbone has local stabilizing effect for RNA structure. N1 position of pseudouridine and water molecule are indicated. Illustration adapted from (Charette & Gray 2000).

It has been proposed that pseudouridines are required for local stabilization of the RNA structures (Hall & McLaughlin 1992; Davis 1995; Auffinger & Westhof 1997, 1998). Stacking interactions and additional hydrogen bonding stabilize the nucleotides surrounding the pseudouridine, making the structure less temperature sensitive (Davis 1995; Newby & Greenbaum 2001). Hyperthermophiles like *Pyrococcus phuriosus*, that have optimum growth temperature about 100ºC, contain significantly increased amount of modified nucleotides in their tRNAs. Although significant increase of pseudouridines was not detected, it must be noted that most of the studies have done by mass-spectrometry (MS)

which cannot distinguish between uridine and pseudouridine (Kowalak *et al.* 1994).

Lack of one pseudouridine in the rRNA usually has no detectable effect on cell's viability, but lack of several pseudouridines seems to have a cumulative effect. Most of the results indicate that lack of Ψ causes little structural rearrangements, which is in agreement with proposal that Ψ can fine-tune the ribosomal structure (King *et al.* 2003; Baxter-Roshek *et al.* 2007). Although cells, lacking few pseudouridines in ribosomal RNA, are not noticeably compromised, they are not able to compete with wild type cells indicating that pseudouridines have small but significant role in RNAs (King *et al.* 2003).

E. coli 16S and 23S rRNAs contain one and 10 pseudouridines, respectively (Ofengand & Del Campo 2004). As discussed in Chapter 1.1, 30S subunit exhibits much higher mobile nature than 50S and probably needs less Ψ-dependent stabilization of the rRNA structure. The sites for Ψ run the gamut from being in a single-stranded or loop region (Ψ746, m^3Ψ1915, Ψ1917, Ψ2504), adjacent to double stranded stem (Ψ516), part of loop closing base pair (Ψ955, Ψ1911, Ψ2457, Ψ2580), or part of base pair in stem (Ψ2604, Ψ2605) (Ofengand & Del Campo 2004). Pseudouridines are mostly found near the end of the RNA helical region (Ofengand & Bakin 1997), and this has been shown to make the loop structure more stable by stabilization of the loop closure (Meroueh *et al.* 2000). Loop closure could be the reason why Ψ at position 1911 has the stabilizing effect on H69 structure, as discussed below. In *E. coli* one pseudouridine in 23S rRNA is further methylated by methyltransferase RlmH (Ero *et al.* 2008; Purta *et al.* 2008a). The pseudouridine is methylated at third position and is located at highly conserved and hyper modified H69 at position 1915 (Kowalak *et al.* 1996). The purpose for the pseudouridine methylation is mysterious because methylation makes the nucleotide more hydrophobic and alters the hydrophilic nature of previously formed pseudouridine. Ψ at position 1915 is formed by multispecific pseudouridine synthase RluD (Huang *et al.* 1998a; Raychaudhuri *et al.* 1998), and it can be speculated that Ψ at position 1915 is accidental co-product of the RluD which has to be corrected with the methylation.

Striking results were obtained when stability of the H69 was studied. Stability of fully modified and completely unmodified H69 is basically the same (Meroueh *et al.* 2000; Sumita *et al.* 2005). Pseudouridine at position 1911 exhibits visible stabilizing effect on H69 but pseudouridines in loop region (1915 and 1917) exhibit destabilizing effect on H69 stability (Meroueh *et al.* 2000). Chemical probing experiments revealed that H69 can exhibit different conformational states and pseudouridines play a regulatory role in switching between these states (Sakakibara & Chow 2011), supporting the molecular switch theory (Charette & Gray 2000). Also NMR results indicate for only subtle difference between the structures of the modified and unmodified H69. Despite of two extra pseudouridines in H69 of *H. sapiens* compared to *E. coli*, the structure of *E. coli* H69 exhibits higher thermal stability (Sumita *et al.* 2005). Thus, pseudouridines have no uniform effect on RNA, and each

pseudouridine has unique local influence on the folded RNA structures. In combination, pseudouridines induce further structural variations of RNA (Meroueh *et al.* 2000). H69 pseudouridines at positions 1911, 1915 and 1917 are linked to effective translational termination probably due to their contribution in helix conformation (Ejby *et al.* 2007).

1.3.2. Pseudouridine synthases (PS)

U to Ψ conversion is an isomerization reaction where C1'-N1 glycosyl bond is cleaved, the uracil base is rotated 180º over the C6-N3 axle while still bound to the enzyme and the new C1'-C5 glycosyl bond is formed (Figure 5). No energy or cofactors are needed for this isomerization reaction in bacteria, where proteins are responsible for both selecting the right substrate uridine and the catalysis of Ψ formation. Notably, *Saccharomyces cerevisiae* pseudouridine synthase Pus1 is the only pseudouridine synthase known so far, which needs Zn^{2+} as an extra factor for maintaining its structure and the catalytic activity (Arluison *et al.* 1998). In eukaryotes and archaea, guide RNAs are required for substrate selection and proteins have only the catalytic role. Guide RNA system has made it possible to fulfill the demand for increased necessity for Ψ, without significantly increasing the metabolic burden. In principle, only one pseudouridine synthase, few auxiliary proteins, and several small guide RNAs are required for the synthesis of all the pseudouridines (Kiss 2001; Ge & Yu 2013).

All pseudouridine synthases are divided into five families: RluA, RsuA, TruA, TruB (Koonin 1996) and TruD (Kaya & Ofengand 2003). All five Ψ synthase families are named according to the first synthase identified in *E. coli* (Koonin 1996). While high conservation of the protein sequences have been shown inside the families, the similarity between the families is limited to short motifs and in case of TruA and TruD, not detectable at all (Koonin 1996; Kaya & Ofengand 2003). Despite sharing little or no sequence homology, PS families possess several conserved sequence motifs and similar tertiary structures (Mizutani *et al.* 2004; Ofengand & Del Campo 2004). All pseudouridine synthases contain catalytic aspartate residue, which is the only absolutely conserved structural element among the families (Del Campo *et al.* 2001; Ferre-D'Amare 2003; Sivaraman *et al.* 2004) and indicates conserved mechanism for the modification. Mutation of the catalytic aspartate completely inactivates the synthase (Huang *et al.* 1998b; Conrad *et al.* 1999; Ramamurthy *et al.* 1999; Raychaudhuri *et al.* 1999; Gutgsell *et al.* 2000; Gutgsell *et al.* 2001).

Two alternative catalytic mechanisms were proposed for the isomerization reaction. Catalytic aspartate performs initial attack either on nitrogen base C6 or on sugar base C1' of the target uridine (Gu *et al.* 1999). Although Santi and co-workers proposed that the C6 is more plausible target, recent crystallographic studies argue against the proposal. Resent data indicate that the catalytic aspartate is in hydrogen bond contact distance with backbone O2' and side chain N3 atoms (Hoang *et al.* 2006; Alian *et al.* 2009).

Pseudouridine synthases induce extensive structural rearrangements upon binding to the substrate, flipping out several bases from stacking interactions, and forming some interactions between the PS and the substrate (Hoang *et al.* 2006; Alian *et al.* 2009). *E. coli* rRNA pseudouridine synthases all contain conserved RLD motif in their catalytic site (Figure 7A). Conserved arginine seems to play a key role in flipping out the substrate uridine (Hoang *et al.* 2006; Alian *et al.* 2009). All PS are believed to use the base flipping mechanism.

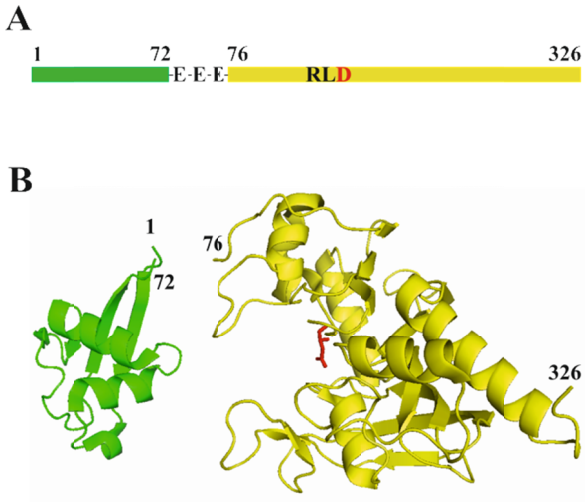

Figure 7. Structure of pseudouridine synthase RluD. Catalytic domain is colored yellow, S4-like domain is colored green, and catalytic aspartate (139) which is located in the bottom of the catalytic cleft is colored red. Numbers are referring amino acids corresponding to S4-like and catalytic domains. **A.** Primary structure of RluD. Linker region, connecting catalytic and S4-like domains contain three glutamic acid residues, shown as "-E-E-E-". **B.** Tertiary structure of RluD. Linker region is not visible due to the highly flexible nature**.** Illustration was created with PyMOL DeLano Scientific (PDB ID: 2IST).

General approach for studying pseudouridine synthases and effect of Ψ in *E. coli* has been gene knock-out experiments. PS deletion always results in lack of specific Ψ. Thus, there is no evidence that different PS can recognize the same uridine as a substrate (Ofengand 2002). However, *E. coli* contains seven rRNA specific PS for formation of all 11 Ψ (Ofengand & Del Campo 2004) (Table 1). Hence, some PS must exhibit multisite specificities and, indeed, pseudouridine synthases RluC and RluD catalyze formation of three Ψ in the 23S rRNA. RluC is specific for positions U955, U2504, U2580 and RluD is specific for positions U1911, U1915, U1917, located at the conserved H69 of the 23S rRNA (Huang *et al.* 1998a). Although RluC and RluD recognize more than one uridine as a substrate, the enzymes are still specific for the 23S rRNA. Pseudouridine syn-

thase RluA is the only PS known that is specific for two different substrate RNAs, because it modifies U746 of the 23S rRNA and U32 of tRNA (Wrzesinski *et al.* 1995). It is interesting to note that all three pseudouridine synthases, exhibiting multisite specificities, belong to the RluA family (Del Campo *et al.* 2001) (Table 1).

Table 1. E. coli rRNA pseudouridine synthases [(1)]

	Substrate RNA	Modification site [(2)]	S4-like tag	Catalytic Asp[(3)]	Time of action[(4)]	PDB ID
RsuA family						
RsuA	16S	516	+	102[(5)]	Early	IKSK
RluB	23S	2605	+	110[(6)]	Early	–
RluE	23S	2457	–	79[(6)]	Early	20ML
RluF	23S	2604	+	107[(6)]	Early	3DH3
RluA family						
RluA	23S/tRNA	746/32	–	16[(7)]	Early	2I82
RluC	23S	955, 2504, 2505	+	144[(8)]	Early	2XPI
RluD	23S	1911, 1915, 1917	+	139[(9)]	Late	1QYU

[(1)] Data collected from (Machnicka *et al.* 2013) and (Labarga *et al.* 2007).
[(2)] Modification sites according to *E. coli* rRNA sequence.
[(3)] Catalytic aspartate which mutation leads to complete inactivation of the enzyme.
[(4)] The *in vivo* assembly stage of the modification synthesis according to (Siibak & Remme 2010)
[(5)] According to (Conrad *et al.* 1999)
[(6)] According to (Del Campo *et al.* 2001)
[(7)] According to (Raychaudhuri *et al.* 1999)
[(8)] According to sequence similarity
[(9)] According to (Gutgsell *et al.* 2001)

One of the most intriguing questions about the pseudouridine synthases is their substrate specificity. Guide RNA system, used by eukaryotic PS-s, is quite well understood (Ge & Yu 2013). In prokaryotes, substrate selection is done in protein level. However, it is still unclear whether the prokaryotic PS proteins recognize a sequence, or structural element of substrate RNA. Multisite specific synthases are good candidates for study of the specificity in protein level. It is confirmed that RluA determines its substrate according to the RNA primary sequence, since crystal structure indicates for direct readout of the RNA sequence by the protein (Hoang *et al.* 2006) and all substrates for the RluA share consensus sequence U/ΨUXXAAA (X can be any nucleotide) (Wrzesinski *et al.* 1995). It is proposed that RluD recognizes the H69 as a structure and isomerizes

all uridines in its loop region (Ofengand 2002). Specificity of RluC is still completely unsolved phenomenon. No sequence or structure similarities have been determined for substrates of the RluC, nor are the substrates close proximity in tertiary structure (Huang *et al.* 1998a).

1.3.3. Pseudouridine synthase RluD

RluD (ribosome large subunit pseudouridine synthase D), previously named as YfiI, belongs to a RluA family, based on its protein sequence (Koonin 1996). RluD was first identified by two independent groups by its *in vitro* activity. RluD is responsible for synthesizing three pseudouridines into the 23S rRNA helix-loop 69 at positions 1911, 1915 and 1917 (Huang *et al.* 1998a; Raychaudhuri *et al.* 1998). As expected, deletion of the RluD-encoding gene, causes lack of all the pseudouridines in H69, but also resulting in severe reduction of growth (Raychaudhuri *et al.* 1998) and defects in ribosome assembly, biogenesis, and function (Ofengand *et al.* 2001; Gutgsell *et al.* 2005). Latter made RluD the only pseudouridine synthase known to be required for ribosomal assembly and function. Considering also that pseudouridines at positions 1915 and 1917 are highly conserved (Chapter 1.3.1) (Ofengand 2002) has made the RluD potentially very interesting object to study.

RluD exhibits high specificity for the positions 1911, 1915 and 1917 *in vivo* but loses some of its specificity *in vitro* (Huang *et al.* 1998a; Raychaudhuri *et al.* 1998; Wrzesinski *et al.* 2000; Gutgsell *et al.* 2005). RluD isomerizes significantly higher amount of uridines on *in vitro* transcribed rRNA at lower Mg^{2+} contestations. Thus, RluD is less specific *in vitro* and isomerizes uridines that are not its natural substrates (Huang *et al.* 1998a; Wrzesinski *et al.* 2000). It can be speculated that rRNA exhibits loosened structure at lowered Mg^{2+} concentrations and more uridines become accessible for the RluD. However, no RluD-dependent pseudouridines are found in tRNA after *in vitro* treatment. Thus, RluD still recognizes only rRNA as a substrate and the specificity is not completely lost (Huang *et al.* 1998a).

In the solution, RluD is a monomeric enzyme, and it contains two major domains, the N-terminal S4-like domain, named so due to its similarity to the ribosomal protein S4, and the C-terminal catalytic domain (Figure 7.A and B). The S4-like and the catalytic domains are connected with a very flexible linker (Mizutani *et al.* 2004; Sivaraman *et al.* 2004) (Figure 7.A). The S4-like domain of the RluD often appears to be disordered in electron density maps. At first, it was speculated that proteolysis occurs during crystallization process, but after determining the molecular mass of the crystalized protein, this speculation was ruled out and the absence of S4-like domain in RluD electron density map was confirmed as a result of the highly flexible nature of the domain (Del Campo *et al.* 2004). It has been proposed that the flexible linker allows the N-terminal S4-like domain of RluD to perform highly specific binding to the correct molecular target without producing very high affinity. In this way, the enzyme can modify

the appropriate three sites within the rRNA efficiently and avoid becoming trapped in unproductive enzyme-product complexes (Mizutani *et al.* 2004). The negatively charged S4-like domain of the RluD has been seen to occupy the positively charged cleft of the catalytic domain in the absence of substrate in the crystal structures. Yet, authors interpreted this as a crystal packing artifact, because in nature, the catalytic cleft is needed for RNA binding (Del Campo *et al.* 2004). However, it cannot be ruled out that the S4-like domain shields the catalytic cleft while the RluD has not performed proper binding to its substrate. RluD, lacking the S4-like domain, has been shown to lose some of its specificity, and could modify U2457 normally modified by RluE *in vitro* (Vaidyanathan *et al.* 2007). This result is in good agreement with the proposal that S4-like domain prevents the catalytic domain from unspecific binding to the rRNA. S4-like domain becomes ordered in crystals upon substrate binding, confirming that it is a RNA binding domain (Aravind & Koonin 1999). Also, if RluD is manually docked in an orientation that positions the uridine at position 1915 into the active site pocket of the RluD, the S4-like domain positions very close to the junction of three helixes (H68, H69 and H70) of 23S rRNA. Thus, this structure is possible recognition site for the RluD by the S4-like domain (Vaidyanathan *et al.* 2007).

Catalytic domain of RluD contains positively charged catalytic cleft with average dimensions 25Å long by 10Å wide by 14Å deep. Catalytic aspartic acid (Asp 139), which is the strictly conserved amino acid in all known pseudouridine synthases (Table 1), is located at the base of the catalytic cleft (Figure 7.B) (Del Campo *et al.* 2004; Sivaraman *et al.* 2004). So far, it hasn't been possible to crystallize the RluD in complex with small substrate analog. The failure is probably because RluD does not have an appreciable affinity for simple substrate analogues under the crystallization conditions (Mizutani *et al.* 2004). Docking experiments have revealed that catalytic cleft of RluD is big enough to accommodate substrate rRNA in several ways and does not provide a complementary surface to the H69 structure. Latter is probably the reason for the multisite specificity of RluD (Del Campo *et al.* 2004).

RluD has been the only rRNA pseudouridine synthase which was determined as significant for normal cell growth rate. Deletion of RluD causes 3–6 times reduction in cell growth and major ribosome assembly defects are seen in sucrose density gradient (Ofengand *et al.* 2001; Gutgsell *et al.* 2005). However, bacterial population lacking RluD and all three pseudouridines in H69 starts to grow normally after longer incubation in solid medium. It was speculated that such reversion occurred due to a second site mutation (Raychaudhuri *et al.* 1998; Gutgsell *et al.* 2005). First compensatory mutation was found from ribosomal termination factor 2 (RF2) at position 172, which is located near to the H69 when RF2 is bound to the ribosome, linking H69 pseudouridines to effective termination of the translation (Ejby *et al.* 2007). Latter is not surprising, because during translation many translation factors such as RF2 (Klaholz *et al.* 2003; Rawat *et al.* 2003), RF3 (Klaholz *et al.* 2004) and RRF (Agrawal *et al.* 2004) (Figure 3) bind very near to the H69. Further studies revealed that several

mutation in RF2 can compensate lack of Ψ in H69, including the mutation T246S which is known to be naturally occurring variation in bacterial RF2 (O'Connor & Gregory 2011). It was revealed that severe growth defect, caused by deletion of RluD, was specific for *E. coli* strain K12 and did not occur in *E. coli* strain B or *Salmonella enterica* (O'Connor & Gregory 2011). K12 strain has been widely used as a model organism for different studies. However, data shows that K12 contains variations in its genome that are unique for the strain (Dreyfus & Heurgue-Hamard 2011). RF2 in *E. coli* K12 contains threonine at position 246 which exhibits significantly lower activity in termination of protein synthesis than RF2 containing serine or alanine at the same position. T249 hasn't been found from any other organism than *E. coli* strain K12 (Dincbas-Renqvist *et al.* 2000). Ribosomal protein S7 contains extension in its C-terminal end which is probably due to the single point mutation in stop codon region. Extended S7 is target for the tmRNA tagging and degradation of the protein in RluD-deficient strain (Schaub & Hayes 2011). S7, containing C-terminal extension, enhances the RF2 T246 phenotypic effects in K12 strain in case of RluD deletion (Dreyfus & Heurgue-Hamard 2011; Schaub & Hayes 2011). Thus, lack of RluD activity has major effects on ribosome assembly and translation termination in the context of these two K12 specific mutations.

It was shown that expression of RluD with mutated catalytic aspartate in slow-growing RluD-deficient cells restores cell's growth. This was sufficient grounds for speculation that RluD has second function in cells, being for example a chaperon (Gutgsell *et al.* 2001). Unfortunately, this effect was not reproducible and was probably an artifact. Authors speculated that the observed effect was due to the reversion caused by the second site mutation in bacterial genome (Gutgsell *et al.* 2005).

2. RESULTS AND DISCUSSION

Objectives of current study

Pseudouridines are the most abundant modification found in rRNA and they are clustered around functionally important regions of the ribosome. RluD isomerizes three uridines in functionally important stem-loop of 23S rRNA, H69. Pseudouridine at position 1917, located in H69, is universally conserved through all kingdoms of life and other Ψs at positions 1911 and 1915 are also highly conserved. Although pseudouridines have been studied for decades, little is known about the function of pseudouridines during translation and how pseudouridine synthases recognize their substrates.

The main aims of the current study were as follows:

1. Definition of the substrate specificity of RluD for positions 1911, 1915 and 1917.
2. To identify which nucleotides in H69 are important for the specificity of the RluD.
3. To identify the time and order dependence of formation of three pseudouridines in H69.

2.1. Substrate specificity of pseudouridine synthase RluD (Ref. I and III)

Pseudouridine synthase RluD isomerizes uridines at positions 1911, 1915 and 1917, all located in highly conserved H69 of 23S rRNA (Huang *et al.* 1998a; Raychaudhuri *et al.* 1998). In order to investigate which elements in H69 determine the specificity of the RluD for its substrates, we used site directed mutagenesis to construct 23S rRNA variants, containing single point mutations at positions A1912, A1913, C1914, U1915, A1916, U1917, A1918 in the H69 loop region, U1911, A1919, and insertion of GC base pair in the stem region. All 23S rRNA variants contained also streptavidin aptamer in H25 for affinity purification (Leonov *et al.* 2003). Constructed *rrnB* operons were cloned under IPTG inducible *tac* promoter. For *in vivo* specificity studies, 23S rRNA variants were expressed in the wild type strain and in the ΔRlmH/ΔRluD double knockout strain for *in vitro* specificity studies. rRNA was extracted from 50S subunits, affinity purified from sucrose density gradient 70S or 50S fractions and analyzed with CMCT/alkali and primer extension method. MG1655 ribosomes or affinity purified 50S subunits containing the streptavidin aptamer served as controls. All 50S subunits used for *in vitro* specificity studies were dissociated from 70S ribosomes to avoid possible effects caused by incomplete assembly. Only ribosomes containing H69 extension by one GC base pair were purified from free 50S pool (Further information Ref. I and Ref. III Materials and methods).

2.1.1. RluD is highly specific for positions 1911, 1915 and 1917 *in vivo*

In order to test the hypothesis that RluD recognizes the H69 as a structure and isomerizes all uridines around the loop region (Ofengand 2002), we constructed 23S rRNA variants A1912U, C1914U, A1916U, A1918U, A1919U. Unfortunately, we were not able to purify ribosomes containing 23S rRNA variant A1918U probably due to their instability in strain used. Notably, none of the three kingdoms of life contains uridine at position corresponding to 1918 of LSU rRNA (Cannone *et al.* 2002). A1918 is part of the hydrogen bonding network in H69 (Schuwirth *et al.* 2005) and it is probable that pyrimidine at position 1918 of 23S rRNA causes instability of the 50S subunit *in vivo* and we were not able to isolate the corresponding 50S subunits. Ofengand has proposed that RluD may be specific for only the H69 and modifies all uridines located at loop region of the H69 (Ofengand 2002). Our results disconfirm this proposal. We have found that RluD is highly specific for only its natural positions 1911, 1915 and 1917 *in vivo*, despite of extra uridines introduced into the H69 loop region (Ref. I Figure 2). Surprisingly, mutations A1916U and A1916G caused loss of RluD specific pseudouridines in free 50S subunits and significant reduction in 70S ribosomes (Ref. I Figure 2 and 4). Effects, caused by mutation A1916 are further discussed in chapter 2.1.3.

2.1.2. RluD exhibits loosened substrate specificity on free rRNA

Previous studies have revealed that RluD isomerizes more uridines on *in vitro* transcribed 23S rRNA during prolonged incubation with RluD, indicating that RluD exhibits unspecific activity on free rRNA (Huang *et al.* 1998a; Wrzesinski *et al.* 2000; Vaidyanathan *et al.* 2007). RluD also isomerizes more uridines in rRNA at lower Mg^{2+} concentrations (Wrzesinski *et al.* 2000). rRNA structure is loosened at lower Mg^{2+} concentrations and RluD is probably able to access uridines, that are normally buried inside the rRNA structure. If this is the case, the catalytic domain of RluD is unspecific and isomerizes every uridine in reach, although with low efficiency. We studied RluD specificity *in vitro* on 23S rRNA using HPLC and CMCT/alkali treatment (Figure 8). RluD makes in total 10–12 pseudouridines into naked 23S rRNA which is either *in vitro* transcribed or extracted from 50S subunit of RluD-deficient strain, under our experimental conditions (Figure 8.A). It must be noted that 23S rRNA, extracted from 50S subunits containes seven pseudouridines before the RluD treatment. Thus, RluD isomerizes about 10–12 uridines in case of *in vitro* transcribed 23S rRNA and about 3–5 uridines in case of 23S rRNA extracted from 50S subunits. Pseudouridine specific bands are visible at positions 1911, 1915 and 1917 in case of 23S rRNA extracted from 50S ribosomes but not in case of *in vitro* transcribed 23S rRNA even after prolonged incubation with RluD (Figure 8.B). Thus, RluD completely loses its specificity on *in vitro* transcribed 23S rRNA. It must be noted that *in vitro* transcribed 23S rRNA contains no modifications and has never been properly folded, suggesting that correct structural elements for binding of the RluD have not been formed.

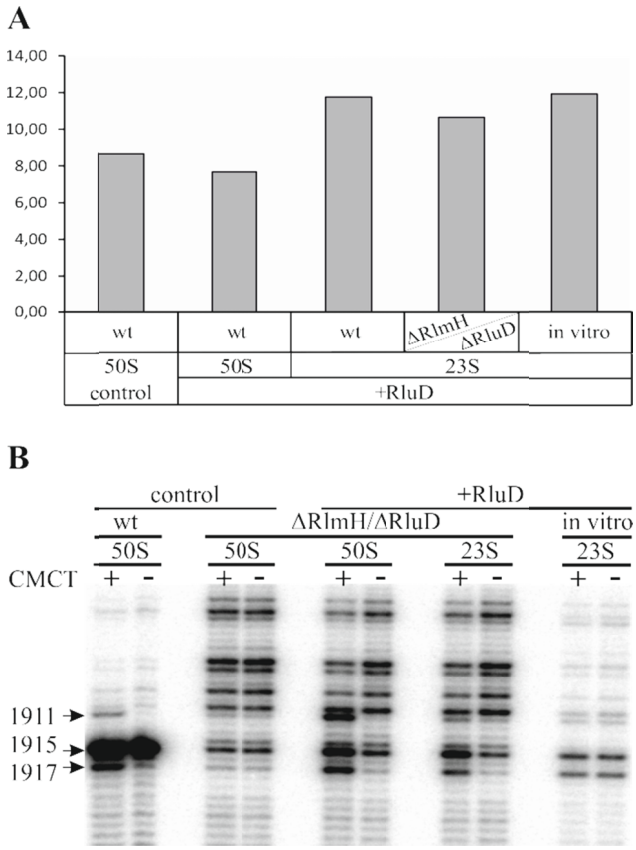

Figure 8. RluD *in vitro* specificity on different 23S rRNAs. **A.** Total amount of pseudouridines in 23S rRNA determined with HPLC. All RluD-modified free 23S rRNAs contain more pseudouridines compared with RluD-treated and untreated 23S rRNAs assembled into 50S subunits. **B.** Mapping of the pseudouridines in the H69 with CMCT/alkali and praimer extension analysis. Wild type (wt) and ΔRlmH/ΔRluD double knockout 50S ribosomes served as a control. ΔRlmH/ΔRluD double knockout 50S subunit, 23S rRNA and *in vitro* transcribed 23S rRNA was treated with RluD *in vitro*. Bands corresponding to positions 1911, 1915 and 1917 are shown. (For details see Ref. I Figure 1).

To our big surprise, uridine at position 1916 becomes substrate for RluD *in vitro*, although pseudouridine specific bands indicate for significantly reduced amount of all RluD-specific pseudouridines, compared to 50S subunits containing wild type 23S rRNA (Ref. III Figure 2B) (Further discussed in chapter 2.1.3). As discussed in chapter 2.1.1, 23S rRNA variant A1916G exhibited similar effect on synthesis of RluD specific pseudouridines as A1916U *in vivo*. Unfortunately, we were not able to isolate 50S containing A1916G rRNA variant, for comparison with A1916U *in vitro*, probably due to the assembly

defects and/or instability of mutant ribosomes in ΔRlmH/ΔRluD double knock-out strain.

Differences between *in vivo* and *in vitro* substrate specificities suggest that RluD may need extra factors for the specificity. To test a possibility that 30S subunit is contributing to substrate recognition of the RluD, we modified 50S subunits with RluD *in vitro* with and without presence of 30S. 12 mM and 20 mM Mg^{2+} was used to minimize association defects of mutant 50S particles. 30S had an inhibitory effect for RluD-directed pseudouridine synthesis on both wild type and mutant 50S subunit containing A1916U rRNA (Ref. III Figure 3). This data suggests that RluD isomerizes its substrates at very late step of 50S subunit assembly (Ref. I Figure 5) but before the first round of translation.

2.1.3. Nucleotide at position 1916 in H69 of 23S rRNA influences the specificity of the RluD

Appearance of RluD specific polymerase stop signals indicate that base alternation at positions A1912, A1913, C1914, A1918 and A1919 have no detectable effect on RluD specificity *in vivo* (Ref. I Figure 2 and 4) or *in vitro* (Ref. III Figure 2B). A1916 was the only position in H69 found to alter the RluD *in vivo* and *in vitro* specificity.

H69 containing uridine at position 1916, turns out to be poor substrate for the RluD *in vivo* (Ref.I Figure 2) and *in vitro* (Ref. III Figure 2B) as judged by the reduced RluD-specific Ψ band intensities. 23S rRNA A1916G and A1916U variants lack RluD specific pseudourdines in the free 50S particles, but contain reduced amount of pseudouridines when entered into 70S pool (Ref. I Figure 2 and 4). This indicates that RluD needs more time for productive binding to A1916G(U) variant of the H69 and that 23S rRNA, which contains Ψ in H69 has a significant advantage in subunit association and initiation of translation. Surprisingly, A1916C substitution mutation caused no effect on the specificity of the RluD (Ref. I Figure 4). Notably, cytidine at position, corresponding to *E. coli* 23S rRNA position 1916, is common in Archaea (Cannone *et al.* 2002).

Base alternation A1916G is the only known mutation in H69 that causes 50S assembly defect according to the sucrose density gradient centrifugation (Liiv *et al.* 2005). It is known from previous studies that loss of pseudouridines, synthesized by RluD, causes 50S assembly defects (Ofengand *et al.* 2001; Gutgsell *et al.* 2005). The effect was later shown to be specific for *E. coli* strain K12 (O'Connor & Gregory 2011), the strain used in our study as well. However, assembly defect, caused by mutation at position 1916, is not caused by the loss of pseudouridines from H69, since mutation A1916U has stronger effect on pseudouridine formation than mutation A1916G (Ref. I Figure 2 and 4) but has no effect on the 50S subunit assembly (Figure 9).

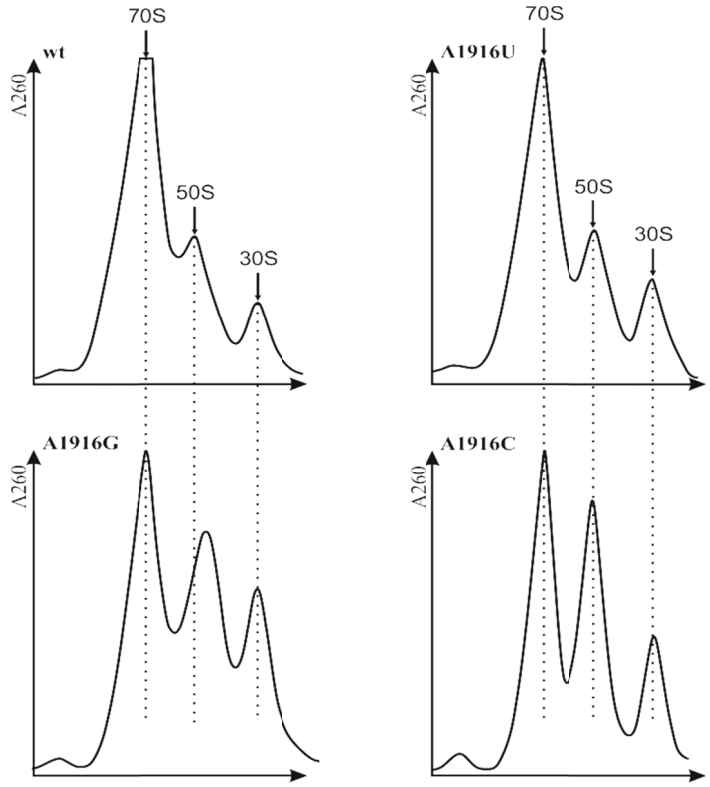

Figure 9. Sucrose density gradient profiles from cells expressing different A1916 rRNA variants. 70S, 50S and 30S ribosomal fractions are indicated. A1916G 50S subunit peak is widened and shifted toward 30S subunit fraction, indicating problems in 50S subunit assembly. A1916C and A1916U are showing increased amount of free subunits but no assembly defects.

Ribosome crystal structure gives a clue why the position 1916 has effect on substrate specificity of the RluD. U1916 is located between two natural substrates for RluD, U1915 and U1917 (Ref. III Figure 5). Also, nucleotide at position 1916 shares the orientation of these uridines, placing the U1916 very near to the catalytic aspartate of the RluD (Ref. III Figure 5). It must also be noted that according to crystal structure, all RluD natural substrates at positions 1911, 1915 and 1917 are located on the side of the H69 that faces the peptidyl-transferase cleft, whereas all other nucleotides at other positions we studied (except 1916), tend to be located on the opposite side of the H69 (Ref. III Figure 5). Position and orientation of A1916 gives enough ground for proposal that A1916 is probably making contact with RluD needed for productive binding of the catalytic domain. Base substitution A1916 to G or U abolishes the contact between RluD and H69 and has therefore negative effect on synthesis of RluD-dependent pseudouridines. U at position 1916 makes no contact with the RluD but is oriented toward the catalytic amino acid, thus it can be isomerized to

pseudouridine. It is known that pseudouridine synthases induce extensive structural rearrangements upon binding to the substrate (Hoang *et al.* 2006; Alian *et al.* 2009) and position 1916 may have a key role in this structural rearrangement.

We speculate that difference between *in vivo* and *in vitro* specificity of RluD at position 1916 is caused by different experimental conditions. RluD acts during the late step of 50S assembly when all LSU proteins are present and final sedimentation coefficient is acquired (Ref. I Figure 5). Formation of 50S particle takes about a minute, but additional 1–2 minutes are needed before freshly assembled 50S subunit participates in translation (Lindahl 1975). Probably final maturation of the 50S ribosomal structure takes place during this time. Thus, RluD has only 1–2 minutes for isomerization of its substrates and there is also much lower RluD concentration inside the cells than in our *in vitro* experiments. Catalytic values for RluD were determined in Ref. II by Rya Ero (Ref. II Table 2) and RluD catalytic values fit perfectly to the 1–2 minute time window for isomerization reactions to occur. Notably, we used much longer incubation time and high enzyme concentrations for *in vitro* isomerization reactions in experiments of the substrate specificity of the RluD.

This data suggests that A1916 is important recognition element for the RluD catalytic domain. Considering that 23S rRNA A1916C substitution retained the specificity of the RluD, we propose that the extra-cyclic amino group of A and C at position 1916 makes a contact with RluD during the binding of the catalytic domain of the enzyme. Lack of the hydrogen bond donor in case of A1916G and A1916U makes the H69 poor substrate for the RluD.

2.1.4. S4-like domain of the RluD is necessary for initial binding to the substrate

We used 50S ribosomes, containing extended H69 stem region by one base pair, to determine whether the length of the stem region is important for the RluD specificity *in vitro*. According to our results, stem extension makes H69 poor substrate for the RluD as judged by the lack of Ψ at position 1911 (Ref. III Figure 2C). This indicates that U1911 becomes out of reach for RluD after stem extension, suggesting that the H69 loop region is not primary specificity determinant for RluD. S4-like domain of the RluD positions close to the base of the H69, containing junction of three helices, helix 68, 69 and 70 when the RluD is manually docked to the ribosomal crystal structure in a way that U1915 is in the catalytic cleft (Vaidyanathan *et al.* 2007). Notably, r-protein S4 has been shown to bind to the rRNA three helix junction (Powers & Noller 1995) and, as RluD S4-like domain is structural analog of the r-protein S4, it is predicted to bind to a helix junction as well.

To test whether the junction of the helices 68, 69 and 70 could be the initial binding site for the RluD, we constructed RluD mutant by deleting two out of three glutamate residues from the linker region which connects the catalytic

domain to the S4-like domain. Shortened RluD couldn't isomerize U at position 1911 revealing the same modification pattern as 23S rRNA with extended H69 stem region (Figure 10). This result is consistent with the proposal that the S4-like domain binds to the 3-helix junction close to the base of the H69.

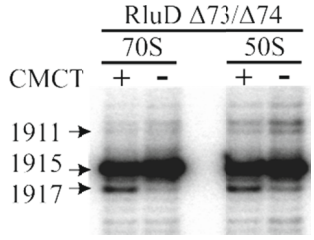

Figure 10. Praimer extension analysis of 23S rRNA H69. 23S rRNA was purified from RluD-deficient cells expressing RluD mutant lacking two glutamate residues from its linker region connecting catalytic and S4-like domains (For further information see Ref. I Figure 1).

Our results suggest for the two-step binding mechanism of the RluD to the ribosome. S4-like domain is responsible for the initial binding of the RluD and is necessary for directing the catalytic domain to the H69. Catalytic domain performs second binding which directs the H69 into the catalytic cleft in its right conformation.

Nucleotide at position 1916 is probably necessary for proper binding of the RluD catalytic domain. Our results support the speculation that A1916 makes contacts with the RluD. Although U at position 1916 is not able to make the contact with RluD catalytic domain, needed for the specificity, still the initial binding of the S4-like domain guides the catalytic amino acid near to the tip of the H69 and causes isomerization of U at position 1916 after prolonged incubation. Lack of the 1916-RluD contact causes unstable binding of RluD to H69, causing reduced efficiency in isomerization of all the uridines. Alternative it is possible that A1916 is important for maintaining the proper conformation of the H69.

Some crystals have revealed that the S4-like domain occupies the catalytic cleft of RluD while the enzyme is not bound to its substrate (Del Campo *et al.* 2004). Authors speculated that this was a crystal packing artifact because S4-like domain is known to bind RNA. Still, it could be possible that S4-like domain occupies the catalytic cleft until it locates its proper rRNA binding site and vacates the catalytic cleft for substrate binding after S4-like domain has performed its initial binding. In this case, lack of proper binding site would completely abolish RluD activity. Our results are in agreement with the assumptions that S4-like domain of RluD is for RNA binding and it is not bound to the catalytic cleft while RluD is not bound to its substrate. It is possible that *in vitro* transcribed 23S rRNA has no proper binding site for the S4-like domain of

RluD that directs the catalytic domain to its substrates. Our results suggest that proper binding site for the RluD S4-like domain becomes available after the 50S subunit is fully assembled, and binding of RluD to its substrate is ineffective before that. This would explain the fact that RluD-synthesized pseudouridines are formed during the late step of the ribosome maturation *in vivo* (Ref. I Figure 5) and why prolonged incubation and excess of the enzyme reduces the RluD specificity on 23S rRNA (Figure 8) and also on 16S rRNA *in vitro* (Huang *et al.* 1998a). It has also proposed that RluD lacking the S4-like domain would isomerize uridines that normally are substrates for other pseudouridine synthases (Vaidyanathan *et al.* 2007). This proposal remains to be examined.

2.2. Time and order dependence of formation of three Ψ's into H69 (Ref. I and II)

All pseudouridine synthases contain universally conserved aspartic acid on the bottom of their catalytic cleft. Mutating this aspartic residue inactivates the enzyme (Del Campo *et al.* 2001; Ferre-D'Amare 2003; Sivaraman *et al.* 2004). Several catalytic mechanisms are proposed for Ψ formation (Gu *et al.* 1999; Hoang *et al.* 2006; Alian *et al.* 2009) but the exact mechanism is still unknown. All pseudouridines in H69 are located very close together in tertiary structure (Ref. III Figure 5) but it is unlikely that RluD isomerizes all three pseudouridines simultaneously because RluD contains only one catalytic aspartate. We can speculate that RluD has an affinity for only one uridine in the H69 and all other pseudouridines are accidental coproducts. This would explain the N3 methylation of Ψ1915 which eliminates the extra hydrogen bond donor occurred with Ψ formation. To test if impeding of one pseudouridine formation inflicts formation of others, we constructed several substitution mutations U1911C, U1915C, U1917C, U1911C/U1915C. Analysis of the mutant 23S rRNAs revealed that particular mutation prevented pseudouridylation only at position of the base substitution but not elsewhere in the H69, indicating that all pseudouridines are synthesized autonomously and independent of each other (Ref. I Figure 3).

2.2.1 RluD isomerizes its substrate uridines concurrently

To study if the synthesis of Ψ in the H69 follows some kind of order, we determined the time course of *in vitro* isomerization of 1911, 1915 and 1917. 50S ribosomes, purified from ΔRlmH/ΔRluD double knockout strain were incubated with RluD for 60 to 140 seconds at 25ºC (Ref. II Figure 5). In our *in vitro* experiments, all pseudouridines in H69 appear concurrently over time upon RluD treatment of the 50S subunit, meaning that they are synthesized at similar rate and stochastically, rather than in any specific order (Ref. II Figure 5). This result is in agreement with our *in vivo* results where we expressed 23S

rRNA variants containing U-to-C transitions at positions 1911, 1915 or 1917 (Ref. I Figure 3). Substitution of uridine by cytidine leads to the loss of pseudouridine at the mutated position but synthesis of other two pseudouridines are not affected (Ref. I Figure 3). Latter indicates that RluD has no strict order for modification of its substrates and isomerization of one uridine is not required for synthesis of others. These results suggest that RluD either makes all pseudouridines upon one productive binding or makes one pseudouridine in random order within every binding to its substrate. Latter suggestion is supported by the results of Del Campo et al, who showed that catalytic cleft of RluD is big enough to accommodate H69 in several ways and does not provide complementary surface to the H69 structure (Del Campo *et al.* 2004).

CONCLUSIONS

Pseudouridines are the most abundant posttranscriptional modifications known and they have been found in functional RNAs like tRNA, rRNA and snoRNA. Pseudouridines are clustered around functionally important rRNA regions. Although Ψ were found over five decades ago (Davis & Allen 1957; Cohn 1960), little is known about their functions in RNA. Pseudouridine synthases are responsible for formation of pseudouridines. The main focus of the current study was substrate specificity of pseudouridine synthase RluD. RluD is known to synthesize three pseudouridines in the conserved helix-loop structure of 23S rRNA, H69. Two pseudouridines, at positions 1915 and 1917, are known to be universally conserved.

Based on the results obtained, we conclude:

I. RluD is specific for positions 1911, 1915 and 1917 of 23S rRNA. RluD retains its *in vivo* specificity for positions 1911, 1915 and 1917 even if extra uridines are introduced into loop region of H69. The data overrules the previous hypothesis that RluD recognizes H69 as a structure and isomerizes all uridines located around the loop region.

II. RluD exhibits loosened specificity on naked 23S rRNA making considerably more pseudouridines into free 23S rRNA than into assembled 50S subunit. This indicates that catalytic domain of RluD exhibits unspecific nature.

III. Position 1916 is the only important recognition element for RluD specificity in H69 loop region. Mutations at position 1916 reduce the RluD activity. A1916 is necessary for making contact with RluD catalytic domain or presenting its substrates in correct way.

IV. RluD exhibits two-step binding to the substrate and S4-like domain is responsible for initial binding of the RluD.

V. All three pseudouridines at positions 1911, 1915 and 1917 appear concurrently and no pseudouridine formation is required for formation of others.

SUMMARY IN ESTONIAN

Multisait spetsiifilise pseudouridiini süntaasi RluD spetsiifika

Iga raku elutegevuse üks olulisemaid protsesse on valgusüntees, mille käigus mRNAs kodeeritud nukleotiidse järjestuse järgi sünteesitakse vastavalt geneetilisele koodile valkude aminohappeline järjestus. Valgusünteesi eest vastutab valkudest ja RNA-st koosnev kompleks, ribosoom. Bakterialne ribosoom koosneb kolmest ribosomaalsest RNA-st (rRNA) ja reast valkudest. rRNA moodustab umbes 2/3 ribosoomi massist ja on evolutsioonis üsna konserveerunud nii nukleotiidse järjestuse, aga eriti ruumilise struktuuri tasemel. Lisaks konserveerunud struktuurile sisaldab rRNA ka mitmeid posttranskriptsiooniliselt modifitseeritud nukleotiide, millest enamus on konserveerunud. Vaatamata sellele, et rRNA modifikatsioonide paiknemine primaar-, sekundaar- ja tertsiaarstrukutuuris on olnud teada juba mõnda aega, on nende funktsiooni kohta väga vähe andmeid.

Pseudouridiinid (Ψ) on enim levinud RNA posttranskriptsioonilised modifikatsioonid looduses. Pseudouridiinid sünteesitakse isomerisatsiooni reaktsiooniga uridiinist, samas kasutamata selleks lisaenergiat ega -faktoreid. Isomerisatsioonireaktsiooni viivad läbi pseudouridiini süntaasid, mis muudavad oluliselt substraadiks oleva nukleotiidi omadusi samas lisamata sellele ühtegi keemilist rühma. Bakterirakkudes vastutab ensüüm nii isomerisatsioonireaktsiooni katalüüsimise kui ka õige uridiini valimise eest.

E. coli ribosoomi suurema subühiku RNA (23S rRNA) sisaldab 10 pseudouridiini, millest üks on veel lisaks metüleeritud. Neid pseudouridiine sünteesivad kuus pseudouridiini süntaasi (RluA-RluF), millest kaks (RluC ja RluD) isomeriseerivad kumbki kolme uridiini. Pseudouridiini süntaas RluD on juba pikka aega olnud teadlaste kõrgendatud tähelepanu all, sest erinevalt enamikust modifikatsiooniensüümidest on RluD multispetsiifiline ensüüm, mis katalüüsib Ψ tekkimise 23S rRNA positsioonidesse 1911, 1915 ja 1917. Need positsioonid asuvad kõik väga kõrgelt konserveerunud rRNA elemendis „juuksenõelas“ 69 (H69). Ribosoomide funktsioneermise seisukohalt on H69 äärmiselt oluline struktuur ning positsioonides 1915 ja 1917 paiknevad Ψ on äärmiselt konserveerunud. Lisaks kõigele on RluD ainuke pseudouridiini süntaas, mille rakust eemaldamine põhjustab raku elutegevuses tõsiseid häireid. Vaatamata varasematele uuringutele ei ole senini suudetud üheselt välja selgitada, et kuidas tunneb RluD ära oma substraadid ja milleks täpselt on vaja tema poolt sünteesitud pseudouridiine?

Käesolevas töös uurisime RluD substraadispetsiifikat *in vivo* ja *in vitro* ning peamised tulemused on järgmised:

1. *In vivo* on RluD spetsiifiline vaid positsioonide 1911, 1915 ja 1917 suhtes. Isegi kui kunstlikult viia H69-sse lisaks uridiine, tunneb RluD substraatidena ära vaid uridiine mis asuvad positsioonides 1911, 1915 ja 1917. See tulemus lükkab ümber varem püstitatud hüpoteesi, et RluD tunneb ära vaid H69 struktuuri ja isomeriseerib ebaspetsiifiliselt kõiki seal leiduvaid uridiine.

2. RluD on *in vitro* oluliselt ebaspetsiifilisem kui *in vivo*, sünteesides valkudega mitteseondunud rRNA-sse oluliselt rohkem pseudouridiine kui valmis ribosomaalsetesse subühikutesse. Seega on RluD katalüütiline osa võimeline ebaspetsiifiliste substraatide sidumiseks ja spetsiifilisuse määramisel on oluline osa ka S4-tüüpi osal ja rRNA struktuuril.
3. H69-a positsioonis 1916 olev nukleotiid on ainuke nukleotiidne determinant, mis mõjutab RluD spetsiifilisust oma substraatide suhtes. Mutatsioonid positsioonis 1916 vähendavad oluliselt RluD katalüütilist aktiivsust. Ilmselt on positsioonis 1916 olev nukleotiid oluline, et kontakteeruda RluD-ga.
4. RluD seondub oma substraadile kahes etapis ja tema S4-sarnane domeen on oluline esmaseks seondumiseks.
5. Kõik RluD poolt sünteesitud pseudouridiinid ilmuvad RNA ahelasse samaaegselt, mis viitab sellele et, erinevate pseudouridiinide süntees on üksteisest sõltumatu. RluD isomeriseerib oma substraadid järjest, ühe seondumise jooksul, või siis isomeriseerib iga seondumisega ühe uridiini. Mõlemal juhul isomeriseerib RluD uridiine suvalises järjekorras.

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Peamised uurimisvaldkonnad:
Ribosoomi biosüntees, rRNA pseudouridiinid ja rRNA pseudouridiini süntaasid.

Publikatsioonide loetelu:

Leppik, M; Peil, L; Kipper, K; Liiv, A; Remme, J: Substrate specificity of the pseudouridine synthase RluD in Escherichia coli. FEBS J 2007, 21: 5759–66.

Ero, R; Leppik, M; Liiv, A; Remme, J: Specificity and kinetics of 23S rRNA modification enzymes RlmH and RluD. RNA 2010, 11:2075–84.

Leppik, M; Ero, R; Liiv, A; Kipper, K; Remme, J: Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in Escherichia coli 23S rRNA. Biochimie 2012, 94(5):1080–9.

Konverentsid:

2008	NordFrosk RNA Network Meeting, Helsinki, suuline ettekanne
2009	Ribosome Synthesis, Regensburg, posterettekanne
2010	Ribosomes 2010, Orvieto, posterettekanne

Õppetöö:

Nukleiinhapete keemia praktikumi juhendamine
Valgu biosüntees, 1 loeng

Juhendamine:

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