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ARTO PULK

Studies on bacterial ribosomes by chemical modification approaches

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CONTENTS

LIST OF ORIGINAL PAPERS

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

I. **Maiväli U., Pulk A., Loogväli E.L., Remme J**. 2002**.** Accessibility of phosphates in domain I of 23 S rRNA in the ribosomal 50 S subunit as detected by R(P) phosphorothioates. Biochim Biophys Acta. 1579(1):1–7.

II. **Pulk A., Maiväli U., Remme J**. 2006. Identification of nucleotides in E. coli 16S rRNA essential for ribosome subunit association. RNA. 12(5):790–6.

III. **Pulk A., Liiv A., Peil L., Maiväli U., Nierhaus K., Remme J**. 2009. Ribosome reactivation by replacement of damaged proteins. Mol Microbiol. 75(4): 801–814.

My contribution to the articles is as follows:

Ref. I carried out most of laboratory experiments, analyzed the data and participated in the modelling of the data into the structure of *D. radiodurans* 50S.

Ref. II carried out all laboratory experiments, analyzed the data, and participated in writing of the manuscript.

Ref. III carried out *in vitro* ribosome recovery experiments, and identification of exchangeable ribosomal proteins by radiolabeling experiments, analyzed the data, participated in writing of the manuscript.

LIST OF ABBREVIATIONS

aa-tRNA	aminoacyl- tRNA
ASF	A-site finger
ASL	tRNA anticodon stem-loop
ATP	adenosine triphosphate
B	bridge
CMCT	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate
CP	central protuberance
cryo-EM	cryo-electron microscopy
CTD	carboxy-terminal domain
DEPC	diethylpyrocarbonate
DMS	dimethylsulfate
EF-G	elongation factor G
EF-Tu	elongation factor Tu
eIF2α	eukaryotic initiation factor 2α
GAC	GTPase-associated center
GAR	GTPase-associated region
G-protein	GTP-binding protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
Hsp70	heat shock protein 70
IF1	initiation factor 1
IF2	initiation factor 2
IF3	initiation factor 3
L-proteins	ribosomal large subunit proteins
LSU	ribosomal large subunit
MIC	minimum inhibitory concentration
mRNA	messenger RNA
NTD	amino-terminal domain
PDB	protein data bank
(p)ppGpp	guanosine pentaphosphate
PTC	peptidyl transferase center
RF3	release factor 3
RP	ribosomal protein
RRF	ribosome recycling factor
SD	Shine-Dalgarno
S-proteins	ribosomal small subunit proteins
SRL	sarcin-ricin loop
SRP	signal recognition particle
TP70	total proteins of 70S ribosome
tRNA	transfer RNA
Ψ	pseudouridine

INTRODUCTION

The ribosome is a macromolecular assembly that is responsible for protein biosynthesis following genetic instructions in all organisms. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. Proteins also have structural or mechanical functions, such as actin and myosin in the muscle and the proteins of the cytoskeleton, which form a scaffold that maintains cell shape. Other proteins are important in cell signaling, immune responses, cell adhesion, and the cell cycle. The ribosome itself consists of 50 to 80 proteins that mainly function as structural proteins by stabilizing and folding of ribosomal RNA. The RNA molecule in the ribosome is the catalytic part of the ribosome that is responsible for decoding the genetic code and catalyzing peptide bond formation between amino acids. In these two main steps of ribosome mediated translation, ribosomal proteins support optimal functioning of the ribosome.

Nowadays there is a wide range of structural data available on ribosomes freezed at the different stages of translation. It is possible to compare biochemical results with the available structural data. But yet, the exact mechanism and which ribosome or ligand components are needed to catalyze peptide bond formation etc. are unknown. Forexample, the peptide bond formation by the ribosome is an aminolysis of an acyl-ester bond in the P site. The reaction begins with a nucleophilic attack of the α-amino group of the aminoacyl-tRNA bound in the A site onto the carbonyl carbon of the peptidyl-tRNA positioned in the P site and it proceeds through a tetrahedral oxyanion intermediate. The O2′ hydroxyl and O3′ oxygen of A76 of the P-site tRNA as well as the α-amino group of the aminoacyl-tRNA are important for peptidyl transfer reaction (Lang et al., 2008; Simonović and Steitz, 2009). It is proposed that a water molecule coordinated by the ribosomal bases (A2602 and U2584) stabilizes the oxyanion of the tetrahedral intermediate in the peptide bond formation (Simonovic and Steitz, 2009). But there is no crystal structure available where this water molecule is resolved. Therefore, higher resolution structures are needed to catch a water molecule in action. Another step forward in structural biology would be high resolution videos of ribosome in action. But this technology is out of reach right now, we only can put together video of freezed ribosomes in the different stages of translation. As ribosome is dynamic structure, lots of interactions in the ribosome brake and form during translation. Therefore, so-called intermediate structures exist between well known stages of translation. To crystallize these short-living intermediates you need to freeze critical amounts of ribosomes in particular conformation. To solve this problem, biochemical studies are needed to produce ribosomes freezed in a specific conformation. Some of ribosome-ligand (antibiotic, mutated factors etc.) complexes or mutated ribosomes can reveal these intermediate structures of ribosome.

However, structures by themselves do not reveal which chemical groups are functionally important. They indicate chemical groups that are potentially close to reaction centres. As the ribosome is a large macromolecular complex, it

contains lots of internal and additional interactions with factors or other ligands. Therefore, biochemical experiments coupled with structural study are needed to investigate the mechansims of specific processes.

The present dissertation focuses on structural aspects of the ribosome and the roles of ribosomal proteins. Ribosome is a macromolecular complex consisting of two subunits and over 30 interactions are formed when these two subunits associate (Gabashvili *et al*., 2000; Yusupov *et al*., 2001; Gao *et al*., 2003; Schuwirth *et al*., 2005). The functional importance of these intersubunit interactions is the focus of paper II.

In order to study RNA backbone interactions in the ribosome, we combined different assays like *in vitro* T7 transcription, *in vitro* 50S reconstitution and primer extension to generate a reliable approach to this issue (paper I).

In addition, because the ribosome is made of many individual proteins, we studied the ability of ribosomal proteins to exchange and restore the function of damaged ribosomes (paper III).

REVIEW OF THE LITERATURE

1. Ribosome structure

Prokaryotic ribosomes sediment at 70S (MW ≈ 2.6 x 10^6), and contain ~54 different proteins, 23S rRNA, 16S rRNA, and 5S rRNA (Moore, 1998). The prokaryotic ribosome contains about two-thirds RNA and one-third protein and consists of two subunits, the larger (50S) of which is approximately twice the molecular weight of the smaller (30S). First ribosome crystals from several organisms were obtained in the early 1990s (Arad *et al*., 1983; Trahhanov *et al*., 1989; Bohlen *et al*., 1991).

The small subunit mediates the interaction between mRNA codons and tRNA anticodons on which the fidelity of translation depends. The large subunit includes the activity that catalyzes peptide bond formation (peptidyl transferase) and the binding site for the G-protein (GTP-binding protein) factors that assist in the initiation, elongation, and termination phases of protein synthesis. A major understanding of the structural-mechanism of translation was achieved a decade ago when high-resolution structures of the 50S and 30S ribosomal subunits were solved (Ban *et al*., 2000; Wimberly *et al*., 2000).

1.1. Structural features of the small ribosomal subunit

30S subunit. The *E. coli* 30S subunit comprises of 16S rRNA (1542 nucleotides, helices 1–45) and 21 proteins (S-proteins). The small ribosomal subunit contains a decoding centre that binds mRNA and tRNA anticodon stem-loops for decoding genetic information.

The secondary structure of 16*S* rRNA (Figure 1) is divided into four recognizable domains, called the 5′, central, 3′-major and 3′-minor domains (Woese *et al*., 1980; Woese *et al*., 1983; Gutell *et al*., 1985). The secondary structure domains of 16S rRNA correspond to the three-dimensional domains that are nearly structurally autonomous (Yusupov *et al*., 2001; Gao *et al*., 2003) (Figure 2). The 5′ domain represents the major part of the body, the central domain most of the platform, and the 3′ major domain constitutes the head (Wimberly *et al*., 2000). The 3′ minor domain is the only significant exception to this rule, as it is part of the body at the subunit interface. The four domains of the 16S rRNA secondary structure radiate from a central point in the neck region of the subunit, and are especially tightly associated in this area, which is functionally the most important region of the 30S ribosomal subunit. This organization suggests that the domains are designed to move relatively to one another during protein synthesis. In particular, the very minimal interaction between the head and the rest of the subunit is consistent with movement of the head during translocation (Frank and Agrawal, 2000; Gao *et al*., 2003; Schuwirth *et al*., 2005; Zhang *et al*., 2009).

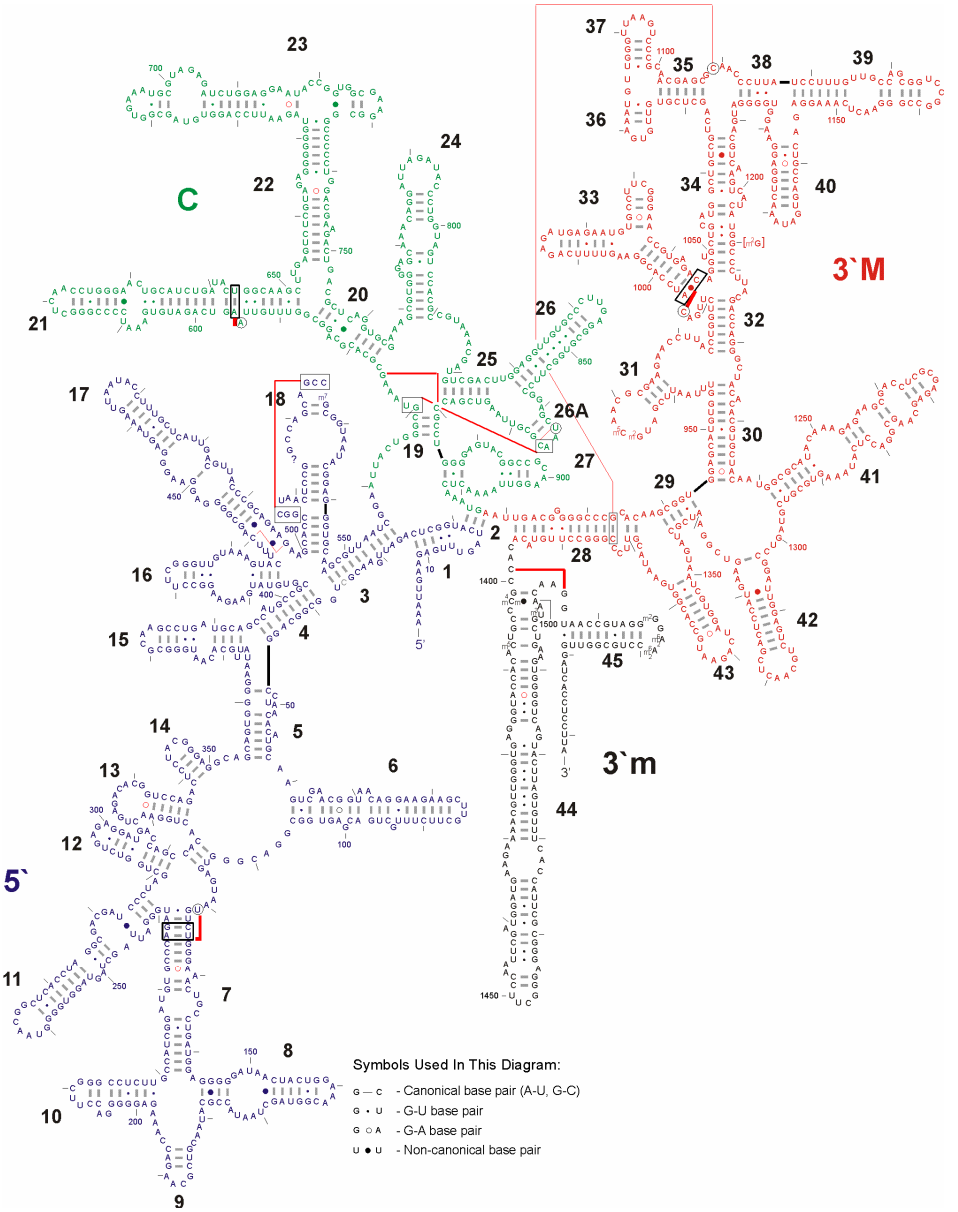


Figure 1. Secondary structure of *Escherichia coli* 16S rRNA, with its 5´, central, 3´-major, and 3´-minor domains shaded in blue, green, red, and black, respectively. 45 helical elements are numbered and used throughout the text. Secondary structure is downloaded from http://www.rna.ccbb.utexas.edu/.

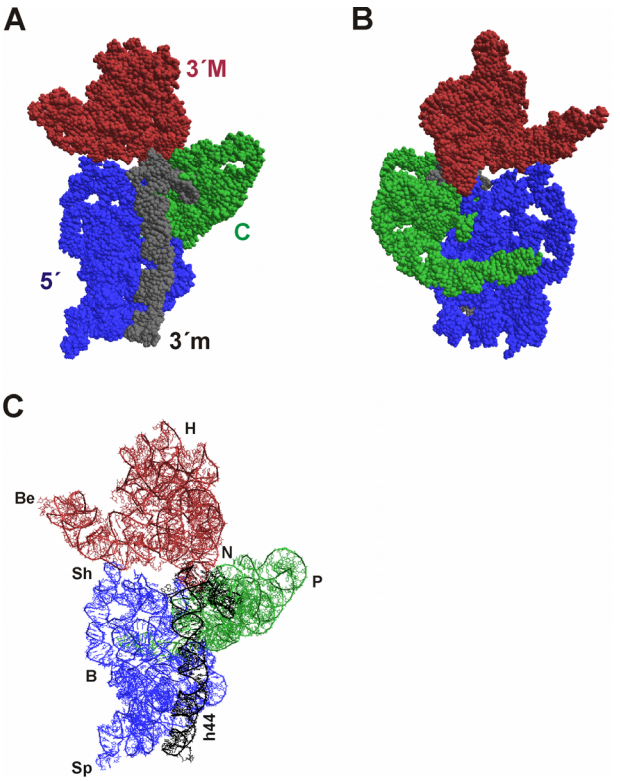


Figure 2. Tertiary structure of *Thermus thermophilus* 16S rRNA. (A) Three-dimensional fold of 16S rRNA in 30S subunit, with its domains coloured as in figure 1. Interface of the 30S subunit faces the reader. (B) Surface-side view of 16S rRNA in the 30S subunit. (C) 30S subunit morphological features. Interface view of 16S rRNA in the 30S subunit. H, head; Be, beak; N, neck; P, platform; Sh, shoulder; Sp, spur; Bo, body and h44. PDB co-ordinate 2WRI is used and modelled by PyMol.

Proteins in the small subunit are concentrated in the head, sides and surface of the 30S subunit (Figure 3). The 30S interface-side is largely free of proteins, with the exception of protein S12 which lies near the decoding site at the top of the long helix 44 (h44) that runs down the interface. Other proteins lie at the periphery of the subunit interface, allowing them to make contact with the 50S subunit.

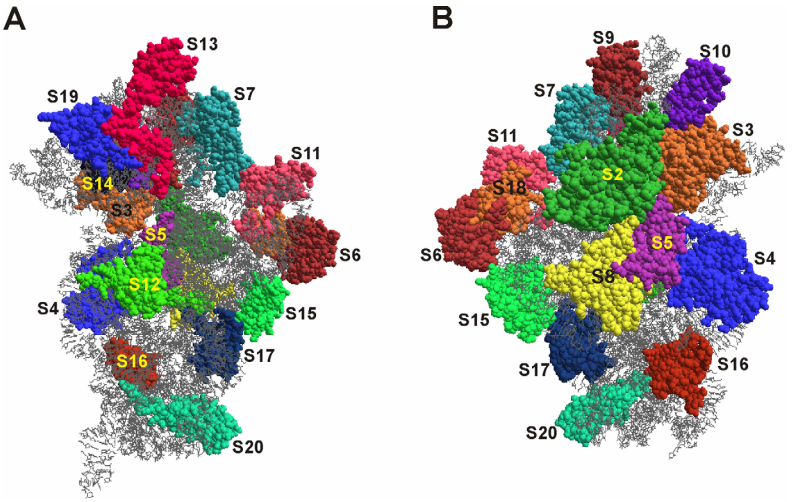


Figure 3. Location of *T. thermophilus* small subunit proteins in the 30S subunit. (A) Interface view of 30S subunit. Small ribosomal proteins are in spacefill and coloured, 16S rRNA is in a gray wireframe structure. (B) Surface view of 30S subunit. PDB coordinate 2WRI is used and modelled by PyMol.

Most of the 16S rRNA may be described as helical (helices 1 to 45) or approximately helical, and it is useful to consider the RNA structure as a three-dimensional arrangement of helical elements. Interactions between helical elements include stacking of neighbouring helical sequences, and horizontal packing of helices, usually between their minor grooves. Short single-stranded rRNA segments make idiosyncratic long-range interactions to stabilize the packing of helical elements. Proteins also help to stabilize the RNA tertiary structure by binding to two or more helical rRNA elements.

The 5′ domain. The 5′ domain is the RNA component of the body (Figure 2). It contains 16S rRNA helices 1–18. The spur (Sp) at the bottom of the 30S is formed by helix 6 (h6), which is known to vary in length across species (Gutell, 1996). Helix 18 is sharply bent to accommodate the functionally important 530 pseudoknot (Wimberly *et al*., 2000) (Figure 4A,B). The universally conserved 530 loop of 16S rRNA plays a crucial role in translation, related to the binding of tRNA to the ribosomal A site (Powers and Noller, 1991) and is involved in the decoding process (Carter *et al*., 2000).

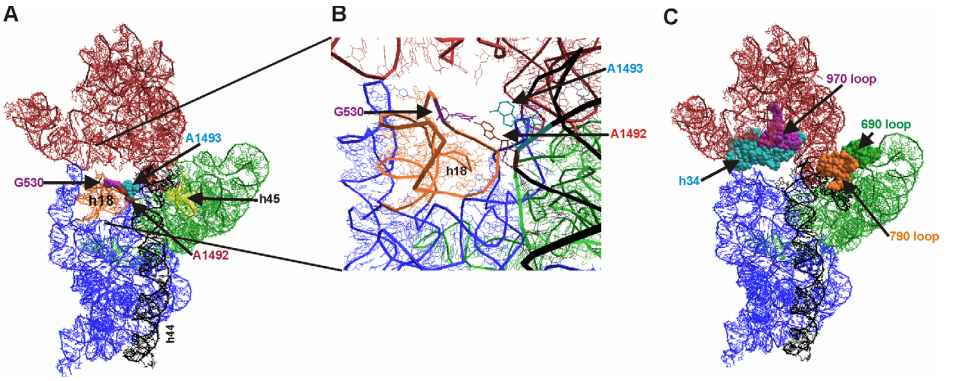


Figure 4. Functionally important rRNA regions in the 30S subunit. *T. thermophilus* 30S subunit (PDB code 2WRI) is in interface view. *E. coli* numbering is used throughout the text (A) Three universally conserved bases (G530, A1492 and A1493) line the minor groove of the codon-anticodon helix in such a way that they monitor correct codon-anticodon interaction. Helix 18 (orange) accommodates the functionally important 530 pseudoknot (in purple spacefill). Helix 44 positions A1492 and A1493 are in red and light-blue spacefill, respectively. The ultimate 16S rRNA helix 45 is in a yellow wireframe structure. (B) Closer look to the decoding region of 30S. Designations are same as in panel A. (C) Important central domain loops (690 and 790) are in green and orange spacefill, respectively. Functionally important 3′-major domain elements (h34 and 970 loop) are in light-blue and purple spacefill, respectively. 16S rRNA domains are coloured as in figure 1.

The central domain. The central domain is the RNA component of the 30S platform (Figure 2). It contains 16S rRNA helices 19–27 (Wimberly *et al*., 2000). The tip of the platform consists of helices 23 and 24, whose conserved hairpin loops (the 690 and 790 loops, respectively) are tightly packed and functionally important (Wimberly *et al*., 2000) (Figure 4C).

The 690 loop (h23) and the adjacent internal loop are protected from chemical modification by proteins S11 and IF3 (Wickstrom *et al*., 1986; Muralikrishna and Wickstrom, 1989; Moazed *et al*., 1995; Powers and Noller, 1995; Agalarov and Williamson, 2000). The 690 loop has also been implicated in subunit association based on hydroxyl radical protection of the loop nucleotides (Merryman *et al*., 1999b). The 690 loop may also interact with P-site bound tRNA as evidenced by protection from chemical modification (Moazed and Noller, 1986). There are indications that the 690 loop is also interacting with E-site tRNA (Yusupov *et al*., 2001). Loop nucleotides G693 and A694 affect binding of the antibiotics pactamycin and edeine, which inhibit initiation of protein synthesis (Egebjerg and Garrett, 1991; Woodcock *et al*., 1991; Mankin, 1997; Oehler *et al*., 1997).

Helix 24 (790 loop) nucleotides 783–793 constitute a major portion of the IF3-binding site (Moazed *et al*., 1995; Dallas and Noller, 2001; Fabbretti *et al*., 2007). Two mutations (U789C and A790G) lie in this region and confer the strongest phenotypes to decrease the affinity of IF3 for the 30S subunit

(Tapprich *et al*., 1989; Qin *et al*., 2007; Qin and Fredrick, 2009). The 790 loop also interacts with the P and E site tRNAs (Yusupov *et al*., 2001).

The 3′ major domain. The 3′ major domain is the RNA component of the head (Stern *et al*., 1988). It contains 16S rRNA helices 28–43 (Wimberly *et al*., 2000). The functionally important helices h31 and h34 are quite irregular and make only rather weak packing interactions with other RNA helices (Figure 4C).

The 970 loop (h31) of *E. coli* 16S rRNA is located near the ribosomal P site and therefore is believed to be intimately involved in translation (Döring *et al*., 1994; Selmer *et al*., 2006; Korostelev *et al*., 2006; Berk *et al*., 2006).

Helix 34 of the 16S rRNA forms part of the decoding region. Genetic studies suggested that mutations in h34 have multiple effects on ribosome function (Moine and Dahlberg, 1994; Kubarenko *et al*., 2006). The conformational flexibility of h34 seems to be important for translocation. The antibiotic spectinomycin, which binds to h34 and presumably interferes with movement of h34 relatively to h35 and h38, inhibits translocation (Peske *et al*., 2004).

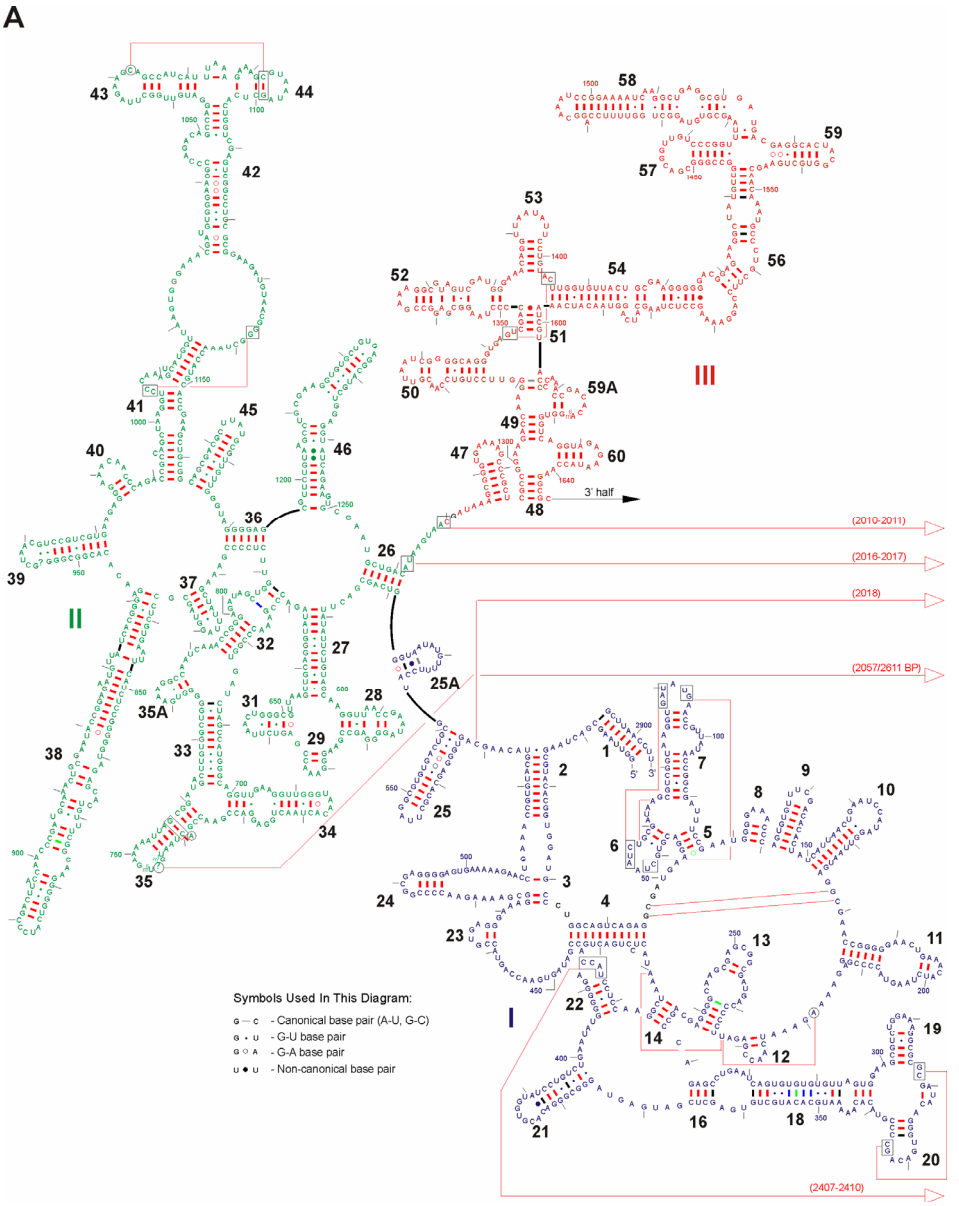
The 3′ minor domain. The 3′ minor domain consists of just two helices (h44 and h45) at the subunit interface (Wimberly *et al*., 2000) (Figure 4A,B). Helix 44 is the longest single helix in the subunit, and stretches from the bottom of the head to the bottom of the body. Helix 44 interacts extensively with the 50S subunit. The penultimate h44 contacts with A- and P-site tRNAs, and is involved in the decoding process during translation by monitoring correct codon-anticodon interaction at A and P sites (Yusupov *et al*., 2001; Ogle *et al*., 2001; Ogle *et al*., 2002). The binding of cognate aminoacyl-tRNA to the 30S subunit induces a change in the conformation of A1492 and A1493, these nucleotides flip out from 16S rRNA helix 44, in an orientation in which they would be able to inspect directly the minor groove of the codon-anticodon helix (Ogle *et al*., 2001) (Figure 4A,B).

The ultimate helix 45 is part of the platform domain of the small subunit and is close to the A site (Figure 4A). It interacts with the 50S subunit (Yusupov *et al*., 2001; Gao *et al*., 2003; Schuwirth *et al*., 2005).

The final important structural region in the 3′ minor domain is the single-stranded 3′ tail of 16S rRNA that contains CCUCC (1535–1539), the anti-Shine-Dalgarno sequence (Shine and Dalgarno, 1974). The Shine-Dalgarno (SD) duplex causes strong anchoring of the 5′-end of mRNA onto the platform of the 30S subunit, with numerous interactions between mRNA and the ribosome (Yusupova *et al*., 2006). The presence of the SD helix reduces the mobility of the head and platform. Thus, positioning of the SD helix may help to fix the orientation of the mobile head of the 30S subunit (Schuwirt *et al*., 2005; Gao *et al*., 2003) for optimal interaction with $tRNA^{fMet}$ at the 30S P site during initiation.

1.2. Structural features of the large ribosomal subunit

50S subunit. The *E. coli* 50S subunit comprises 23S rRNA (2904 nucleotides), 5S rRNA (120 nucleotides) and 33 proteins. The secondary structure of the 23S rRNA divides it into six secondary structure domains (Glotz *et al*., 1981; Branlant *et al*., 1981) (Figure 5), each of which has a highly asymmetric tertiary structure (Penczek *et al*., 1999; Ban *et al*., 1999; Cate *et al*., 1999; Ban *et al*., 2000) (Figure 6A,B). The large ribosomal subunit (LSU) proteins (L-proteins) are dispersed throughout the structure and are mostly concentrated on its surface (Figure 6C,D). However, they are largely absent from the 30S subunit interface and the active site of the 50S subunit peptidyl transferase, the regions of the subunit that are of primary functional significance to protein synthesis (Ban *et al*., 2000; Yusupov *et al*., 2001; Schuwirth *et al*., 2005). Despite the organization of large subunit RNAs at the secondary structure level, in the three dimensional structure, the large subunit is a single, gigantic domain. In this respect, it is different from the small subunit. This qualitative difference between the two subunits may reflect a requirement for conformational flexibility that is greater for the small subunit. The ratchet-like movement describes mostly changes in the small subunit, rotation of 30S subunit head domain and other parts (Gao *et al*., 2003; Zhang *et al*., 2009). In case of the 50S subunit, large-scale movements are missing (Gao *et al*., 2003). Most of the regions of the rRNA in the large subunit appear to be less mobile than average, generally moving less than 3Å during the translation cycle, in contrast to the behavior of 16S rRNA. The exceptions are helices 43, 44 (L7/L12 stalk), 76 (L1 stalk), and 86 of 23S rRNA and 5S rRNA showing relatively large movements (Gao *et al*., 2003).


A
I
II
III
3' half
(2010-2011)
(2016-2017)
(2018)
(2057/2611 BP)
(2407-2410)
Symbols Used In This Diagram:
- Canonical base pair (A-U, G-C)
- G-U base pair
- G-A base pair
- Non-canonical base pair

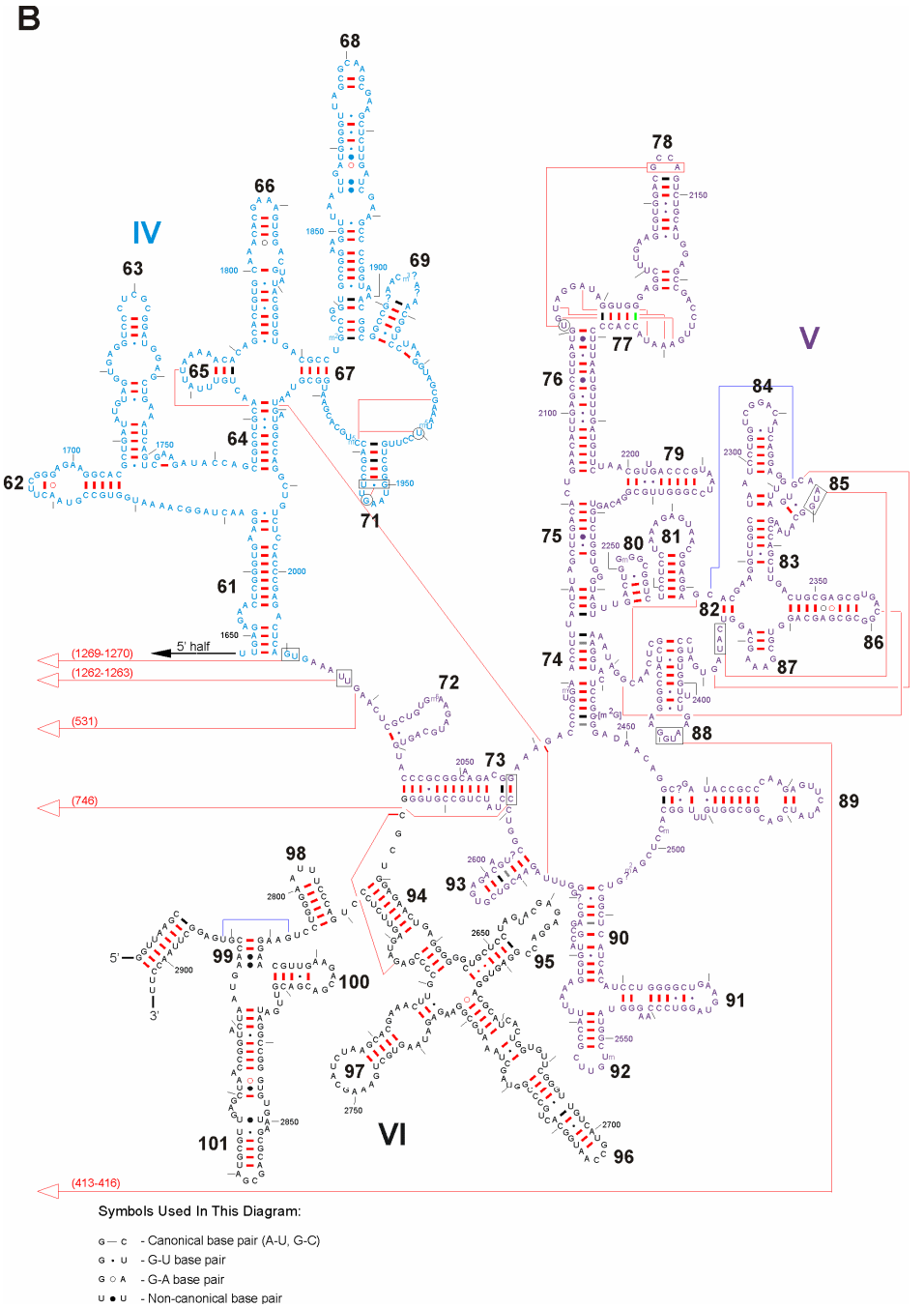


Figure 5. Secondary structure of *Escherichia coli* 23S rRNA. (A) Secondary structure of 5′ half of 23S rRNA. Domains I (blue), II (green) and III (red). (B) Secondary structure of 3′ half of 23S rRNA. Domains IV (light-blue), V (violet) and VI (black). 101 helical elements are numbered and used throughout the text. Secondary structure is downloaded from http://www.rna.ccbb.utexas.edu/.

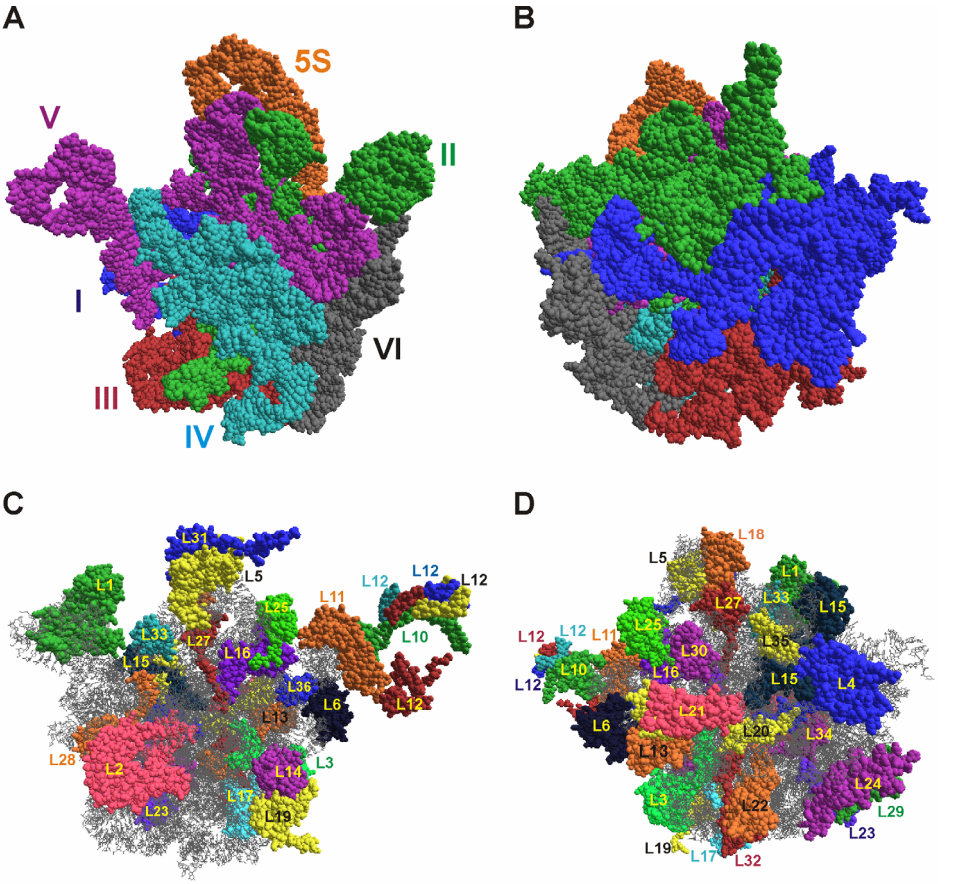


Figure 6. Tertiary structures of *T. thermophilus* 23S rRNA and 5S rRNA. (A) Three-dimensional fold of 23S rRNA and 5S rRNA in 50S subunit, with its domains coloured as in figure 5 and 5S rRNA (orange). 50S is in interface view. (B) The surface-side view of 23S rRNA in the 50S subunit. (C) The interface side view of 50S subunit with r-proteins. The RNA of the subunit is shown in gray wireframe and proteins are in colour spacefill. (D) The surface-side view of 50S subunit with r-proteins. PDB co-ordinate 2WRJ is used and modelled by PyMol.

L1 stalk. Helix 76 of domain V of the 23S rRNA belongs to the so-called L1 stalk along with helices 77, 78, and protein L1 (Figure 7). The mobility of the L1 stalk has been inferred from its different locations in different cryo-EM (Gomez-Lorenzo *et al*., 2000; Gao *et al*., 2003; Valle *et al*., 2003b) and crystal structures (Harms *et al*., 2001; Yusupov *et al.,* 2001). It has been proposed to facilitate the release of the E-site tRNA (Agrawal *et al*., 1999b; Gomez-Lorenzo *et al*., 2000; Yusupov *et al*., 2001; Harms *et al*., 2001) and to be actively involved in the translocational movement of tRNA from the P site to the E site (Valle *et al*., 2003b).

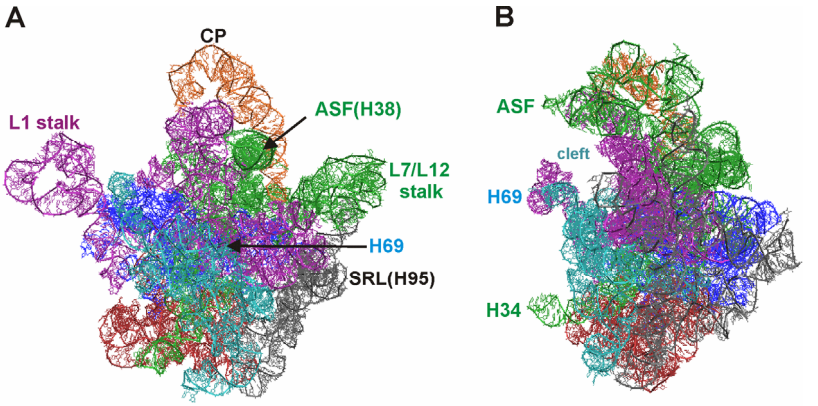


Figure 7. The 50S subunit morphological features. *T. thermophilus* 50S (PDB code 2WRJ). *E. coli* numbering is used throughout the text. (A) In this view, the surface of the subunit that interacts with the small subunit faces the reader. The L7/L12 stalk is to the right, the L1 stalk is to the left, and the central protuberance (CP) is at the top. Functionally important helical features in domains II (H38, called also A-site finger), IV (H69) and VI (H95, called also sarcin-ricin loop) are indicated by arrows. 23S rRNA domains are coloured as in figure 5. (B) 50S ribosome viewed from the L7/12 side. 23S rRNA helices H34, H38 and H69 extend out of 50S subunit, and interact with 30S subunit. Helices 67 to 71 of domain IV form the front rim of the cleft.

L7/L12 stalk. Another important part in the 50S subunit is the L7/L12 stalk (Figure 7). The ribosomal stalk complex in *Escherichia coli* consists of L10 and four copies of L7/L12, and is largely responsible for binding and recruiting translation factors. It has been shown that the major translation factors (IF2, EF-Tu, EF-G and RF3), which catalyze different steps of translation in bacteria, bind to the same region of the CTD of L12 (Helgstrand *et al*., 2007). Helices 43 and 44 that form the RNA part of the L7/L12 stalk-base constitute another flexible region in the 50S subunit (Harms *et al*., 2001; Agrawal *et al*., 2001; Gao *et al*., 2003; Valle *et al*., 2003a). Helices 42–43 of 23S rRNA, the associated proteins L11 and L10 and L7/L12 protein form the GTPase-associated center (GAC) (Li *et al*., 2006; Connell *et al*., 2007). GAC shares a common binding locus on the ribosome for GTP-associated factors (IF2, EF-G, EF-Tu, RF3).

Polypeptide exit tunnel. On the way out, all newly synthesized polypeptides, large and small, hydrophobic and hydrophilic, positively and negatively charged, must pass through the ribosomal nascent-peptide exit tunnel. Cryo-electron microscopy and ribosome crystallographic studies have shown the existence of this polypeptide exit tunnel (Frank *et al*., 1995b; Nissen *et al*., 2000). Starting at the peptidyl-transferase center and ending at the solvent side of the large ribosomal subunit, the exit tunnel ~100 Å in lenght defines the path for the nascent polypeptide out of the ribosome. The tunnel wall, is primarily built of RNA (23S rRNA domains I and III), and is "non-sticky" (Nissen *et al*., 2000). The "non-sticky" character of the tunnel wall reflects a lack of polarity.

The tunnel surface is largely hydrophilic and includes exposed hydrogen bonding groups from bases, backbone phosphates, and polar protein side chains (Nissen et al., 2000). There is growing amount of evidence indicating that some polypeptides can specifically interact with the tunnel, and these interactions might affect translation by stalling the ribosome on its tracks on mRNA (Morris and Geballe, 2000; Tenson and Ehrenberg, 2002; Jenni and Ban, 2003; Mitra *et al*., 2006; Mankin, 2006).

At approximately one-third of the tunnel length, away from the peptidyl-transferase center (20–35 Å), the nascent peptide reaches a constriction formed by the tunnel walls. The extensions of two ribosomal proteins, L22 and L4, are exposed here in the lumen from opposite walls of the tunnel. In bacteria, the region of the tunnel between the peptidyl-transferase center and the constriction seems to be the most crucial for the functional interactions of the ribosome with the nascent peptide. Additionally, the exit tunnel is encircled by proteins L23, L24 and L29, which are involved in factor docking, such as TF (trigger factor) (Kramer *et al*., 2002; Ludlam *et al*., 2004; Ferbitz *et al*., 2004; Baram *et al*., 2005), SRP (signal recognition particle) (Schaffitzel *et al*., 2006) and SecY (protein-conducting channel, consisting of the membrane proteins SecY, SecE, and SecG) (Osborne *et al*., 2005; Menetret *et al*., 2007).

Central protuberance (CP). The central protuberance is located between the L1 and L7/L12 stalks, forming a pronouced head-like structure (Figure 7). It makes contact with the head of the 30S subunit (Yusupov *et al*., 2001). In *E. coli* ribosomes, the CP consists of 5S rRNA, 23S rRNA (domains II and V) and r-proteins L5, L18, L25 and L31 (Gao *et al*., 2003).

In the 70S ribosome, a long helical 23S RNA arm of helix 38 reaches from the right side of the central protuberance of the 50S subunit to the middle of the head of the 30S subunit (Figure 7). Sometimes, H38 is called A-site finger (ASF), because it interacts with the elbow of A-site tRNA (D and T loops) (Ban *et al*., 2000).

5S rRNA and 23S rRNA do not interact extensively with each other. 5S rRNA consists of three stems radiating out from a common junction called loop A (Ban *et al*., 2000). The few RNA/RNA interactions that occur involve the backbones of the helix 4/5 arm of 5S rRNA and helix 38 of 23S rRNA (Yusupov *et al*., 2001). 5S rRNA binding to the large ribosomal subunit appears to depend on its extensive interactions with proteins that act as modeling clay, sticking it to the rest of the ribosome (Ban *et al*., 2000). The precise function of 5S rRNA in protein synthesis is not fully understood. Biochemical studies with *E. coli* ribosomes led to the hypothesis that 5S rRNA acts as a physical transducer of information, facilitating communication between the different functional centers and coordinating the multiple events catalyzed by the ribosome (Bogdanov *et al*., 1995; Dokudovskaya *et al*., 1996). The molecule itself does neither directly contact P- or A-site bound tRNAs, nor is it a component of the peptidyltransferase, decoding, or elongation factor binding centers. However, it is uniquely positioned in a way as to be able to connect all of these components with one another (Dinman, 2005).

1.2.1. Domain composition of the 23S rRNA

Domain I. Domain I lies in the back of the large ribosomal subunit, behind and below the L1 region (Ban *et al*., 2000) (Figure 6A,B). It is formed by the 23S rRNA helices 1 to 25 (Figure 5A). Helix 1 is essential for assembly of the large ribosomal subunit (Liiv *et al*., 1996). Large ribosomal subunit assembly occurs during transcription of 23S rRNA, and those proteins whose binding sites are closest to the 5′-end of 23S rRNA may assemble earlier than those located closer to the 3′-end (Klein *et al*., 2004). This indicates that assembly of 50S subunit begins from domain I. The ribosomal protein L24 interacts entirely with domain I and is thought to be critical in ribosome assembly (Cabezón *et al*., 1977; Nowotny and Nierhaus, 1982; Dabbs, 1982; Skinner *et al*., 1985; Klein *et al*., 2004).

Domain II. Domain II is the largest of the six 23S rRNA domains, accounting for most of the surface of the particle (Figure 6A,B). It is formed by the 23S rRNA helices 26 to 46 (Figure 5A). As mentioned above, helices 42–44 in domain II form the rRNA part of of the L7/L12 stalk, and helix 38 forms ASF. The third region (helix 32 to 35.1) points directly towards the small subunit and its terminus, the loop of stem-loop 34, interact directly with the small ribosomal subunit (Ban *et al*., 2000; Culver *et al*., 1999) (Figure 7B).

Domain III. Domain III is a compact globular domain that occupies the bottom left region of

the subunit in the intersubunit face (Ban *et al*., 2000) (Figure 6A,B). It is formed by the 23S rRNA helices 47 to 60 (Figure 5A). The most extensive contacts of domain III are with domain II, but it also interacts with domains I, IV, and VI. Unlike all the other domains, domain III hardly interacts with domain V at all (Ban *et al*., 2000).

Domain IV. Domain IV accounts for most of the interface of the 50S subunit that contacts the 30S subunit (Ban *et al*., 2000) (Figure 6A). It is formed by the 23S rRNA helices 61 to 72 (Ban *et al*., 2000; Gao *et al*., 2003) (Figure 5B). Helices 67 through 71 constitute the most prominent feature of domain IV, the front rim of the cleft (Ban *et al*., 2000) (Figure 7B). Helix 69 in the middle of this ridge interacts with the long penultimate stem of 16S rRNA in the small ribosomal subunit (Ban *et al*., 2000; Yusupov *et al*., 2001; Gao *et al*., 2003). Additionally, the minor groove of helix 69 of 23S rRNA, which forms intersubunit bridge B2a, interacts with the minor groove of the D stem of P-tRNA, extending into the A site where its conserved loop interacts with almost the same features of the D stem of A-tRNA (Yusupov *et al*., 2001).

Domain V. Domain V, which is sandwiched between domains IV and II in the middle of the subunit, is known to be intimately involved in the peptidyl transferase activity of the ribosome, forming the peptidyl transferase center (PTC) (Ban *et al*., 2000; Selmer *et al*., 2006) (Figure 6A,B). It is formed by the 23S rRNA helices 72 to 93 (Ban *et al*., 2000; Gao *et al*., 2003) (Figure 5B). Structurally, this domain can be divided into three regions. The first starts with helix 75 and ultimately forms the binding site for protein L1. The second, which

consists of helices 80 to 88, forms the bulk of the central protuberance region and is supported at the back by 5S rRNA and domain II. The third region, which includes helices 89 to 93, extends toward domain VI and helps to stabilize the elongation factor-binding region of the ribosome.

Domain VI. The smallest domain in 23*S* rRNA, domain VI, forms a large part of the surface of the subunit immediately below the L7/L12 stalk (Ban *et al*., 2000) (Figure 6A,B). It is formed by the 23S rRNA helices 94 to 101 (Ban *et al*., 2000; Gao *et al*., 2003) (Figure 5B). The most interesting region of this domain is the sarcin-ricin loop (SRL) (stem-loop 95) (Ban *et al*., 2000; Yusupov *et al*., 2001) (Figure 7A). The SRL is essential for factor binding, interacts with the G domains of the elongation factors. Ribosomes can be inactivated by the cleavage of single covalent bonds in this loop (Glück *et al*., 1992).

1.3. Intersubunit bridges

The intersubunit bridges are important for maintaining the overall architecture of the ribosome (Frank *et al*., 1995a; Cate *et al*., 1999; Gabashvili *et al*., 2000), but are also expected to play a role in the dynamics of translation (Frank and Agrawal, 2000, 2001; Zhang *et al*., 2009). The bridges at the subunit interface bury more than 6000 $Å^2$ of solvent-accessible surface area (Brünger *et al*., 1998). Using the 5.5 Å resolution crystal structure of the 70S ribosome from *T. thermophilus*, Yusupov and coworkers (2001) identified more than 30 individual intersubunit interactions spread among 12 bridges (Table 1) (Figure 8).
For each bridge identified in the *T. thermophilus* ribosome, a counterpart exists in the *E. coli* structure (Gao *et al*., 2003). In terms of their locations, RNA helices and proteins involved, many bridges in *E. coli* are virtually identical to the bridges found in *T. thermophilus*. The high similarity suggests that the intersubunit bridge regions are highly conserved across species, and that their atomic makeup is essential for ribosome function. Most of the bridges are RNA-RNA bridges, while a second class of bridges are RNA-protein bridges, and only one bridge is formed between proteins.

Table 1. Intersubunit bridges (Yusupov et al., 2001). Bridges are numbered B1a, B1b, etc. rRNA nucleotide numbers are according to *E. coli* numbering. Molecular contacts are scored in parentheses as follows: M, major groove; m, minor groove; L, loop; B, backbone; Lm refers to the minor groove side of the loop; LB to the loop backbone; NH2 term, NH_2-terminal tail; COOH-term, COOH-terminal tail; Hm24e, protein L24e of the *Haloarcula marismortui*.

Bridge	Type	30S subunit		50S subunit	
		16S rRNA helix or S protein	RNA or protein positions	23S rRNA helix or L protein	RNA or protein positions
B1a	Prot-RNA	S13	92–94	H38(L)	886–888
B1b	Prot-Prot	S13	NH2-term	L5	134–53
B2a	RNA-RNA	H44(m)	1408–410, 1494–1495	H69(Lm)	1913–1914, 1918
B2b	RNA-RNA	H24(m,LM)	784–785,794	H67(m), H69(M)	1836–1837, 1922
	RNA-RNA	H45(LM,Lm)	1516–1519	H71(M), H69(B)	1919–1920, 1932
B2c	RNA-RNA	H24(Bm)	770–771	H67(B)	1832–1833
	RNA-RNA	H27(Bm)	900–1	H67(B)	1832–1833
B3	RNA-RNA	H44(m)	1484–1486	H71(m)	1947–1948, 1960–1961
B4	RNA-RNA	H20(m)	763–764	H34(Lm)	717–718
	Prot-RNA	S15	40–44, COOH-term	H34(LB,LM)	713, 717
B5	RNA-RNA	H44(m)	1418–1419	H64(m)	1768–1769
	RNA-Prot	H44(B)	1420–1422	L14	44–49
	RNA-RNA	H44(B)	1474–1476	H62(Bm)	1689–1690
	RNA-RNA	H44(B)	1474–1476	H64(m)	1989
B6	RNA-RNA	H44(m)	1429–1430, 1474–1476	H62(m)	1689–1690, 1702–1705
	RNA-prot	H44(B)	1431	L19	(Hm24e:R44)
B7a	RNA-RNA	H23(L,m)	698,702	H68(m)	1848–1849, 1896
B7b	RNA-Prot	H23(M,m)	712–713	L2	162–164, 172–174, 177–178
	RNA-Prot	H24(M,m)	773–776	L2	177–178, 198–202
B8	RNA-Prot	H14(LM)	345–347	L14	116 –119

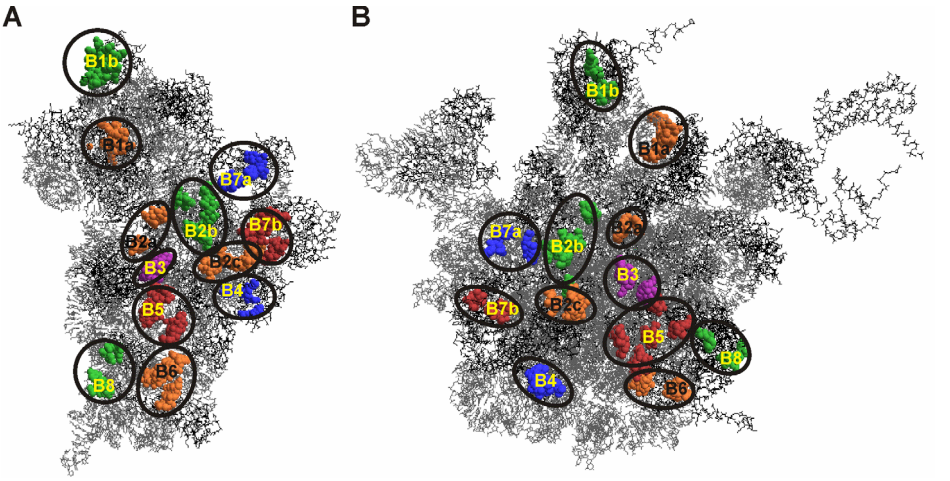


Figure 8. Bridges between the 30S subunit and the 50S subunit. (A) Contacts at the interface of the 30S subunit. Bridges are in spacefill and color-coded. Rest of the subunit is in gray wireframe structure. (B) Contacts at the interface of the 50S subunit. Bridges are color-coded as in panel (A). Bridge contacts from Gao et al., 2003 are modeled to *T. thermophilus* 30S (PDB code 2WRI) and 50S (PDB codeWRJ) subunits by PyMol.

RNA-RNA bridges. Most of the RNA-RNA bridges are stable and rather do not change during translation. The bridge B3 is the largest RNA-RNA bridge according to its connection surface area. In bridge B3, two sheared base pairs, G-A (G1417-A1483) in h44 of 16S rRNA and G-C (G1959-C1947) in H71 of 23S rRNA, form a type I A-minor interaction (Schuwirth *et al*., 2005) (Figure 9A). The same type of A-minor interaction occurs with A1418 of 16S rRNA, where A1418 of 16S rRNA interacts with G1948, and C1958 of 23S rRNA (Figure 9B). As observed in other type I A-minor interactions (Ogle et al., 2002), close packing takes place that stabilizes these structures. This bridge is essential for subunit association (Pulk *et al*., 2006), modification of the N1 positions of A1418, and A1483 with DMS in 30S subunits strongly interferes with 70S ribosome formation.

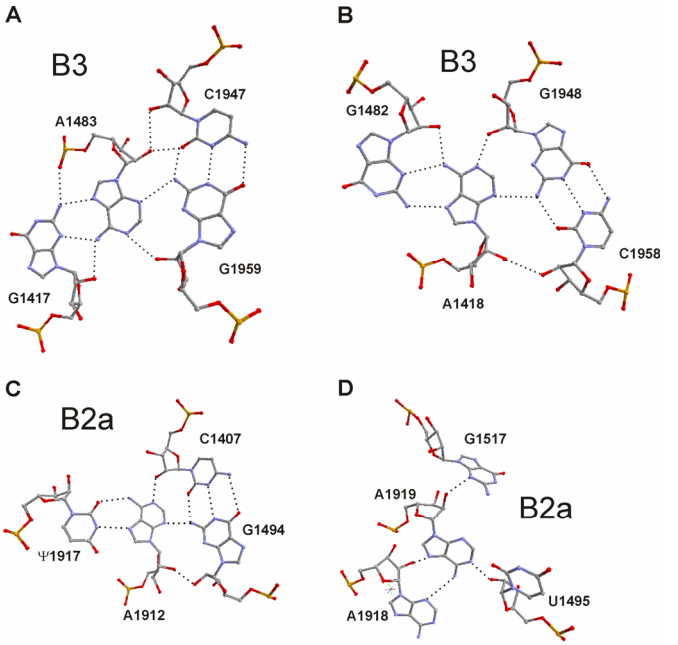


Figure 9. Molecular interactions in the intersubunit bridges. (A) Molecular interactions in bridge B3. A1483 of 16S rRNA form a type I A-minor interaction with C1947, and G1959 of 23S rRNA. (B) Molecular interactions in bridge B3. A1418 of 16S rRNA forms a type I A-minor interaction with G1948, and C1958 of 23S rRNA. (C) Molecular interactions in bridge B2a. A1912 of 23S rRNA forms a type I A-minor interaction with C1407, and G1494 of 16S rRNA. (D) A1919 of 23S rRNA interacts with U1495, and G1517 of 16S rRNA. *E. coli* 30S (PDB code 2AVY) and 50S (PDB code 2AW4) co-ordinates are used and modelled by PyMol.

Bridges B2a and B4 occur between the 30S platform and the 50S subunit H69 or H34, respectively. These bridges are essential for subunit association (Maiväli and Remme, 2004; Pulk *et al*., 2006). Bridge B2a occurs at the functional center of the ribosomal interface and is immediately adjacent to the mRNA decoding site, between the top of h44 in 16S rRNA and H69 in 23S rRNA. It extends under the P site toward h45 and h24 (Yusupov *et al*., 2001; Stark *et al*., 2002; Schuwirth *et al*., 2005). The large subunit part of the bridge B2a, H69 moves laterally with respect to the small subunit by 6 to 8 Å during the ratchet-like motion of the small subunit during translocation (Valle *et al*., 2003a). Additionally, ribosome recycling factor causes the tip of H69 to peel away from the 30S subunit as part of the subunit dissociation process (Agrawal *et al*., 2004; Gao *et al*., 2005; Wilson *et al*., 2005). In the H69, A1918 and A1919 form an A-A dinucleotide platform (Cate *et al*., 1996), where A1919 is projected into the minor groove of h44 near bases U1406/U1495, where it also interacts with the base of G1517 (Schuwirth *et al*., 2005) (Figure 9D). Nucleotide A1912 of bridge B2a stacks on A1918 and forms a distorted

reversed-Hoogsteen base pair with Ψ1917, projecting A1912 into the minor groove of base pair C1407/G1494 in h44 of 16S rRNA (Schuwirth *et al*., 2005) (Figure 9C). The involvement of all three N1 positions of A1912, A1918, and A1919 in packing interactions is consistent with interference of subunit association when these residues are N1-methylated by dimethyl sulfate (Maiväli and Remme, 2004).

In bridge B6, interactions between h44 in 16S rRNA and H62 in 23S rRNA bury a large surface area that is almost entirely solvated (Schuwirth *et al*., 2005). The minor grooves of h44 and H62 contact each other barely and leave a 6 Å gap that can accommodate a monolayer of water molecules. Interestingly, many other bridges between the center of the small subunit platform and the large subunit are also highly solvated (Yusupov *et al*., 2001). In these solvent spaces, phosphate groups from both subunits lie within 4 to 6 Å of each other and accommodate a water molecule layer. The high level of solvation at the subunit interface may be necessary to allow ratcheting during translocation, where the relative orientation of the two subunits may change by 7^{o} to 10^{o} (Valle *et al*., 2003a; Spahn *et al*., 2004; Schuwirth *et al*., 2005).

RNA-protein bridges. H34 in 23S rRNA and protein S15 in the small subunit are involved in formation of bridge B4 (Culver *et al*., 1999). H34 extends from the interface of the 50S subunit by about 30 Å and has been observed in different orientations in the isolated 50S subunit structures (Ban *et al*., 2000; Harms *et al*., 2001) (Figure 7B). H34 is 60 Å away from the centre of the small subunit or ratcheting pivot point, which may explain the need for its flexibility in order to maintain intersubunit interactions (Schuwirth *et al*., 2005). H34 position A715 is involved in a U-turn which packs against a hydrophobic surface on S15 (Schuwirth *et al*., 2005). U-turns represent an important class of structural motifs in the RNA structures, wherein a uridine is involved in a sharp change in the direction of the polynucleotide backbone (Quigley and Rich, 1976; Pley *et al*., 1994; Doudna, 1995; Huang *et al*., 1996; Stallings and Moore, 1997). In addition, Gln39 and Arg88 of S15 interact with the minor and major grooves of the H34 loop nucleotides, respectively. Methylation of the N1 position of A715 has been shown to interfere with subunit association (Maiväli and Remme, 2004). Since position N1 of A715 is not in direct contact with any residue of either subunit, but is 4 to 5 Å away from the guanidinium group of Arg52 in S15 (Schuwirth *et al*., 2005), the interference may therefore be due to a positive charge on 1-methyladenosine (Macon and Wolfenden, 1968), which would lead to charge-charge repulsion with Arg52 (Schuwirth *et al*., 2005).

1.3.1. Bridge contacts in the translating ribosome

RNA-protein and protein-protein bridges are mainly located at the periphery of the ribosome and interactions between these bridges change during translation (Gao et al., 2003). In contrast, RNA-RNA bridges are mainly located at the centre of the ribosome and do not change a lot during translation (Gao et al., 2003). Recently, Zhang and colleagues (2009) determined a new ribosome

structure, the so-called intermediate state structure, where ASL (tRNA anti-codon stem-loop) analogs are positioned in a way that if the full length tRNAs are modelled into an intermediate state structure, these full length tRNAs would occupy positions between the classical and hybrid states. The classical state represents a ribosome structure with tRNAs in the A/A or P/P sites, and the hybrid state represents a ribosome structure where tRNAs are in the A/P or P/E sites of the small and the large subunit, respectively. Comparison of this intermediate structure (R_2) to post-initiation (classical state, R_0) or pre-translocation (hybrid, R_F) state structures, revealed that the intermediate structure is more similar to the hybrid state structure (tRNAs in the A/P or P/E sites) than to the classical state structure (tRNAs in the A/A or P/P sites) (Figure 10). In the new conformation, the small subunit is rotated by 3° to 6° relative to its position in a post-initiation state of the ribosome, in which initiator tRNA is bound to the P site (Gabashvili *et al*., 2000; Berk *et al*., 2006). In ribosomes that are occupied by tRNA at the hybrid P/E binding site (R_F) (Frank *et al*., 2007; Connell *et al*., 2007; Agirrezabala *et al*., 2008), the small subunit is rotated by an additional 2° to 4° relative to the rotational state of this intermediate state (R_2) (Zhang *et al*., 2009) (Figure 10). Comparing ribosome structures between different stages of translation may help to reveal the precise ratcheting mechanism where ribosome subunits rotate with respect to each other, and movement of the head domain of the small subunit.

In state R_2, the central contacts or "bridges" between the ribosomal subunits are nearly indistinguishable from those observed in ribosomes in state R_0 (Zhang *et al*., 2009). The only change that occurs is with the central bridge B2a (Zhang *et al*., 2009). In B2a, nucleotide A1913 in 23S rRNA and nucleotides A1492 to A1493 in helix h44 of 16S rRNA adopt different conformations depending on the tRNA occupancy of the A site. To maintain contacts in the bridges at the centre of the interface during subunit rotation, helix h44 in the small subunit bends near 16S rRNA helix 14 (Zhang *et al*., 2009).

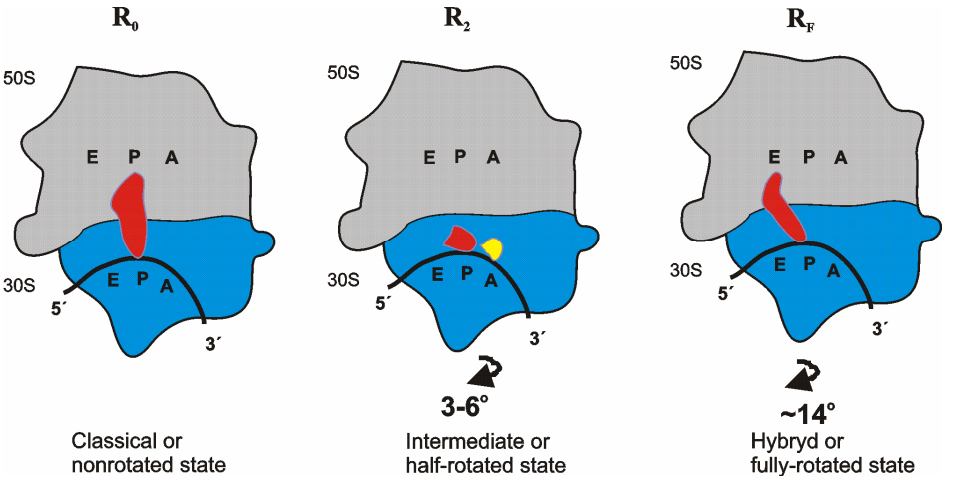


Figure 10. Schematic of tRNA binding states on the ribosome. View of the bacterial 70S ribosome, composed of the small (30S) ribosomal subunit and the large (50S) ribosomal subunit. The small subunit of the ribosome (blue) can rotate from a starting conformation seen in post-initiation and termination states (state R_0) to a fully rotated conformation seen in elongation, termination, and recycling steps of translation (state R_F). In the transition of the ribosome to the fully rotated state, tRNAs shift from binding in the A/A and P/P sites (30S subunit and 50S subunit, respectively) to occupy hybrid binding sites (A/P and P/E for 30S/50S sites). Rotations of the head domain of the small ribosomal subunit is shown by arrow. In state R_0, the head domain is centered over the P site (~0° rotation). Rotations of the head domain toward the E site of up to 14° have been observed (Spahn et al., 2004; Shuwirth et al., 2005). In the new conformation (R_2), the small subunit is rotated by 3° to 6° relative to its position in a post-initiation state (R_0) of the ribosome, in which initiator tRNA is bound in the P site (Zhang et al., 2009). In the intermediate structure, the ASL analogs are positioned in a way that if the full length tRNAs are modelled into an intermediate state structure, these full length tRNAs would occupy positions between the classical and hybrid states.

In contrast, key bridges (B4 and B7a) between the platform of the 30S and the 50S subunit are shifted halfway in the intermediate state compared to their position in the fully-hybrid state model (Zhang *et al*., 2009). The small subunit platform bridge B7a involves the only cross-subunit base stacking interaction, between A702 in h23 of 16S rRNA and A1848 in H68 of 23S rRNA (Schuwirth *et al*., 2005) (Figure 11). N1 position of A702 interacts with N2 position of G1846 in H68 of 23S rRNA in the nonrotated ribosomes (Schuwirth *et al*., 2005; Zhang *et al*., 2009). The interface in this region shifts by at least 6 Å laterally with respect to H68 (Valle *et al*., 2003a; Spahn *et al*., 2004), and this contact breakes during translocation. Intersubunit rotation exposes nucleotide A702, whereas in the classical state (nonrotated state) models (Berk *et al*., 2006; Selmer *et al*., 2006; Laurberg *et al*., 2008; Weixlbaumer *et al*., 2008; Korostelev *et al*., 2008) it is buried in the minor groove of H68 in 23S rRNA (Figure 11). Additionaly, nucleotide A702 is protected from chemical probes when tRNAs

are bound in the A/A and P/P sites (Moazed and Noller, 1989). However, when tRNAs occupy the hybrid binding sites (A/P and P/E), nucleotide A702 becomes exposed to chemical probes and bridge B7a is rearranged (Frank *et al*., 2007; Schuwirth *et al*., 2005; Connell *et al*., 2007; Agirrezabala *et al*., 2008). Methylation of the N1 position of A702 has been shown to interfere with subunit association (Pulk *et al*., 2006).

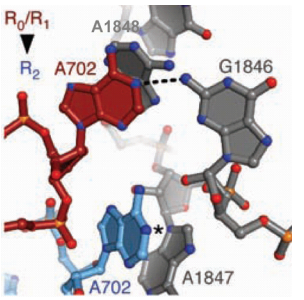


Figure 11. Molecular interactions in bridge B7a. Bridge B7a in state R_2 (rotated state, or intermediate state) (Zhang et al., 2009) compared to states R_0 (nonrotated state, initiation-like complex) (Laurberg et al., 2008; Weixlbaumer et al., 2008; Korostelev et al., 2008) and R_1 (pretranslocation complex) (Selmer et al., 2006). Nucleotide A702 of 16S rRNA in the 30S subunit (light blue) and nucleotides in H68 of 23S rRNA in the 50S subunit (gray) are shown for state R_2. Nucleotide A702 in state R_0 or R_1 is shown in red. The N1 position of A702 that would be methylated by dimethylsulfate is marked with an asterisk (Moazed and Noller, 1989). Adapted from Zhang et al., 2009.

Bridge B4 component S15 is also shifted halfway to its position in fully-hybrid state (Zhang *et al*., 2009). Most of the platform of the small subunit does not make direct contacts with the large subunit, indicating that limited contacts probably allow large shifts in the platform domain of 30S subunit.

This limited contact principle also holds with the small subunit head domain. Contacts between the 30S subunit head domain and the 50S subunit have been shown to adopt many different configurations.

Protein-protein bridge. The only protein-protein bridge in the ribosome is B1b. B1b is formed between protein L5 in the central protuberance of the 50S subunit and the N-terminal lobe of protein S13 in the 30S head domain (Frank *et al*., 2007; Berk *et al*., 2006; Selmer *et al*., 2006). In the hybrid state of the ribosome, the head domain of the 30S subunit is shifted in a way that protein S13 forms a key interaction between its long central α-helix and protein L5 in the large subunit (Hoang *et al*., 2004; Cukras and Green, 2005; Frank *et al*., 2007). In the intermediate state, the key interaction between proteins L5 and S13 is essentially indistinguishable from that in the fully-rotated ribosome (Zhang *et al*., 2009). Contacts between L5 and S13 rearrange from classical to hybrid state, and these changes support head domain rotation.

It is proposed that during ratcheting, which combines intersubunit rotation and rotation of the small subunit head domain, key bridges between the ribosomal subunits rearrange in a stepwise manner. Ratcheting likely begins with the 30S subunit body, continuing with the 30S platform and head domains, and completes with rearrangement of the central bridges (Zhang *et al*., 2009). Such a stepwise rearrangement would assist the ribosome in making large shifts at the interface without fully destabilizing the subunits. In addition, the multiple conformations of the head domain of the 30S subunit would help to position tRNAs on the ribosome during ratcheting (Spahn *et al*., 2004; Schuwirth *et al*., 2005; Frank *et al*., 2007). Fluctuation of P site tRNAs between the P/P and P/E states (Korostelev *et al*., 2006; Cornish *et al*., 2008) would not require complete rotation of subunits. Movement of P-tRNA acceptor end to the 50S E-site may be completed in this intermediate state.

2. Ribosomal proteins

Ribosomal RNA has an essential role in ribosomal function, such as decoding and peptidyl-transferase activity, but the ribosomal proteins are nevertheless important for the assembly and optimal functioning of the ribosome.

The 5S, 16S, and 23S rRNAs in *E. coli* are 120, 1542, and 2904 nucleotides in length, respectively. In *E. coli* there are 21 r-proteins in the small subunit (S1–S21) and 33 r-proteins in the large subunit (L1–L36). L7/L12 is the only r-protein in the ribosome that is present as more than one copy per ribosome. L7 is the *N*-acetylated form of L12, and together with L10 forms the pentameric complex L10 × $(L7/L12)_4$ in *E. coli* which was referred to as L8 in the past.

The prokaryotic ribosome contains about two-thirds RNA and one-third protein. In contrast, mitochondrial ribosomes contain two-thirds of protein and one-third RNA (Mears et al., 2002). Mitochondrial ribosomes have longer versions of r-proteins than cytosolic ribosomes but also contain additional organelle specific r-proteins, whereas mitochondrial rRNAs are significantly shorter or absent (5S) (Mears *et al*., 2002). Mitochondrial ribosomes are significantly larger than bacterial ribosomes, and loss of rRNA in mitochondrial ribosomes is compensated by the presence of additional r-proteins in the equivalent positions of the rRNA (Sharma *et al*., 2003). It seems that r-proteins in mitochondrial ribosomes have taken over the role of rRNA to some extent, especially for many intersubunit bridges (Sharma *et al*., 2003).

R-proteins obtained their numbers according to their arrangement on a two-dimensional polyacrylamide gel, as large acidic proteins have small numbers and small basic proteins have large numbers (Kaltschmidt and Wittmann, 1970). Most of the r-proteins are very basic (average pI ~10.1 compared to pI = 4 to 5 for most translation factors), suggesting that a general function of r-proteins may be to counteract the negative charges of the phosphate residues in the rRNA backbone. Exceptions are S1, S6 in the small subunit and the L7/L12 in the large subunit. These acidic r-proteins mainly interact with the other r-

proteins: L7/L12 interacts with L10, S6 majority contacts are with the S18, while S1 interacts with S21, S11 and S18.

One of the most surprising features of ribosomal proteins found in the crystal structures of ribosome subunits is the finding that almost half of the proteins have globular bodies with long extensions that penetrate deeply into the ribosome core (Ban *et al*., 2000; Wimberly *et al*., 2000; Harms *et al*., 2001; Selmer *et al*., 2006; Schuwirth *et al*., 2005). It has been proposed that these extensions that are most often disordered in solution play a key role in ribosomal assembly (Klein *et al*., 2004; Brodersen *et al*., 2002). Many of the ribosomal proteins are also involved in translational regulation by binding to operator sites located on their own messenger RNA (Zengel and Lindahl, 1994).

2.1. Ribosome assembly

Ribosome assembly is a process were r-proteins bind to the rRNA, conformational changes take place during ribosome assembly and intermediate ribosome particles form. Protein binding leads to particles of increasing compactness. The binding of many proteins depends on prior binding of other proteins. Primary binding proteins bind directly and independently to rRNA. Many ribosomal proteins have an RNA chaperone activity that may be important for assembly of ribosomal proteins (Semrad *et al*., 2004). *In vivo*, the early assembly reactions already start with a small number of r-proteins shortly after rRNA synthesis. Ribosomal assembly *in vivo* is coupled with transcription, and takes only few minutes. However, ribosome reconstitution *in vitro* takes several hours with the need of several steps of incubation at high temperature and high salt concentration (Nomura, 1973; Lietzke and Nierhaus, 1988).

2.1.1. *In vitro* reconstitution of 30S

Reconstitution of the 30S subunit is simpler and faster than of the 50S (Williamson, 2003; Talkington *et al*., 2005; Kaczanowska and Ryden-Aulin, 2007). 30S subunit reconstitution *in vitro* requires 16S rRNA and 21 small subunit proteins, where the secondary structure in the 16S rRNA is stabilized by Mg^{2+} ions, but tertiary folding depends on the proteins (Moazed *et al*., 1986). The protein binding sites are created as the rRNA folds. At low temperatures (0–15^{o}C) incomplete particles form with an altered sedimentation coefficient (21S). Heating (42^{o}C) these intermediate particles (RI) shifts their sedimentation coefficient to 26S (RI*) and enables them to complete assembly at low temperatures. Thus, the rate-limiting step in 30S subunit reconstitution is the transition RI $\rightarrow$ RI* and important conformational changes in the rRNA occure with this transition. The standard RI$\rightarrow$RI* mechanism, whereby assembly stalls at the 21S intermediate at low temperatures, implies that the late proteins have lower rates of binding than the early proteins at low temperatures, while the binding rates for all proteins are more similar at 40 °C, where assembly

proceeds smoothly. Protein binding is slow at 15 °C, requiring more than two days to proceed to completion (Talkington *et al*., 2005). The overall activation energy for 30S assembly is 38 kcal/mol (Traub and Nomura, 1969; Talkington *et al*., 2005). The magnitude of the activation energy corresponds to melting of ~ 4 RNA base pairs (Xia *et al*., 1998). The activation energies for the late binding proteins are somewhat larger than for the early binding proteins, but the correlation is poor, and the differences in activation energies (24–44 kcal/mol) are insufficient to produce stalling of reconstitution at low temperature (Talkington *et al*., 2005). The activation energies do not vary with temperature changes and thus the rate-determining step is the same for each protein at high and low temperatures. This means that not a single step is responsible for the apparent activation energy of overall assembly. The slowly-binding proteins, which include both those that precede the canonical RI→RI* transition and those that follow it, do not have the highest avtivation energies (*E*a's), and therefore the last steps of assembly are not more temperature-dependent than the earlier steps. It was further revealed that the final stages of reconstitution are limited by multiple different transitions (Talkington *et al*., 2005). The classical RI→RI* mechanism is not adequate to explain the rates and activation energies for binding of the individual proteins observed in the pulse-chase quantitative mass spectrometry (PC/QMS). The 21S particle from low temperature reconstitution is not a true assembly intermediate. Yet, the reason why 21S particles are retrieved from sucrose gradient purification of low-temperature assembly reactions is that a diverse collection of unstable particles that are in the process of assembling all sediment at ~ 21S. There are different kinds (rRNA fold and protein composition) of assembled 21S particles. This is in agreement with earlier observations of RI, where variable RIs have been found and where observations have been made showing that some pre-RI proteins bind only transiently at the RI stage (Held and Nomura, 1973). It is likely that weakly bound proteins dissociate to different extents during RI assembly, so that the binding of some "pre-RI" proteins (particularly S5, S12, and S19) is observed to be slow by PC/QMS.

Since r-proteins that belong to the same assembly group (Figure 12) do not share the same activation energy, the binding of proteins within a given group is not entirely limited by a single RNA folding step. Assembly occurs via a variety of local transitions rather than a single rout, global step allows for the various subunits in a population to assemble into the native structure by a variety of routes. Reconstituted RI and RI* footprinting results show that conformational changes are scattered throughout the 16S rRNA sequence (Holmes and Culver, 2004). This indicates also that many local conformational changes may take place in parallel during late stages of assembly. Therefore, the RI→RI* folding pathways have been expanded to folding landscapes that can be traversed by any of a variety of parallel pathways (Dill and Chan, 1997; Pan *et al*., 1997; Rook *et al*., 1998; Woodson, 2008; Adilakshmi *et al*., 2008).

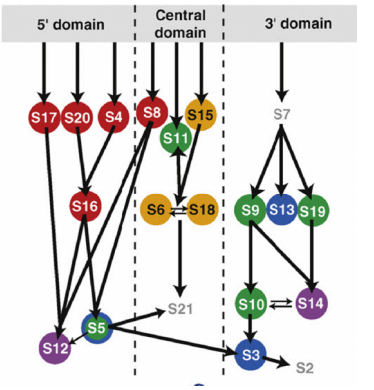


Figure 12. Nomura 30S subunit assembly map is colored by the protein binding rates at 37°C: red, ≥20 min^{-1}; orange, 8.1–15 min^{-1}; green, 1.2–2.2 min^{-1}; blue, 0.38–0.73 min^{-1}; purple, 0.18–0.26 min^{-1} (adapted from Woodson et al., 2008).

The 30S assembly landscape model states that all possible conformations of the 16S rRNA map onto a free energy surface, but in the absence of proteins, the native 30S conformation is energetically unfavourable (Figure 13). Folding can proceed along many pathways to the native state because the landscape is composed of many local and modest barriers. Once RNA folding produces a new binding site, protein binding creates new downhill directions by which further RNA folding can proceed. Each protein binding event further stabilizes the native 30S conformation, until all assembly pathways converge at this state. Despite the changes in the landscape that accompany protein binding, the heights of the various barriers encountered on any particular pathway appear to be quite similar.

The ribosomal proteins do not have an absolute dependence on each other for binding, but rather can bind in a variety of orders (Nomura, 1973). Assembly via a global rate-limiting step, which would be represented by a bottleneck on the landscape, could bring assembly to a standstill under non-optimal conditions. Assembly through a landscape of different barriers would mean that slowing any one of the steps would dercrease speed, but not completely stall assembly. RNA and protein chaperones are expected to play a role in assembly, and the protein chaperone DnaK has been specifically implicated in aiding 30S assembly (Alix and Guerin, 1993; Maki *et al*., 2002; Maki *et al*., 2003). The landscape model predicts that there are many folding transitions that are points at which chaperones might assist.

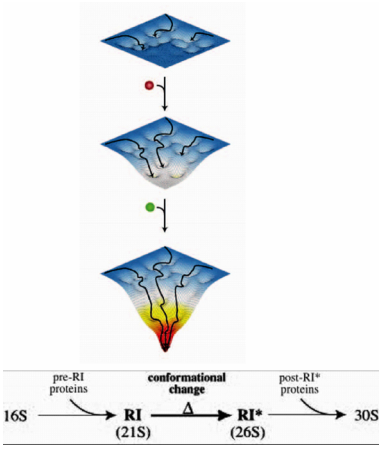


Figure 13. An assembly landscape for 30S assembly. The horizontal axes of the surface corresponds to 16S rRNA conformational space, and the vertical axis is free energy. The native conformation of the 16S rRNA adopted in the 30S subunit is located at the bottom corner. In the absence of proteins, this is not the lowest-energy conformation of the RNA. Parallel folding pathways are indicated by the arrows on the energy surface. Local folding creates protein binding sites, and major changes in the landscape accompany protein binding (coloured spheres). Sequential protein binding eventually stabilizes the native 30S conformation. All pathways converge on this point, and there is no bottleneck through which all folding trajectories must pass (adapted form Talkington *et al*., 2005).

30S subunit assembly plasticity has been shown with ribosomal protein S15 (Bubunenko *et al*., 2006). *In vitro* reconstitution experiments have shown that S15 is a primary binding protein that orchestrates the assembly of ribosomal proteins S6, S11, S18, and S21 (Mizushima and Nomura, 1970; Held *et al*., 1974) (Figure 12). These proteins and the 16S rRNA part form the platform of the 30S subunit. *E. coli* with an in-frame deletion of the S15 gene (rpsO) are viable, although at 37°C this ΔrpsO strain has an exaggerated doubling time compared to its parental strain. In the absence of S15, the remaining four platform proteins are assembled into ribosomes *in vivo*, and the overall architecture of the 30S subunits formed in the ΔrpsO strain at 37°C is not altered. 30S subunits lacking S15 appear to be defective in subunit association *in vivo* and *in vitro*. The strain is also cold sensitive, indicating ribosome biogenesis defects at low temperature. The cold-sensitive phenotype is typical for bacterial strains with ribosomal assembly defects (Guthrie *et al*., 1969; Dammel and Noller, 1993). Under nonideal conditions S15 is critical for assembly. The viability of this strain indicates that *in vivo*, functional populations of 70S ribosomes must form in the absence of S15 and that 30S subunit assembly show high plasticity, as expected according the landscape assembly model.

2.1.2. *In vitro* reconstitution of 50S

Assembly of the large ribosomal subunit requires the coordinate association of two rRNAs and 33 ribosomal proteins. *In vivo*, additional ribosome assembly factors, such as RNA helicases, small GTPases, pseudouridine synthetases, chaperones (Hsp70) and RNA methyltransferases, are also critical for ribosome assembly (El Hage *et al*., 2001; Alix and Nierhaus, 2003; Maki *et al*., 2003; Semrad *et al*., 2004; Maki and Culver, 2005; Al Refai and Alix, 2008). The *in vivo* assembly of ribosomes in *E. coli* takes only a few minutes at 37 ^{o}C (Lindhal, 1975), whereas *in vitro* the two-step procedure for the total reconstitution of the large ribosomal subunit requires very long incubation times at high temperatures (20 min/44 ^{o}C + 90 min/50 ^{o}C).

An assembly map was constructed in the 80´s for the 50S subunit of *E. coli* (Röhl and Nierhaus, 1982; Herold and Nierhaus, 1987) (Figure 14). Three reconstitution intermediates have been found: RI50(1) 33S, RI50*(1) 41S, RI50(2) 48S and 50S. The RI50(l) particle already forms at 0^{o}C (in 30 min) under the ionic conditions of the first step (4 mM Mg^{2+}) of the two-step reconstitution procedure (Dohme and Nierhaus, 1976). RI50(1) contains r-proteins L1, (L2), L3, L4, (L5), L7/12, L9, L10, L11, (L13), L15, L17, (L18), L20, L21, L22, L23, L24, (L26), L29, (L33), and (L34) (proteins given in parentheses were found in substoichiometric amounts). Five r-proteins essential for the early assembly reaction (RI50*(1) formation) bind exclusively near the 5'-end of the 23S RNA. These five essential r-proteins are L4, L20, L22 and L24 that bind on the first rRNA domains. In addition, L3 is considered to be the initiator protein that binds at the 3'-end of the 23S rRNA. The existence of two major protein assembly centres (L24 and L3) located at the ends of the 23S rRNA has been confirmed by reconstitution experiments using separate transcripts of the six major structural domains of 23S RNA (Ostergaard *et al*., 1998). This study indicates that the two centres assemble independently of each other around protein L24 and L3. Following this step five primary binding proteins L3, L4, L20, L22 and L24 play an essential role in the assembly of the first reconstitution intermediate.

Like during 30S subunit assembly, where the primary binding protein S15 deficient *E. coli* strain survives and the overall architecture of the 30S subunits formed in the ΔrpsO strain at 37°C is not altered (Bubunenko et al., 2006), the same phenomenon occurs with the initiation protein L24 (Dabbs, 1982; Franceschi and Nierhaus, 1988). A mutant lacking the assembly-initiator protein L24 shows distinct phenotypic features (temperature sensitivity, growth rate reduced by a factor of 6 at permissive temperatures below 34 degrees C, underproduction of 50S subunits). R-protein L20 can replace L24 for the initiation of assembly of 50S subunit (Franceschi and Nierhaus, 1988). This suggests that assembly landscape model is valid also for 50S assembly, where the assembly of a particle can proceed along many possible pathways.

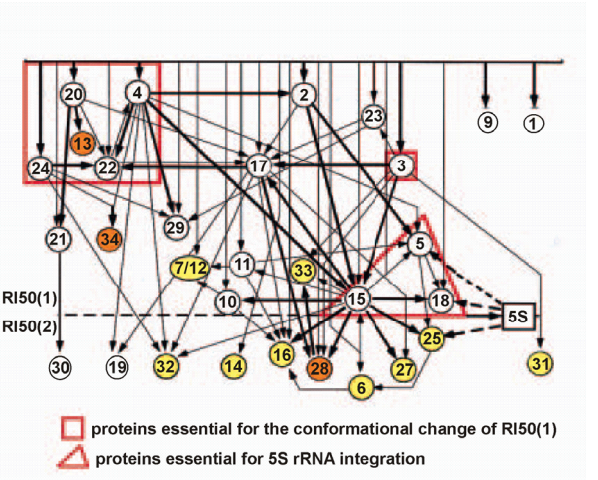


Figure 14. The *in vitro* assembly map of the 50S ribosomal subunit. Arrows indicate interactions between components. Thick and thin arrows indicate that binding of large ribosomal subunit protein is strongly or weakly dependent on other r-protein, respectively. Proteins enclosed by the red rectangle are essential for early assembly. Proteins enclosed by the red triangle are essential for 5S rRNA integration. Components below the dotted line are not present on the early RI50(1) particle (adapted from Charollais et al., 2003).

In vitro 50S reconstitution continues from RI50(1) (33S) proceeding to RI50*(1) (41S) formation. RI50*(1) is formed when RI50(1) particles are incubated at 44°C in the presence of 4 mM Mg^{2+}. Addition of 10 r-proteins L6, L14, L16, L19, L25, L27, L28, L30, L31 and L32 to the RI50*(1) particles at 44°C, 4 mM Mg^{2+} results RI50(2) (48S) particles and increasing temperature/ ionic strenght (50°C, 20 mM Mg^{2+}) completes 50S reconstitution (Dohme and Nierhaus, 1976).

2.2. Ribosomal protein tails and their role in subunit assembly

Almost half of the proteins have globular bodies with long extensions (tails) that penetrate deeply into the ribosome core (Ban *et al*., 2000; Wimberly *et al*., 2000; Harms *et al*., 2001; Selmer *et al*., 2006; Schuwirth *et al*., 2005). In fact, all S-proteins (except S4 and S15) and about 50% of the L-proteins contain extensions, which are either "random coil" located at N- or C-termini or α-helix or β-sheet in the central region of the protein (Wilson and Nierhaus, 2005). Although the biological role of the extensions is still unclear, based on the crystal structures of the ribosome subunits it has been postulated that they could participate in ribosome assembly (Klein *et al*., 2004). The extensions of ribosomal proteins often lack an obvious tertiary structure and many regions are

also missing significant secondary structures. The globular domains of r-proteins are found on the surface of a particle, while extensions penetrate deeply into the subunit and intertwine with rRNA helices. The lack of structure of the proteins that contain extensions makes them hard to crystallise in their free state. If crystallisation is possible, the extensions are generally not visible in the electron density map since they are disordered. The extensions of r-proteins are basic and flexible, a property that makes them candidates for assembling RNA segments during rRNA folding. In both subunits, these extensions have a distinctive amino acid composition and they differ from the globular domains mainly in glycine (13.7% vs. 8%), arginine (15.9% vs. 7.5%) and lysine (12.7% vs. 5.1%) (Brodersen *et al*., 2002; Klein *et al*., 2004). The basic nature of the extensions enables them to neutralize the highly negatively charged RNA backbone and the higher glycine content is supposed to increase their flexibility and to avoid steric clashes in tightly packed RNA regions. The finding of extensions in proteins (L3, L4, L22 and L20) essential for the formation of the first intermediate RI50(1) during *in vitro* reconstitution experiments has suggested that they may participate in ribosome assembly. Co-folding or disorder/order transition in r-proteins extensions would help to avoid the kinetic traps that frequently impede the correct RNA folding during the course of ribosome assembly (Tompa and Csermely, 2004).

The structure of the protein extensions within the crystal of the ribosome particles provides a view of the final product of the assembly. Recent genetic, biochemical and structural data have shown that r-protein extensions are not systematically required for the subunit assembly (Brodersen *et al*., 2002; Hoang *et al*., 2004; Zengel *et al*., 2003; Timsit et al., 2009). For example, the fact that some r-proteins that possess extensions are not essential for ribosome assembly indicates that they are not strictly correlated with an assembly function. It is also important to note that in the 30S subunit none of the primary binding proteins have extended basic tails and they appear to be typical globular proteins (Brodersen *et al*., 2002). In addition, in the 50S particle the assembly initiator protein L24 does not have an extension that penetrates the ribosome. L24 is bound at the ribosome surface similarly to the other globular domains of other ribosomal proteins.

Deletion mutants of r-proteins extensions should answer the question of their function in assembly. The effect of deletion of the extensions of L4 and L22, two primary binding proteins that are essential for the 50S subunit assembly, has been tested *in vivo* (Zengel *et al*., 2003). This study has shown that the extended loop of L4 and β-hairpin of L22 are not only dispensable for assembly into 50S ribosomal particles but also for the proper assembly of proteins that bind later in 50S assembly pathway. These experiments provide a clear demonstration that the globular domains of these two proteins are sufficient to initiate the assembly of the large 50S particles. In consequence, this finding does not support the general concept that extensions of ribosomal proteins play a role in ribosome assembly. Another study has also shown that C-terminal tails of S9 and S13 are not essential for ribosome functions (Hoang *et al*., 2004).

L20, which is one of the most basic proteins of the eubacteria, is a primary binding protein that belongs to the five proteins essential for the first reconstitution steps *in vitro* (Nowotny and Nierhaus, 1980). L20 can also replace the assembly initiator protein L24 for the initiation of assembly at low temperatures (Franceschi and Nierhaus, 1988). L20 has also been shown to be essential *in vivo*, as a deletion within its gene is lethal (Guillier *et al*., 2005). More importantly, deletion experiments have shown that the N-terminal extension is strictly required for normal ribosome assembly (Guillier *et al*., 2005). Thus, L20 is the sole example for which the extension is strictly required for the assembly of the large ribosome subunit *in vivo*. Biochemical data therefore clearly indicate that all the extensions of ribosomal proteins are not required for assembly and extensions may have other functions in the translating ribosome.

2.3. Functions of individual r-proteins

Some r-proteins have an essential function in the assembly of ribosomal subunits, but after the ribosome is fully assembled, these r-proteins are not ultimately important for catalytical functioning of ribosomes. They stay in the ribosome to improve the stability of particle. It seems that proteins S6, S9, S13, S17, S20, L1, L9, L11, L15, L19, L24, L27 to L30, and L33 neither are essential for the translational function of the ribosome, because *E. coli* strains lacking these r-proteins are viable (Dabbs, 1986; Herr *et al*., 2001). As the ribosome is a large multicomponent macromolecule, these single r-protein deletions may be compensated by other elements of the ribosome *in vivo*. It is possible that if two or more non-essential r-proteins are simultaneously deleted from genome, together these r-protein deficient strains do not survive *in vivo*. *In vitro* experiments show different kinds of functions (translation accuracy, tRNA interaction, subunit association, subunit assembly, translation control etc) for these *in vivo* non-essential r-proteins. Therefore, these r-proteins facilitate ribosome functioning by fine-tuning different stages of ribosome mediated protein synthesis and ribosome formation.

2.3.1. S1 and mRNA binding

S1 is the largest *E. coli* r-protein, with 557 amino acids and a molecular weight of 61,558 Da. S1 is important but not essential for viability of *E. coli*. S1 interacts with both the ribosome and mRNA. This allows it to function in the initiation of translation possibly by catching the mRNA and directing it to the ribosome. S1 is weakly bound to ribosomes and thus can readily exchange between free ribosomes. A pool of about 10 to 20% of cellular S1 is found in the post-ribosomal supernatant, whereas most of the r-proteins do not have a detectable pool (Ulbrich and Nierhaus, 1975). In contrast to polysomes, free ribosomes do not contain a full complement of S1 (Subramanian, 1983).

S1 contains six repeats of the so-called S1 motif which is a five-stranded antiparallel β-barrel RNA binding motif (Bycroft *et al*., 1997). The S1 motif is also found in the translation initiation factors, the bacterial IF1, eukaryotic eIF2α and polynucleotide phosphorylase. S1 is known to directly bind to RNA, even in the absence of the ribosome, although with low specificity and a relatively low binding constant ($\sim 3 \times 10^6$ M^{-1}) (Subramanian, 1983). S1 NTD is important for ribosome binding, while the middle three S1 motifs appear to have a role in mRNA binding.

S1 is located at the junction between the head, platform, and body of the 30S subunit (Golinska *et al*., 1981; Capel *et al*., 1988; Walleczek *et al*., 1990). In the 30S subunit, S1 is surrounded by a number of r-proteins (S2, S6 to S9, S11, and S18) with extensive contacts to r-proteins S21, S11, and S18. S1 also contacts 16S rRNA, predominantly helices h26 and the ultimate h45 that contains the anti-Shine-Dalgarno sequence. The platform placement of S1 in the 30S subunit, and crosslinks to the 5′ end of an mRNA (Czernilofsky *et al*., 1975) indicate that the major function of S1 might be to bring the mRNA onto the 30S subunit, thereby assisting subsequent mRNA interactions between the SD sequence in the mRNA with the 3′ end (anti-SD) of 16S rRNA in order to position the AUG at the ribosomal P-site (Subramanian, 1983). This is consistent with the observation that translation of leaderless mRNAs does not require S1 (Moll *et al*., 2002), since they do not depend on SD interactions.

2.3.2. mRNA entry site and ribosomal proteins S3, S4 and S5

The ribosome covers approximately 35 to 40 nucleotides of an mRNA (Beyer *et al*., 1994). The ribosomal entry site for the mRNA and the following upstream tunnel is a functional region dominated by ribosomal proteins S3, S4, and S5 (Figure 15). Basic residues (mainly Arg and Lys) from these r-proteins protrude into the subunit and form a tunnel like structure with a diameter of ~15 Å (Yusupova *et al*., 2001; Takyar *et al*., 2005). The minimum secondary structure of RNA, a double helix with a diameter of 20 Å, is not capable to enter the mRNA entry site. It is therefore clear that any secondary structure present in mRNA has to be melted in order to allow the mRNA to pass through the entry pore. It has long been known that the translating ribosome is able to unwind RNA/RNA duplexes with a stability lower than $\Delta G = -60$ kcal x mol^{-1} (Kozak, 1989). Takyar and co-workers (2005) demonstrated that the ribosome itself is capable of melting double-stranded RNA with a length of up to 27 base pairs (Tm = 70°C). Consistent with this finding was the observation that mutations of the basic residues in S3 and S4 (but not S5) that protrude into the mRNA entrance pore severely impaired the ribosome helicase activity. The ribosomal helicase activity was shown not to require energy in form of GTP or ATP, instead the driving force was the translocation reaction. The movement of mRNA and two tRNAs from the A-P to the P-E sites was sufficient to unwind secondary structure in the mRNA (Takyar *et al*., 2005).

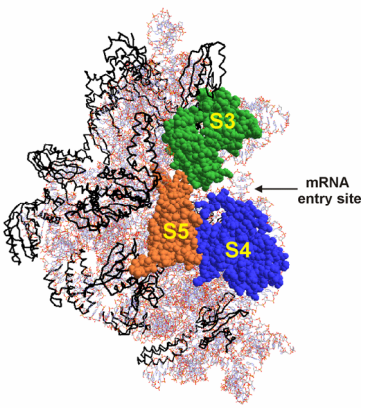


Figure 15. mRNA entry site in the crystall structure of the *Escherichia coli* 30S subunit (PDB accession code 2AVY). Ribosomal proteins S3, S4 and S5 form mRNA entrance channel. Ribosomal RNA is in a wireframe structure, r-proteins (S3, S4 and S5) are in spacefill, and all other r-proteins are in black wireframe (used PyMol).

2.3.3. Translation accuracy and ribosomal proteins S12, S4 and S5

Mutations in many ribosomal components can impair ribosomal accuracy. Mutations in tRNAs, EF-Tu, rRNAs and r-proteins (S7, S11, S17, L6, and L7/L12) have been shown to negatively affect fidelity. Strongest translational accuracy effects have been seen in r-proteins S12, S4, and S5 and in h27 of 16S rRNA.

Many S12 mutations confer resistance to streptomycin (Biswas and Gorini, 1972; Garvin *et al*., 1973; Garvin *et al*., 1974; Kurland *et al*., 1990). Most of these S12 mutations have associated phenotypes, being either dependent on the antibiotic for cell growth (SmD) or resistant but hyperaccurate in translation (restrictive SmR). Streptomycin is an error-inducing aminoglycoside antibiotic. Mutants conferring resistance to, or even dependence on, streptomycin have an increased accuracy and, in most cases, a slower elongation rate. S12 mutations and the omission of this protein from *in vitro* reconstituted 30S subunits lead to an increase in accuracy, suggesting that wild-type S12 increases the rate of translation at the cost of accuracy. The highly conserved residue Ser46 of *E. coli* S12 participates in the recognition of the middle base-pair of A-site codon-anticodon interaction and Pro44 indirectly monitors the third base-pair position (Ogle *et al*., 2002). In an antibiotic-free environment, these resistant bacteria are expected to be at a disadvantage compared with the sensitive strains because of their lowered fitness (Maisnier-Patin et al., 2002). Compensation is of special interest with regard to the potential reversibility of antibiotic resistance, as antibiotic-resistant bacteria may adapt genetically to the costs by acquiring mutations that restore fitness.

Second-site compensating mutations that phenotypically reverse streptomycin dependency and hyperaccuracy have been isolated and localized to rpsD and rpsE, the genes for the ribosomal small subunit proteins S4 and S5 (Rosset

and Gorini, 1969; Bjare and Gorini, 1971; Piepersberg et al., 1975). Mutations in the S4 and S5 genes increase misincorporations, a 10-fold reduction in translation accuracy (Kurland *et al*., 1990). The resulting phenotype is termed *ram* (ribosomal ambiguity mutants) and cells displaying the *ram* phenotype remain viable despite a high error rate in protein synthesis.

Selection of the correct tRNA by the ribosome triggers a transition of the 30S subunit from an open to a closed form (Ogle *et al*., 2002). The closed form is required for accommodation of the aa-tRNA to the A site after the decoding step. Transition into the closed form involves disruption of multiple interactions at the interface between S4 and S5, and establishment of salt-bridge interactions between S12 and either h44 or h27 of the 16S rRNA. *Ram* mutations in S4 and S5 would also lead to disruptions at this interface of these proteins, suggesting that the observed error-prone ribosomes are in the closed form, and mutations in S12 that block salt-bridge formation may destabilize the closed form thus confering resistance to streptomycin.

The presence of an E-site tRNA has also been shown to influence the accuracy of A-site decoding (Geigenmüller and Nierhaus, 1990) and maintenance of the ribosomal reading frame (Marquez *et al*., 2004), suggesting that interactions in the E-site may also contribute to the transition between open to closed forms. This allosteric linkage between A- and E-sites may also explain how the binding of the antibiotic edeine within the E-site of 70S ribosomes can cause severe translational misreading at the ribosomal A-site (Dinos *et al*., 2004). It has been shown that r-proteins S7 and S11 can influence translational fidelity from their position in the E-site (Robert and Brakier-Gingras, 2003). S7 and S11 form part of the binding site of the anticodon loop of the E-tRNA, and mutations that disrupt the interface between S7 and S11 led to readthrough, frameshifting and mis-incorporation events similar in extent as those seen in the presence of streptomycin (Robert and Brakier-Gingras, 2003).

2.3.4. P-site tRNA stability and L9

L9 has two globular RNA binding domains, separated by a remarkably long and invariant nine-turn α-helix, with the NTD (1 to 52) showing structural homology to r-proteins L7/L12 and L30 (Hoffman *et al*., 1994; Hoffman *et al*., 1996). L9 binds at the base of the L1 stalk, whereas the L9 NTD domain contacts predominantly H76, and the CTD (59 to 149) extends more than 50 Å away from the surface of the subunit (Yusupov *et al*., 2001). An intresting phenomenon with L9 is observed in ribosomes of *E. coli* and *T. thermophilus*. In all previously reported crystal forms of ribosomes from both *E. coli* and *T. thermophilus* (Schuwirth *et al*., 2005; Korostelev *et al*., 2006; Selmer *et al*., 2006; Gao *et al*., 2009), a contact with ribosomal protein L9 from a neighboring molecule would result in a steric clash with a bound GTPase factor. This interaction of L9 is so strong that attempts to crystallize EF-G in complex with the ribosome resulted in a high-resolution crystal form in which the L9 contact had displaced the factor from the ribosome (Selmer *et al*., 2006; Gao *et al*., 2009). To

crystallize EF-G in complex with the ribosome the L9 gene must be truncated (Gao *et al*., 2009). The portion of the L9 gene coding from residue 56 to the C-terminus was deleted by replacement with a kanamycin resistance gene by homologous recombination (Gao *et al*., 2009). Interestingly, the N-terminal residues of L9 were not visible in the structure, suggesting that it was either degraded or not incorporated into ribosomes.

Mutations of certain amino acids in the CTD of L9 have been shown to stimulate translational bypassing by 10-fold (*hop-1* phenotype) (Adamski *et al*., 1996). Deletion of the gene coding for the ribosomal protein L9 (*rplI*) has been found to produce two- to threefold increase in -1 frameshifting and hopping over stop codons, leading to the suggestion that L9 may influence mRNA movement through the ribosome, rather than P-site tRNA stability, as thought at first (Herr *et al*., 2001).

It seems unlikely that L9 can directly contact the P-site tRNA, as seen from crystal structures. One possibility is that the CTD of L9 makes contact with the head of the L1 stalk and influences tRNA stability by altering the orientation of the stalk relatively to the ribosome. This is supported by toeprinting experiments, whereas the CTD of L9 was found to bind 5′ to nucleotide 2179 (H77) near to the L1 binding site (Adamski *et al*., 1996). Therefore, L9 may adopt different orientations during translation to influence tRNA stability. It should be mentioned that L9 is not present in archaea or eukaryotes and therefore probably represents a fine-tuning mechanism specific to bacteria (Herr *et al*., 2001; Lieberman *et al*., 2000).

2.3.5. tRNA interactions with r-proteins

2.3.5.1. L16 and L27 interactions with A- and P-site tRNA

L16 and L27 are globular r-proteins that have extensions toward the PTC. Chemical modification of L16 at its single histidine residue at position 13 greatly reduces peptide bond formation (Baxter *et al*., 1980; Maimets *et al*., 1983; Tate *et al*., 1987). L16 makes potential contacts with the elbow region (TΨC arm) of both A- and P-site tRNAs. *E. coli* Arg10 and His13 in the N-terminus of L16 interact with the backbone of C63 P-tRNA, whereas Arg55 and His13 are within hydrogen bonding distance to the backbone of U52-G53 of an A-tRNA (Nishimura *et al*., 2004).

It was found that several inhibitors of peptidyl transferase activity could be crosslinked to L27 (Sonenberg *et al*., 1973; Tejedor and Ballesta, 1985; Arévalo *et al*., 1989; Bischof *et al*., 1995; Colca *et al*., 2003), among which was puromycin (Nicholson and Cooperman, 1978), a mimic of the aminoacyl-adenosine moiety of aminoacyl-RNA (Monroe and Marcker, 1967). Deletion of the gene for protein L27 led to a severe growth defect, a sharp decline in peptidyl transferase activity and a decrease in the binding of aminoacyl-tRNA to the A-site (Wower *et al*., 1998). These dramatic effects suggest that L27, the most basic protein in the *E. coli* ribosome, may facilitate peptide bond formation by

influencing the organization of the 23S rRNA, or helping to position the tRNA molecules, at the peptidyl transferase center. Truncations of L27 suggest that the N-terminal amino acids may come within close proximity to the aminoacyl moiety of the P-tRNA (Maguire *et al*., 2003). In the *D. radiodurans* 50S subunit, the flexible N-terminus of L27, specifically the highly conserved lysine residues at positions 4 and 5 of L27 contact the backbone of G4-A5 of the acceptor stem of tRNA.

It was shown that the extensions of L16 and L27 interact with the ribosome recycling factor (RRF) (Wilson *et al*., 2005). Since both, RRF and L27 are only present in bacteria (and organelles), the function of L27 may be associated with ribosome recycling rather than with tRNA binding. In contrast, L16 has a homolog in archaea and eukarya called L10e, and therefore may play a more fundamental role in translation. However, the loop in L16 that contacts RRF has no equivalent in L10e. Interestingly, mutations in L16 give rise to evernimicin resistance, an oligosaccharide antibiotic, that has been proposed to inhibit 70S initiation complex formation in an IF2-dependent manner (Belova *et al*., 2001), suggesting a possible link between L16 and translation initiation.

2.3.5.2. S9, S13 and L5 interactions with A- and P-site tRNA

An interesting r-protein is S13 that likely plays a functional as well as structural role at the small subunit interface. Based on X-ray crystallographic evidence this protein is positioned to the subunit interface in the head of the small subunit (Wimberly *et al*., 2000). There, S13 contacts the large subunit of the ribosome in the central protuberance and forms two specific bridges, B1a and B1b, which were identified first by cryo-electron microscopy (cryoEM) (Frank *et al*., 1995a; Lata *et al*., 1996; Gabashvili *et al*., 2000). B1a connects the middle of S13 (around residue 93) to helix 38 (ASF) of the 23 S rRNA and B1b connects the N-terminus of S13 to the large subunit protein L5 (Figure 16). These regions of the large subunit, helix 38 and protein L5, are of particular interest because they make direct contacts with the A- and P-site tRNAs. The C-terminus of S13 contacts the anticodon stem of the P-site tRNA around nucleotide 36. In addition, lysine 120 of protein S13 interacts with the A-site tRNA backbone around position 41 (Yusupov *et al*., 2001). At its elbow, a β-hairpin loop of protein L5 (positions 54–66) interacts with the T-loop of P-tRNA at the minor-groove face of C56 (Yusupov *et al*., 2001).

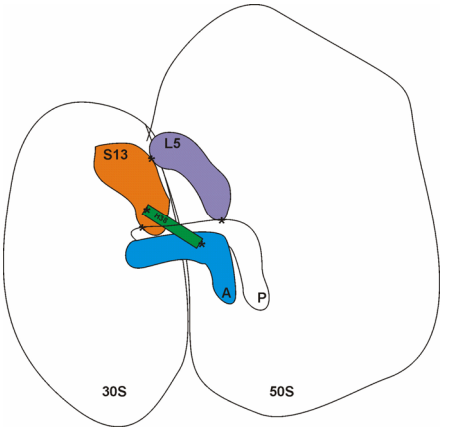


Figure 16. Schematic description of the interactions that ribosomal protein S13 makes with both, large subunit and tRNA elements. Those contacts that are supported by structural analysis are indicated with a star (Yusupov et al., 2001); C-terminal residues of S13 contact the P-site tRNA anticodon region, helix 38 of 23 S rRNA contacts S13 and the A-site tRNA elbow through bridge 1a and large ribosomal subunit protein L5 connects S13 to the P-site tRNA elbow region through bridge 1b (adapted from Cukras and Green, 2005).

Ribosome crystal structures have revealed that S9 and S13 have C-terminal tails which, together with several features of 16S rRNA, contact the anticodon stem-loop of P-site tRNA (Wimberly *et al*., 2000; Yusupov *et al*., 2001). All tail deletions, including double mutants containing deletions in both S9 and S13, were viable, showing that *Escherichia coli* cells can synthesize all of their proteins by using ribosomes that contain 30S P sites only composed of RNA. However, these mutants have slower growth rates, indicating that the tails may play a supporting functional role in translation (Hoang *et al*., 2004). *In vitro* assays indicate that the C-termini of S9 and S13 contribute significantly to binding of P-tRNA to isolated 30S subunits, although the importance of the tails varies for different tRNA species (Hoang *et al*., 2004). The lysine-rich tail of S13 is phylogenetically somewhat variable than the tail of S9 which is highly conserved. The C-terminal arginine (128) of S9 appears to interact with phosphate 35 in the anticodon of P-tRNA, and is universally conserved (Yusupov *et al*., 2001). These two P-tRNA phosphates (35 and 36) were identified in phosphorothioate-interference experiments to be important for binding to the 30S P-site (Schnitzer and Ahsen, 1997).

2.3.5.3. E-site tRNA interactions with L1 and S7

The tRNA exit (E) site has been implicated in several ribosomal activities, including translocation, decoding, and maintenance of the translational reading frame. The important E-site element is a two-domain protein L1 (Nevskaya *et al*., 2006). Its binding region (H76–77) on 23S rRNA interacts with the elbow of E-tRNA (Yusupov *et al*., 2001). Two regions in the 50S subunit that change their positions as part of the translational elongation cycle are the L1 and L11 arms (Schuwirth *et al*., 2005). The L1 arm is thought to influence movement of tRNA into and out of the E-site (Yusupov *et al*., 2001; Valle *et al*., 2003b). E-tRNA interacts also with the protein S7 in the small subunit head (Yusupov *et al*., 2001). The C-terminal α-helix of protein S7 packs against the backbone of the anticodon stem, whereas the S7 β-hairpin is positioned at the Watson-Crick face of the E-tRNA anticodon. The blockage of the exit path for the E-tRNA by protein S7 and by L1 and its rRNA binding site requires that one or both of these structures move to allow release of the deacylated tRNA (Yusupov *et al*., 2001). There is an enormous 70 Å difference in the tip position of the L1 stalk in the pre- and post-translocational states of the ribosome (Beckmann *et al*., 2001; Spahn *et al*., 2001).

Ribosomes obtained from *E. coli* strains lacking L1 display a 40% to 60% reduced activity in polypeptide synthesis, which can be rescued by the addition of L1 (Wilson and Nierhaus, 2005). Deletion of ribosomal protein L1 stabilizes the classical tRNA state by disrupting tRNA binding in the E-site and hybrid tRNA configurations were significantly destabilized (Munro *et al*., 2007). By using single-molecule fluorescence resonance energy transfer (smFRET), an interaction between the ribosomal L1 stalk and the newly deacylated tRNA is established spontaneously upon peptide bond formation (Fei *et al*., 2008). In the absence of elongation factor G, the entire pretranslocation ribosome fluctuates between just two states: a nonratcheted state, with tRNAs in their classical configuration and no L1 stalk-tRNA interaction, and a ratcheted state, with tRNAs in an intermediate hybrid configuration and a direct L1 stalk-tRNA interaction (Fei *et al*., 2008). The L1 stalk-tRNA interaction persists throughout the translocation reaction, suggesting that the L1 stalk acts to direct tRNA movements during translocation. The structure of L1 in complex with its own mRNA has been determined revealing remarkable similarity with the L1-rRNA complex (Nevskaya *et al*., 2005). However, the mRNA has a shortened loop B compared to the equivalent region in the rRNA, which removes the potential for a number of hydrogen bonds with L1. This may explain the 5- to 10-fold higher affinity of L1 for the rRNA than the mRNA.

Introducing a deletion in rpsG (S7) that truncates the beta-hairpin of ribosomal protein S7 (S7DeltaR77-Y84) increases both –1 and +1 frameshifting but does not increase miscoding, thus providing evidence that the 30S E-site plays a specific role in frame maintenance (Devaraj *et al*., 2009). S7 is also important for 30S assembly, all of the proteins that reside in the head of the 30S subunit, except S13, have been shown to be part of the S7 assembly branch, that is, they all depend on S7 for association with the assembling 30S subunit (Grondek and

Culver, 2004). R-proteins S9, S19, S14, S10, S3, and S2 depend on S7 for their association with the assembling 30S subunit (Mizushima and Nomura, 1970; Held *et al*., 1974; Nowotny and Nierhaus, 1988).

2.3.6. L7/L12 stalk and factor binding

L7/L12 forms the pentameric complex L10 × $(L7/L12)_4$. The acidic L7/L12 (pI ~4.9) is the only protein present in four copies per *E. coli* ribosome. In the thermophilic bacterium *Thermus thermophilus* a heptameric L10 x $(L7/L12)_6$ complex exists (Ilag *et al*., 2005). L7/L12 do not interact with rRNA, but two dimers bind independently to the C-terminal region of L10 (positions 71 to 164) to form the pentameric complex. L7/L12 can be selectively removed from ribosomes by washing with 50% ethanol and 1M ammonium chloride, a process which results in loss of factor-dependent GTPase activity. L7/L12 can then be rebound to restore activity (Kischa *et al*., 1971; Hamel *et al*., 1972).

The complex (L10 × $L7/L12_4$) has a high stability that partially resists even 6 M urea and was considered to be an individual protein termed L8 in the early days of ribosome research (Pettersson *et al*., 1976). L7 is the N-acetylated form of L12 (Gordiyenko *et al*., 2008). Increased acetylation of L12 NTD occurs during the stationary growth phase in *E. coli* (Gordiyenko *et al*., 2008). Growing cells in minimal medium results in deficient removal of N-terminal methionine in approximately 50% of the L12 population while processed L12 is almost 100% acetylated. Interaction between L7 and L10 is tighter relative to that between L12 and L10 (Gordiyenko *et al*., 2008). Since acetylation is predominant when cells are grown in minimal medium, the modification is part of the cell's strategy to increase stability of the stalk complex under conditions of stress.

The L7/L12 proteins contain three functional regions: a N-terminal domain (NTD, residues 1–37), a very acidic C-terminal domain (CTD, residues 50–120) and a flexible hinge-region (residues 38–49). NTD is required for protein-protein interaction and dimerization. NTD binds L7/L12 to r-protein L10, and dimerization is essential for this process. CTD improves ribosome binding to the elongation factors and stimulates their GTPase activities (Kischa *et al*., 1971; Dey *et al*., 1995). The hinge-region facilitates independent movements of the N- and CTD domains. L7/L12 flexibility prevents their structure determination. In the *Thermus thermophilus* 70S × $(tRNA)_3$ crystal structure, only one dimer of L7/L12 has been modelled onto the ribosome (Yusupov *et al*., 2001). Deletions within the L7/L12 hinge region lead to translational misreading, deceased factor binding and a lowered rate of protein synthesis (Gudkov *et al*., 1991). The hinge region seems to be important in maintaining the flexibility of the CTD of L7/L12 required during elongation (Peske *et al*., 2000).

L12 binds directly to the factors IF2, EF-Tu, EF-G, and RF3 from *E. coli* (Helgstrand *et al*., 2007). Ribosomes containing only a single L7/L12 dimer (made by removing 10 amino acids from the C-terminus of L10) supported EF-

G-dependent GTP hydrolysis and protein synthesis *in vitro* with the same activity as that of two-dimer particles (Griaznova and Traut, 2000). This means that at least one dimer is needed to support translation efficently.

In hybrid ribosomes that were generated in *E. coli*, L7/L12, L10, and L11, were replaced by their eukaryotic counterparts from rat P1/P2, P0 and L12, respectively (Uchiumi *et al*., 2002). Both, the *in vitro* translation and GTPase activity of the resultant hybrid ribosomes were strictly dependent on the presence of the eukaryotic elongation factors, EF2 and EF1a. Mutating two EF-G glutamic acid residues (224 and 228) to lysine (E224K and E228K) within helix AG' caused large defects in GTP hydrolysis and smaller defects in ribosomal translocation (Nechifor *et al*., 2007). Removal of L7/L12 from the ribosome strongly reduced the activities of wild type EF-G but had no effect on the activities of the E224K and E228K mutants. These results provide evidence for functionally important interactions between helix AG' of EF-G and L7/L12 of the ribosome.

2.3.7. L11 and GTPase-associated centre

The L11 protein is located at the base of the L7/L12 stalk of the 50S subunit of the *Escherichia coli* ribosome (Agrawal *et al*., 2001; Gao *et al*., 2003; Schuwirth *et al*., 2005). L11 is a highly conserved, 14.8 kDa ribosomal protein that is associated with the functional center, the so called GTPase-associated centre (GAC). Protein L11 and the antibiotic thiostrepton bind cooperatively to the highly conserved 58 nucleotide long target segment (nucleotides 1051–1108 region) of the 23S rRNA of the ribosome (Thompson *et al*., 1979; Ryan *et al*., 1991). In addition to L11 and the 58 nucleotide segment of 23S rRNA, the GTPase-associated region contains another highly conserved stretch of 23S rRNA, the α-sarcin/ricin stem-loop (H95) (Ryan *et al*., 1991).

Protein L11 consists of two domains: a C-terminal domain is responsible for the tight interaction with the rRNA that is connected through a flexible linker to the N-terminal domain, and a NTD which is required for the co-operative binding of the antibiotic thiostrepton. Thiostrepton is one of the most effective blockers of translocation (Vazquez, 1979). Since thiostrepton also blocks the factor-dependent GTPase, the L11-binding site on 23S rRNA was identified as part of the "GTPase-associated center". Mutations in the NTD (P22S/T in *E. coli* L11) confer resistance to thiostrepton, although not by affecting interaction of thiostrepton with the rRNA, but perhaps by allowing L11 the freedom to move despite the presence of thiostrepton (Porse *et al*., 1998). Interestingly, in bacterial (and archaeal) ribosomes, on which thiostrepton is active, Pro22 (Pro18 in archaea) in L11 is conserved, whereas at the equivalent position in eukaryotic L11, this proline is not conserved. This is consistent with the natural resistance of eukaryotic ribosomes to thiostrepton (Wilson and Nierhaus, 2005). Mutations in the 23S rRNA, 2′-*O*-methylation (natural resistance mechanism of thiostrepton-producing *Streptomyces* strains) or mutation of A1067 (as well as 1095) confer resistance to thiostrepton (Hummel and Böck, 1987; Rosendahl

and Douthwaite, 1993; Mankin *et al*., 1994; Cameron *et al*., 2004). These two 23S rRNA positions interact directly with thiostrepton by forming a binding pocket for thiostreptone (Lentzen *et al*., 2003). A1067 and, more weakly, A1096 are also protected by EF-G●GTP from chemical modification (Cameron *et al*., 2002). Furthermore, A1067U mutation impairs the function of EF-Tu and EF-G (Saarma *et al*., 1997). Thiostrepton has been shown to reduce the binding affinity of EF-G for the ribosome and therefore decreases the EF-G dependent GTPase activity (Cameron *et al*., 2002). Each one of the two elongation factors, EF-G and EF-Tu, is supposed to recognize one specific conformation of the GAC, thus producing a different chemical modification protection pattern in this region (Bowen *et al*., 2005). In spite of its structural and functional relevance, protein L11 is not absolutely essential for ribosome activity since bacterial strains lacking this protein are viable, although they grow very poorly (Cundliffe *et al*., 1979).

The crystal structure of the large ribosome subunit from *Deinococcus radiodurans* in complex with the antibiotic thiostreptone reveals binding within a cleft located between the ribosomal protein L11 and helices 43/44 of the 23S rRNA, and an overlap of the EF-G domain V, thus explaining how this class of drugs perturbs translation factor binding to the ribosome (Harms *et al*., 2008). The L11-NTD constitutes ~50% of the buried surface area of thistreptone, thereby explaining why the absence of L11 dramatically reduces the affinity of thiostreptone for the ribosome to confer resistance (Cundliffe *et al*., 1979; Thompson *et al*., 1979; Harms *et al*., 2008). The thiazole rings THZ6 and THZ14 of thiostreptone stack upon Pro22 and Pro26, respectively, within L11-NTD, and therefore mutations, for example to Ser, Leu, or Arg at these positions, or deletion of neighboring residues Ala20-Pro21 could abolish this type of interaction (Harms *et al*., 2008). More likely is that the mutations have a global influence on the conformation of the L11-NTD by disrupting the proline-rich helix α1, explaining why mutations at Pro23 can also confer resistance, even though this residue does not come within 7Å of thiostreptone (Harms *et al*., 2008). As mentioned above, in eukaryotic organisms, the Pro residues in L11-NTD are not conserved; specifically, Pro22, Pro23, and Pro56 are usually Ser, Ala, or Thr at the equivalent positions. Since mutations at positions Pro22 and Pro23 (to Ser or Thr) as well as Pro56 (to His) confer resistance to thiostreptone in various bacteria (Cameron *et al*., 2004), the natural thiopeptide resistance of eukaryotes can be explained by presence of Ser, Ala, and Thr at these positions. However, it should be noted that 90%–98% of known eukaryotic 23S rRNA sequences also contain a guanine at the position equivalent to *E. coli* A1067, and as mentioned above A1067G mutations confer thiostreptone resistance in bacteria and archaea. Thus eukaryotes can be considered to be ‘‘double’’ protected through the presence of rRNA as well as protein differences. The mechanism by which thiopeptides inhibit EF-G action, is that thiostreptone sterically clashes with EF-G by mimicking regions of EF-G domain V (Harms *et al*., 2008).

It has been proposed, that L11 acts as a molecular switch to control L7 binding and plays a pivotal role in positioning one L7-CTD monomer on the G′

subdomain of EF-G to regulate EF-G turnover during protein synthesis (Harms *et al*., 2008). The first switch in L11, switch 1, is an interdomain event, which is to exist on the basis of the flexibility of the L11-NTD (Porse *et al*., 1998; Wimberly *et al*., 1999). Switch 1 involves displacement of the L11-NTD with respect to the L11-CTD and controls the widening and closure of the cleft present between the L11-NTD and H43/44 of the 23S rRNA ("on" and "off" positions). Switch 1 is stabilized in the ''on'' position upon EF-G binding in a way that the open conformation allows proper insertion and accommodation of domain V of EF-G within the cleft of the L11-NTD and H43/44. Thiostreptone interacts with both H43/44 and L11-NTD locking switch 1 in the ''off'' position by restricting the L11-NTD movement that leads to cleft widening (Harms *et al*., 2008). The second switch in L11, switch 2, is an intradomain event that involves a conformational change within L11-NTD, enabling it to promote a stable interaction between L11-NTD and L7-CTD. Switch 2 is in the ''off'' position in the presence of thiostreptone. In the absence of thiostreptone, switch 2 is in ''on'' position, so the interaction between the L11-NTD with the L7-CTD helps the L7-CTD to contact with the G´ subdomain of EF-G.

As elongation factors are loaded onto the ribosome, the L11-NTD and H43/44 oscillate between ''off'' and ''on'' positions. In case of EF-G, this transition is accompanied by the hydrolysis of GTP that has been shown to occur as soon as EF-G interacts with the ribosome. The release of Pi and translocation, which are much slower steps (Rodnina *et al*., 1999; Seo *et al*., 2004, 2006; Savelsbergh *et al*., 2005), are facilitated by the involvement of L7/L12 (Savelsbergh *et al*., 2005). In this respect, the activation of both molecular switches identified in L11 plays an important role: first, turning on switch 1 ensures the proper insertion and accommodation of domain V of EF-G into the widened cleft present at the L11-NTD and H43/44. Switch 2 promotes a stable interaction of the L7-CTD with the L11-NTD, such that L7-CTD is optimally positioned to contact the G´ subdomain of EF-G (Gao et al., 2009). L7 has been shown to be important for stimulating Pi release, but not GTP hydrolysis *per se* (Savelsbergh *et al*., 2005), suggesting that the interaction of the L7-CTD with the G´ subdomain of EF-G will trigger conformational changes within the GTP-binding pocket of EF-G to allow Pi release (Harms *et al*., 2008).

Another role for L11 in bacteria has been discovered during conditions of starvation. When nutrient levels are low, deacylated tRNA binds to the A-site of the ribosome and stalls translation. L11 detects the presence of the uncharged tRNA and signals this event to the stringent factor, RelA, which responds by catalyzing the synthesis of the alarmone (p)ppGpp (Wendrich *et al*., 2002). This signal molecule downregulates transcription of components of the translational apparatus and upregulates transcription of metabolic enzymes. This tight coupling of translation and RNA synthesis is termed the stringent control. Mutants lacking L11 (*relC*) do not show the stringent response under starvation conditions, a phenotype that is called "relaxed".

2.3.8. Polypeptide exit tunnel proteins L22 and L4

The L22 protein contains globular surface domains and a elongated C-terminal 'tail' that reach into the core of the large subunit to form part of the lining of the peptide exit tunnel. The polypeptide exit tunnel has a length of 100 Å and a diameter of approximately 10 Å at its narrowest up to 20 Å at its widest points (Yonath *et al*., 1987; Frank *et al*., 1995b; Ban *et al*., 2000). Structures show that the wall of the tunnel is composed of nucleotides from domains I through V of the 23S rRNA, as well as of the extensions of r-proteins L4 and L22 (Ban *et al*., 2000; Harms *et al*., 2001) (Figure 17). The extension of the bacterial-specific protein L32 also reaches the same tunnel region.

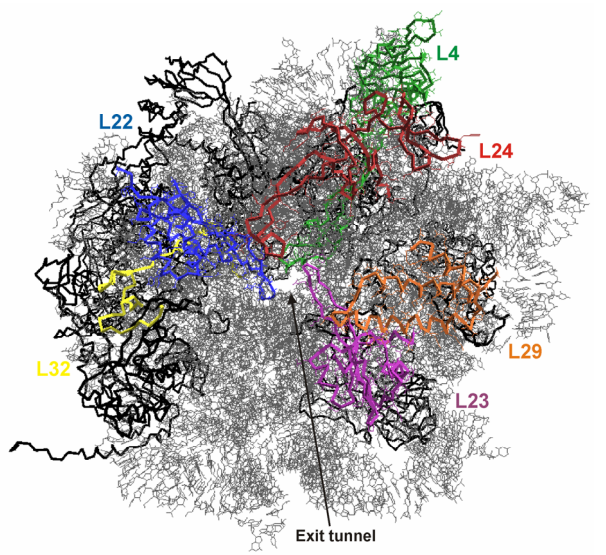


Figure 17. Ribosomal proteins located in the ribosomal exit tunnel. View through the tunnel from the cytoplasmic side of the *Thermus thermophilus* 50S subunit (PDB accession code 2WRJ) showing the positions of the r-proteins located at the exit site (L4 green, L22 blue, L23 purple, L24 red, L29 orange and L32 yellow). L4 and L22 have extensions that reach to the polypeptide exit tunnel. Ribosomal RNA is in grey wireframe structure and other r-proteins are colored as a black.

Mutations in the tail regions of L4 and L22 confer macrolide resistance in a variety of pathogenic and nonpathogenic bacteria (Wittmann *et al*., 1973; Pardo and Rosset, 1977; Chittum and Champney, 1994; Franceschi *et al*., 2004; Zaman *et al*., 2007). Macrolides consist of a 14- to 16-member lactone ring with different appended sugars and comprise a key group of inhibitors of bacterial translation by blocking the peptide exit tunnel (Vester and Douthwaite, 2001; Vimberg *et al*., 2004; Lovmar *et al*., 2006; Roberts, 2008). Insertion and deletion mutations that confer resistance to macrolides have been found at many positions along the L4 and L22 loops, but missense mutations tend to be

localized to L4 Gln62-Gly66 and L22 Arg88-Ala93 (Canu *et al*., 2002; Peric *et al*., 2003; Bogdanovich *et al*., 2006; Corcoran *et al*., 2006; Cagliero *et al*., 2006). In *E. coli*, two erythromycin-resistant mutants have been found. Strain N281 contains a deletion removing Met-Lys-Arg (MKR) corresponding to codons 82–84 of L22, and strain N282 contains a change from lysine to glutamine at codon 63 of L4 (Apirion, 1967; Wittmann *et al*., 1973; Chittum and Champney, 1994). N281, containing the mutant L22 protein, can still bind the antibiotic (Chittum and Champney, 1994). It was thought that the L22 mutation widens the tunnel in a way that erythromycin binds but the nascent polypeptide is able to pass by (Gabashvili *et al*., 2001). In a crystal structure of the *E. coli* ribosome, the MKR sequence is part of an extended L22 loop, which together with a similar loop in protein L4 forms a narrow constriction in the exit tunnel (Schuwirth *et al*., 2005; Voss *et al*., 2006) (Figure 18).

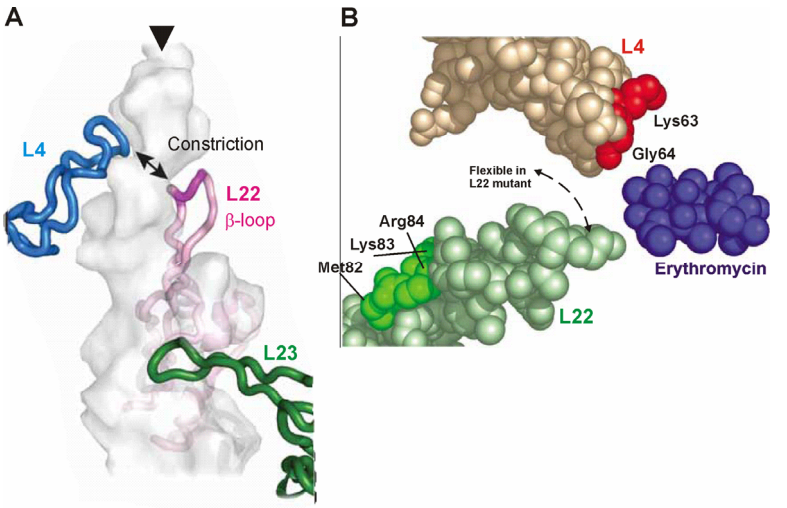


Figure 18. The path of the nascent chain through the ribosomal exit tunnel. (A) Outside view of the ribosomal tunnel (transparent gray surface). The loops of ribosomal proteins L4 (blue) and L22 (pink-magenta) form the narrowest constriction along the tunnel (arrow) (adapted from Kramer et al., 2009). (B) Localization of the erythromycin resistance mutations. The positions of the mutated amino acids in L4 and L22 that leads to erythromycin resistance in relation to the erythromycin-binding site. A dashed arrow indicates the flexibility of the β-hairpin in the L22(Δ82–84) mutant (Tu et al, 2005) (adapted from Lovmar et al., 2009).

It is further known that the ΔMKR mutation affects translation of certain mRNAs by reducing programmed ribosome stalling (Nakatogawa and Ito, 2002; Vazquez-Laslop *et al*., 2008). It is possible that AcrAB and/or TolC efflux pump activities are increased through direct effects on translation of these proteins or indirect effects on translation of a regulator. AcrA and TolC antibodies did not reveal up-regulation of these proteins in the ΔMKR strain.

Thus, although the mechanism by which the ΔMKR mutation increases macrolide resistance remains to be determined, it seems plausible that L22-mediated ribosome stalling plays a role in determining the balance of cell envelope components, which, in turn, affects macrolide resistance by altering the efficiency of efflux pumps.

Recently, another group showed a five-fold reduction in erythromycin affinity to the ribosome by the ΔMKR mutation (Lovmar *et al.*, 2009). Recent atomic resolution X-ray crystal data on the ΔMKR ribosome (Tu *et al*., 2005) suggest that the cryo-EM interpretation (ΔMKR widens peptide exit tunnel) may be an over simplification. Tu and co-workers, show that the peptide loop in L22, that is part of the peptide exit tunnel constriction, becomes bent and more flexible by the ΔMKR alteration. Accordingly, it is likely that the apparent broadening of the exit tunnel seen by cryo-EM is caused by a loss of visible density due to the flexibility of the L22 loop. Lovmar *et al*., (2009) suggest that the increased freedom of movement of the L22 constriction loop by ΔMKR alteration results in a distribution of conformations that obstruct the transport of erythromycin and perhaps other drugs through the tunnel.

Furthermore, 50S subunit assembly is inhibited in the L22 mutant at high erythromycin concentrations (Chittum and Champney, 1994). In contrast, peptidyltransferase activity and decoding are unaffected in the L22 mutant (Wittmann *et al*., 1973; O'Connor *et al*., 2004).

L4 Lys63Glu mutation alters the structure of domain V within 23S rRNA and significantly decreases ribosome affinity for erythromycin (Chittum and Chapney, 1994; Gregory and Dahlberg, 1999; Wittmann *et al*., 1973; Gabashvili *et al*., 2001).

This narrow L22 and L4 region of the polypeptide exit tunnel has also been pointed out as a region that in special cases may regulate translation *via* interaction with specific sequences (effector sequence) within the nascent chain under translation. Such sequence-specific interactions between the exit tunnel and nascent peptides suggest that the ribosome can recognize signals in the nascent chain and use them for translational regulation (Nakatogawa *et al*., 2004; Murakami *et al*., 2004; Trabuco et al., 2010).

2.3.9. Ribosomal protein mediated docking of ribosome-associated factors

Concurrently with protein synthesis by the ribosome, nascent polypeptides are subjected to enzymatic processing, chaperone-assisted folding or targeting to translocation pores at membranes. The ribosome serves as a platform for the spatially and temporally regulated association of enzymes, targeting factors and chaperones that act on the nascent polypeptides emerging from the exit tunnel.

It is believed, that in the bacteria the first chaperone that interacts with the nascent chain is the trigger factor (TF). This factor was first identified based on its ability to maintain the precursor of the outer membrane protein A in a non-aggregated form (Lill *et al*., 1988). TF (the *tig* gene product) is a 48 kDa

protein, with peptidyl-prolyl *cis*/*trans* isomerase activity (PPIase) and chaperone-like function (Crooke and Wickner, 1987; Hesterkamp *et al*., 1996). Based on recent crystal structures, TF has an elongated shape, with the peptidyl-prolyl *cis*/*trans*-isomerase (PPIase) domain and the N-terminal ribosome binding domain positioned at opposite ends of the molecule and between them the C-terminal domain with two arms (Kramer *et al*., 2002; Blaha *et al*., 2003; Kristensen and Gajhede, 2003; Merz *et al*., 2008). Ribosome binding is mediated by the N-terminal residues of TF, specifically by a loop region consisting of amino acids Phe-44, Arg-45, and Lys-46 that contacts the L23 protein in the 50S ribosomal subunit (Kramer *et al*., 2002). The TF N-terminal domain interacts also with protein L29, and the 23S rRNA near the peptide exit tunnel (Ludlam *et al*., 2004; Ferbitz *et al*., 2004; Baram *et al*., 2005). The function of the PPIase domain remains unclear. Although prolyl-*cis*/*trans*-isomerase activity has been detected *in vitro*, the domain is dispensable for TF function *in vivo* (Genevaux *et al*., 2004; Kramer *et al*., 2004). The C-terminal region forms the body of TF with two protruding arms and was shown to constitute the main module for its chaperone activity (Merz *et al*., 2006). Approximately half the cellular TF in *E. coli* is bound to the ribosome, near the nascent peptide exit tunnel, while the other half is free in the cytosol (Hesterkamp *et al*., 1996; Kramer *et al*., 2002). TF has a general role in cytosolic protein folding that overlaps partially with that of the Hsp70 chaperone system, DnaK, DnaJ, and GrpE (Deuerling *et al*., 1999; Teter *et al*., 1999). The Hsp70 family of molecular chaperones, such as DnaK, requires the action of co-chaperone members of the DnaJ protein family to both, transfer specific substrates to DnaK and stimulate its ATPase activity. It has been shown that TF competes with DnaK in chaperoning of newly synthesized proteins (Deuerling *et al*., 1999; Teter *et al*., 1999), because deletion of the *tig* gene increases the binding of DnaK to nascent polypeptides from 15% to about 40%. Deletion of the *tig* gene alone does not show any apparent growth defect, while the *dnaK-tig* double mutant exhibits synthetic lethality, causes misfolding and aggregation of several hundred different newly synthesized proteins, suggesting that at least one of these chaperones is required for bacterial survival (Deuerling *et al*, 1999; Teter *et al*, 1999). However, at the lower temperatures (below 30^{o} C) cells can be adapted to double mutation (Genevaux *et al*., 2004; Vorderwülbecke *et al*., 2004). Other chaperones, such as SecB and GroEL may partially compensate for the loss of TF and DnaK under these conditions (Genevaux *et al*., 2004; Vorderwülbecke *et al*., 2004).

The molecular mechanism of the action of TF on translating ribosomes is described by Merz *et al*., 2008. The nascent polypeptides first contact the N domain of TF, then use the entire C domain by passing through the narrow region formed by the C-terminal arms and finally reach the area of the distally located PPIase domain. The minimal length of an extended nascent chain to reach a distinct crosslinker position can be estimated on the basis of the TF crystal structure docked on the ribosome. Theoretically, 43 residues are necessary to contact the position of the first crosslinker within TF's N domain, approximately 63 residues are necessary to proceed to the C domain between

the arms and around 85 residues are required to reach the PPIase domain. This implies that nascent chains initially follow a rather defined path alongside the TF interior in an unfolded conformation (Merz *et al*., 2008). When the polypeptide is further elongated (over 90 aa), two different scenarios could be envisioned. In the first scenario, the polypeptide may continue to traverse through the TF interior upon elongation and perhaps exit in the area of the PPIase domain, whereas in the second scenario, the polypeptide could start to accumulate inside TF. Additionally, previous studies have shown that ribosome-bound TF protects nascent polypeptides up to a size of about 41 kDa against proteolysis *in vitro* (Hoffmann *et al*., 2006; Tomic *et al*., 2006). These two scenarios may both occure depending on the nature of a nascent chain including its hydrophobicity and folding kinetics. The binding of an unfolded polypeptide stretch could delay folding and allow binding of downstream factors such as DnaK or GroEL. On the other hand, the accumulation of a nascent polypeptide with multiple transient interactions to TF would significantly reduce the available conformational freedom of the unfolded polypeptide and could thereby promote the formation of intramolecular contacts within the nascent chain, which are known to drive the folding of extended polypeptides towards their native structure (Jahn and Radford, 2007).

The half-life of the TF-ribosome-nascent chain complex ranges between approximately 15 and 50 s depending on the length and hydrophobicity of the nascent polypeptide exposed at the ribosomal exit (Kaiser *et al*, 2006; Rutkowska *et al*, 2008).

Nascent chains need to be processed by enzymes, including the removal of the N-terminal formyl group by protein deformylase (PDF) in bacteria and the cleavage of the N-terminal methionine by aminopeptidases (MAPs). This processing has been shown to occur co-translationally as soon as the nascent chains reach a length of 40–60 aa (Housman *et al*., 1972; Ball and Kaesberg, 1973). EM structures localized nascent chains of such a length exclusively in the area of the TF N-domain, which is accessible through large lateral gaps on both sides and therefore could allow PDF and MAP to approach nascent polypeptides during their progression through the TF interior (Merz *et al*., 2008). The same entry might be used by the signal recognition particle (SRP) as SRP co-translationally binds nascent chains as soon as their signal sequence has a distance of 40–60 amino acids from the ribosomal peptidyl-transferase centre (Ullers *et al*., 2006). The SRP complex recognizes nascent chain-bearing specific signal sequences as they emerge from the tunnel. SRP binds to the ribosome, interacts with the signal sequence and directs the translating ribosome to the docking site on the membrane. Concomitant binding of TF and SRP might be possible. In the presence of TF, SRP might be flexibly attached only to L23 and L29, as observed for the 70S ribosome complex without a nascent chain (Schaffitzel *et al*., 2006). Such a mode might allow SRP to sample the nascent chain even in the presence of TF. Upon recognition of the signal sequence, SRP would rearrange and TF may associate with the ribosome.

The biosynthesis of secretory proteins requires that they are transported across the plasma membrane in prokaryotes or across the endoplasmic reti-

culum (ER) membrane in eukaryotes. Transport occurs through a protein-conducting channel formed by a heterotrimer of membrane proteins, called the Sec61 complex in eukaryotes and the SecY (SecYEG) complex in prokaryotes (Johnson and Waes, 1999; Osborne *et al*., 2005). This protein-conducting channel consists of the membrane proteins SecY, SecE, and SecG as core components. Transportation of proteins through the membrane begins from a ribosome-nascent chain complex interacting with the signal recognition particle which then binds to the membrane via the SRP receptor (Luirink and Sinning, 2004; Halic *et al*., 2006). Beneath the ribosome, a SecY or Sec61 complex contacts with the proteins L23, L29, and L24, or the rRNA helices H7, H50 and H59 (Menetret *et al*., 2007). After that, the nascent chain is transferred from SRP into the channel. Secretory proteins move completely through the channel, while transmembrane segments of membrane proteins exit through a lateral gate into the lipid phase (Martoglio *et al*., 1995; Heinrich *et al*., 2000). Beside SRP, additional factors interact with Sec complex, one of them being the ATPase SecA (van der Does *et al.*, 1996) and membrane protein YidC (Scotti *et al.*, 2000). YidC is implicated in the lateral release of a transmembrane domain from the protein-conducting channel into the lipid phase (Beck *et al.*, 2001; Urbanus *et al.*, 2001).

2.3.10. Other functions and activities of r-proteins

Ribosomal proteins (RPs) are abundant RNA-binding proteins found in every cell. It seems likely that they will be recruited to carry out many auxiliary functions. Recent eukaryotic proteomic studies have revealed that proteasome inhibition causes r-proteins to accumulate in the nucleoplasm and nucleoli, whereas in response to actinomycin D (selectively blocks RNA Pol I transcription in the nucleolus and therefore also ribosome biogenesis), r-proteins are rapidly dispersed and degraded in the nucleoplasm (Andersen *et al*., 2005). Ribosomal proteins are expressed at high levels beyond that required for ribosome-subunit production and accumulate in the nucleolus more quickly than all other nucleolar components (Lam *et al*., 2007). This is balanced by continual degradation of unassembled ribosomal proteins in the nucleoplasm, thereby providing a mechanism for mammalian cells to ensure that ribosomal protein levels are never rate limiting for the efficient assembly of ribosome subunits. It appears that cells produce and import r-proteins into the nucleus and excess of r-proteins are degraded in the nucleoplasm by nuclear proteasomes. The r-proteins which are incorporated into the ribosome subunit and exported to the cytoplasm remain stable. It can be assumed that there is a pool of un-associated r-proteins in the nucleus and cytoplasm, free to perform other functions (Lindström, 2009). R-proteins could transiently or stably interact with RNA/DNA structures or other proteins. For example, RPS3 (in *E. coli* S3) and RPS6 can interact with Hsp90 that maintains a minor stable pool by protecting them from ubiquitination and proteasomal degradation (Kim *et al*., 2006). Inhibition of Hsp90 with the drug geldanamycin releases RPS3 which instead associates with

Hsp70. Some research groups have shown that several r-proteins possess additional ‘‘extra-ribosomal” functions in cellular apoptosis, transcription/ translation, mRNA processing, DNA repair, development and tumorigenesis (reviewd in Wool, 1996).

DNA repair activity of RpS3. RpS3, a 26.7 kDa protein, is a member of the small ribosomal subunit known to be involved in the initiation of translation in eukaryotes (homolog to bacterial r-protein S3) (Westermann *et al*., 1979; Tolan *et al*., 1983; Polakiewicz *et al*., 1995). Interestingly, it was reported that this protein possesses an extra-ribosomal function by which DNA damage caused by UV irradiation is repaired (Kim *et al*., 1995; Yacoub *et al*., 1996; Jung *et al*., 2001; Lee *et al*., 2002). The significance of this DNA repair activity acting on 8-oxoguanine is shown by the ability of S3 to rescue the H_2O_2 sensitivity of an *E. coli* mutM strain (defective for the repair of 8-oxoguanine) and to completely abolish the mutator phenotype of mutM caused by 8-oxoguanine-mediated G-->T transversions (Yacoub *et al*., 1996). DNA endonuclease repair activity and ribosome incorporation ability appear to be regulated by phosphorylation and methylation of the protein (Kim *et al*., 2009; Shin *et al*., 2009). Most of the rpS3 pool is located in the ribosome while the minority exists in free form in the cytoplasm (Kim *et al*., 2010). In addition, cells from Xeroderma pigmentosum group D (XPD) patients that are subject to high incidence of skin cancer show abnormal endonuclease activity. Since the profile of rpS3 endonuclease activity appears to differ in XPD cells compared to healthy cells (Kim *et al*., 1995), there is a probability that the defective function of rpS3 is related with XPD disease. Furthermore, it was reported that interaction between rpS3 and Hsp90 is necessary for ribosomal protein protection from degradation (Kim *et al*., 2006).

Ribosome assembly is guarded by RPs. Increasing evidence indicates that surveillance of ribosome assembly plays an important role in a cell’s self-evaluation, in which defects in ribosome synthesis can lead to cell-cycle arrest or apoptosis through extraribosomal functions of RPs. 5S rRNA assembles with L5 and L11 as a complex before being inserted into the large ribosomal subunit (Steitz *et al*., 1988; Zhang *et al*., 2007). In murine cells L5 and 5S rRNA can associate with the MDM2 proteins (mouse double minute homolg 2), as well as with MDM2-p53 complexes (Marechal *et al*., 1994). Overexpression of the mdm-2 gene can increase the tumorigenic potential of cells (Fakharzadeh *et al*., 1991), thus qualifying it as an oncogene. The identification of MDM2 (and its human ortholog HDM2) as an E3 ligase responsible for the ubiquitination of p53, leading to its rapid degradation (Vazquez *et al*., 2008), suggested a functional role for the interaction of L5 with MDM2. Subsequently, it was shown that L11 could bind HDM2, confining it to the nucleolus, and that overexpression of L11 could lead cells to apoptoses due to the accumulation of p53, whose E3 ligase was unavailable to initiate its destruction (Lohrum *et al*., 2003; Dai *et al*., 2004; Zhang *et al*., 2003). Knockdown of L5 or L11 impairs p53 accumulation (Lohrum *et al*., 2003; Sun *et al*., 2008). L23 can also bind MDM2 and overexpression of L23 causes cell-cycle arrest or apoptosis (Dai *et al*., 2004). Intriguingly, siRNA-mediated suppression of L23 synthesis also

leads to p53 accumulation and its downstream effects (Dai *et al*., 2004; Jin *et al*., 2004). It has now been shown that a constituent of the small ribosomal subunit, S7, can also interact with the MDM2-p53 complex, again protecting p53 so that its effective concentration rises (Chen *et al*., 2007).

Accumulation of RPs can occur due to defects in ribosome assembly caused by an imbalance among RPs, caused by an imbalance between RPs and rRNA, or caused by a defect in one of the hundred or more proteins that catalyze the assembly process. Such an accumulation of any of several RPs can lead to accumulation of p53, either slowing p53 degradation by sequestering MDM2, or stimulating p53 translation. RPs serve an extraribosomal function as guards to warn of defects in ribosome assembly.

Developmental defects caused by r-proteins. Mutations in either the S19 or S20 genes can lead to developmental defects in mice, including abnormal melanocyte proliferation and red blood cell hypoplasia (McGowan *et al*., 2008). Although engineered knockouts of relatively few mouse RP genes have been reported, most of these are embryonic lethal even as heterozygotes (e.g. S6) (Panic *et al*., 2006).

Other cases of insufficient RPs leading to developmental defects have been reported. Deletion of a portion of chromosome 5, usually in older individuals, leads to severe anemia and to a propensity to progress to acute myeloid leukemia. This 5q-syndrome is due to the loss of one copy of the gene for S14 (Ebert *et al*., 2008).

Diamond-Blackfan anaemia and S19. Diamond-Blackfan anaemia (DBA) is a rare inherited red cell hypoplasia characterised by a defect in the maturation of erythroid progenitors and in some cases associated with malformations, therefore the body´s bone marrow produces little or no red blood cells. Patients have an increased risk of solid tumors. Mutations have been found in several RP genes, i.e RPS19, RPS24, RPS17, RPL5, RPL11, RPL35A (Gazda *et al*., 2008). S19 mutations are the cause of 25% of the cases of Diamond-Blackfan anemia (Draptchinskaia *et al*., 1999).

Human ribosomal protein S4 is encoded by the X and Y chromosomes. *RPS4* genes encode for the ribosomal small subunit protein 4 (29kD; 263 amino acids), a protein involved in mRNA binding and located at the 40S/60S subunit interface of the small ribosomal subunit (Nygard and Nika, 1982). *RPS4* is found on autosomes in all vertebrates except mammals, which all have an X-linked copy (*RPS4X*) (Fisher et al., 1990). Human S4 is the only RP encoded by the X and Y chromosomes. RPS4Y and RPS4X proteins differ in 19 of 263 amino acids (Fisher *et al*., 1990). Both genes are widely transcribed in human tissues, suggesting that the ribosomes of human males and females are structurally distinct. Turner's syndrome, characterized by short stature, degeneration of the gonads, and frequent intrauterine lethality, has been identified by insufficiency of S4 in females due to the failure to exempt the S4 gene from X inactivation (Fisher *et al*., 1990). In human lineage, *RPS4Y* is duplicated and the Y chromosome therefore carries a third functional paralog: *RPS4Y2*, which presents a testis-specific expression pattern (Skaletsky *et al*., 2003; Andrés *et al*., 2008). However, nothing is yet known about *RPS4Y2* essentiality, or about

its functionality. It might be possible that *RPS4Y2* gene had accumulated mutations that would have improved an extra-ribosomal function already present in the gene. Wool (1996) even speculated that RPS4 could be involved in the regulation of development. The existence of two paralogous copies is a unique feature of human RPS4 compared to other ribosomal proteins, and the presence of three copies is even more surprising (Andrés *et al*., 2008).

RPs feed-back regulation. The feed-back regulation of ribosomal proteins by their own gene expression is an important extraribosomal function (Nomura *et al*., 1984). The general mechanism is that a r-protein coded by a cistron of a polycistronic mRNA binds to the first ribosomal initiation site of its polycistronic mRNA, thus preventing the translation of the whole mRNA. Tranlational regulation has been shown with r-proteins S1 (S1 operon), S2 (S2 operon), S4 (alpha operon), S7 (*str* operon), S8 (*spc* operon), S15 (S15 operon), S20 (S20 operon), L1 (L11 operon), L4 (S10 operon), L10 and L12 (L10 operon), and L20 (thrS/L20/pheS operon). The overall organization of r-protein genes in their operons is shown in the Table 2. Genes for the 54 ribosomal proteins are organized into at least 16 operons and 4 monocistrons.

Table 2. Genes organization of ribosomal proteins in *Escherichia coli* operons or monocistronic genes. The order of genes in the operons corrsponds to their order in this table. Genes for the 50 ribosomal proteins are organized into at least 16 operons. In addition to r-proteins many operons contain genes for essential cellular processes including protein secretion, DNA replication, transcription and translation.

Operon	**Genes in the operon** gene product (gene)
rpoBC	L11 (rplK) L1 (rplA) L10 (rplJ) L7/L12 (rplL) RNA polymerase, beta subunit (rpoB) RNA polymerase, beta prime subunit (rpoC)
Str	S12 (rpsL) S7 (rpsG) EF-G, elongation factor G (fusA) EF-Tu, elongation factor Tu (tufA)
S10	S10 (rpsJ) L3 (rplC) L4 (rplD) L23 (rplW) L2 (rplB) S19 (rpsS) L22 (rplY) S3 (rpsC) L16 (rplP) L29 (rpmC) S17 (rpsQ)

Operon	**Genes in the operon** gene product (gene)
Spc	L14 (rplN) L24 (rplX) L5 (rplE) S14 (rpsN) S8 (rpsH) L6 (rplF) L18 (rplR) S5 (rpsE) L30 (rpmD) L15 (rplO) preprotein translocase membrane subunit (secY)
alpha	L36 (rpmJ) S13 (rpsM) S11 (rpsK) S4 (rpsD) RNA polymerase, alpha subunit (rpoA) L17 (rplQ)
S1	Cytidine monophosphate kinase (cmk) S1 (rpsA)
S2	S2 (rpsB) EF-Ts, elongation factor Ts (tsf)
S6	S6 (rpsF) S18 (rpsR) L9 (rplI)
S15	S15 (rpsO) Polynucleotide phosphorylase (pnp)
L20	L35 (rpmI) L20 (rplT)
L13/S9	L13 (rplM) S9 (rpsI)
trmD	S16 (rpsP) important for maturation of the head domain of the 30S subunit (rimM) tRNA-m^{1}G37 methyltransferase (trmD) L19 (rplS)
L21/L27	L21 (rplU) L27 (rpmA)
L28/L33	L28 (rpmB) L33 (rpmG)
MMS (macromolucular synthesis)	S21 (rpsU) DNA primase (dnaG) RNA polymerase sigma-70 subunit (rpoD)
L32	L32 (rpmF) fatty acid/phospholipid synthesis protein (plsX) 3-oxoacyl-[acyl-carrier-protein] synthase III (fabH) malonyl-CoA-[acyl-carrier-protein] transacylase (fabD) 3-oxoacyl-[acyl-carrier-protein] reductase (fabG)
S20	S20 (rpsT)
L25	L25 (rplY)
L31	L31 (rpmE)
L34	L34 (rpmH)

S1 and degradosome. Danchin (1997) suggested that S1 may act as an RNA-binding protein presenting mRNA to a degradation complex. The multi-functional ribonuclease RNase E and the 3'-exonuclease polynucleotide phosphorylase (PNPase) are major components of an *E. coli* ribonucleolytic "machine" that has been termed the RNA degradosome. PNPase processively cleaves single-stranded RNA substrates in the 3′-to-5′ direction using inorganic phosphate to attack the phosphoester linkage at the 3′ terminus and liberate nucleoside diphosphate (Nurmohamed *et al*., 2009). Under conditions of excess nucleoside diphosphate and low concentrations of phosphate, PNPase catalyses the reverse reaction to add 3′ extensions to transcripts (Mohanty and Kushner, 2000; Jarrige *et al*., 2002; Lin-Chao *et al*., 2007). Besides PNPase and RNase E the degradosome comprises a DEAD-box RNA helicase (RhlE) (Py *et al*., 1996), and, most interestingly enolase, an enzyme involved in intermediary metabolism and providing phosphoenolpyruvate during glycolysis (Miczak *et al*., 1996). The sequence similarity between S1 and PNPase, propose that since S1 is a mRNA binding factor and is similar to RNA helicases, it could act as an RNA-binding protein presenting mRNA to a degradation complex comprising PNPase. S1 can indeed be a component of a RNA helicase as it is subunit of the bacteriophage Qβ replicase (Senear and Steitz, 1976). The selection pressure has linked S1 to function in mRNA degradation and associated S1 to the *cmk* (cytidine monophosphate kinase) gene product (Table 2), because this results in the same general function, the generation of CDP (Danchin, 1997). Indeed, the *cmk* gene product is an enzyme which synthesizes CDP from CMP, and the *rpsA* gene product, perhaps through an RNA helicase function, permits PNPase to degrade mRNA to NDPs. Expression of the *cmk-rpsA* operon would thus permit synthesis of CDP at a level required for appropriate DNA synthesis.

S10 and NUS complex. Studies of bacteriophage λ transcription identified a NUS (N utilization) complex necessary for certain transcription termination events during bacteriophage λ infection. One component of the NUS complex, the host NusE protein, is in fact S10 (Friedman *et al*., 1981). Recent structural work has shown that S10, together with NusB, another host protein, interacts with specific regions (BoxA) of λ transcripts and can do so only when it is not associated with the ribosome (Luo *et al*., 2008). S10 has a globular portion that sits at the ribosome surface and an extended loop that penetrates into the 30S ribosomal subunit. The latter is essential for ribosome function but not for NUS activity. The NUS complex can effect either termination or antitermination, depending on the context (reviewed in Roberts *et al*., 2008). Since BoxA is strictly conserved in all seven *rrn* operons of *E. coli* (Berg *et al*., 1989), the NUS complex functions as an antiterminator for rRNA transcription. Thus, the presence of S10 in the NUS complex provides one way in which rRNA and RPs can be coupled, i.e., a deficiency of S10 will lead to less antitermination and less rRNA, and *vice versa* (reviewed in Squires and Zaporojets, 2000).

UV-B damaged ribosome repair by r-proteins. Ultraviolet-B (UV-B, 280–315nm) photons can cause substantial damage in biomolecules, as it is well established for DNA. Recent depletion of stratospheric ozone by chloro-fluorocarbons and other pollutants has increased terrestrial UV-B levels with

potentially deleterious consequences for all living organisms and particularly for plant development and physiology (Ballare´ et al., 2001; Searles et al., 2001; Paul and Gwynn-Jones, 2003). Plants have evolved UV-induced mechanisms of protection and repair, such as accumulation of UV-absorbing pigments and use of UV-A (315–400 nm) photons to repair UV-B induced DNA damage (Stapleton and Walbot, 1994; Britt, 1996). In flowering plants, flavonoids, including anthocyanins, accumulate in the vacuoles of epidermal cells where they attenuate the UV component of sunlight with minimal absorption of photosynthetically active radiation (Stapleton and Walbot, 1994; Landry et al., 1995). UV-B also stimulates production of ROS (Arnots and Murphy, 1991; Dai et al., 1997) and antioxidant defences (Rozema et al., 1997; Jansen et al., 1998). This radiation can also damage proteins and lipids directly (Gerhardt et al., 1999). Experimentally, UV has been extensively used to analyze ribosome structure *in vitro*, because crosslinks can be introduced at points of close contact between proteins, within ribosomal RNA, and between proteins and rRNA, tRNA, or mRNA (Brimacombe et al., 1990; Noah et al., 2000).

UV-B photons cause ribosomal damage by crosslinking RNA to ribosomal proteins in irradiated maize leaves (Casati and Walbot, 2004). UV-B damaged ribosomes may be degraded followed by rapid synthesis of new ribosomes during recovery or r-protein exchange may overcome UV-B inflicted damage. In maize (Zea mays) leaves, UV-B radiation damages ribosomes by crosslinking cytosolic ribosomal proteins S14, L23a, and L32, and chloroplast ribosomal protein L29 to RNA (Casati and Walbot, 2004). Ribosomal damage accumulated during a day of UV-B exposure correlated with a progressive decrease in new protein production; however, *de novo* synthesis of some ribosomal proteins is increased after 6 h of UV-B exposure (Casati and Walbot, 2004). Transcripts for several ribosomal proteins such as S4, S7, S8 S15, S19 and L5, L6, L10A, L10 (QM), L11, L17, L18, L25, L27, L31, P0, together with transcripts for histones and chaperones, are examples of genes up-regulated (greater than 2-fold) by UV-B (Casati and Walbot, 2003). After 16 h without UV-B, damaged ribosomes were eliminated and translation was restored to normal levels. The increase in transcription of translation-related genes is probably the consequence of ribosomal damage by UV-B, resulting in a 50% reduction in protein synthesis (Casati and Walbot, 2004). After UV-B exposure a 45% increase in *de novo* ribosomal proteins synthesis is seen compared to synthesis in control plants when equal amounts of proteins were compared (Casati and Walbot, 2004). New ribosomes that are synthesized within this recovery period can overcome the results of UV-B mediated crosslinking within organellar and cytosolic ribosomes (Casati and Walbot, 2004) or r-protein exchange can restore UV-B damaged ribosomes. These data show that UV-B induces the *de novo* synthesis of ribosomal proteins, and this is observed despite the overall decrease in translation. Despite the presence of significant ribosome damage and a decrease in translation, physiological parameters, such as photosynthesis and pigment levels, were not significantly affected by the UV-B treatment employed (Casati and, Walbot, 2004).

In animal cells ribosomal protein S6 phosphorylation has been implicated as one route to translational up-regulation by UV-B of mRNAs coding for the components of protein synthesis apparatus. Phosphorylation of ribosomal protein S6 and p70 S6 kinase has also been reported in maize (Williams et al., 2003; de la Cruz et al., 2004; Casati and Walbot, 2004). An increased translation of mRNAs encoding ribosomal proteins and translation factors has been suggested as an additional mechanism for recovery from ribosome damage in animal cells (Meyuhas et al., 1997; Brenneisen et al., 2000). Phosphorylation of the ribosomal protein S6 and activation of the corresponding ribosomal protein S6 kinase signaling pathway occurs upon UV-B irradiation (Brenneisen et al., 2000; Nomura et al., 2001). Brenneisen et al. (2000) demonstrated that the activity of p70 ribosomal S6 kinase is increased in cultured human dermal fibroblasts after UV-B irradiation; they hypothesized that the p70 ribosomal S6 kinase is an essential component of a DNA damage-dependent signaling pathway. Furthermore, Nomura et al. (2001) confirmed that UV-B induces activation of ribosomal p70 S6 kinase in cultured mouse epidermal cells.

RESULTS AND DISCUSSION

Ref I. Using phosphorothioate nucleosides for studying rRNA backbone interactions

Both, the backbone RNA interactions and the base interactions are important for structural stability and function of RNA. Chemical modification of bases with reagents like DMS, CMCT, ketoxal, DEPC have been used to study the importance of individual bases. ENU (N-ethyl-N-nitrosourea), which ethylates randomly the phosphate oxygens of nucleic acids, has been used for backbone probing (Vlassov *et al*., 1980; Vlassov *et al*., 1981). Fe^{2+}-EDTA complex has proven to be a useful reagent to probe the solvent accessibility of the backbone (Moser and Dervan, 1987; Heilek and Noller, 1996; Moine *et al*., 1997; Brunel and Romby, 2000).

The roles of individual nucleobases in rRNA during ribosome functioning have been analysed by site-directed mutagenesis and chemical probing methods (Green and Noller, 1997). We wanted to devise a method to study RNA backbone interactions of the large ribosomal subunit by using phosphorothioate approach. The phosphorothioate approach has previously been used for small RNAs like tRNA (Dabrowski *et al*., 1995; Schnitzer and von Ahsen, 1997) or 5S rRNA (Shpanchenko *et al*., 1998). This approach was introduced by Eckstein and co-workers in 1991. We employed the technique to study 23S rRNA domain I. Iodine can trigger cleavage of the sugar-phosphate backbone at a phosphorothioted position (Schatz *et al*., 1991). Iodine is small enough to intrude into the ribosome, and if a specific phosphorothioated nucleotide is not in interaction with its thioated position then iodine can trigger cleavage of the sugar-phosphate backbone. The sulphur in a phosphorothioated nucleotide has similar chemical properties as oxygen in the normal nucleic acid chain (Figure 19). There are two stereoisomeric oxygens in the phosphate backbone of a nucleic acid, the Rp and Sp oxygens. The Sp (but not Rp) stereoisomer of α-phosphorothioate nucleoside triphosphates can be incorporated into RNA transcripts, accompanied with inversion into the Rp configuration (Griffiths *et al*., 1987).

Reconstitution of functional 50S subunits from *in vitro* transcribed 23S rRNA has been reported for *Thermus aquaticus* (Khaitovich *et al*., 1999). This method allows preparation of ribosomal subunits containing 23S rRNA with artificially modified nucleotides. Incorporation of Rp-phosphorothioate substitutions in to *Thermus aquaticus* 23S rRNA transcripts was accomplished during *in vitro* T7 RNA polymerase transcription by adding a 5% of a single α-phosphorothioate nucleotide triphosphate. 5S rRNA was transcribed without α-phosphorothioate nucleotide triphosphates. 50S ribosomal subunits were reconstituted using phosphorothioate-containing transcripts of *T. aquaticus* 23S rRNA, *in vitro* transcribed *T. aquaticus* 5S rRNA and the large subunit protein fraction (TP-50) which was extracted from native 50S subunits. Reconstituted particles were characterized by sucrose gradient centrifugation. Reconstituted

particles formed a single peak, which is similar to the native 50S (see Fig. 1 of paper I). Reconstituted subunits sediment at the same rate as native *T. aquaticus* 50S subunits.

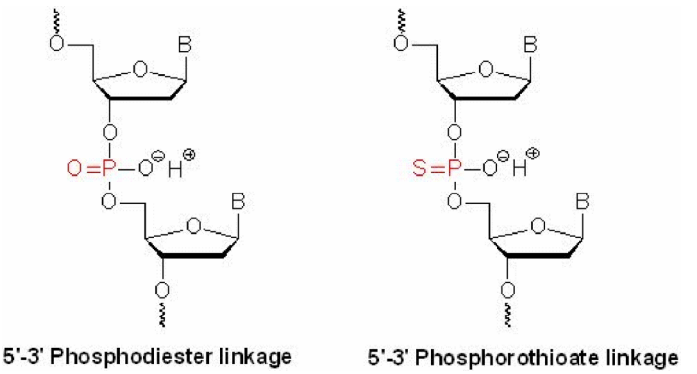


Figure 19. Phosphorothioate backbone modification is a linkage where a non-bridging oxygen on the phosphate linkage is replaced with a sulfur atom. This substitution has only a relatively small effect on the oligonucleotide structure (binding affinity to oligonucleotides is slightly reduced), but this disadvantage is outweighed by a greater resistance to enzymatic hydrolysis.

Functional activity of the reconstituted ribosomal particles was assayed by the peptidyl transferase reaction where puromycin acts as the acceptor substrate and formyl-Met-tRNA as the donor substrate. When unmodified 23S rRNA transcript was reconstituted, the peptidyl transferase activity of the resulting 50S subunits was 14% as compared to the native *T. aquaticus* 50S subunits. 23S rRNA modified with 5% of α-phosphorothioates and incorporated into 50S particles, exhibited 13–16% relative peptidyl transferase activity (see Table 1 of paper I). Therefore, the presence of phosphorothioate substitutions at 5% of the corresponding nucleotide in the 23S rRNA did not influence the functional activity of the reconstituted 50S subunits.

23S rRNA domain I is important for 50S assembly. Four primary binding r-proteins (L4, L20, L22, and L24) bind to domain I (Nowotny and Nierhaus, 1982; Ostergaard *et al*., 1998). To analyze accessibility of phosphates in the 23S rRNA domain I (positions 1–580), we used iodine treatment and subsequent reverse transcriptase-directed primer extension. Primer extension stop sites were specific to the phosphorothioate nucleotides in the 23S rRNA as nucleotide-specific signals were detected in all four thioate-substituted 23S rRNA samples upon treatment with iodine. In order to identify nonbridging phosphate oxygen accessibility, we compared negative controls (thioate-substituted 23S rRNA is not treated with iodine) to 50S subunits that contain thioate substitutions and were treated with iodine. A specific set of phosphate groups in 23S rRNA was clearly less accessible to iodine in the 50S subunit as compared to the free rRNA. These phosphates were taken to be protected when at least a two-fold,

reproducible reduction in iodine-induced rRNA cleavage occurred. 280 positions were accessible to iodine in the reconstituted 50S (see Fig. 4 of paper I) and 80 positions were protected (see Fig. 4 of paper I). Comparing *Deinococcus radiodurans* 50S subunit (Harms *et al*., 2001) crystal structure data with our 23S rRNA domain I footprinting data reveals that most of footprinting protections are caused by the shielding of specific r-proteins (Figure 20). Remaining positions that were not detectable are either masked from analysis by secondary-structure or lack of a sufficiently strong iodine-dependent stop signal.

A dozen positions, that were accessible in naked 23S rRNA, failed to give a consistent footprint in the reconstituted 50S subunits. Their presence may be indicative of certain heterogeneity in the reconstituted 50S population, or of the inherent difficulties in the primer extension protocol.

This footprinting technique that we have developed is suitable for large RNA-protein complexes, such as the ribosome. The crystal structure of the *D. radiodurans* 50S subunit (Harms, *et al*., 2001) allowed us to model our protection data from *T. aquaticus* (see Fig. 4 of paper I) into the 50S structure of *D. radiodurans* (see Fig. 5 of paper I). *D. radiodurans* and *T. aquaticus* are phylogenetically related belonging to the same phylum (Weisburg *et al*., 1989). High-resolution crystal structures are available of the *D. radiodurans* 50S subunit (Harms *et al*., 2008). Only α-carbons of the r-proteins were determined by Harms *et al.,* 2001, but with new structures available whole r-protein sidechains are analyzable in the *D. radiodurans* 50S subunit (Harms *et al*., 2008). Modelling protections into *D. radiodurans* 50S subunit (Harms *et al*., 2008) (PDB code 2ZJP) shows that most of the phosphate backbone protections are positioned close to r-proteins (Figure 20). From 80 protections observed in the primer extension analysis, 57 were close enough to interact with r-proteins. Nine r-proteins (L4, L13, L20, L22, L23, L24, L29, L32 and L34) are close enough to interact with nonbridging phosphate oxygens (Figure 20). According to our analysis, the base of helix 1 is protected by L13. Protections in helix 2 are caused by L20 and L32. Helix 3 is contacted by L20. Helix 7 is in proximity of L29 and L24. Helix 11 is partly protected by L34, helix 19 by L4 and L24, helix 23 by L23, L22 and L34, helix 24 by L22 and L24, in the base of helix 25 by L13 and L20 (Figure 20).

Some protections (23 of 80) in helices 3, 5, 7, 11, 18, 19, 22, 24, and 25 are likely caused by shielding by rRNA (Figure 20). We conclude that the most common reason for iodine protection of the rRNA backbone is shielding by protein.

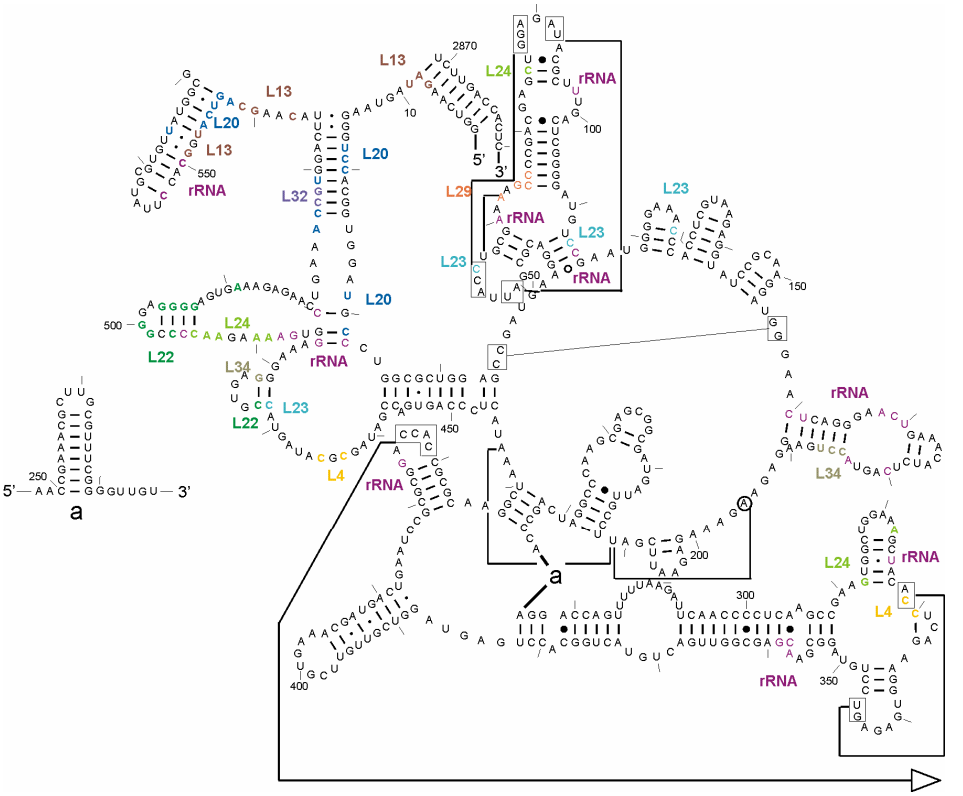


Figure 20. Protected positions (see Fig. 4 of paper I) are modelled into secondary structure of *D. radiodurans* 23S rRNA domain I. Shielding type (protein or RNA) of protections are determined by modelling protected positions into *D. radiodurans* 50S (PDB code 2ZJP) crystal structure by PyMol. Most of the protections are caused by the shielding of specific r-proteins (L4 yellow, L13 brown, L20 blue, L22 green, L23 light-blue, L24 light-green, L29 orange, L32 violet, and L34 light-brown). Remaining protections are shielded by RNA (red).

The apparent protection of specific phosphates could be caused by functional interference of phosphorothioates during ribosome reconstitution. If a modified nucleotide at a particular position prevents the 23S rRNA molecule to be incorporated into the 50S subunit, the position would show up as an apparent protection site in the primer extension gel. To test this possibility, we analyzed modification interference at two L24 binding regions, at positions 65–160 and 470–540 (Nowotny and Nierhaus, 1982). Modifications at the L24 binding site could potentially disrupt L24 binding to 23S rRNA and thereby reconstitution of the 50S subunit, resulting in modification interference to 50S reconstitution. Phosphorothioate modifications in the region C65–G160 and C479-C540 of 23S rRNA do not interfere with the incorporation of 23S rRNA into 50S subunits (see Fig. 3 of paper I).

A similar phosphorothioate-substitution approach was used by Ghosh and Joseph (2005) to identify nonbridging phosphate oxygens within 16S rRNA that are important for the *in vitro* assembly of the *Escherichia coli* 30S small ribosomal subunit and for its association with the 50S large ribosomal subunit. The 30S small subunit was reconstituted from phosphorothioate-substituted 16S rRNA and small subunit proteins. Analysis of the selected population shows that phosphate oxygens at specific positions in the 16S rRNA are important for either subunit assembly or for binding to the 50S subunit. However, several of the phosphate oxygens identified as important assembly positions do not participate in any interaction within the mature 30S subunit, suggesting that they play a role in the early steps of the 30S subunit assembly. Ghosh and Joseph (2005) describe many of phosphate oxygen protections that interact with ribosomal proteins and several phosphate oxygens that interact with metal ions. The only Rp-phosphorothioate substitution that is not tolerated in the 70S ribosome is C770 in 16S rRNA. Therefore, nonbridging phosphate oxygen C770 may inhibit subunit association (Ghosh and Joseph, 2005). They also observe that bridges in h44 of 16S rRNA do not involve contacts between the phosphate oxygens. Their study shows that there are not much of important phosphate backbone interactions. They were able to detect only one interfering position for the 70S reassociation. At the same time, the phosphorothioate-substitution approach is good tool for studying RNA backbone interactions for protections and in appropriate conditions suitable for detection of interfering positions.

Ref II. Important 16S rRNA positions for 70S ribosome formation

An important step in the translation initiation is ribosomal subunit joining. The two subunits are held together with interactions that form bridges between subunits. Intersubunit bridges are divided into stable and labile bridges according to their nature during translation. Interactions between subunits may change or even break during translation but most of these interactions remain stable. Unchanging stable interactions are mostly RNA-RNA interactions, located centrally in the subunits, and changing interactions are RNA-protein or protein-protein interactions that are placed peripherally (Yusupov *et al*., 2001; Gao *et al*., 2003; Schuwirth *et al*., 2005; Zhang *et al*., 2009). The two subunit rotation with respect to each other takes place numerous times during translation, and is called ratcheting. Ratcheting occurs in all stages of translation: initiation, elongation, termination, and ribosome recycling (Frank *et al*., 2007) and is targeted by clinically useful antibiotics (Johansen *et al*., 2006; Ermolenko *et al*., 2007). Ribosomal subunits must be held together to accomplish protein synthesis but on the other hand the ribosome must be dynamic. There must be balanced placement of interactions between subunits. One group of interactions are stable and do not brake or change during translation, and another group of interactions are unstable and change to accomplish dynamic movement of two subunits.

In this work we wanted to know if there are 16S rRNA positions that are indispensable for subunit joining. There are more than 30 individual interactions between the 30S and the 50S subunits. By modification interference, Maiväli and Remme (2004) identified three adenosines in the *E. coli* 23S rRNA, N1-methylation of which strongly reduced the ability of 50S subunits to form 70S ribosomes. These adenosines, which are essential for subunit association *in vitro*, have been assigned to intersubunit bridges B2a (A1912 and A1918) and B4 (A715) (Yusupov *et al*. 2001; Gao *et al*. 2003). Here we extend the modification interference studies of 70S ribosome formation to cover the *E. coli* 16S rRNA.

The idea of the modification interference methodology is to assemble functional complexes using randomly modified macromolecules, and subsequently physically separate active and inactive subpopulations. Interfering positions are positions whose modifications are not tolerated in the active subpopulation and therefore these interfering positions accumulate to the inactive subpopulation. The function of the macromolecule in question was ribosomal subunit reassociation ability. The subpopulation that we chose to modify was the ribosomal 30S subunit. We used RNA specific modifying chemicals: DMS (dimethyl sulfate; methylates N1 positions of adenosines, N7 positions of guanosines, and N3 positions of cytosines) or CMCT (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate; modifies N1 and N3 positions of uracils and N1 positions of guanosines). Modified 30S subunits were reassociated with unmodified 50S subunits to form 70S ribosomes. Resulting ribosomal populations were separated by sucrose gradient centrifugation, and 70S (active) or 30S (inactive) populations were collected. The 16S rRNA was purified from the 30S and 70S gradient fractions, and 16S rRNA positions 1–1507 were scanned for DMS- or CMCT-specific reverse transcriptase stops. Modifications which were present in the 30S subunits but strongly reduced in the 70S ribosomes were designated as interfering with 70S ribosome formation.

While using standard conditions for CMCT modification resulting in no more than a few modifications per 16S rRNA molecule (Stern *et al*. 1988), we titrated DMS to ensure that a minimal modification level would still be detectable by primer extension, thus decreasing the danger of overmodification with DMS and causing large-scale structural rearrangements in the structure of the 30S subunits. The chosen conditions (4 mM DMS) do not affect the sedimentation of the 30S subunits. If the chemical modification levels used resulted in nonspecific rupture of the 30S structure, the resulting ribosomes would very likely be inactive in translation. We used a poly(U)-directed cell-free translation system to test the activity of 30S subunits previously subjected to various concentrations of DMS. In no case did chemical modification reduce the level of poly-Phe synthesis (see Table I of paper II).

189 modifications of 16S rRNA were detected in free 30S subunits. Modification of 16S rRNA at six positions was absent or considerably reduced in the 70S population (see Fig. 3 of paper II). 16S rRNA adenosines 702, 1418, and 1483 exhibited DMS-specific primer extension stops in the 30S fractions, while lacking stops in the 70S fractions. Therefore, methylation of each of these bases

interfered with the ability of 30S subunits to reassociate with 50S subunits to form 70S ribosomes. Similarly, CMCT modification of 16S rRNA uridines 793, 1414, and 1495 interfered with 70S reassociation. We conclude that 16S rRNA bases at positions A702, U793, U1414, A1418, A1483, and U1495 are functionally important for the association with 50S subunits.

All modified bases interfering with formation of 70S ribosomes are located in or near the known intersubunit contact areas (Table. 1; Gabashvili *et al*. 2000; Yusupov *et al*. 2001; Gao *et al*. 2003; Schuwirth *et al*., 2005). It is possible that bases whose modification interferes with 70S ribosome formation make direct contacts with components of the 50S subunit or modification of the bases can disturb the local conformation of the 16S rRNA. Five interfering positions out of six can be assigned to the five distinct intersubunit bridges (A702 to B7a, U793 to B2b, A1418 and A1483 to B3, and U1495 to B2a) as defined by X-ray crystallography and cryo-EM (Table 1; Yusupov *et al*. 2001; Gao *et al*. 2003; Schuwirth *et al*. 2005).

Methylation of A702 at the N1 position by DMS strongly interfered with 70S ribosome formation. A702 in bridge B7a involves the only cross-subunit base stacking interaction, between A702 in h23 of 16S rRNA and A1848 in H68 of 23S rRNA (Schuwirth *et al*., 2005) (Figure 11). N1 position of A702 directly interacts with the N2 position of G1846 in H68 of 23S rRNA (Schuwirth *et al*., 2005; Zhang *et al*., 2009) (Figure 11). The platform of the 30S subunit rotates in ratchet-like movement, and exposes nucleotide A702 in 16S rRNA to solvent, whereas it is buried in the minor groove of H68 in 23S rRNA (Zhang *et al*., 2009). In 16S rRNA, nucleotide A702 is protected from chemical probes when tRNAs are bound in the A/A and P/P sites (Moazed and Noller, 1989). However, when tRNAs occupy hybrid binding sites (A/P and P/E), nucleotide A702 becomes exposed to chemical probes (Moazed and Noller, 1989) and bridge B7a is rearranged (Frank *et al*., 2007; Connell *et al*., 2007; Agirrezabala *et al*., 2008; Zhang *et al*., 2009). This base is increasingly protected from DMS modification by the 50S subunit as subunit reassociation time was extended implying that formation of bridge B7a is a late event in 70S ribosome formation (Hennelly *et al*. 2005). Bridge B7a is important for holding subunits together in translating ribosomes, and on the other hand it is important for rearrangements that occur during translocation of tRNAs. Our results indicate that bridge B7a contributes significantly to 70S association also in the absence of tRNA.

Methylation of the N1 positions of A1418 and A1483 of helix 44 strongly interferes with 70S reassociation. A1483 is involved in the intersubunit bridge B3 (Yusupov *et al*., 2001; Gao *et al*., 2001). Initially A1418 was identified as a component of bridge B5 (Yusupov *et al*., 2001; Gao *et al*., 2003) but later it was determined as a part of bridge B3 (Schuwirth *et al*., 2005). In the low-resolution structures (Yusupov *et al*., 2001; Gao *et al*., 2003) only backbone of rRNA is seen and exact base localization is not known. Therefore, A1418 is placed in bridge B5 because the closest 23S rRNA positions in H64 are 1718–1719 (Yusupov *et al*., 2001; Gao *et al*., 2003). In the high-resolution structure of the *E. coli* ribosome (Schuwirth *et al*., 2005) where whole base localization is seen,

A1418 forms a A-minor interaction with G1948 and C1958 (Schuwirth *et al*., 2005) (Figure 9B). N1 position of A1418 directly interacts with 2´OH of G1948 in H71 of 23S rRNA. 23S rRNA position G1948 is part of bridge B3 (Yusupov *et al*., 2001; Gao *et al*., 2003), therefore looking from the 23S rRNA side, this interaction A1418-G1948 may also be assigned to bridge B3.

A1483 also forms a A-minor interaction with 23S rRNA positions C1947 and G1959 (Schuwirth *et al*., 2005) (Figure 9A). N1 position of A1483 directly interacts with 2´OH of G1959 in H71 of 23S rRNA (Schuwirth *et al*., 2005). A1418 N1 and A1483 N6 lie close together (~3 Å) on the same surface of helix 44 (Schluenzen *et al*. 2000; Wimberly *et al*. 2000) (see Fig. 4C of paper II). Both nucleobases (A1418 and A1483) are in direct contact with 23S rRNA. However, the conformations of bridges B3 and B5 do not appear to be significantly changed in the ratchet-like intersubunit movement of the ribosome effected by EF-G-GTP binding (Gao *et al*. 2003; Spahn *et al*. 2004). These two nucleotides are positioned close together in the ribosome structure and are responsible for maintaining the ribosome in associated form during translation.

The CMCT modification of N3 position of the 16S rRNA nucleotide U1414 strongly interferes with 70S association. U1414 is too far from the 50S subunit to make an intersubunit contact. It is not far (~9.3 Å) from A1483 (B3) and forms a base pair with G1486 in 16S rRNA (Schluenzen *et al*. 2000; Wimberly *et al*. 2000) which, in turn, is a part of bridge B3 (Yusupov *et al*., 2001) (see Fig. 4C of paper II). U1414 by itself is not interacting with 50S subunit but is maintaining the functional structure of the intersubunit bridge B3.

CMCT modification of N3 position of the 16S rRNA nucleotide U793 interferes with 70S formation. U793 is close to bridge B2b while the closest 23S rRNA position is C1920, and the distance between O4 position of U793 and backbone phosphate oxygen of C1920 is 3.8 Å (Schuwirth *et al*., 2005). C1920 is part of bridge B2b which interacts with h45 of 16S rRNA (Yusupov *et al*., 2001). U793 neighbours A792 and A794 in h24 of 16S rRNA are also part of intersubunit bridge B2b (Yusupov *et al*., 2001; Gao *et al*., 2003. U793 may possibly be involved in a transient interaction with the 50S subunit but more reasonable is that U793 is involved in structural maintenance of bridge B2b.

A third CMCT modification which interferes with 70S formation is U1495 of the 16S rRNA. U1495 is part of intersubunit bridge B2a (Yusupov *et al*., 2001; Gao *et al*., 2003; Schuwirth *et al*., 2005). 2´OH position of U1495 directly contacts with N1 position of A1919 (Schuwirth *et al*., 2005) (Figure 9D). Maiväli and Remme, (2004) showed that modification of N1 positions of A715, A1912 and A1918 of the 23S rRNA interferes 70S formation. However, a closer look on the autoradiograph reveals that the modification of 23S rRNA position A1919 (Maiväli and Remme, 2004) (Figure 21) is the same as for position A1918. We speculate that modification of N1 position of A1919 may interfere as well with 70S formation.

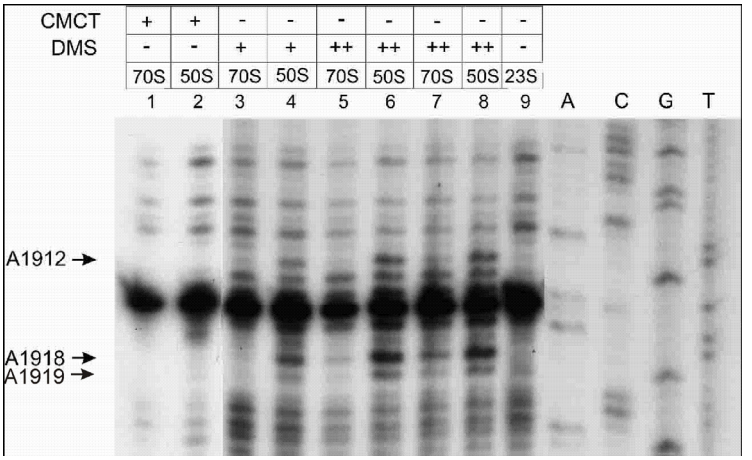


Figure 21. Reverse transcriptase analysis of the positions of the DMS and CMCT modifications in the 23S rRNA. Positions A1912 and A1918, whose modification interfere with 50S reassociation, are denoted by arrows. Modification of 23S rRNA position A1919 is the same as of position A1918, and modification of N1 position of A1919 may interfere with 70S formation. The dideoxy sequencing lanes are indicated by A, C, G, and T. (+) 17 mM DMS; (++) 85 mM DMS. (Lanes *1–6*) selection experiments done in 6 mM $MgCl_2$; (lanes *7*,*8*) selection experiments done in 13 mM $MgCl_2$ (adapted form Maiväli and Remme, 2004).

This intersubunit interaction between U1495 of the 16S rRNA and A1919 of the 23S rRNA is important for 70S formation. U1495 stacks with its neighbour position G1494 which in turn interacts with A1912 of the 23S rRNA (Figure 9C). N1 modification of A1912 of the 23S rRNA interfere 70S formation (Maiväli and Remme, 2004). Additionally, A1918 and A1919 of the 23S rRNA form an A-A dinucleotide platform (Cate *et al*., 1996; Schuwirth *et al*., 2005) (Figure 9D). Moreover, point mutations A1912G, C1917C, and A1919G of the helix–loop 69 of 23S rRNA have a severe effect on the translational activity both *in vivo* and *in vitro*, further emphasizing importance of the B2a bridge in ribosome function (Liiv *et al*. 2005; Kipper *et al*., 2009). Tight packing interactions that take place in this B2a bridge region are important for 70S ribosome formation. The relatively bulky CMCT modification of U1495 may interfere with bridge B2a formation of the 70S ribosome.

Modification of the N1 position of A702, A1418, and A1483 with DMS, and of the N3 position of U793, U1414, and U1495 with CMCT in 30S subunits strongly interferes with 70S ribosome formation. Five of these positions localize into previously recognized intersubunit bridges, namely, B2a (U1495), B2b (U793), B3 (A1483; A1418), and B7a (A702). The remaining position displaying interference, U1414, forms a base pair with G1486, which is a part of bridge B3. These four intersubunit bridges are essential for reassociation of the 70S ribosome, thus forming the functional core of the intersubunit contacts.

Ref III. Ribosome reactivation by replacement of damaged proteins

Ribosomes account for as much as 30% of total cell mass, with up to 10^5 and 10^6 ribosomes per cell in bacteria and mammalia, respectively (Bashan and Yonath, 2008). Protein synthesis is the most energy-demanding process in the cell, accounting for more than 90% of energy consumption in the bacterium *E. coli* (Molin *et al.*, 1977). A large proportion of this energy is used to build the ribosome itself. Consequently, when r-proteins are damaged in the ribosome, it should be energetically more favourable to replace them with newly synthesized proteins, rather than to synthesize new ribosomes. It is known that ribosomal proteins in the ribosome are main targets to oxidation in the yeast cell (Mirzaei and Regnier, 2007). Stressing yeast cell cultures with hydrogen peroxide leaves 86% of the proteins in yeast ribosomes carbonylated, thus making ribosomal proteins the most heavily oxidized class of proteins (Mirzaei and Regnier, 2007). Oxidative stress caused by exposure to H_2O_2 results in a rapid and reversible inhibition of protein synthesis (Shenton and Grant, 2006). Yeast cells can adapt to oxidative stress by altering global transcription, including genes encoding antioxidants and other metabolic enzymes (Gasch *et al*., 2000; Causton *et al*., 2001). H_2O_2 causes a dose dependent inhibition of protein synthesis mediated in part by Gcn2-dependent phosphorylation of eIF2α (Shenton *et al*., 2006). Translation of certain mRNAs was maintained or increased following oxidative stress indicating that translational control is a key component of the cellular response to oxidative stress (Shenton *et al*., 2006).

To study r-protein exchange and what function it may have, we used the protein-specific chemical reagent N-ethylmaleimide (NEM) that alkylates thiol groups of cysteines. It was shown that ribosomes which were treated with various sulfhydryl specific reagents are less active in protein synthesis (Heintz *et al*., 1966; Traut and Haenni, 1967; McAllister and Schweet, 1968; Retsema and Conway, 1969). Therefore, if r-proteins are exchangeable then this exchange may recover the ribosomes function in translation. The damaged r-proteins are replaced by native r-proteins and the translational activity of the ribosome would recover. Treatment of ribosomes with low concentrations of p-chloromercuribenzoate, NEM or dithiobis-(2-nitrobenzoic acid) (Heintz *et al*., 1966; Traut and Haenni, 1967; McAllister and Schweet, 1968; Retsema and Conway, 1969) led to a 40–80% loss of ribosomal activity in amino acid incorporation in poly(U)-directed synthesis of poly-Phe. A NEM treated ribosome preparation was found to be inhibited by 60% in the assay for poly-Phe formation (Traut and Haenni, 1967). The inhibition was the same whether the ribosomes were incubated with NEM under conditions giving complete association into 70S particles, or dissociation into 50S and 30S subunits (Traut and Haenni, 1967).

In our assay, NEM-treated ribosomes exhibited progressive inhibition of poly(U) dependent poly-Phe synthesis, with maximal inhibition at 20 mM NEM (see Fig. 3A of paper III). The translational activity of ribosomes is inhibated about 60% compared with NEM untreated ribosomes. This result is in good

agreement with earlier observations (Traut and Haenni, 1967). 30S subunits were inactivated by 50% in the same concentration range (see Fig. 3A of paper III). A slightly lower level inactivation of 30S subunits compared to 70S ribosomes suggests that modifications of proteins from both subunits are responsible for the loss of catalytic activity of the ribosome. Based on these initial data, 10 mM NEM was used in the following modification experiments.

To test whether the functional activity of inactivated ribosomes can be restored by replacement of damaged ribosomal proteins, NEM-treated ribosomes were incubated with total ribosomal proteins (TP70) under ribosome reconstitution conditions (Lietzke and Nierhaus, 1988) and tested for their catalytic activity in poly(U) translation. A twofold increase in translational activity was observed when NEM inactivated 70S ribosomes were treated with ribosomal proteins (see Fig. 3B of paper III). In contrast, incubation of NEM-treated ribosomes with NEM-treated TP70 did not restore the functional activity of ribosomes (see Fig. 3B of paper III).

Incubation of chemically damaged 30S subunits with TP30 (total proteins of 30S subunit) increased translational activity from 60% to 75% (see Fig. 3C of paper III). The final activity for both 70S ribosomes and 30S subunits was the same (~75%). 30S subunits exhibited slightly smaller restoration of their functional activity. This suggests that the replacement of damaged ribosomal proteins from both 30S and 50S subunits is required for the functional rescue of the ribosome.

We titrated NEM-treated ribosomes with different concentrations of TP70 to find out optimal concentartions for the restoration of ribosomal function (see Fig. 4 of paper III). We used 10 A_{260} units of NEM-treated 70S ribosomes which were incubated with 0–12 equivalent units (EU) of TP70. Translational recovery reached a plateau value of twofold activation at a ribosome to ribosomal protein molar ratio of 1:1. This indicates that at optimal r-protein concentration there is a twofold molar excess of r-proteins over rRNA, because one set of proteins is present in the ribosomes and a second set is added *in trans*.

Next, we used different incubation conditions to show that ribosome repair is different from ribosome reconstitution. Ribosome reconstitution *in vitro* takes several hours and needs several steps of incubation at high temperature (47^{o} C), presence of Mg^{2+} (20 mM) and high salt concentration (400 mM NH_4Cl) (Lietzke and Nierhaus, 1988). Surprisingly, the recovery of ribosomes function was not dependent upon the temperature at which r-protein exchange was performed (see Fig. 5 of paper III). Lack of temperature dependence from 32°C to 52°C suggests that ribosome reactivation by added r-proteins does not involve considerable reorganization of the global ribosome structure. In addition, ribosome reactivation levels did not change when damaged ribosomes were incubated with r-proteins at reduced Mg^{2+} and NH^{4+} concentrations (10 mM and 100 mM respectively) (see Fig. 5 of paper III). Therefore, ribosome reactivation does not follow the rules of total reconstitution. Ribosome reactivation by protein exchange makes use of preformed ribosomal structures.

In order to identify exchangeable r-proteins, the protein fractions of *E. coli* ribosomes (TP70) were [^{35}S]-radiolabelled. *E. coli* cells were grown in MOPS

minimal medium with [^{35}S] labelling mixture (containing labelled Met and Cys). Labelled 70S ribosomes were collected, and [^{35}S]-labelled r-proteins (TP70) were extracted from ribosomes. Native ribosomes were incubated with [^{35}S]-TP70 at a 1:1 molar ratio. Ribosomes were subsequently purified from unbound r-proteins by sedimentation through a sucrose cushion. Ribosomal proteins were identified by using two-dimensional gel electrophoresis and visualized by Coomassie staining and autoradiography. Incorporation of [^{35}S]-labelled r-proteins into unlabelled ribosomes indicate r-protein exchangeability. About 20% of input radioactivity was incorporated into purified ribosomes in separate experiments. Ribosomal proteins L1 (L2), L9, L10, L11, L16, L17 and L22 of the large and S2 (S4), S9, S13, S20 of the small ribosomal subunit were identified as exchangeable r-proteins (see Fig. 1 of paper III). The Met/Cys content of an r-protein affects the sensitivity autoradiography to detect its presence. Ribosomal proteins L20, L24 and L33 do not contain any Met or Cys and therefore could not be detected using autoradiography. Protein S1 was lost during sample preparation, and proteins S5, L6, L7/L12, L35 and L36 were not resolved on the two-dimensional gels (see Fig. 1A of paper III).

In the next experiment ribosomal protein exchange was monitored using a quantitative mass-spectrometric approach. *E. coli* MRE600 cells were grown in the presence of [^{15}N]H_4Cl as the only nitrogen source. Ribosomes were prepared from [^{15}N]-labelled cell mass, and used in the exchange experiment. Equimolar quantities of [^{14}N] TP-70 and [^{15}N] 70S ribosomes were incubated for 30, 60 or 120 min at 47°C. The ratio of [^{14}N] and [^{15}N] r-proteins was determined by quantitative mass-spectrometry of the ribosome and corresponding supernatant fractions. Ribosomal protein exchange was evident from the incorporation of [^{14}N]-proteins into ribosomes and the concomitant release of [^{15}N]-proteins (see Fig. 2 of paper III). Four small subunit proteins S1, S2, S13 and S21 and eight large subunit proteins L1, L9, L10, L11, L7/L12, L20, L31 and L33 were found to be exchangeable (see Fig. 2 of paper III). The threshold level of exchange was taken 10% during 120 min incubation. Proteins S13 and L11 exhibited clear time dependence of exchange (see Fig. 2 of paper III). Control experiments revealed that the exchange of proteins S1 and L33 occurred during sample processing, as the exchange level of these proteins was not dependent upon incubation. The difference between the radioactivity and mass spectrometry results is in part due to technical reasons. Proteins S1 and L7/L12 were not resolved using two-dimensional gel-electrophoresis, and proteins L20 and L33 do not contain Met or Cys residues, so are therefore not visible using autoradiography. Protein S21 has one Met, therefore the labeling level for S21 is too low to detect the radioactivity signal by autoradiography even in the exponential phase ribosomes (see Fig. 1D of paper III).

We used another modifying agent, diethylpyrocarbonate (DEPC), which reacts with a histidine residue, forming a N-carbethoxyhistidyl derivative (Miles and Kumagai, 1974; Hirs and Timasheff, 1977). This compound covalently modifies histidines and makes them unable to be protonated. On the other hand, DEPC also modifies DNA/RNA at the N7 position of A's and G's by carbethoxylation (Ehresmann *et al*., 1987; Childs *et al*., 2002). Modification of the

histidyl residue on L16 (Baxter and Zahid, 1978; Sumpter *et al*., 1985; Baxter and Zahid, 1986; Tate *et al*., 1987) and also those of L2 (Dohme and Fahnestock, 1979) inactivates the peptidyltransferase centre. Protein L16 or a proteolytic fragment of L16 lacking nine amino acids at the N-terminus can restore activity to ribosomal subparticles whereas a fragment lacking a further six amino acids, including the single histidine residue, does not (Remme *et al*., 1983; Maimets *et al*., 1983). When L16 is missing from 50S reconstituted subunits the particle is inactive for releasefactor-mediated peptidyl-tRNA hydrolysis (Tate *et al*., 1983). Ribosomes lacking L16 cannot form a peptide bond with an aminoacyl-tRNA fragment as the acceptor substrate, but they readily can with puromycin, suggesting that L16 may be essential for correct positioning of the aminoacyl stem of tRNA in the A site (Maimets *et al*., 1984). The presence of L16 in the reconstitution mix is important for assembly of the peptidyltransferase centre (Tate *et al*., 1987). It is likely that L16 is loosely associated with the reconstituted subunits but upon their isolation from the reconstitution mixture by gradient centrifugation the protein dissociates from the particles (Tate *et al*., 1987). An earlier observation indicated that the association of L16 with the ribosome was accompanied by a conformational change (Teraoka and Nierhaus, 1978) and it seems likely that when L16 is absent or not tightly bound, the peptidyltransferase centre is not in its optimum state for the reactions to proceed (Tate *et al*., 1987).

Modifying ribosomes with 0.01% DEPC decreases the ribosomes translational activity by approximately 70 % (Figure 22A). When DEPC modified ribosomes are treated with r-proteins, we can see 10–14% translational recovery (Figure 22B). The recovery effect of DEPC modified ribosomes is 3-fold smaller (10 %) than with NEM treated ribosomes (30 %). We argue that lower recovery extent of the DEPC treated ribosomes is due to the ability of DEPC to modify RNA. The small recovery effect may be part of the L16 exchange, because it is known that chemical modification of L16 at its single histidine residue at position 13 greatly reduces peptide bond formation (Tate *et al*., 1987), and L16 is exchangeable in radioactive labeling experiment (see Fig. 1B of papaer III).

Most of these exchangeable r-proteins belong to a group of loosely bound r-proteins that can be removed from the ribosome using high salt concentrations (Homann and Nierhaus, 1971; Moore *et al.*, 1975). We treated ribosomes with 2M LiCl that removed r-proteins S2, S3, S5, S9, S10, S13, S14, S20, L1, L2, L5, L6, L9, L10, L11, L15, L16, L18 and L23 from ribosomes. These removable r-proteins are referred to as ‘split proteins’ (spLi), while those that remain bound to the ribosome are termed ‘core proteins’ (cLi). When NEM-modified ribosomes were incubated with a fourfold molar excess of LiCl split r-proteins, a 2.6-fold increase in translational activity was observed (from 35% to 88.5% 70S; see Fig. 6 of paper III). Incubation of NEM-damaged ribosomes with LiCl core r-proteins did not lead to a significant rescue of damaged ribosomes (see Fig. 6 of paper III). Therefore, only loosely bound r-proteins are responsible for the reactivation of chemically inactivated ribosomes.

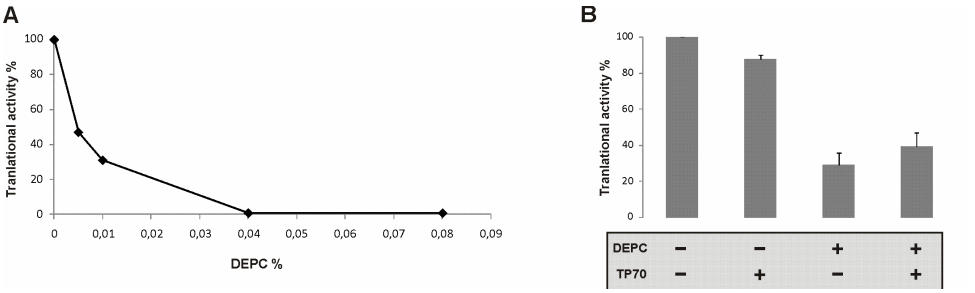


Figure 22. Treatment of DEPC-damaged ribosomes with ribosomal proteins. (A) DEPC-modification of 70S ribosomes inhibits poly(U) dependent translation. Translational activity is normalized to the unmodified 70S ribosomes, 100% activity is equivalent to 60 pmol of incorporated Phe per 70S ribosome. (B) Incubation of DEPC-modified 70S ribosomes with TP70. Translational activity is normalized to unmodified native 70S ribosomes. DEPC modified ribosomes recovery effect (10 %) is 3-fold smaller than with the NEM treated ribosomes (30 %) (see in paper III) in poly(U) dependent translation. 70S ribosomes were modified with 0.01% DEPC where indicated, and ribosomes were subsequently incubated for 90 min at 47^0 C with TP70.

We also studied r-protein exchange in *E. coli* stationary phase cells. *E. coli* cells were cultivated for 52 h into the stationary phase, where *de novo* ribosome synthesis is negligible and the level of protein synthesis is low (Molin *et al.*, 1977). Radioactive labeling of newly synthesized proteins was then performed by adding a [^{35}S] Met/Cys mixture. After 3 h 70S ribosomes were isolated by sucrose gradient centrifugation, r-proteins were extracted, and radioactively labelled proteins were identified by two-dimensional gel-electrophoresis and subsequent autoradiography. Specific sets of labelled r-proteins were incorporated into ribosomes (see Fig. 7 of paper III). Proteins L1, L5, L10, L11, L30, L31, S2 and S5 were consistently strongly labelled in ribosomes. Proteins L9, L13, L15, L16, L17, L19, L22, S4 and S9 were less strongly labelled, whereas the labeling efficiency of S8 and L14 varied from strong to weak between experiments. The fact that some r-proteins were labelled shows that ribosomal proteins are translated during the stationary phase. The set of exchangeable proteins *in vivo* is similar to the corresponding *in vitro* set. In conclusion, we show that ribosomal proteins can be exchanged in stationary phase ribosomes, suggesting that ribosomes subjected to stressful conditions can be repaired in this way.

There is a total number of ~17 or ~27 thiol groups in 30S and 50S subunits, respectively (Bakardjieva and Crichton, 1974). Ribosomal proteins that react with NEM have been identified (Rosenberg *et al*., 1973; Bakardjieva and Crichton, 1974). S18 and S1 are most reactive towards NEM in the 30S subunit (Rosenberg *et al*., 1973). S2 is slightly more reactive when it is in the 70S ribosome, and S21 has some reactivity toward NEM (Rosenberg *et al*., 1973). S12 is also modified by NEM according to Bakardjieva and Crichton (1974). 50S subunit proteins that are mostly modified by NEM are L27 and L17 (Rosenberg *et al*., 1973). Slightly less reactive are L2, L10, L11, and L28

(Rosenberg *et al*., 1973; Bakardjieva and Crichton, 1974). Two other proteins from the 50S subunit (L5 and L9) are also labeled to some extent by [^{14}C]-NEM (Rosenberg *et al*., 1973). There are a number of other proteins which react with NEM in 50S subunits, such as L6 and L15 (Bakardjieva and Crichton, 1974). Up to 15 different r-proteins can be alkylated by NEM. Eight of these NEM modified r-proteins are exchangeable in our exchange assay, and are potential candidates for recovery of NEM modified ribosomes.

The regulation of protein synthesis in response to oxidative stress induced by exposure to H_2O_2 has been analyzed in the yeast *Saccharomyces cerevisiae* (Shenton *et al*., 2006). It is known that ribosomal proteins are the most heavily oxidized class of proteins (Mirzaei and Regnier, 2007). Typically, cells respond to stress conditions by invoking complex regulatory mechanisms, including global inhibition of translation (Clemens, 2001; Proud, 2005). This reduction in protein synthesis may prevent continued gene expression during potentially error-prone conditions as well as allow for the turnover of existing mRNAs and proteins, whilst gene expression is reprogrammed to deal with the stress. All aerobic organisms are exposed to reactive oxygen species (ROS), such as H_2O_2, the superoxide anion, and the hydroxyl radical, during the course of normal aerobic metabolism or following exposure to radical-generating compounds. Yeast cells can adapt to oxidative stress by altering global transcription, including genes encoding antioxidants and other metabolic enzymes (Gasch *et al*., 2000; Causton *et al*., 2001).

Expression levels of ribosomal proteins are decreased under conditions of oxidative stress (Shenton *et al*., 2006). Only certain mRNAs are translationally maintained following oxidative stress. Translationally up regulated genes belong to antioxidants, metabolic enzymes, transport class of genes. In the presence of higher concentrations of ROS (e.g. 2 mM H_2O_2), there are also up regulated genes that are involved in ribosome biogenesis and rRNA processing. Few r-proteins are expressed but their expression is 2-fold down regulated under oxidative stress conditions (Shenton *et al*., 2006). Interesting is that these translationally down regulated r-proteins show mRNA copy numbers which are many folds up-regulated. Transcriptionally up-regulated r-proteins are RPS0A (similar to *E. coli* S2; 20 fold up-regulation of mRNA), ASCI (40S subunit protein; 14.8 fold up-regulation of mRNA), RPL7A (similar to *E. coli* L30; ~6.1 fold up-regulation of mRNA), RPL18B (similar to *E. coli* L15; 3.6 fold up-regulation of mRNA), MNP1 (similar to *E. coli* L7/L12; 1.9 fold up-regulation of mRNA) and RPL4A (similar to *E. coli* L4; 1.6 fold up-regulation of mRNA). Three r-proteins which are translationally down-regulated but transcriptionally maintained are GO45 (homolog to *E. coli* L36), MRPL8 (similar to *E. coli* L17) and IMG1 (similar to *E. coli* L20). Increased transcript levels in the absence of active translation may therefore provide a source of mRNAs that can become rapidly translated once the stress is removed. High transcript levels of certain r-proteins may be needed for replacement of damaged r-proteins when stress conditions dissapear. Most of these yeast r-proteins that are transcriptionally up-regulated are also similar to the *E. coli* exchangeable set of r-proteins.

Table 3. Exchangeable ribosomal proteins identified form three approaches. (I) *In vitro* [^{35}S] labelling approach: 70S ribosomes were incubated with [^{35}S]-labelled r-proteins and the presence of a radioactive protein in the gel was taken to indicate its incorporation into the ribosome and thus exchangeability. (II) *In vitro* ^{15}N/^{14}N-labelling approach: [^{15}N] 70S ribosomes were incubated with equimolar quantities of [^{14}N] TP-70. The ratio of [^{14}N] and [^{15}N] r-proteins was determined by quantitative mass-spectrometry of the ribosome and corresponding supernatant fractions. Exchange threshold level for individual proteins was taken 10 %. (III) *In vivo* stationary phase [^{35}S]-labelling: *E. coli* cells were cultivated for 52 h into the stationary phase, where *de novo* ribosome synthesis is negligible and protein synthesis low (Molin *et al.*, 1977). Radioactive labelling of newly synthesized proteins was then performed by adding a [^{35}S] Met/Cys mixture. Autoradiographs from samples obtained from stationary phase cultures revealed the incorporation into ribosomes of a specific subset of labelled proteins. +++; exchange is strong, ++; exchange is medium, +; exchange is weak, -; no exchange is seen, nd; not detectable by approach.

Exchangeable r-proteins	*In vitro* [^{35}S]-label	*In vitro* ^{15}N/^{14}N-label	*In vivo* stationary phase [^{35}S]-label
L1	+++	++	+++
L2	+++	–	–
L5	–	–	+++
L9	+++	++	+
L10	+++	++	++
L11	+++	+	+++
L7/12	nd	++	nd
L13	++	–	+
L14	–	–	++
L15	–	–	+
L16	+++	–	+
L17	++	–	+
L19	–	–	+
L20	nd	+	nd
L22	+	–	+
L31	–	++	+++
L30	–	–	+++
L33	nd	++	nd
S1	nd	+++	nd
S2	+++	++	++
S4	++	–	+
S5	–	–	+++
S8	–	–	++
S9	+++	–	+
S13	++	+	+
S20	+++	–	+
S21	nd	++	nd

The sets of proteins that are exchangeable *in vitro* and *in vivo* are similar but not identical (Table 3). Exchange of a protein *in vivo* depends on the availability of free r-proteins in stationary phase cells. The fact that proteins S2, S4, S5, S8, S9, L1, L5, L9, L10, L11, L13, L14, L15, L16, L17, L19, L22, L30 and L31 are exchangeable *in vivo* means that they must be translated *de novo* in the stationary phase in sufficient quantities. In living cells various macromolecular factors could influence the protein exchange process, e.g. translation factors, tRNA, mRNA, chaperons and proteases. Notably, we discovered that proteins L5, L30 and S5 are readily exchangeable *in vivo,* but not *in vitro.* Therefore, it is possible that replacement of these proteins in living cells is catalysed by extraribosomal factors.

CONCLUSIONS

I. We have developed a method for incorporation of nucleoside phosphorothioates into the functionally active *Thermus aquaticus* 23S rRNA. Accessibility at 23S rRNA phosphates 1–580 in the reconstituted 50S subunit was analyzed. 280 positions were accessible to iodine in the reconstituted 50S and 80 positions were protected. The majority (57 of 80) of the phosphate backbone protections are positioned close to r-proteins, and some (23 of 80) of protections are likely caused by shielding by rRNA. The phosphorothioate-substitution approach is suitable for footprinting of various ligand-ribosome complexes and for functional studies in the modification interference assay.

II. Methylation of 16S rRNA adenosines 702, 1418, and 1483 at the N1 position interferes with the ability of 30S subunits to reassociate with 50S subunits to form 70S ribosomes. All three adenosines are involved in the intersubunit interactions that form bridges B7a and B3, respectively. Modification of 16S rRNA uridines 793, 1414, and 1495 at the N3 position interfered with 70S reassociation. Interfering uridines are close to or part of the intersubunit bridges B2b, B3 and B2a, respectively. We can conclued that the intersubunit bridges B2a, B2b, B3 and B7a are essential for ribosome subunit association.

III. Incubation of chemically inactivated ribosomes with total ribosomal proteins led to reactivation of translational activity. Ribosomal proteins S1, S2, L1, L7/12, L9, L10, L11 and L33 are among the most readily exchangeable proteins. Their exchange is evident from three approaches: *in vitro* [^{15}N]-labelling approach, *in vitro* [^{35}S]-labelling approach, and *in vivo* [^{35}S]-labelling approach. The results show that the damaged ribosomes can be repaired by mean of protein exchange.

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SUMMARY IN ESTONIAN

Bakteri ribosoomide uurimus keemilise modifitseerimise meetoditega

Ribosoom on suur makromolekulaarne kompleks, mis kodeerib päriliku informatsiooni valgulisse olemusse. Enamus valke on ensüümid, mis osalevad biokeemiliste protsesside katalüüsimisel, kiirendades reaktsioonide kulgu tuhandeid kordi. Samas on valkudel struktuursed ja mehaanilised funktsioonid. Osad valgud on olulised rakkudevahelises suhtlemises (signaalvalgud), immuunvastuse kujunemises, rakkude kontakteerumises üksteisega ja rakutsüklis. Ribosoom ise koosneb samuti paljudest väikestest (30–500 aminohapet) valkudest ja RNA-st. Eeltuumsete organsimide ribosoom koosneb kahest alamühikust, väikesest (30S) ja suurest (50S) alamühikust. Väike alamühik koosneb ühest RNA molekulist (16S rRNA, 1542 nukleotiidi) ja 21-st ribosoomi valgust (S1-S21). Ribosoomi suur alamühik koosneb kahest RNA molekulist (5S rRNA, 120 nukleotiidi ja 23S rRNA, 2904 nukleotiidi) ja 33-st ribosoomi valgust (L1-L36). Valgusünteesi algfaasis kaks ribosoomi alamühikut ühinevad ja moodustavad funktsionaalselt aktiivse ribosoomi (70S). Ribosoomi kahte alamühikut hoiavad koos ~ 30 erinevat ühendust, mis on jagatud 12 silla (B1a-B8) vahel.

Ribosomaalne RNA osaleb ribosoomi kahe olulise aktiivsuse tagamisel, geneetilise informatsiooni kodeerimisel väikeses alamühikus ja peptiidsideme sünteesis suures alamühikus. Ribosomaalsetel valkudel (r-valgud) on peamiselt strukturaalne funktsioon, tagamaks ribosomaalse RNA korrektse voltumise ja stabiliseerides ribosoomi kolmemõõtmelist struktuuri. Samas aitavad ribosoomi valgud kaasa ribosoomi optimaalseks funktsioneerumiseks.

Minu dissertatsioon keskendub ribosoomi struktuurile ja ribosoomi valkude funktsionaalsusele.

I. Ribosoomides on ribosomaalne RNA keerukalt kokkuvoltunud ja moodustab tuhandeid interaktsioone RNA ja r-valkude erinemate keemiliste gruppide vahel. RNA koosneb nukleotiididest, mis läbi fosfodiestersidemete moodustavad RNA polümeerse ahela. Iga nukleotiid koosneb lämmastikalusest ja suhkur-fosfaat selgroost. Lämmastikaluses olevate keemiliste gruppide interaktsioonide ja funktsioonide uurimiseks on kasutatud keemilise modifitseerimise meetodit. RNA keemilise modifitseerimise tulemusena ei ole spetsiifiline keemiline grupp võimeline osalema ühenduste loomises teiste läheduses olevate aatomitega ja seda on võimalik detekteerida praimer ekstensiooni meetodiga. RNA fosfaat-selgroog sisaldab samuti keemilisi gruppe, mis moodustavad intensiivselt interaktsioone naaberaatomitega. Näiteks osalevad ribosomaalse RNA ja tRNA suhkur-fosfaat selgroo 2´OH rühmad peptidüültransferaasse tsentri moodustamises ja seeläbi mängivad olulist rolli peptiidsideme sünteesis. Ühtegi head meetodit ei leidu uurimaks suurte RNA molekulide fosfaat-selgroo mittesildavate hapnike interaktsioone. Käesolev töö kirjeldab eksperimentaalset süsteemi, mis võimaldab uurida RNA suhkur-fosfaat

selgroo interaktsioone 23S rRNA-s. T7 RNA polümeraasi vahendusel on *in vitro Thermus aquaticus´*e 23S rRNA ahelasse lülitatud α-fosforotioaat nukleosiidid, kus mittesildava hapniku asemel on väävli aatom. Väävli asendus ei muuda oluliselt RNA fosfaat-selgroo keemilisi omadusi, väävel on sarnane vesinksideme aktseptor kui hapnik. Kui vastav mittesildav hapnik osaleb interaktsioonis naaberaatomitega, siis sellisel juhul joodiga töötlemisel ei katke RNA fosfaat-selgroog. Kui aga konkreetne mittesildav hapnik ei osale interaktsioonis, siis joodiga töötlemisel katkeb spetsiifiliselt vastava positsiooni juurest suhkur-fosfaat selgroog. Katkenud RNA ahelat on võimalik detekteerida pöördtranskriptaasi vahendatud praimer ekstensiooniga. Uurimaks ribosoomis olevaid fosfaat-selgroo interaktsioone, rekonstrueerisime me *T. aquatucus´*e modifitseeritud 23S rRNA-st, natiivsest 5S rRNA-st ja r-valkudest aktiivsed ribosoomi 50S alamühikud. Rekonstrueeritud 50S alamühikuid töötlesime joodiga, et detekteerida 23S rRNA I domääni (1–580 nukleotiidi) suhkur-fosfaat selgroo mittesildavate hapnike kaitstust joodi eest. 280 positsiooni olid joodile kättesaadavad ja 80 olid kaitstud. Enamus kaitstud positsioonidest (57) olid protekteeritud r-valkude poolt ja osa (23) ribosomaalse RNA enda poolt. Kokkuvõtteks võib öelda, et välja töötatud meetodit on võimalik kasutada RNA suhkur-fosfaat selgroo interaktsioonide uurimiseks, substraatide sidumiskohtade määramiseks ja individuaalsete positsioonide mõju määramiseks valgusünteesi erinevates etappides.

II. Valgusünteesi initsiatsiooniks on vaja, et ribosoomi kaks alamühikut kontakteeruks omavahel ja moodustaks funktsionaalse 70S ribosoomi. Selles töös uuriti *Escherichia coli* ribosoomi väikese alamühiku RNA (16S rRNA) lämmastikaluste funktsionaalset rolli ribosoomi alamühikute omavahelisel seondumisel. RNA lämmastikaluste keemilise modifitseerimise meetod võimaldab uurida spetsiifiliste RNA positsioonide olulisust konkreetse funktsiooni tagamisel. Selleks me modifitseerisime 30S alamühikuid kemikaalidega DMS (dimetüülsulfaat) ja CMCT (karbodiimide). Peale 30S alamühiku modifitseerimist assotseerisime modifitseeritud 30S alamühikud natiivsete (mittemodifitseeritud) 50S alamühikutega, et uurida mod-30S alamühikute võimet assotseeruda suure alamühikuga. Praimer ekstensiooni meetodit kasutades detekteerisime 16S rRNA-s kuus positsiooni (A702, A1418, A1483, U793, U1414 ja U1495), millede modifitseerimine takistab alamühikute assotseerumist. Detekteeritud positsioonid paiknevad tuntud alamühikute vahelistes sildades. Seega alamühikute assotsiatsioonil mängivad olulist rolli sillad B2a (U1495), B2b(U793), B3 (A1418, A1483, U1414) ja B7a (A702).

III. Kolmandas töös uuritakse sellist fenomeni nagu r-valkude võimet välja vahetuda ja selle tulemusena taastada inaktiivsete ribosoomide funktsioon. *E. coli* ribosoomid koosnevad 54-st erinevast r-valgust. Ribosoomide biosüntees kiires kasvufaasis kulutab enamuse raku energiast, seega kui ribosoomi valgud ribosoomis saavad kahjustada (hapniku radikaalid ja muud keemilised ühendid) ja ribosoomide vahendatud valgusünteesi optimaalne aktiivsus väheneb, siis oleks rakul energeetiliselt kasulik toota

uued r-valgud ja vahetada kahjustatud r-valgud ribosoomis välja uute funktsionaalselt aktiivsete valkude vastu. Enamus r-valkude massist asetseb ribosoomide pinnal ja võiks oletada, et r-valkudel võiks olla ka RNA kaitsefunktsioon, kuna enamus funktsionaalselt oluline RNA asetseb ribosoomide sisemuses. Seega selline r-valkude välja vahetumise võime võimaldab ribosoomidel optimaalselt funktsioneerida ja rakkudel üle elada raskeid tingimusi.

Ribosoomide modifitseerimine valgu-spetsiifilise kemikaaliga NEM (N-etüülmaleimiid) vähendab ~ 60% ribosoomide võimet osaleda rakuvabas polü(U) vahendatud polü(Phe) sünteesis. NEM-modifitseeritud ribosoomide (35% aktiivsust võrrelduna mittemodifitseeritutega) töötlemine ribosoomi valkudega (TP70) taastab ligi 2 korda (75% aktiivsust) ribosoomide võimet osaleda valgusünteesil.

On teada, et kui ribosoome inkubeerida kõrge kontsentratsiooniga soolalahuses (2M LiCl), siis osad r-valgud dissotseeruvad ribosoomilt. Neid kergesti ära tulevaid r-valke kutsutakse „split" (lõhenenud) valkudeks ja neid mis jäävad peale soolapesu RNA-ga seotuks „core" (tuumik) valkudeks. NEM-modifitseeritud ribosoomide töötlemine „split" valkudega taastas ligi 2.6 korda ribosoomide valgusünteesi aktiivsust. Kusjuures „core" valgud ei suutnud inaktiivsete ribosoomide valgusünteesi aktiivsust taastada. Seega „split" valgud on vastutavad ribosoomide aktiivsuse taastamisel.

Ribosoomis välja vahetuvate valkude kindlaks tegemiseks kasutasime kahte *in vitro* meetodit, nii radioaktiivset märgistamist kui ka raskete isotoopide eristamise meetodit. Esimesel juhul detekteeriti välja vahetuvad valgud kahe-dimensionaalsel valgu geelelektroforeesi ja autoradiograafia abil. Teisel juhul kasutati kvantitatiivset mass-spektromeetriat. Ribosoomi valgud S2, L1, L7/12, L9, L10, L11 ja L33 on kõige kergemini vahetuvad r-valgud.

Lisaks sellele detekteerisime me *in vivo* statsionaarses faasis *E. coli´*s välja vahetuvad r-valgud, mis osutusid enam-vähem samadeks kui *in vitro* detekteeritud. Seega, meie tulemused näitavad, et kahjustatud ribosoome on võimalik parandada valkude asendamise teel.

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Best wishes and gratitudes to my parents and brothers, who have supported me through life. Finally, all the love goes to Riina.

PUBLICATIONS

CURRICULUM VITAE

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II. Scientific and researcher activity

Main research interests:
My main research interest has been to study bacterial ribosome translation by using chemical modification approaches. Ribosomal intersubunit contacts and ribosomal protein functionalities have been in intrest.

List of publications:

Maivali Ü., Pulk A., Loogväli E.L., Remme J. 2002. Accessibility of phosphates in domain I of 23 S rRNA in the ribosomal 50 S subunit as detected by R(P) phosphorothioates. Biochim Biophys Acta. 1579(1):1–7.

Pulk A, Maiväli U, Remme J. 2006. Identification of nucleotides in E. coli 16S rRNA essential for ribosome subunit association. RNA. 12(5):790–6.

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II. Teaduslik ja arendustegevus

Peamised uurimisvaldkonnad: peamiseks uurimisvaldkonnaks on olnud *Escherichia coli* ribosoomide RNA ja valkude roll translatsioonil.

Publikatsioonide loetelu:

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Pulk A, Maiväli U, Remme J. 2006. Identification of nucleotides in E. coli 16S rRNA essential for ribosome subunit association. RNA. 12(5):790–6.
Pulk A., Liiv A., Peil L., Maiväli U., Nierhaus K., Remme J. 2009. Ribosome reactivation by replacement of damaged proteins. Mol Microbiol. 75(4): 801–814.

III. Saadud uurimistoetused ja stipendiumid

Muu teaduslik organisatsiooniline ja erialane tegevus (konverentside ettekanded, osalemine erialastes seltsides, seadusloome jms.):
Konverentsid:

2007	4th International PhD Student Symposium „Horizons in Molecular Biology" , Göttingen, Saksamaa, 14–16 september
2008	13th Annual Meeting of the RNA Society, Berliin, Saksamaa, 28. juuli – 3. august

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