DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
97

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97

STUDIES ON THE STRUCTURE-FUNCTION RELATIONSHIP OF THE BACTERIAL RIBOSOME

ÜLO MAIVÄLI

TARTU UNIVERSITY
PRESS

Department of Molecular Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation is accepted for commencement of the degree of Doctor of Philossophy (in molecular biology) on August, 27th, 2004 by the council of the Institute of Molecular and Cell Biology, University of Tartu.

Opponent: Prof. A.A. Bogdanov (PhD), University of Moscow, Russia.

Commencement: Room No 217, Riia 23, Tartu 08.27.2004, at 09.15

Tartu Ülikooli Kirjastus
www.tyk.ut.ee
Tellimus nr. 331

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I. **Maiväli, Ü.**, Saarma, U., Remme J. (2001) Mutations in the *Escherichia coli* 23S rRNA increase the rate of peptidyl-tRNA dissociation from the ribosome. Mol Biol. 35, 569–574.

II. **Maivali, Ü.**, Remme J. (2004) Definition of bases in 23S rRNA essential for ribosomal subunit association. RNA. 10, 600–604.

III. **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, 1–7.

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Additional publication: Gunnery S., **Mäivali Ü.**, Mathews M.B. (1997) Translation of an uncapped mRNA involves scanning. J Biol Chem. 272, 21642–6.

LIST OF ABBREVIATIONS

aa-tRNA	—	aminoacyl-tRNA
A site	—	ribosomal site for aminoacyl-tRNA binding (acceptor site)
CMCT	—	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate
CP	—	central protuberance
Cryo-EM	—	cryo-electron microscopy
DMS	—	dimethyl sulfate
EF	—	elongation factor
E site	—	ribosomal site for deacylated tRNA binding (exit site)
Fus	—	fusidic acid
GAC	—	GTPase-associated centre
GEF	—	GTP exchange factor
G-protein	—	a member of the GTP-binding protein family
IF	—	initiation factor
P site	—	ribosomal site for peptidyl-tRNA binding (donor site)
PTC	—	peptidyl transferase centre
Poly(Phe)	—	poly-phenylalanine
Poly(U)	—	poly-uridine
p-tRNA	—	peptidyl-tRNA
RF	—	release factor
RRF	—	ribosome release factor
rRNA	—	ribosomal RNA
r-protein	—	ribosomal protein
SRL	—	Sarcin-ricin loop
WC	—	Watson-Crick

INTRODUCTION

Not since the discoveries of general principles of conveying genetic information from DNA to protein by the mid-1960s, has there been a more exciting time for ribosome studies. The last four years have brought us the first realistic atom-scale models of ribosomal particles that allow resolution of some long-standing questions about the structure and function of the ribosome. Perhaps more importantly, the resolved ribosomal structures allow formulate questions more precisely. That is the best guarantee of informative answers. The potential of the new kind of structure-derived hypothesis is already becoming apparent in the studies of ribosomal catalytic mechanisms, decoding, fidelity, and translocation. Attesting to the recent rejuvention of the ribosome field is the recent plenitude of "reviews of everything", which attempt to cover most aspects of the basic ribosomal working cycle (Ramakrishnan 2002, Moore and Steitz 2002, Wilson et al. 2002, Wilson and Nierhaus 2003, Bashan et al. 2003b). Yet, as of 2003, none of the basic steps of protein synthesis is satisfactorily understood at the atomic level. Also, the quantitative modelling of protein synthesis is still at a rather embryonic stage, and would greatly benefit from any descriptive advances concerning the basic mechanisms of translation. The rate of accumulation of new knowledge in the ribosome field is likely to increase during the next few years.

The history of ribosome research may well start with the electron microscopic work of Claude who in the late-1930s co-isolated with tumor-inducing Rous sarcoma viruses quite harmless and ubiquitous particles of similar size (reviewed in Tissieres 1974). He called them "small granules" and later "microsomes". In the early 1940s Jeener and Brachet showed that microsomes always contained RNA. At the same time, Brachet and Caspersson found strong correlation between protein synthesis activity of a given cell type, and the amount of its cytoplasmic RNA. In mid-1950s electron-microscopists Palade and Siekevitz found, that microsomes in all tissues examined were ribonuclear particles about 200Å in diameter, rich in RNA, and of high density. Clear demonstration that ribosomes were the site of protein synthesis came in the mid-1950s from the cell-free rat liver translation system, which was developed in the laboratory of Paul Zamecnik (for an exiting history of the Zamecnik lab see Rheinberger 1997) and a few years later from cell-free bacterial systems (Tissieres 1974). The name "ribosome" was introduced in 1958 by Roberts because the old terms were somewhat confusing and the word "has a pleasant sound" (Nomura 1989). Perhaps the most important year in translation studies was 1961. This year saw the discovery of mRNA by Jacob and Monod (Jacob and Monod 1961, Judson 1996). The poly-uridine-dependent incorporation of phenylalanine into peptides was accomplished also in 1961 by Matthei and Nirenberg, and soon led to the full description of the genetic code

by several labs (Judson 1996, Nirenberg 2004). By 1962 all major components of the translational elongation machinery had been identified. Polyribosomes on mRNA were described in 1962 by ultracentrifugation and electron microscopy in Alex Rich's lab (Warner and Knopf 2002). The peptide transfer model reaction with puromycin as the acceptor substrate was introduced by Gilbert in 1963 and was extensively studied by Monro throughout the 1960s (Maden 2003). The classical model of ribosomal elongation cycle involving two tRNA binding sites was proposed by Watson in 1964 (Nomura 1989). During the same year Davies, Gilbert and Gorini demonstrated misreading of the genetic code induced by the ribosome binding antibiotic, streptomycin. Basic mechanisms of initiation and termination and the factors responsible for the full cycle of translation were elucidated in the second half of the 1960s by several labs (Nomura 1989).

Thus the structural and biochemical experiments have defined a number of biochemical properties for the ribosome, to which more up-to-date treatment is given in the following chapters.

This dissertation intends to tread the interface between the structure and function of the ribosome. By discussing both structural and functional data, I hope to convince the reader that neither approach alone can be sufficient for the satisfactory understanding of protein synthesis. Furthermore, the shape of things to come in the ribosome field will not resemble the simple sum of the two complementary approaches. Clearly, structural studies have fancied the results of the biochemical work in choosing, which complexes to study and how to interpret the results. On the other hand, designing biochemical experiments without reference to published structures might be unwise, to say the least.

REVIEW OF LITERATURE

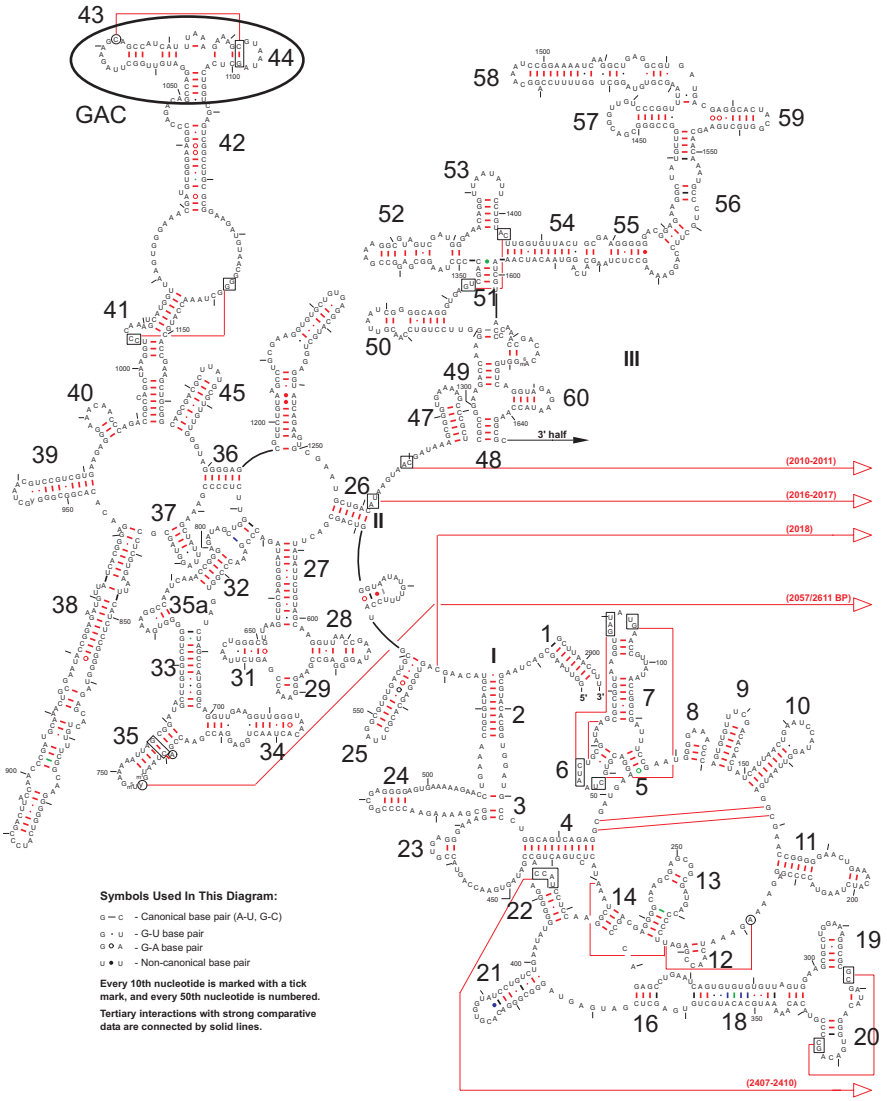
Chapter 1. General features of translation

The translation of mRNA into protein is ribosome's business. It is achieved through a series of tightly orchestrated manoeuvres that use several non-ribosomal factors as catalysts and add up to a translational cycle (Fig 3a). All ribosomes consist of two unequal subunits containing 3 RNA molecules (which are further fragmented in many organisms, notably in eukaryotes), and a number of different r-proteins. The most thoroughly studied example, *Escherichia coli* 70S ribosome, consists of 50S and 30S subunits. 50S contains 23S and 5S ribosomal RNAs and 34 ribosomal proteins, while 30S contains 16S rRNA and 21 r-proteins (see figs 1 and 2 for the secondary structures of 23S rRNA and 16S rRNA). The 70S ribosome has a molecular weight of 2.6 MDa and diameter of 200–250 Å. About two thirds of its mass is RNA and one third is protein.

The large subunit is responsible for the catalytic activities of the ribosome. It alone is sufficient for the catalysis of peptide bond formation *in vitro* (Maden 2003). It also contains the GTPase-associated centre (GAC), which is necessary for the binding and activation of the ribosome-associated G-proteins (EF-G, EF-Tu, IF2, RF3, Tet(O); Ramakrishnan 2002). The small subunit is responsible for the decoding of the genetic cipher and transmitting (rather mysteriously) the news of the correct codon-anticodon interaction to the GAC of the 50S subunit, thus coordinating the movement of the ribosome through the translational cycle (Ogle et al. 2003, Bashan et al. 2003b). The ribosome has at least three separate tRNA binding sites, each spanning the two subunits (Marquez et al. 2002, Yusupov et al. 2001). The A site binds aminoacyl-tRNA (aa-tRNA), the P site binds peptidyl-tRNA (p-tRNA) or deacylated tRNA, and the E site binds deacylated tRNA (Rheinberger 1991). During elongation there are always at least two tRNAs in the ribosome, in either P and E sites or in the P and A sites (Remme et al. 1989, Marquez et al. 2002). Negative allosteric co-operativity has been proposed to occur between E and A sites, according to which the E site tRNA is released upon the binding of tRNA to the A site (Marquez et al. 2002). In addition, positive co-operativity exists in the PTC between tRNA binding to the P and A sites (Bourd et al. 1983).

Unlike the catalysis of peptidyl transfer and the decoding of mRNA codons, the translocation of peptidyl-tRNA from the acceptor site (A site) to the donor site (P site) of the ribosome, and deacylated tRNA from the P to exit site (E site), seems not to be the exclusive property of either subunit (Joseph 2003). The need to taxi tRNAs through the ribosome is likely a reason for the two-subunit nature of the ribosome (Spirin 2002).

Secondary Structure: large subunit ribosomal RNA - 5' half



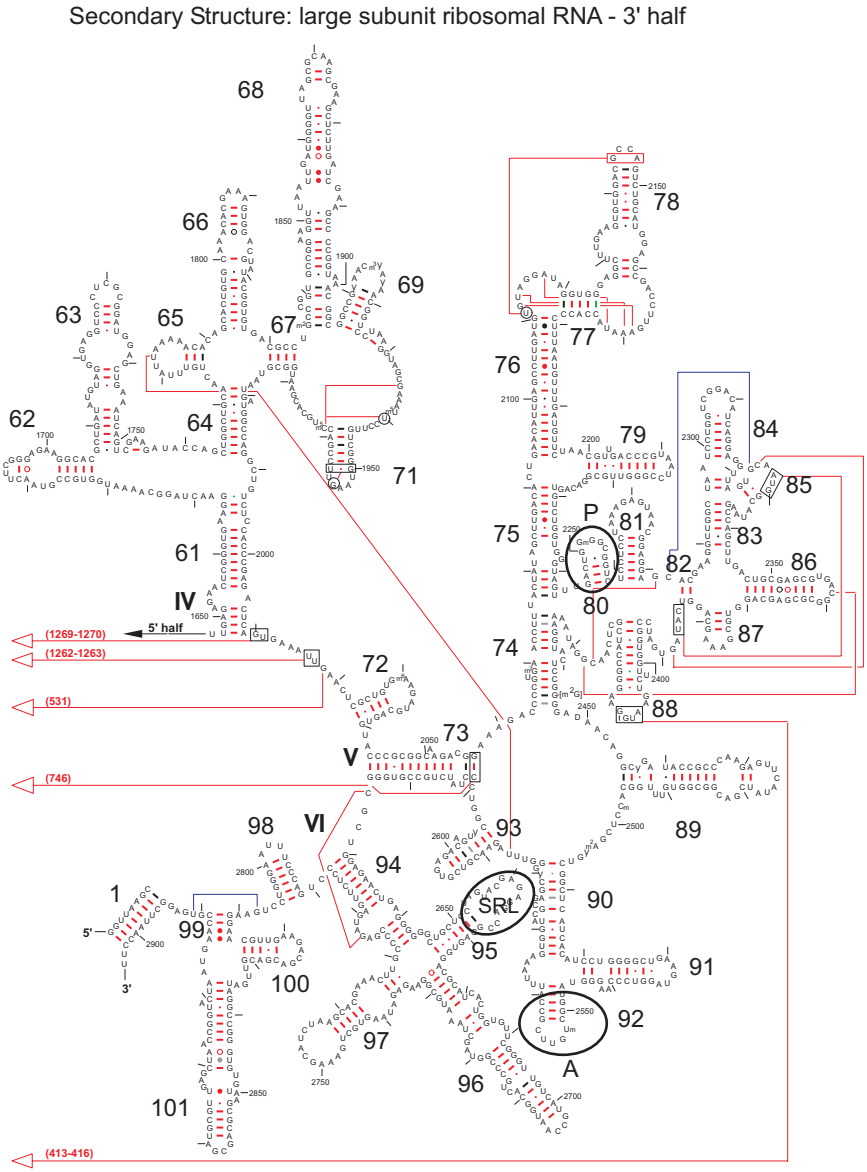


Figure 1. Secondary structure diagram of the *Escherichia coli* 23S rRNA (URL:http://www.rna.icmb.utexas.edu/). Helices are numbered as for the model of the archeal *D. mobilis* 23S rRNA (Leffers et al. 1987). A – the A loop, P – the P loop, SRL – the sarcin-ricin loop, GAC – the part of the GTPase-associated centre that comprises the thiostrepton resistance mutation A1067U.

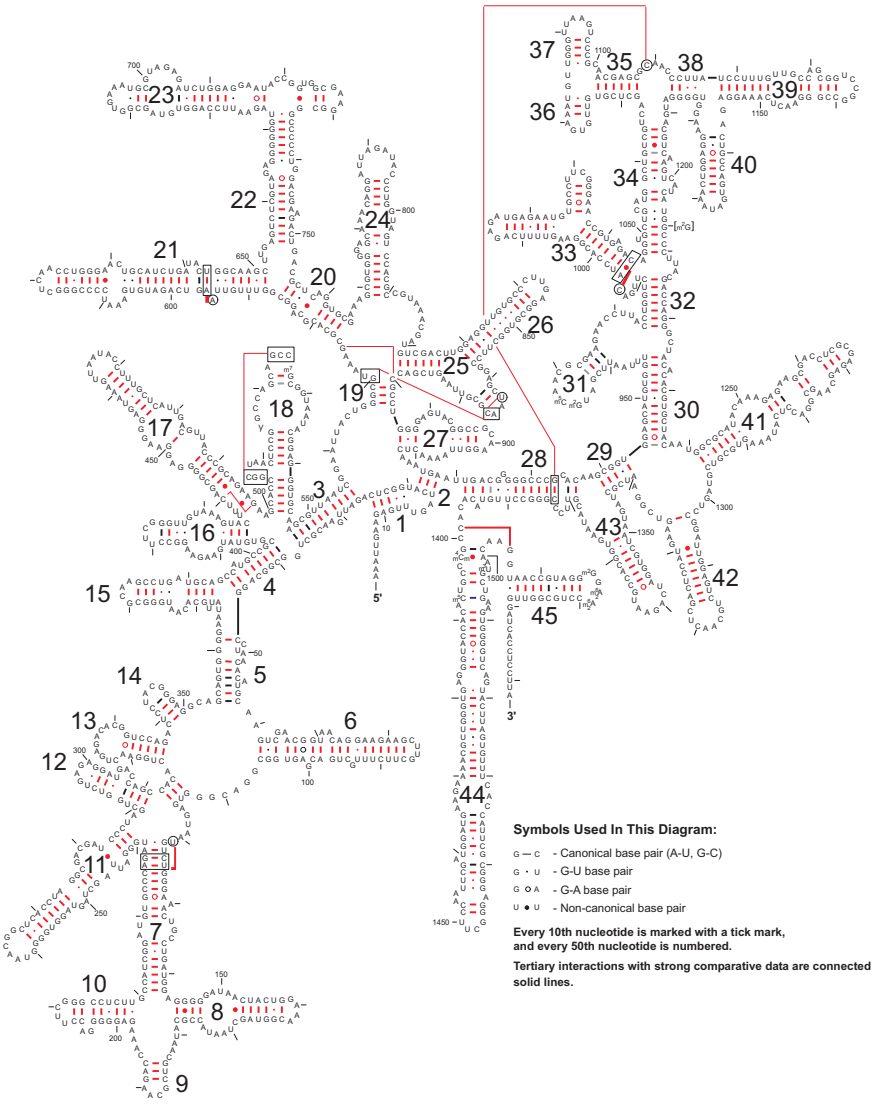


Figure 2. Secondary structure diagram of the *Escherichia coli* 16S rRNA (URL:http://www.rna.icmb.utexas.edu/).

There are two more important points to remember. First, in the test tube, given the correct substrates (mRNA and tRNAs), all steps of the translational cycle can be performed in the absence of translation factors (Chetverin and Spirin 1982). This suggests that translation factors act as enzymes, increasing the rates of different steps of the translation cycle by lowering the activation energies, without changing the basic reaction mechanisms (Spirin 2002). Secondly, ribosomal A site and the GAC, which seem to be functionally associated with it, can accommodate many substrates. They include the tRNAs, IF1, IF2, EF-G, EF-Tu, RF1, RF2, RF3, RRF, RelA, RelE, Tet(0) and tmRNA (Table 1). The identity of the mRNA codon in the A site of the 30S subunit and the presence or absence of peptide on the tRNA in the P site seem to act in concert in programming the A site/GAC (Zavialov et al. 2002, Zavialov and Ehrenberg 2003). The programming of the A site is the key to the sequence of events that comprise the translational cycle. Competition for a single binding site indicates that there can be no single pathway of translation. At every step many distinct substrates compete for binding, and who wins depends not only on the pre-programming of the binding site, but also on the relative affinities and concentrations of the competing substrates. For example, RelE protein, which is active in starved bacteria and helps to adapt to hunger by cleaving mRNAs in the ribosome's A site, efficiently competes with RF1 for the stop codon UAG (Pedersen et al. 2003). tmRNA, whose accepted function is to rescue stalled ribosomes with empty A sites from broken mRNAs, can also successfully compete with RF2 for the weak stop codon UGA and with the cognate ternary complex for the rare arginine codon AGG in the A site (Collier et al. 2002, Hayes et al. 2002). Such tmRNA action is likely preceded by the cleavage of mRNA in the A site (Sunohara et al. 2004). Many programmed recoding events (frame-shifts and ribosomal hopping on mRNA) are also brought about by induced ribosomal pausing on sense codons where the ribosome has to "choose" between different reaction pathways (Namy et al. 2004). This once more underlines the need for serious quantitative models of translation. Intuitive approaches to the translational "cycle" no longer give an entirely satisfactory description to the experimental facts that are collected daily.

On a more mundane level, such a multitude of substrates for a single binding site dictates some overall similarity in their general shape. Hence the molecular mimicry hypothesis, which in this case may be formulated as follows: the shapes of all the A site substrate proteins converge on tRNA (see Nissen et al. 2000b for review). The most discussed example of molecular mimicry comes from the comparison of structures of the aa-tRNA/EF-Tu/GTP ternary complex and the EF-G (Aevarsson et al. 1994, Czworkowski et al. 1994, Nissen et al. 1995). Domains III-V of EF-G mimic the shape of the tRNA, domain IV is the mimic for the tRNA anticodon stem-loop, both in shape and in overall charge distribution (Fig 3b).

Table1. Programming of the ribosomal A site for factor binding

factor	Ribosomal programming	Function of the factor
IF1	Except for mRNA, empty 30S A site.	Mediates $tRNA_i^{fMet}$-IF2-GTP binding to the P site, excludes elongation tRNA binding to the A site.
IF2/GTP	IF1 in the 30S A site. Exact binding site is unknown but probably includes the A site (Roll-Mecak et al. 2000).	Brings fMet-tRNA to the P site of the 30S subunit.
aa-tRNA/EF-Tu/GTP	Sense codon in the A site, tRNA in the P site. EF-Tu binds GAC. GTPase indirectly activated by codon-anticodon interaction.	aa-tRNA decodes sense codons, EF-Tu is involved in proofreading. GTP hydrolysis releases EF-Tu.
EF-G/GTP	Deacylated tRNA in the P (or E/P) site (Zavialov and Ehrenberg 2003). Binds both GAC and the A site (Wilson and Noller 1998).	Translocation of tRNAs to the E and P sites, subunit dissociation (with RRF). Role of GTP hydrolysis controversial (see below).
RF1	UAA, UAG codons in the A site	Induces peptide transfer to water.
RF2	UAA, UGA codons in the A site	Induces peptide transfer to water
RF3/GDP	tRNA in P site, RF1 or RF2 in A site. RF1 or RF2 acts as GEF for RF3, but only if there is deacylated tRNA in the P site (Zavialov et al. 2002).	RF3-GTP removes RF1 or RF2 from the ribosome. GTP hydrolysis releases RF3.
RRF	70S with deacylated tRNA in the P site.	Dissociates ribosomal subunits (in association with EF-G).
IF3	30S with deacylated tRNA in P site. Location of the binding site is controversial, but likely includes P and E sites (Dallas and Noller 2001).	Removes deacylated tRNA from the P site. Subunit anti-association factor. Selects for $tRNA_i$.
Ribosomal protection proteins (EF-G homologues), incl. Tet(O)/GTP	Tetracycline bound to the open A site of the post-translocational ribosome (Chorpa and Roberts 2001, Connell et al. 2003).	Removal of tetracycline from the A site.

Table 1 (continuation)

factor	Ribosomal programming	Function of the factor
RelA	Exact binding site not known, probably at least partially in the A site. Deacylated tRNA in the A site (necessary for (p)ppGpp synthesis, not RelA binding, for which mRNA with otherwise empty A site is sufficient; Wendrich et al. 2002).	(p)ppGpp synthesis that leads to stringent response.
RelE	Cleaves UAG stop codon (and to a lesser extent UAA and CAG) in the A site. Exact binding requirements and binding site is not known (Pedersen et al. 2003).	During stress or the stringent response the antitoxin RelB is removed and RelE action leads to transient inhibition of translation.
Ala-tmRNA/SmpB/EF-Tu/GTP	Empty A site (preferably not containing mRNA).	Release of unfinished peptides from incomplete mRNAs and labelling for their subsequent degradation (Withey and Friedman 2003, Haebel et al. 2004).
$tRNA^{Sec}$/SelB/GTP	UGA stop codon at the A site, SECIS sequence immediately after the UGA (Namy et al. 2004).	Selenocystein incorporation into proteins. SelB is an EF-Tu analogue.
SRP	Nascent peptide with signal sequence emerging from the peptide channel. The Alu domain contacts GAC, thus excluding ternary complex binding (Hallic et al. 2004).	Directs nascent proteins for membrane transport, Alu domain blocks translational elongation (Nagai et al. 2003).

Tethered hydroxyl-radical cleavage-based mapping of EF-G into the 23S rRNA and cryo-EM studies of the ribosome-EF-G complexes have revealed that EF-G domains, which mimic tRNAs, indeed bind to the A site of the ribosome (Wilson and Noller 1998, Valle et al. 2003c). In addition to EF-G, translation factors IF2, RF2 and RRF have all been implicated as tRNA mimics (Nissen et al. 2000b).

From similarities of shape, one is tempted to infer similarities in function. This, however, is evidently a dangerous thing to do. Apparently, the molecular mimicry hypothesis has been a very useful tool for sowing confusion into the ribosome field (Ehrenberg and Tenson 2002, Nakamura and Ito 2003). For example, in the case of RRF, the biochemical and cryo-EM studies do not support the inferences about the binding and the mechanism of action of the

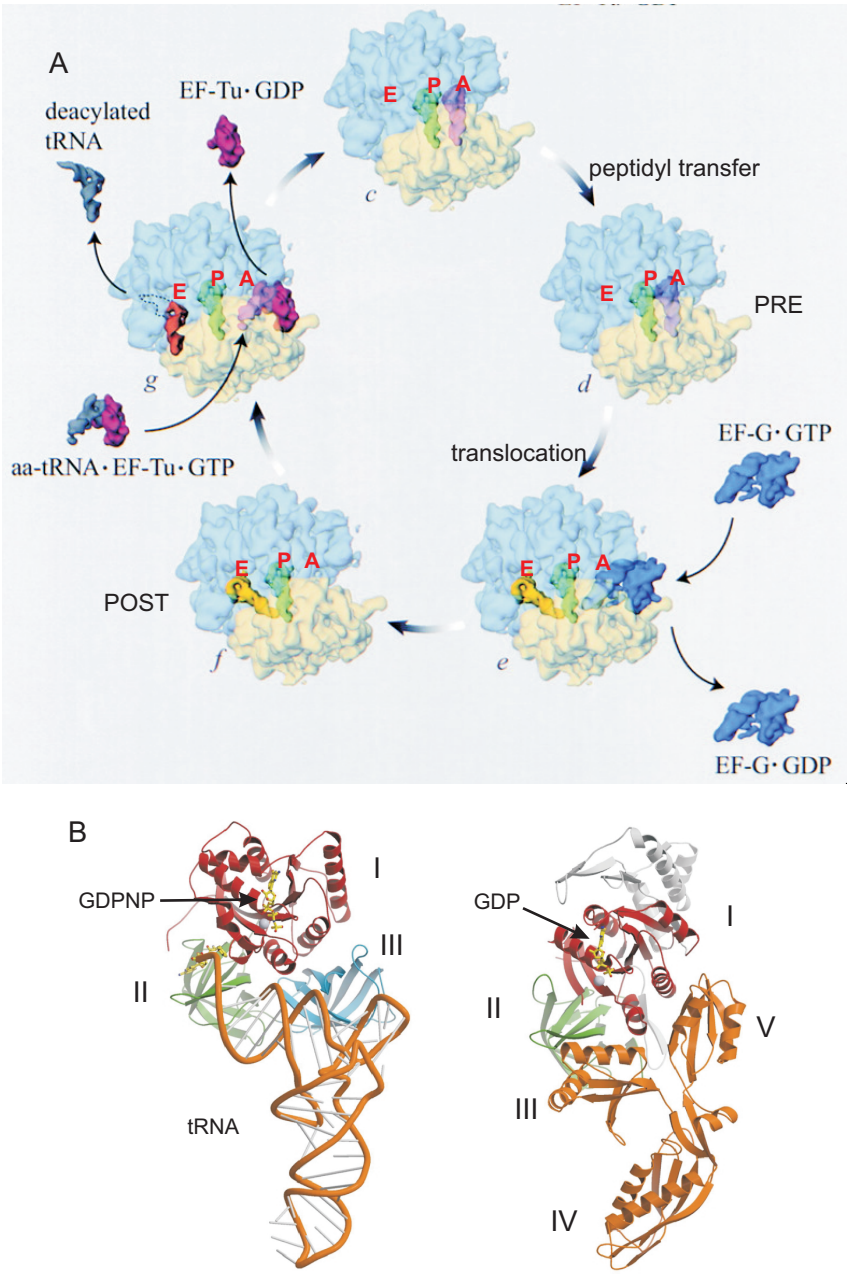


Figure 3.

A. A cryo-EM based illustration of the elongation cycle of translation (adapted from Agrawal et al. 2000). The 50S subunit is light blue and 30S subunit is yellow. PRE is pre-translocational state, where tRNAs are shown in conventional A/A (p-tRNA) and P/P (deacylated tRNA) binding states. POST is posttranslocalional state of the ribosome, where p-tRNA is in the P site and deacylated tRNA in the E site. g, c- denote decoding (for a detailed scheme see Fig. 8). d- EF-G-GTP binding and translocation.

B. Molecular mimicry between AA-tRNA/EF-Tu/GTP ternary complex, on the left, and EF-G/GDPNP, on the right (adapted from Andersen et al. 2003). Protein domains are marked by roman numerals. GDPNP, the non-hydrolysable analogue of GTP, and GDP are in yellow and marked by arrows.

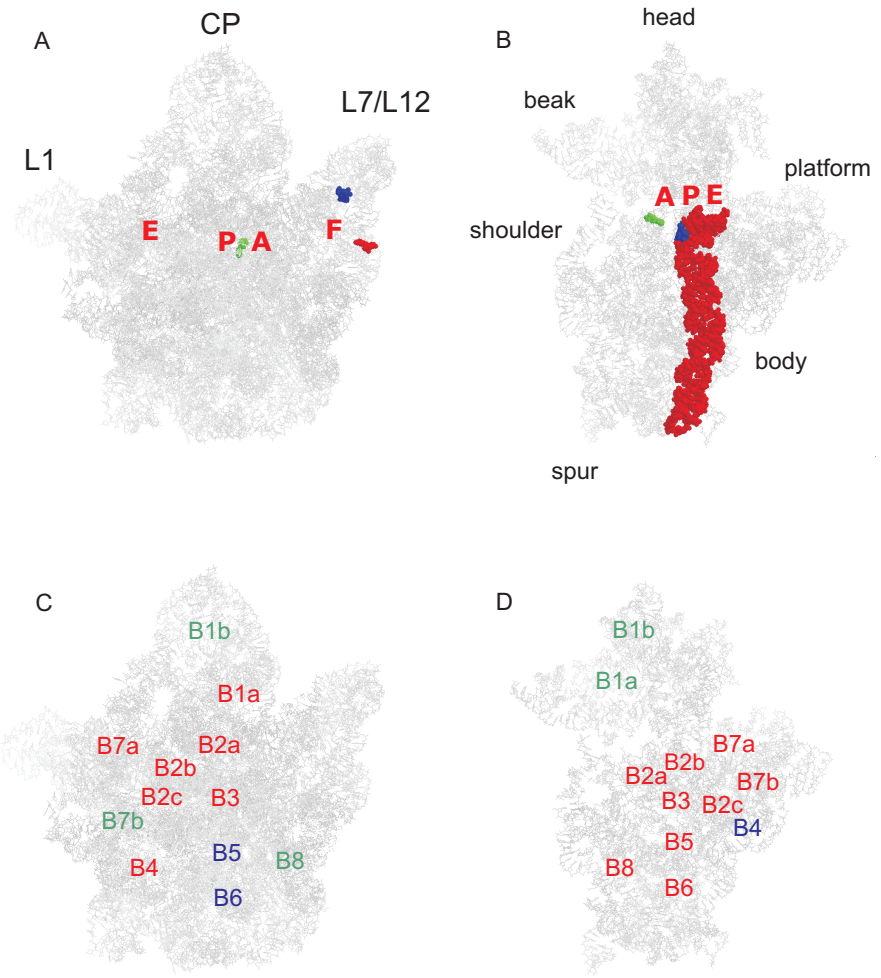


Figure 4.
A. Landmarks of the 50S subunit. 23S rRNA nucleotides 2451 (green), 1067 (blue) and 2661 (red) are highlighted in spacefill. A2451 has been implicated in PT. Mutations at position 1067 confer resistance to thiostrepton, an EF-G and IF2 inhibitor (Brandi et al. 2004). Position 2661 is the target for sarcin and ricin, both inhibitors of translocation. CP central protuberance. F factor binding site.
B. Landmarks of the 30S subunit. 16S rRNA helix 44 is in red spacefill, nucleotides 1492-1493 (blue) and 530 (green) are also highlighted in spacefill. See chapter 4 for discussion of their role in decoding.
C and D. Locations of intersubunit bridges are shown in 50S and 30S subunits . RNA-only contact areas are in red, protein-only contact areas are in green and contact areas containing both RNA and protein are in blue.

RRF, which were derived using the idea of molecular mimicry (Nakamura and Ito 2003, Agrawal et al. 2004).

Unlike the A site binding factors, proteins that bind to the GAC are all homologous in their GTP-binding domains, therefore having by neccesity similar shapes and function (Caldon et al. 2001). All GTPases seem to have similar working cycles, consisting of three functional states. First, the nucleotide-free state is inactive. Second, the GTP-state is functionally active having altered conformation and increased affinity to effector molecules. Third, the GDP-state, after GTP hydrolysis and P_i release, leads to the release of the G-protein from its functionally active complex with the effector molecule (Caldon et al. 2001). Some G-proteins need GEFs (GTP Exchange Factors) for catalysing dissociation of GDP and subsequent association of GTP. For example, EF-Ts is the GEF for EF-Tu. Interestingly, the ribosome appears to be both the GEF (in the RF1/RF2 bound state) and the effector molecule for RF3 (Zavialov et al. 2002, see also Table1 and Chapter 3).

Chapter 2. The Structure of the ribosome

Although the first ribosomal crystals of potential usefulness were grown by two groups in the 1980s, they diffracted to relatively low resolutions of 10–12 Å (Trakhanov et al. 1987, Yonath 2002). By 1991, crystals diffracting to 3 Å were obtained. Unfortunately, they exhibited various defects hampering their usefulness (Moore and Steitz 2003). Anyway, the solution to the ribosome crystallography (phasing) problem had to wait for improvements in general crystallography techniques (Ban et al. 1998, Cate et al. 1999, Clemons et al. 1999, Tocilj et al. 1999, Moore and Steitz 2003). Also, medium-resolution cryo-EM reconstructions that emerged in the late-1990s had to be used to locate the heavy atom clusters, introduced for phasing purposes (Steitz and Moore 2003).

Meanwhile, serious attempts were made to model the ribosomal structure using neutron scattering (Capel et al. 1988, Stern et al. 1988), the rather inaccurate results of which ultimately found their way into textbooks (Lewin 1994). Also, the ribosomal structure was probed by low-resolution electron microscopy (Frank et al. 1989) and by chemical and enzymatic probing (Noller 1991, Green and Noller 1997). This activity also led to heroic efforts of modelling of the ribosomal subunits (Mueller and Brimacombe 1997, Brimacombe et al. 1990), which were nevertheless not much used in constructing the more recent medium- and high-resolution structures (but see Mueller et al. 2000). However, the chemical crosslinking and footprinting studies, along with mutagenesis experiments, defined ribosomal components, which comprise the catalytic and substrate binding regions of the ribosome (Green and Noller 1997, Wilson et al. 2002). These results are generally in good agreement with high-resolution X-ray structures (Sergiev et al. 2001a, b) and where not, may well give us important clues about conformational states that are not captured by the

rigid crystallized complexes (Gabashvili et al. 2003). Moreover, footprinting of tRNAs before and after peptide transfer led to the hybrid state model of tRNA transit through the ribosome (Fig 5; Moazed and Noller 1989b); a model, which has been instrumental in designing and interpreting biochemical (Wower et al. 2000, Zavialov and Ehrenberg 2003, Sharma et al. 2004) and structural experiments (Schmeing et al. 2003, Valle et al. 2003c,). Yet, it is likely, that had the hybrid states model not been born from the footprinting experiments, it would be unborn still. Presumably because of experimental impurities, most relevant medium-resolution ribosome structures fail to exhibit the hybrid states (Agrawal et al. 1999b, 2000, Valle et al. 2003c), and some that do, seem to do so inappropriately (Valle et al. 2003c).

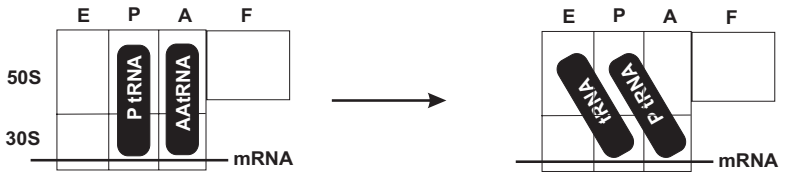


Figure 5. The hybrid states model. After peptidyl transfer from the P site bound p-tRNA to the A site bound AA-tRNA, both tRNAs retain their contacts with the 30S subunit, while on the 50S subunit move to the E and P site, respectively. A – acceptor site, P – donor site, E – exit site, F – factor binding site.

Before wandering into the maze of atomic-scale ribosomal models, let us consider the landmarks of the ribosomal structure. The large subunit is roughly a hemisphere about 250 Å in diameter with three projections protruding radially from the edge of its flat intersubunit face (Moore and Steitz 2003b). The large central protuberance (CP) is in the middle, the L7/L12 stalk at 2 o'clock and the L1 stalk at 10 o'clock (Fig 4a). This is the frequently presented "crown view" of the 50S subunit. The peptidyl-transferase centre (PTC), comprising the 50S-parts of the A, P and E sites, is located below the CP at about the centre of the intersubunit face. The peptide exit tunnel starts from the back wall of the PTC very close to the P site and leads to the opposite side of the hemisphere. The A site is closer to the L7/L12 stalk and P site is approximately in the centre of the intersubunit surface of the 50S . The E site lies close to the L1 stalk. Molecular interactions between tRNAs and the 50S subunit are discussed in Chapter 4. The GAC that binds the GTP-binding domains of ribosome-associated G-factors is located at the base of the L7/L12 stalk. The GAC involves 23S rRNA helices 43 and 44 and the proteins L11 and L7/L12. The G-factor binding centre (the F site in Fig 4a) also includes the sarcin-ricin loop (SRL; helix 95 of 23S rRNA domain IV, Fig 1) (Wilson and Noller 1998). Because no high-resolution structures of ribosomal complexes with G-factors are available, the

exact contacts between G-factors and their binding site(s) remain unclear. The mechanism of GTPase activation by the ribosome is as yet unknown.

The 30S subunit, seen from the intersubunit side, has a somewhat anthropomorphic appearance (Fig 4b). It sports a beaked and movable head on a thin neck (consisting of a single 16S rRNA helix), a shoulder, and a platform on the opposite sides on the top of the body. The mRNA and the A, P and E sites wind around the neck of the 30S (Yusupova et al. 2001). To illustrate how the two ribosomal subunits fit together, their contact areas, which form the intersubunit bridges, are enumerated in Figure 4c and d.

First atom-level X-ray crystallography based models of the ribosomal subunits and a slightly lower-resolution model of the 70S ribosome appeared by the turn of the century. They include a 5.5 Å resolution model of *Thermus thermophilus* 70S ribosomes (Yusupov et al. 2001), two independently constructed ~3 Å resolution models of the *T. thermophilus* 30S subunit (Wimberly et al. 2000, Schluenzen et al. 2000), a 2.4 Å model of the *Haloarcula marismortui* 50S subunit (Ban et al. 2000) and a 3.1 Å model of the *Deinococcus radiodurans* 50S subunit (Harms et al. 2001). Collectively this work suggests some general conclusions about the structure of the ribosome.

1. Both subunits and the 70S ribosome are inherently asymmetric. This means, that no axis of symmetry can be drawn across the ribosome. The exception seems to be the partial symmetry found in tRNA transit area in the 50S subunit, also encompassing the peptidyl transferase region (Bashan et al. 2003a, b).
2. The PTC consists entirely of RNA. This is the conclusive proof that ribosome is indeed a ribozyme. Most r-proteins are peripheral (in ribosome structure, as well as functionally).
3. The 50S subunit has a compact structure, where different 23S rRNA domains are hardly separable. The exception is the 5S rRNA, which forms the CP of the 50S subunit. Such a monolithic structure must be relatively rigid. Potentially flexible parts are located at the beginning (L7/L12 stalk) and at the end (L1 stalk) of the tRNA transit path, apparently reflecting the need to regulate tRNA movement through the ribosome (Bashan et al. 2003a, b).
4. The 30S subunit is considerably more flexible, undergoing conformational changes upon 70S association and decoding. 30S proteins S12, S4 and S5 are likely to be important players in this conformational flexibility. Secondary structure domains of the 16S rRNA are separable into different tertiary structural domains of the 30S subunit. The 5' domain forms the bulk of the body, the 3' major domain forms the bulk of the head and the central domain forms the platform. The only exception is the 3' minor domain (helix 44), which forms a part of the body at the subunit interface.
5. In the three-dimensional structure, many of the single-stranded loop regions in the rRNA secondary structure turned out to be slightly irregular double-stranded extensions of neighboring regular helices (Wilson et al. 2002).

Interactions between the helical elements include vertical co-axial stacking forming long quasi-continuous helical structures, and horizontal packing of helices. From the 36 helical elements in the 30S subunit, 13 are groups of co-axial stacked helices (Wilson et al. 2001). So, the ribosomal RNA is more helical than appears from the secondary structure diagrams. Extensive helical packing is the key to the compactness and rigidity of the ribosomal particles.

6. The tertiary structure of rRNA is built of a limited number of secondary structure modules including the A-minor, kink-turn, U-turn, S-turn, hook-turn, and tetraloop motifs (Hermann and Patel 1999, Moore 1999, Klein et al. 2001, Nissen et al. 2001, Moore and Steitz 2003). By recent work on the ribosomal structure, the sheer amount of known high resolution 3D RNA structure has increased ten-fold. It is therefore instructing that the kink-turn is actually the only entirely new RNA secondary structure element discovered from the ribosomal structures. Besides, the kink-turn motif appears to need auxiliary factors (proteins or metal ions) for its stability and is therefore not a *bona fide* secondary structure element at all (Goody et al. 2004). This suggests that the repertoire of RNA secondary structure modules may be (nearly) completely described (but see Sigel et al. 2004). The most important secondary structure module in rRNA (besides hairpin structures) seems to be the A-minor motif, which often stabilizes long-range interactions by the hydrogen-bonding of the adenines with minor grooves of the base-pairs (mostly G:C) of neighbouring helices. In the ribosome the A-minor interaction is considerably more frequent in stabilizing remote RNA-RNA interactions than long-range base pairing. In *Haloarcula marismortui* 23S RNA 186 adenines are involved in A-minor interactions (Nissen et al. 2001).
7. Ribosomal proteins by themselves do not form recognizable domains in the 30S or the 50S subunits. Their globular domains are in the exterior of the particle and frequent long flexible tails lead into the interior, where they intertwine with RNA helices. In the 50S of *H. marismortui* there are 12 proteins with such tails (Klein et al. 2004). The globular domains tend to be acidic whereas the flexible tails are highly basic. All of the 50S proteins, except for L12, interact directly with RNA and 23 out of the 30 remaining 50S proteins interact with more than one domain of 23S rRNA. R-proteins tend to recognize the sugar-phosphate backbone rather than bases. The primary function of r-proteins seems to be the stabilisation of the RNA structure. Moreover, flexible tails of r-proteins may actively promote the ribosomal assembly process (Jakovljevic et al. 2004). While the 30S proteins stabilize 16S rRNA domains, the 50S proteins are more involved in the stabilization of 23S rRNA interdomain interactions. When the protein components of the two 50S stalks are not included, every 50S protein contacts in average 2.6 different RNA domains (Moore and Steitz 2003).

Although the highest resolutions of crystallography-derived ribosomal subunit models (2.4 – 3 Å) are sufficient for relatively independent atom-scale modelling, there is paucity of functionally relevant ribosomal complexes at that resolution range. As of 2003, there is high-resolution data for at least 26 different antibiotics soaked into the 50S or 30S crystals (see Chapter 9, Table 8), but only for two translation factors: IF1 (Carter et al. 2001) and IF3 (Pioletti et al. 2001). Furthermore, the IF3 data has been questioned on account of the 30S crystal-packing that would preclude the soaked-in IF3 from binding to the 30S area deemed to be the most likely binding area by chemical footprinting (Dallas and Noller 2001) and cryo-EM (McCutcheon et al. 1999).

In addition, all high resolution work uses tRNA fragments as substrates, which are likely to exhibit somewhat different binding from the full-length tRNAs (Bashan et al. 2003a). The 5.5 Å resolution model of the 70S ribosome, that does use full-length tRNAs (Yusupov et al. 2001), is not fully interpretable without using the data from higher resolution subunit structures. In addition, the 70S crystals contain three tRNAs per ribosome (Yusupov et al. 2001), whereas native polysomes do not exceed two tRNAs per ribosome (Remme et al. 1989). These complaints about potential tRNA misbinding are, of course, relevant if the mechanism of ribosomal catalysis is to be deduced from ribosomal crystals.

Medium resolution (about 10–20 Å) single-particle cryo-electron microscopy comes to rescue where crystals are unavailable or packing interactions inconvenient (Frank 2002). This method does not require crystals, instead it uses extremely fast cooling of complexes in aqueous solution and computational analysis of large numbers of EM images of single particles, which results in an averaged image of the complex of interest. The analysis of single particles in solution allows greater flexibility in preparing truly interesting ribosomal complexes. Of course, for the averaging to produce a single image, the complexes still have to be homogeneous and sufficiently stable. The resulting image may be enhanced by fitting of the crystallography-derived higher-resolution models of structural elements (i.e. RNA helices) into the electron density map (Spahn et al. 2000). One should also bear in mind, that although the microscopists use two different criteria for resolution determination (Malhotra et al. 1998, Frank 2002), neither corresponds directly to the criterion used by crystallographers. Furthermore, whatever resolution is used by the electron microscopists, it is by definition an average of a continuum of resolutions with which different parts of the complex are resolved (Frank 2002). So, it is possible that important details can be seen “below” the stated resolution threshold. For example, Valle et al. (2003b) claim to see from their stated 9 Å resolution map two consecutive bases flipping out of an RNA helix (exemplified by a hole in the electron density of the helix). This implies a considerably higher resolution (perhaps around 5 Å) in that particular region of the ribosome.

Chapter 3. From termination to initiation

The train of events that leads from the stop codon dependent termination of translation to the ribosome's competence for a new round of initiation, has been elucidated in detail by biochemical work, that started in the 1960s (Kisselev and Buckingham 2000). While the sense codons are decoded by cognate tRNAs, stop codons in the ribosomal A site are recognized by proteinaceous factors RF1 or RF2. In bacteria UAA/UAG attracts RF1 and UAA/UGA RF2. In *Eukarya*, *Archaea* and mitochondria there is only a single RF1-type protein for stop codon recognition. Chemical cross-linking and mutagenesis studies give good reasons to think that RF1/RF2 directly interacts with stop codons (reviewed in Kisselev et al. 2003). Mutagenesis experiments have even led to the proposal of a protein anticodon, a linear sequence of amino acids that decodes the stop codons (Nakamura et al. 2000). However, this model remains to be controversial (Kisselev et al. 2003).

The stop codon-RF1/RF2 interaction, which takes place in the 30S subunit, leads to the transfer of the nascent peptide from tRNA to water. This takes place in the PTC of the 50S subunit. It is not known how RF1/RF2 carries the news of the stop codon to the PTC and what role (if any) it might have in catalysing the termination reaction itself. Another intriguing phenomenon is the large conformational change of RF2 on its binding to the ribosome, which was postulated after comparing the X-ray structure of *E. coli* RF2 (Vestergaard et al. 2001) with medium-resolution cryo-EM reconstitutions of the 70S-RF2 complex (Rawat et al. 2003, Klaholz et al. 2003). Also, the primary and tertiary structures of RF1/RF2s of different kingdoms (and mitochondria) seem to be quite different (Kisselev et al. 2003). This observation adds to the mystery of how exactly this factor acts in translational termination (Brodersen and Ramakrishnan 2003).

After the RF1/RF2 has helped to hydrolyse the nascent peptide, thus removing it from the P site bound tRNA, the RF1/RF2 itself must be removed from the ribosome (Fig 6). This is the function of the small G-protein RF3 (Freistroffer et al. 1997). This protein is unique in that it enters the ribosome in its inactive GDP-form and is converted to the active GTP -form only when RF1/RF2 is present in the A site and the nascent peptide is no longer attached to the P site tRNA (Zavialov et al. 2001, 2002). The action of RF3 leaves the 70S ribosome with unoccupied A site and deacylated tRNA in the P site (Kisselev and Buckingham 2000).

The next step is the dissociation of the ribosome into 50S and 30S-mRNA-tRNA complexes by the concerted action of ribosome recycling factor (RRF) and EF-G-GTP (Pavlov et al. 1997, Karimi et al. 1999). RRF is a wonderfully tRNA-like protein in shape (Selmer et al. 1999, Kim et al. 2000, Toyoda et al.

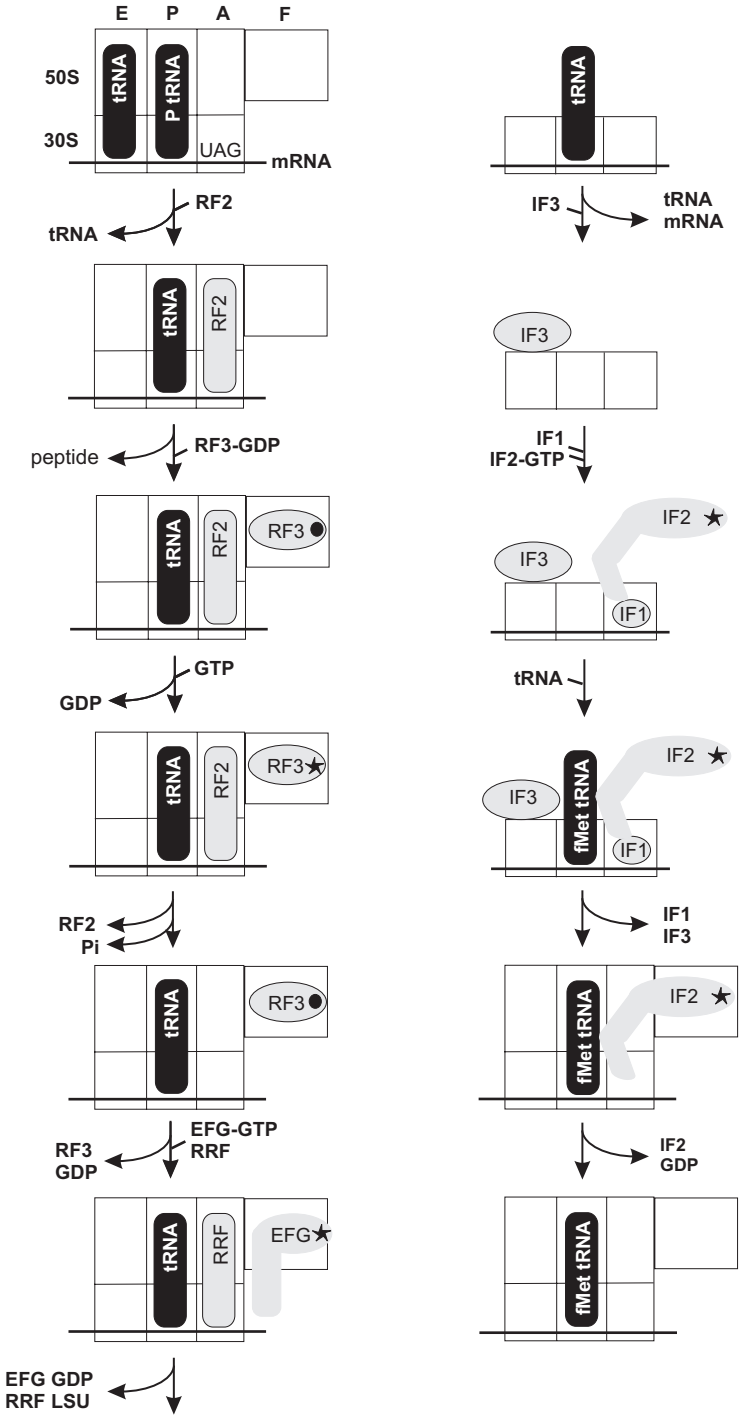


Figure 6. The sequence of events in the termination/initiation of bacterial protein synthesis. Filled circle denotes GDP, filled star denotes GTP.

2000, Yoshida et al. 2001), but apparently not in function (Lancaster et al. 2002, Nakamura and Ito 2003). How it catalyses the subunit dissociation remains unknown. From the tRNA analogy one might expect, that EF-G would translocate the RRF from A to P site and thus eject the deacylated tRNA from the ribosome (Hirokawa et al. 2002). Presumably, this would effect the subunit dissociation. However, after subunit dissociation, mRNA and tRNA remain bound to the 30S subunit and IF3 is required to displace the tRNA (Karimi et al. 1999). IF3 acts as a bridge between the termination and initiation (Fig 6). It acts as an anti-association factor (Sabol et al. 1970, Subramanian et al. 1970), presumably by blocking the 50S binding area in the 30S subunit (Dallas and Noller 2001). IF3 blocks the 70S association when initiator tRNA is absent from the 30S pre-initiation complex (Antoun et al. 2004). In initiation it increases accuracy by increasing ternary complex dissociation rates for both non-initiation codons and non-initiator tRNAs (Risuleo et al. 1976, Hartz et al. 1989, Sussman et al. 1996).

The 30S-IF3 complex recruits a start codon to the P site, IF1 to the A site and IF2-GTP to contact both sites. IF2 then recruits fMet-tRNA into the P site (Gualerzi et al. 2000). The binding sites of IF1, IF2 and IF3 on the 30S subunit were mapped by chemical footprinting (Marzi et al. 2003, Dallas and Noller 2001), crystallography (Carter et al. 2001) and cryo-EM reconstruction (McCutcheon et al. 1999).

Next, the 50S subunit associates and removes IF1 and IF3, resulting in the 70S initiation complex (Gualerzi et al. 2000). Subsequent to GTP hydrolysis, the IF2 dissociates from the ribosome (Antoun et al. 2003). This leaves the A site free to bind the aa-tRNA-EF-Tu-GTP ternary complex; after formation of the first peptide bond the elongation phase of protein synthesis begins.

Interestingly, the binding of IF1 to the 30S A site leads to conformational changes in the 30S similar to those, observed in 70S association (rotation of head towards the A site), and the A site decoding by cognate tRNA (flipping out of A1492 and A1493) (Carter et al. 2001, Ogle et al. 2001, Vila-Sanjurjo et al. 2003). Perhaps IF1 stabilises a transition state in subunit association and thus helps to lower the activation energies for the 50S binding. Consistently with this hypothesis, it increases 70S exchange rate *in vitro* (Grunberg-Manago et al. 1975). Also, IF1 enhances IF2 binding to the 30S (Stringer et al. 1977), probably resulting in a physical contact between them (Marzi et al. 2003, Carter et al. 2001, Boileau et al. 1983). IF2, the largest of the translation factors, accelerates the base-pairing of initiator tRNA anticodon with the initiation codon (Gualerzi and Pon 1990, Meunier et al. 2000).

Thus, termination/initiation adds up to a multi-step process, which is driven by several extra-ribosomal factors.

Chapter 4. Selection of tRNAs by the ribosome and the accuracy of protein synthesis

The frequencies of amino acid mis-incorporation into proteins range from $6x10^{-4}$ – $5x10^{-3}$ *per* codon for internal codons (Rodnina and Wintermeyer 2001a). To a casual observer such a frequency of errors might seem too small and too big at the same time. Too big, because in an average 500 amino acids long protein, every fifth molecule should contain amino acid substitutions (Kurland et al. 1996). In a particle as big as the ribosome almost every molecule should contain errors of protein incorporation. This, however, seems not to be a problem, at least partly due to the convenient structure of the genetic code, which is robust in terms of sensitivity to point mutations. The probability to end up with an amino acid with unaltered charge is around 70% (Kurland et al. 1996). In addition, most of the protein molecules with a random single amino acid replacement are (nearly) as active as the wild-type molecules (Kimura 1985).

Why, then, may the observed frequency of translation errors be called too small? According to Linus Pauling, enzyme's ability to discriminate between structurally similar substrates might be intrinsically limited by the energy differences ($\Delta\Delta G_b$) between these substrates, when bound to the enzyme in appropriate transition states (Pauling 1958, Gorini 1971). However, since cognate and near-cognate tRNAs differ only in one nucleotide of the anticodon loop (other differences may also exist but are not used in differentiating the binding to the ribosome), the $\Delta\Delta G_b$ may be only about 5.5 kcal/mol (Hopfield 1974). Indeed, the replacement of A:U with G:U pair destabilizes pairing in RNA duplex less than tenfold (Xia et al. 1998, Mathews et al. 1999). This is clearly insufficient to explain the known error-rates in protein synthesis. The situation is even harder to explain in DNA replication, where the error rate is about 10^{-9} *per* nucleotide. Such discrepancies between the theory and experimental facts are sometimes described as the "Pauling's paradox". Moreover, the full utilisation of binding energies in substrate discrimination requires, that the binding of the competing substrates is allowed to reach equilibrium (Blomberg et al. 1980). This clearly is not the case in protein synthesis (Gromadsky and Rodnina 2004a).

Counting the differences of binding energies alone is justified, if it is presumed, that the rates of catalysis do not depend on the identities of substrate (Blomberg et al. 1980, Fersht 1999, pp. 377–380). As will be seen below, this assumption does not hold for the ribosome. The rates of GTP hydrolysis on EF-Tu and peptide transfer are limited by preceding structural arrangements of the ribosome, which are influenced by the nature of substrate (Gromadsky and Rodnina 2004a). This means that the maximum energy difference ($\Delta\Delta G_{max}$) between the cognate and non-cognate substrates should include terms for both

the binding energy difference ($\Delta\Delta G_b$) and the activation energy difference ($\Delta\Delta G_a$) for the competing substrates:

$$\Delta\Delta G_{max} = (\Delta\Delta G_b + \Delta\Delta G_a)_{max}.$$

To overcome Pauling's paradox and to reconcile the facts with the theory, Hopfield (1974) and Ninio (1975) evoked a theory of kinetic proofreading. Kinetic proofreading is a general mechanism for obtaining higher accuracy from a given discrimination energy between correct and incorrect substrates. It is achieved by presenting each substrate molecule to the enzyme more than once, before the productive reaction step. For increasing accuracy this way, two conditions must be met. Firstly, the topology of the reaction pathway of the proofreading enzyme must be branched. The simple Michaelis-Menten pathway is not branched.

$$E+S \leftrightarrow ES \leftrightarrow (ES)' \ldots \leftrightarrow E+P$$

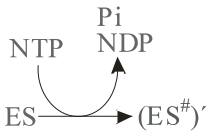
A substrate molecule S bound to the enzyme is either converted to product P or released as S with no change in it or in any other co-substrate. For proofreading the pathway must be branched, such as this:

$$\begin{array}{c} E+S \leftrightarrow ES \rightarrow (ES^{\#})' \leftrightarrow E+P \\ \downarrow \\ E+S^{\#} \end{array}$$

$(ES^{\#})'$ is a high energy intermediate, and the reverse reaction to ES is negligible. This way the two substrate selection steps [ES and $(ES^{\#})'$] are separated and can use the same $\Delta\Delta G_b$ twice for accurate substrate selection. This driven kinetic pathway using a high-energy intermediate theoretically achieves an error fraction equal to one reached by doubling the difference in binding energy between cognate and near-cognate substrates. For this to be achieved, the second requirement must be met. Namely, the step

$$ES \rightarrow (ES^{\#})'$$

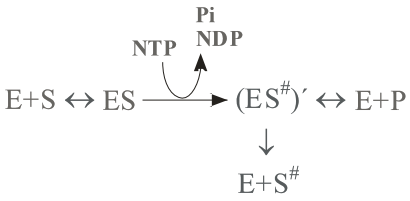
should be made irreversible by coupling with degradation of a high-energy molecule (such as GTP) into a lower energy-state, such as GDP (Ehrenberg and Blomberg 1980).



Therefore, proofreading requires a branched pathway and positioning of the branch after the energy-use step. If the branched reaction pathway was fully reversible, proofreading would be hampered by the backflow from the initial selection E+S↔ES, which would draw the reaction flow backwards from the second intermediate ($ES^{\#}$)' and from the actual proofreading reaction

$$(ES^{\#})' \leftrightarrow E+S^{\#}.$$

The abstract proofreading scheme



can be adapted to translation as follows: E is ribosome, S is aa-tRNA-EF-Tu-GTP ternary complex, ($ES^{\#}$)' is ribosome-aa-tRNA complex after GTP hydrolysis on EF-Tu, $S^{\#}$ is discarded aa-tRNA (which has much lower affinity for the ribosome than ternary complex), P is the newly made p-tRNA. Discarding of the incorrect ternary complex thus constitutes the initial selection step and discarding of the incorrect aa-tRNA constitutes proofreading. An experimental test of the proofreading is to look if the incorporation of incorrect amino acids into protein consumes statistically more GTP than incorporation of correct amino acids. This has indeed been experimentally verified (Ruusala et al. 1982).

Using kinetic proofreading, any process could theoretically be made infinitely accurate by adding additional branchings with coupled energy-consuming reactions. This would, of course, lead to infinite energy consumption and infinite time before product formation.

There is good evolutionary reason to suppose that replication of DNA must be near the threshold of practically feasible accuracy, because any inaccuracy will be propagated into offspring. On the other hand, errors in translation lead to changes in protein sequences, which are unique to a single protein molecule and do not propagate. Indeed, protein synthesis is about 10^5-fold more error-prone than DNA replication (for discussion of mechanisms for such a high fidelity of DNA replication, see Goodman and Fygenson 1998, Kool 2002). This, in turn, implies that translation could be made more accurate, if there was need. Indeed, hyper-accurate ribosomes have been isolated in laboratory. Antibiotic kasugamycin and mutations in ribosomal proteins S12, S17 and L6 increase the accuracy of protein synthesis (Yarus and Thompson 1983). 23S rRNAs, with mutations at positions 2583 (located in the PTC) and 2661 (located in the SRL) are hyper-accurate in protein synthesis (Saarma and Remme 1992, Bilgin and Ehrenberg 1994). In addition, the deficiency of a hypermodified adenosine at

position 37 of some tRNA species leads to increased accuracy by more aggressive proofreading, leaving the initial selection unchanged (Diaz and Ehrenberg 1991). The lack of this tRNA modification results in a two-fold increase in GTP hydrolysis *per* peptide bond formation. In contrast, the hyper-accurate 2661C mutation in the 23S rRNA G-factor binding region does not change the GTP consumption of the ribosome but reduces the binding rate of cognate ternary complex to the A site (Bilgin and Ehrenberg 1994). This mutation does not compromise bacterial growth, unless combined with a hyper-accurate S12 mutation, an unviable combination (Tapprich and Dahlberg 1990). The hyperaccurate S12 mutations themselves also exhibit increased initial selection (Bilgin et al. 1992). The classic error-restrictive mutants of S12 were originally selected as resistant to the error-inducing antibiotic streptomycin (Gorini 1974). Some error-restrictive S12 mutants are actually streptomycin addicts for growth (SmD and SmP phenotypes). Streptomycin decreases the accuracy of protein synthesis by influencing both initial selection and proofreading (Gromadsky and Rodnina 2004b).

So, it is possible to increase translational accuracy by increasing proofreading efficiency (which also results in the increased rejection of cognate tRNAs) or by increasing initial selection (with concomitant increase in the rejection frequency of cognate ternary complexes). Note that the latter appears to bring the Pauling's paradox back to the table. There are two ways around this. Selection, that uses differences in the binding energies of the substrates, needs for its maximal efficiency that the system is at equilibrium. The decoding process is far from equilibrium, probably because of the requirement for speed (Gromadsky and Rodnina 2004a). So by increasing the time, which the system spends in initial binding (ES), the selectivity could in principle be increased. The reduction in k_{cat}/K_M for the 2661C hyperaccurate mutant ribosome-ternary complex interaction supports this hypothesis (Bilgin and Ehrenberg 1994). The second possibility is to take a second look at the concept of substrate. If the selection worked not by comparing binding energies of different anticodons to the mRNA-ribosome complex, but by binding the codon-anticodon complex to the ribosome instead, there would be more in the substrate conformation by which to differentiate between competing substrates. Because the mRNA is already bound, this scheme of improvement requires the enzyme to change conformation from unproductive to productive state upon tRNA binding. Indeed, such an induced fit has been described in the 30S subunit in response to the correct WC-geometry of the cognate codon-anticodon pairs (Ogle et al. 2002; see below). Experimental corroboration comes from the finding that a 2'-deoxy base in the A site codon, but not in the P site codon, affects tRNA binding (Potapov et al. 1995). If the stability of base pairing was the sole requirement for tRNA recognition, such a result would be unlikely. It is currently unclear how changes in the 50S (for example the 23S rRNA mutation at position 2661of the SRL) could affect the productive complex formation by induced

fit in the 30S, but it is not unlikely that they do so (Vila-Sanjurjo et al. 2003, O'Connor et al. 1993).
The path of the mRNA in the 70S ribosomes has been studied by medium-resolution (5–7 Å) X-ray crystallography (Yusupova et al. 2001, Yusupov et al. 2001). About 30 nucleotides of mRNA are tightly bound in a groove around the neck of the 30S subunit. Only about 8 of these around A and P sites are exposed and bond almost exclusively to the 16S rRNA. The simultaneous binding of the A and P site tRNAs is made possible by a 45° kink and, in addition, a 26° angle from a plane between the A and P site codons (Fig 7).

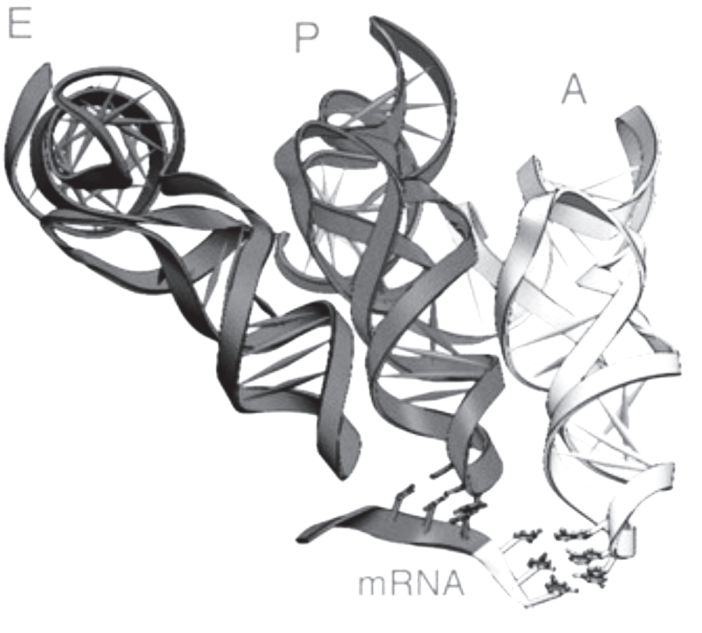


Figure 7. Conformation of mRNA and the A, P, and E site tRNAs in the 70S ribosome (reproduced with permission from Yusupov et al. 2001). The E site tRNA is not cognate to the E site mRNA codon.

The decoding of sense codons takes place in the ribosomal A site in several elementary steps (Rodnina and Wintermeyer 2001a; Fig 8, table 2).

1) Initial rapid but labile binding of aa-tRNA-EF-Tu-GTP ternary complex is codon-independent (k_1=140 $\mu M^{-1}s^{-1}$, k_{-1}=85 s^{-1}; Gromadsky and Rodnina 2004a). Initial binding was inferred from the rapid rise in fluorescence of proflavin-labelled aa-tRNA upon mixing with pre-programmed ribosomes (Pape et al. 1998). This step does not lead to differentiation between cognate and non-cognate tRNAs.
2) In case of cognate ternary complex, codon recognition follows (k_2=190 s^{-1}, k_{-2}=0.23 s^{-1}; Gromadsky and Rodnina 2004a). Codon recognition was

measured by further increase in proflavin fluorescence (Pape et al. 1998). If single-mismatch-containing near-cognate codon-anticodon pairs are studied, k_2 remains 100 s^{-1} but k_{-2} increases (in the case of CUC codon and CAA anticodon) to 80 s^{-1} (Gromadsky and Rodnina 2004a). Error-inducing antibiotics paromomycin and streptomycin significantly reduce k_{-2} (Pape et al. 2000, Gromadsky and Rodnina 2004b). The preferential discarding of ternary complexes with mismatches in codon-anticodon pairing is, in fact, primary selection of tRNA (Ninio 1974, Hopfield 1975, Rodnina and Wintermeyer 2001b). In free solution, replacement of the A:U with the G:U pair destabilizes pairing in RNA duplex less than tenfold (Xia et al. 1998, Mathews et al. 1999). Explanation of the observed 350-fold primary selection level (Gromadsky and Rodnina 2004a) requires inspection of the codon-anticodon duplex in the context of the ribosome. Fortunately, 30S complexes with short messages and cognate or near-cognate tRNA anticodon-loop analogues in the A site have been crystallised and solved to 3Å resolution (Ogle et al. 2001, 2002). The A site of the 30S subunit consists of four movable domains (head, shoulder platform and helix 44), which upon tRNA binding come together by induced fit to form a closed complex (Ogle et al. 2001, 2002, 2003). 16S rRNA nucleotides A1492, A1494 flip out of the internal loop of helix 44 and G530 of the shoulder switches from syn to anti conformation. This way A1493 recognizes the minor groove of the first base-pair of the codon-anticodon helix by A-minor interaction. The second base-pair is also contacted by A-minor interaction by A1492. G530 also interacts with the minor groove of the second base-pair. A1492 and G530 are locked in position by secondary interactions with S12, from the gene of which several hyper-accurate mutations have been isolated (see above). The third pair is not geometrically recognized, allowing latitude for wobble base-pairing. Its minor groove is not contacted by the ribosome. The geometry of unconventional base-pairs in the first two codon positions cannot be recognized; the wobble geometry of the G:U pair displaces the U of the codon into the minor groove precluding the A-minor interactions. The lack of WC base-pairing geometry precludes the induced fit, despite the favourable codon-anticodon interaction energy (Ogle et al. 2002, 2003).

3) Codon-anticodon interaction induces the activation of the GTPase of the GAC (k_3), followed by GTP hydrolysis (k_{GTP}) (Pape et al. 1998). k_3 is measured indirectly by changes in the fluorescence of the labelled GTP, k_{GTP} is measured more directly, using biochemical methods. The physical nature of the GTPase activation in the 50S subunit is obscure but (since it is dependent on the codon-anticodon interaction) could conceivably be induced by conformational changes in the 30S upon decoding (Ogle et al. 2001). This possibility is supported by the discovery that some hyper-accurate S12 mutations result in uncoupled GTPase activity on EF-Tu (Bilgin et al. 1992). Also, error-inducing antibiotics paromomycin and

streptomycin that cause conformational change in the 30S subunit, also lead to large changes in GTPase activation rate k_3, which occurs in the 50S subunit (Pape et al. 2000, Gromadsky and Rodnina 2004b, table 2). Alternatively, the signal could be mediated by deformations in the A site bound tRNA (Valle et al. 2003b, Yarus et al. 2003). Indeed, intactness of the backbone of aa-tRNA is required for EF-Tu GTPase activation (Piepenburg et al. 2000). In the cognate case $k_3+k_{GTP}=260\ s^{-1}$, while the near-cognate rate is $0.4\ s^{-1}$ (Gromadsky and Rodnina 2004a). This can be interpreted as a preferential selection of cognate tRNA by induced fit, where cognate and near-cognate substrates differentially activate conformational change of the enzyme from a catalytically inactive state to an active state (Pape et al. 1999). The enzyme specificity is a function of both substrate binding and catalytic rate (Fersht 1999, p. 377). Therefore, k_{cat}/K_M is the important kinetic constant in determining specificity. Accordingly, Gromadsky and Rodnina (2004a) discovered by the kinetic analysis of decoding, that the observed 350-fold difference in the ΔG-s of cognate and near cognate tRNAs (excemplified by k_{-1}) is not directly used in selection, due to high rate of GTP hydrolysis which does not allow the binding to reach equilibrium. Initial selection is therefore entirely kinetically controlled by k_3 and is due to 650-fold lower GTP hydrolysis on near-cognate ternary complex (Gromadsky and Rodnina 2004a).

4) After GTP hydrolysis and instantaneous P_i release, conformational change of EF-Tu is supposed to occur with the rate $k_4=60\ s^{-1}$ (Pape et al. 1998). This is inferred from the time left over from the next step in decoding, the accommodation of tRNA in the A site. The dissociation of EF-Tu-GDP from the ribosome, which happens after conformational change in the EF-Tu, is slow ($3\ s^{-1}$) and occurs independently of the accommodation (Rodnina and Wintermeyer 2001a). Cognate and near-cognate tRNAs induce EF-Tu conformational change with similar rates (Table 2). Antibiotic kirromycin that binds EF-Tu, allows GTP hydrolysis, but appears to block the ensuing conformational change in EF-Tu, and thus freezes the factor in the ribosome (Parmeggiani and Stewart 1985, Valle et al. 2003b). Several high-resolution crystal structures of the EF-Tu both in GTP and GDP-form and of ternary complexes are available and show a large conformational change upon GTP hydrolysis (reviewed in Andersen et al. 2003).
5) Accommodation (k_5), in turn, is inferred from the reduction of fluorescence of the proflavin-labelled tRNA (and is therefore experimentally connected with measurements of k_1 and k_2). Its rate is $7\ s^{-1}$ for the cognate tRNA, and $0.1\ s^{-1}$ for the near-cognate tRNA (Pape et al. 1998, 1999). This is another potential step of selection by induced fit. In accommodation, tRNA 3'-end must move from the GAC to PTC, while the codon-anticodon duplex presumably remains fixed in the 30S A site (Yusupov et al. 2001, Valle et al. 2003c). This implies movement of tRNA acceptor arm by more than 50

Å (Noller et al. 2002). Cryo-EM reconstructions have demonstrated that stably A site bound aa-tRNA-EF-Tu-GDP-kirromycin pre-accommodation complex is deformed in its tRNA moiety, if compared to free tRNA in solution or in the ribosomal A site (Valle et al. 2003c, Stark et al. 2002). The process of accommodation could well use the energy stored in the tRNA deformation itself. This would make tRNA a molecular spring. Because in the ternary complex the anticodon end of tRNA is rotated in relation to accommodated tRNA, the accommodation must include the rotation of the anticodon arm of tRNA. It is not known for certain, if base-pairing with the codon is retained during this rotation, but identical chemical footprints of tRNA on the 16S rRNA before and after accommodation support this hypothesis (Powers and Noller 1994). If accommodation fails, aa-tRNA dissociates from the ribosome. Perhaps, because near-cognate tRNA is less tightly bound in the 30S decoding site (Ogle et al. 2002, 2003), it dissociates more readily during the toilsome accommodation process.

6) tRNA dissociation rate is very low in the cognate case ($<0.3\ s^{-1}$), but quite high in the near-cognate case ($7\ s^{-1}$). The rejection of aa-tRNAs is essentially irreversible, because the ternary complex has both much higher affinity to the A site and higher cellular concentration than aa-tRNA (Rheinberger 1991). This is another instance of tRNA selection in the ribosome. Taken together with accommodation, they constitute the proofreading step in tRNA selection, which is kinetically separated from the initial selection by the GTP hydrolysis in EF-Tu (Ninio 1974, Hopfield 1975, see above). This ireversible GTP-consuming step allows to use the free energy of the ribosome-codon-anticodon complex twice in selection of the same aa-tRNA. Using high-fidelity *in vitro* assays, proofreading was estimated to result in about 15-fold discrimination of near-cognate tRNA, which is comparable to 30-fold discrimination by initial selection (Gromadsky and Rodnina 2004a). Such twofold selection results in the accuracy of about 10^{-3} per codon for near-cognate amino acid mis-incorporation, which is not very different from translational fidelities *in vivo* (Gromadsky and Rodnina 2004a).

The 30S crystal structures with cognate and near cognate tRNA anticodon stem-loops (ASLs) bound to the A site are attractive, because they offer insight into the mechanism of action of error-inducing antibiotics as well as several error-inducing and error-restrictive 16S rRNA mutations (Ogle et al. 2002, 2003). In the presence of the error-inducing antibiotic paromomycin near-cognate ASLs can be stably bound to the 30S A site, concomitantly with the induced fit. Paromomycin appears to functionally mimic the cognate codon-anticodon interacrtion by inducing the induced fit, which leads the conformation of the 30S towards the active state. Paromomycin binds to the 16S rRNA helix 44 and

Table 2. Rate constants of the elementary steps of decoding.

	low accuracy conditions				high accuracy conditions			
AC	UUU			AAA	UUU		CUC	
tRNA	Phe	Leu^2 (GAG)		Phe	Phe		Phe	
antibiotic	–	–	PARO	–	–	STR	–	STR
k_1	110	110	140	60	140	190	140	190
k_{-1}	25	25	25	25	85	80	85	80
k_2	100	100	**37**		190	**40**	190	**70**
k_{-2}	0,2	**17**	**3,5**		0,23	**<0,01**	**80**	**5**
k_3	500	**50**	**>500**	**0,005**	260	**2,2**	**0,4**	**2,1**
k_4	60	50	**6**					
k_5	7	**0,1**	**1**					
k_6	3	2	nd					
k_7	<0,3	**6**	**0,9**					
ref	1	2	2	5	3	4	3	4

See main text and Figure 8 for description of the individual steps. Low accuracy conditions refer to buffer conditions where10 mM $MgCl_2$ and no polyamines are used. High accuracy conditions refer to 3.5 mM $MgCl_2$, 0.5 mM spermidine. AC — anticodon, PARO — paromomycin, STR — streptomycin. Ref 1. — Pape et al. 1998, Ref 2 — Pape et al. 2000, 3 — Gromadsky and Rodnina 2004a, 4 — Gromadsky and Rodnina 2004b, 5 — Rodnina and Wintermeyer 2001a.

flips out from the internal loop residues A1492 and A1493, which would otherwise be flipped out by cognate tRNA binding. By doing so, paromomycin presumably lowers the activation energy of the near-cognate tRNA binding and therefore of the induced fit. The outcome is that the near-cognate tRNA will happily sail through the decoding process resulting in mis-incorporation of an amino acid. Paromomycin affects the rates of almost every step during ribosomal aa-tRNA selection and, in particular, accelerates both GTP hydrolysis and accommodation (Table 2, Pape et al. 2000). Another error-inducing antibiotic, streptomycin, would also stabilize the closed form of the 30S by stabilizing S12 interaction with helix 27 region of the 30S subunit (Ramakrishnan 2002). Streptomycin binding site in the 30S is close to paromomycin but does not overlap with it (Carter et al. 2000). Yet, unlike paromomycin, that raises the GTPase activation rate k_3 of the near-cognate ternary complex to

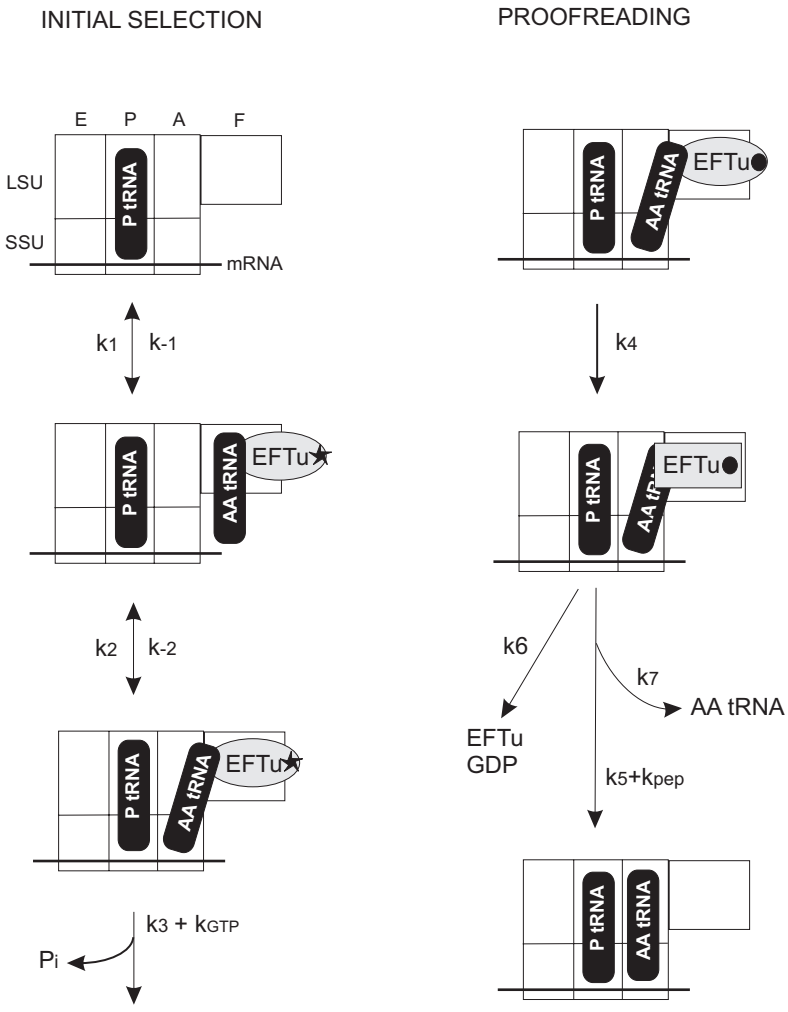


Figure 8. Elementary steps of decoding according to Rodnina and Wintermeyer (2001). Filled star denotes GTP and filled circle denotes GDP. The following are the rate constants of individual elementary steps of decoding. k_1 is initial binding, k_2 is codon recognition, k_3 is GTPase activation, k_{GTP} is GTP hydrolysis, k_{Pi} is dissociation of pyrophosphate, k_4 is conformational change of EF-Tu, k_6 is EF-Tu-GDP dissociation from the ribosome, k_7 is AA-tRNA rejection from the ribosome, k_5 is accommodation of tRNA in the A site and k_{pep} is peptidyl transfer. Initial selection comprises steps up to (and including) k_{GTP} and proofreading comprises steps k_4-k_7.

cognate levels, streptomycin lowers the k_3 of the cognate ternary complex to the near-cognate level (table 2). Mutations in the 30S proteins S4 and S5 are error-prone and can rescue the effects of S12 error-restrictive mutations (Kurland et al. 1996). Yet, S12 does not directly interact with S4 or S5, which are located at the opposite side of the 30S shoulder domain. Instead, S4 and S5 interact with each other and the error-prone mutations are predicted to disrupt this interaction. The S4/S5 interaction in the body/shoulder region is normally disrupted during cognate aa-tRNA binding and the resulting induced fit (Ogle et al. 2002). The mutations in the S4/S5 interaction area are therefore likely to increase the time 30S subunit spends in the closed (productive) form. Similar mechanism has been proposed to lie behind the error-prone phenotype of mutations in the interface of the 30S proteins S7 of the head and S11 of the platform (Robert and Brakier-Gingras 2003). The error-restrictive mutations in S12 are clustered in the region of the protein that interacts with the 16S rRNA helices 27 and 44 only after the domain closure in induced fit. These mutations are likely to destabilize the closed form. Mutations in the 16S rRNA helix 27 can either increase or decrease accuracy (Lodmell and Dahlberg 1997), presumably by influencing S12 interactions in the closed form of the 30S subunit (Rodriguez-Correa and Dahlberg 2004). So, by changing the stability of the open (non-productive) and closed (productive) form of the 30S, the accuracy of translation can be tuned at will.

A question that can not be satisfactorily answered at present time is how alterations in the PTC and GAC can influence misreading in the decoding centre of the 30S subunit? Mutations in 23S rRNA can both increase (Saarma and Remme 1992, Bilgin et al. 1994) and decrease (O'Connor and Dahlberg 1993, 1995, Gregory et al. 1994) translational accuracy. Mutations in tRNA 3'-CCA end (O'Connor et al. 1993) and PTC inhibitors chloramphenicol and oxazolidinones can increase miscoding, such as frameshifting and nonsense suppression (Thompson et al. 2002). One can only surmise that the news of alterations in tRNA contacts with the 50S subunit can be somehow transmitted to the decoding centre in the 30S subunit. Although it is clear that the 50S association itself can change the conformation of the 30S subunit (Vila-Sanjuro et al. 2003), the useful details are still veiled by insufficient resolution of the structural studies of 70S ribosomes.

Chapter 5. The peptidyl transferase center and its abilities

The PTC is a cavity in the interface side of the 50S subunit consisting of RNA and containing the mouth of the peptide tunnel (Bashan et al. 2003a, b, Nissen et al. 2000a). Yonath and co-workers have detected an approximate two-fold symmetry of two groups of about 90 nucleotides in the PTC, relating the backbone fold and base conformation (Bashan et al. 2003a, b). The inner shell

of the symmetry region contacts the 3'-ends of tRNAs bound at the A and P site, and presumably catalyses peptide transfer. The inner shell consists of a number of bases of the central loop of 23S rRNA domain V, the A and P loops (that is, the loops of 23S rRNA helices 80 and 92, which contact 3'$C_{74}C_{75}A_{76}$ of the P and A site tRNAs, respectively), and helices 89, 93 (Bashan et al. 2003b).

The interactions between tRNAs and the ribosome have been extensively studied by chemical footprinting (Moazed and Noller 1989a, b, Noller 1993), cross-linking (Barta et al. 1984, Steiner et al. 1988), mutagenesis (Saarma and Remme 1992, Gregory et al. 1994, Porse and Garrett 1995, Porse et al. 1996, Saarma et al. 1998), fluorescence methods (Odom et al. 1990), modification interference (von Ahsen and Noller 1995, Bocchetta et al. 1998), and by X-ray crystallography (Yusupov et al. 2001). Different methods of study agree that except for the anticodon stem, which is bound to the 30S, the rest of the tRNA is closely contacted by the 50S subunit (Yusupov et al. 2001, Schafer et al. 2002). Single-stranded 3'-CCA ends of the A and P site tRNAs are precisely fixed not only by direct interactions with 23S rRNA, but possibly also by ribosomal contacts with the acceptor helices and with other structural features of tRNAs (Bashan et al. 2003a, Youngman et al. 2004). In this context it is worth mentioning, that peptidyl transfer catalysed by the 70S ribosomes is about 10^4 times faster than the reaction catalysed by the 50S subunit (Moore and Steitz 2003). Also, it is possible that the association of the 70S may cause small but catalytically highly significant changes in the structure of the PTC. Interestingly, the binding of the A and P site tRNAs to 70S ribosomes and aa-tRNA 3'-fragments to the 50S subunits is a positively cooperative process, implying conformational rearrangements in the PTC upon tRNA binding (Bourd et al. 1983).

The conformation of the A site tRNA is indistinguishable from the structure of a free tRNA, the P site bound tRNA is slightly kinked around the junction of D and anticodon stems and the E site tRNA is greatly distorted, including the conformation of its anticodon loop (Yusupov et al. 2001). Interestingly, when the acceptor stems of the A and P site tRNAs are related by translation (i.e. can be moved from the A to the P site without rotation), the CCA-ends are related by about 180 degrees rotation (Hansen et al. 2002b). This puts strain on the tRNA nucleotides C72-C74, resulting in another potential molecular spring with implications for translocation; and presumably reflects the tight interaction of the CCA-ends with their respective binding sites.

In the P site, the C74-G2252 base pair has been postulated from X-ray and mutagenesis studies (Samaha et al. 1995, Ban et al. 2001, Fig 9b). In addition, C75 forms a WC pair with G2251 of the P loop (Hansen et al. 2002b). The CCA-tail may also contact 23S rRNA bases A2602 and U2585 and form backbone-backbone contacts with the stem of the P loop (helix 92) (Yusupov et al. 2001). Mutations at both nucleotides are detrimental to the peptide release reaction, but not so to the peptidyl transferase when full tRNAs are used as substrates (Youngman et al. 2004). The 50S protein L5 interacts with the T loop

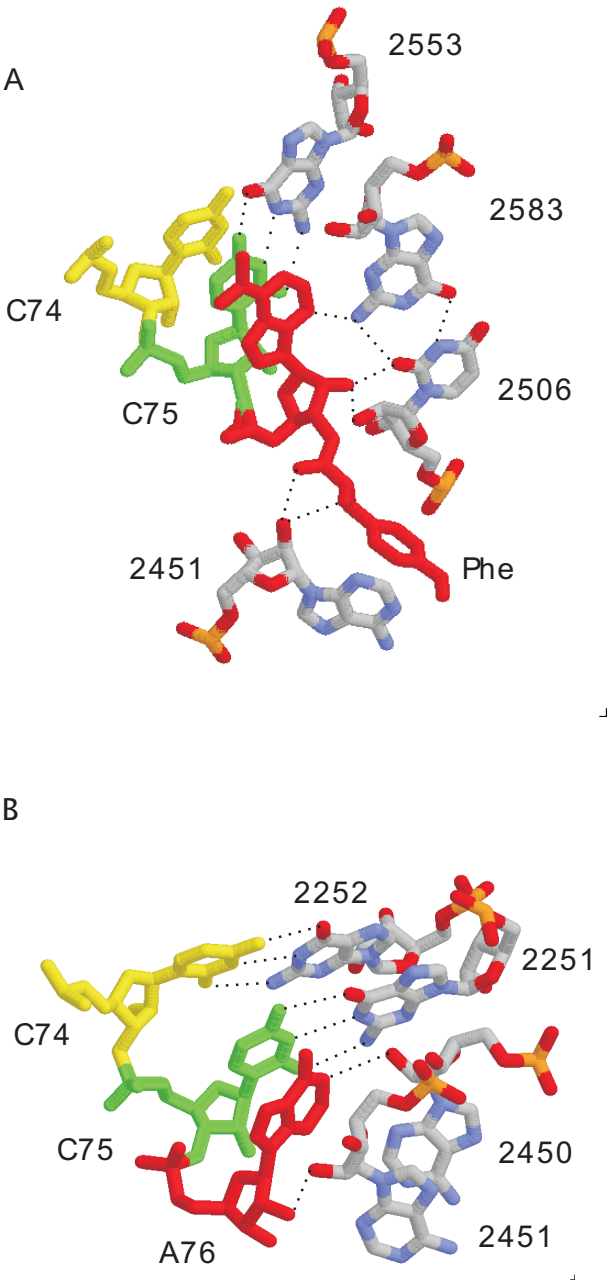


Figure 9.
A. A selection of potential hydrogen bonds of the CCA-end of A site bound tRNA with the *H. marismortui* ribosome (Hansen et al. 2002, PDB accession number 1KQS).
B. A selection of potential hydrogen bonds of the CCA-end of P site bound tRNA with the *H. marismortui* ribosome (Hansen et al. 2002, PDB accession number 1KQS).

of the P site bound tRNA. The minor groove of the D stem of the P site tRNA is contacted by the minor groove of helix 69 of 23S rRNA. Meanwhile, the minor groove of the D stem of the A site tRNA is contacted by the loop of helix 69. This wondrous helix-loop not only simultaneously binds both tRNAs but also contacts helix 44 of 16S rRNA and thus forms the intersubunit bridge B2a (Yusupov et al. 2001, Gao et al. 2003). Moreover, since the helix-loop 69 has a different conformation in the unliganded 50S subunits (Harms et al. 2001) it could be involved in mediating signals between the subunits (for example, between the decoding centre of the 30S and the GAC of the 50S).

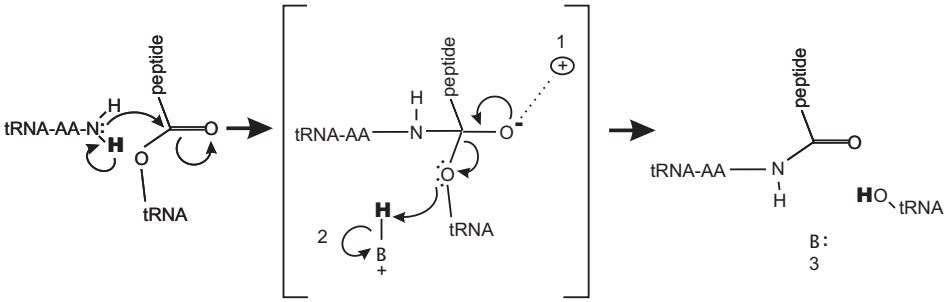
The 3' CCA-end of the A site bound tRNA is also fixed by base pairing of the C75, this time with G2553 of the 23S rRNA A loop (Kim and Green 1999, Nissen et al. 2000a, Fig 9a). The A76 gives a type I A-minor interaction with the U2506:G2583 base pair (Nissen et al. 2001). The attacking α-amino group of the amino acid residue hydrogen-bonds with N3 of A2451 and the 2'OH of the A76 ribose of the P site tRNA (Hansen et al. 2002b). The acceptor stem of the tRNA is positioned by an interaction with the 1942-loop and helix 89 of 23S rRNA, which also helps to fix the CCA (Yusupov et al. 2001). The A site tRNA also contacts bridge B1a (helix 38) and protein L16.

The E site bound tRNA is also stabilized by ribosomal contacts with the acceptor stem, this time with helix 68 of 23S rRNA (Yusupov et al. 2001, Bocchetta et al. 2001). This interaction seems to be functionally important in translocation (Feinberg and Joseph 2001). The CCA end is buried in a pocket that is clearly separate from the A and P sites and is extensively contacted by various ribosomal components (Yusupov et al. 2001). While the acceptor stems of the A and P site tRNAs come within 5 Å from each other, the acceptor end of the E site tRNA lies nearly 50 Å from that of the P site tRNA. Another important interaction occurs with protein L1, which may act as a gatekeeper, regulating the release of tRNA from the E site (Yusupov et al. 2001, Harms et al. 2001).

The actual reaction that is catalysed by the PTC, is the transfer of the activated peptidyl residue from the peptidyl-tRNA in the P site to the aminoacyl residue in the A site. The same reaction centre is able to catalyse the transfer of peptidyl residue to water during the termination (Vogel et al. 1969).

The classical period of the PTC studies started in the mid-1960s with the use of an analogue of the 3' terminus of the aa-tRNA (puromycin) and the fragment CACCA-fMet as substrate analogues, fit to react in the presence of alcohol in isolated 50S subunits (reviewed in Maden 2003). The PTC studies thrived by the use of modified substrate analogues, classical methods of enzyme kinetics and selective disruption and reconstruction of the ribosomal PTC (reviewed in Krayevsky and Kukhanova 1979, Chladek and Sprinzl 1985, Lieberman and Dahlberg 1995). What is noteworthy from these studies is the remarkably wide substrate- and reaction- specificity of the PTC: it can catalyse the formation of ester, thioester, thioamide and phosphinamide bonds and

alcoholysis in the presence of ethanol or methanol. The classical era of PT-studies ended by the advent of detailed crystallographic studies of the PTC in complexes with substrate- and reaction-intermediate analogues (Hansen et al. 2000, 2002b, Bashan et al. 2003a).



1 electrostatic stabilization of oxyanion
2 general base
3 general acid

Figure 10. Mechanism of the peptidyl transfer reaction. Possible scenarios of catalysis by general acid/base catalysis and oxyanion stabilisation are illustrated.

The peptidyl transfer is a nucleophilic attack of the α-amino group of the aa-tRNA on the ester carbonyl group of peptidyl-tRNA (Fig 10; see Green and Lorcsh 2002, Rodnina and Wintermeyer 2003, Parnell and Strobel 2003 for reviews). The nucleophilic attack of the α-amino group leads to the initial protonated tetrahedral intermediate, which forms by deprotonation of the tetrahedral intermediate (Fig 10). Breakdown of the tetrahedral intermediate is achieved by donating a proton back to the leaving oxygen to form the products: P-site deacylated tRNA and A site p-tRNA. So the question is: how can the PTC catalyse this process? In order to answer this, one must first ask, what are the catalytic strategies, that are open to RNA-enzymes (ribozymes) and what constraints do the available crystal structures and the results of biochemical work place on ribosomal catalysis? There are three broad strategies open for RNA catalysis (Lilley 2003, Jenny and Ban 2003). It should be noted that the mechanism of enzymatic catalysis can largely be explained through the stabilization of the transition state (Garcia-Viloca et al. 2004), of which the examples below are but specific instances.

1) General acid/base catalysis. For a peptide bond to be formed, at least three protons must move. A proton must be lost from the ammonium ion (at pH 7, RNH_3^+), having no lone free pair of electrons on the nitrogen, and therefore impotent for the nucleophilic attack (Green and Lorsch 2002). Secondly, a proton must be lost from the initial protonated tetrahedral

intermediate, and thirdly, a proton must be picked up by the oxygen atom of the leaving group (the ribose of the P site tRNA). In general acid/base catalysis the ribosome would facilitate the movements of those protons (Fig 10). Based on the results of co-crystallization of the 50S subunit with a tetrahedral reaction-intermediate analogue (Welch et al. 1995), the N3 position of a conserved A2451 was suggested to act as the catalytic base. Upon approach to the transition state it supposedly snatches a proton from the amino group and, during subsequent breakdown of the tetrahedral intermediate, donates it back to the leaving oxygen (Nissen et al. 2000a). The problem with the N3 of adenines is, that in solution its pK_a <1 would not allow its protonation. Muth et al. (2000) purported to show by the pH-dependence of the dimethylsulfate-reactivity of the N1 of A2451, that its pK_a in the ribosome must be around 7.6 (while in solution it has a pK_a of 3.5), therefore suitable for extracting and giving away protons. Inconveniently, this conclusion was overthrown by the observations that DMS-modification at A2451 occurred only in the inactive population of the 50S subunits (Bayfield et al. 2001). Also, DMS-modification of A2451 of organisms other than *E. coli*, which was used in all previous experiments, does not exhibit any meaningful pH-dependence, except that the pH-shift may well change the conformation of the PTC (Muth et al. 2001, Xiong et al. 2001). Moreover, reassessment of the reaction trajectory points the oxyanion of the tetrahedral intermediate away from A2451 in the transition state (Hansen et al. 2002b). This means that the tetrahedral intermediate analogue, which was used in the co-crystallization of the 50S subunits (Nissen et al. 2000a), is not a realistic creation, and that the A2451 cannot act as a general acid in proton donation. Meanwhile, Katunin *et al*. (2002) deduced from the pH profile of the reaction kinetics that a single ribosomal residue with pK_a 7.5 catalyses the reaction more than 100-fold (they also found that mutation of A2451U inhibits the reaction about 100-fold and changes the pH-profile of the reaction). Altered pH-profile of the A2451U mutant naturally once more leads thoughts to its proposed role in the general acid/base catalysis of the peptidyl transfer. Unfortunately, these results are also consistent with the induction, by a pH-shift, of a conformational change in the PTC from a low-activity state to a high-activity state, which is inhibited in the mutant 2451 background (Rodnina and Wintermeyer 2003). Indeed, pH-dependent rearrangements in the PTC have been demonstrated (Bayfield et al. 2001). Although the mutations at A2451 inhibit the PT model-reaction with the minimal A site substrate, puromycin, by two orders of magnitude (Katunin et al. 2002, Youngman et al. 2004), they retain almost full peptidyl transferase activity when ternary complex is used as the A site substrate (Youngman et al. 2004). Therefore, the possibility of general acid/base catalysis in the ribosome remains open.

2) Charge stabilization. Stabilization of the charged oxyanion of the reaction intermediate by juxtaposition of a positive charge would certainly help in

catalyzing the reaction. Presently, the only candidate for the oxyanion stabilization is U2585, which could conceivably move into the proximity of the oxyanion to fill that role (Hansen et al. 2002b). Mutations of this base confer dominant lethality when co-expressed with wild type rRNA and are usually inactive in the puromycin reaction; except the U to G mutation, which is 36% active (Porse et al. 1996). In addition, the U to A mutant is somewhat impaired in the binding of tRNA fragment CCACCA-N-Ac-Met (Green et al. 1997) and the CMCT-modification of U2585 interferes with tRNA binding to the P site of the 50S subunit (Bocchetta et al. 1998). Of course, defects of mutants in substrate binding need not say anything about the possible role of the wild type base in catalysis (Kraut et al. 2003). Charge stabilization in the course of peptidyl transfer, therefore, cannot be excluded at this stage, but still awaits experimental corroboration.

3) Catalysis by correct positioning of the substrates uses the binding energies of the substrates to facilitate the trajectory into the transition state, by straining the substrate into a more transition state-like conformation. This classic concept of Haldane and Pauling can be reformulated as saying that the transition state makes better contacts with the enzyme than does the substrate, so that the full binding energy is not realized until the transition state is reached (Fersht 1999, pp. 369). This way the enzyme stabilizes transition state more strongly than the ground state, and therefore lowers the activation energy of the reaction. Binding of the substrates by the ribosome also makes the reaction effectively unimolecular and possibly excludes water from the active site, thus lowering the reaction activation energy barrier by reducing the entropic penalty of peptide synthesis (Fersht 1999, pp. 72). Recently, experimental studies of Sievers *et al.* (2004) led to the notion that ribosomal catalysis of peptide transfer has no enthalphic component whatsoever, and is therefore driven entirely by the reduction of the entropic penalty of the reaction. If true, this seems to discredit any idea of ribosomal catalysis by charge stabilization or by a general acid/base. Catalytically advantageous positioning of substrates is currently the most popular theory for explaining the ribosomal catalysis, as the close positioning of the A and P site substrates is very clear from the crystal structures (Hansen et al. 2002b, Bashan et al. 2003a). For example, three RNA groups have the potential of aligning the reactive α-amino group for attack: the 2'-OH of A76 of the P site tRNA, the N3 and the 2'-OH of A2451.

In conclusion, it seems possible that more than one way of catalysis is used by the ribosome, as is done by other ribozymes (Lilley 2003). If the extent and speed of the progress of elucidation of the mechanisms of catalysis of small ribozymes is to be taken into account, we may never know for sure how the ribosome does it (Doherty and Doudna 2001, Lilley 2003). Technologically, the elucidation of catalytic mechanisms of the ribosome will depend on precise

incorporation of various nucleotide analogues into the PTC. This, in turn, requires the development of really good and versatile ribosomal reconstitution systems that use *in vitro* synthesized 23S rRNA.

Chapter 6. The peptidyl-tRNA in the ribosome and beyond

What is the fate of the nascent peptide in the ribosome? From crystallographic studies, it appears that the only way out for an elongating nascent peptide is through a tunnel, whose entrance is located at the back wall of the PTC cavity and the exit is on the back side of the 50S (that is, maximally away from the intersubunit area) (Hansen et al. 2000, Harms et al. 2001). Its length is about 100 Å, the narrowest point is only 10 Å wide (this tightly fits the diameter of an α-helix) and the average width is 15 Å. It is mostly straight, except for a bend, 20–35 Å from the PTC. The wall of the tunnel is composed of both RNA and protein and is largely hydrophilic and non-charged. Therefore, the tunnel is likely filled with water. There are no large patches of hydrophobicity to interact with the hydrophobic portions of nascent peptides. For energetic reasons it might be important to reduce the contacts of nascent peptides with the tunnel wall to a minimum. Nevertheless, there are several nascent peptides that are apparently capable of functional (regulatory) interactions with the ribosomal tunnel (Lovett and Rogers 1996, Tenson and Ehrenberg 2002), and it is not unlikely that elements of the protein L22 in the tightest constriction of the tunnel might gate the tunnel in response to certain nascent peptide sequences (Bashan et al. 2003b). Recently, it has been suggested that the transmembrane signal sequences of nascent peptides interact co-translationally with the tunnel near its beginning and consequently fold into α-helices inside the tunnel (Woolhead et al. 2004). In addition, several antibiotics, including the macrolides, act by binding to and thus gating the tunnel near its entrance at the PTC (Table 8; Harms et al. 2003). The mode of action of these antibiotics seems to be inducing the drop-off of short peptidyl-tRNAs from the ribosome (Menninger and Otto 1982, Rheinberger and Nierhaus 1990, Tenson et al. 2003). There is evidence that *in vitro* translated fluorochrome-tagged homopolymers of polylysine exit the ribosome directly (presumably through intersubunit space), without entering the tunnel (Picking et al. 1991). If any natural nascent protein could imitate this behavior, its synthesis would be impervious to antibiotics, which block the ribosomal tunnel, i.e. erythromycin. So far, there are no strong candidates for bypassing the tunnel among naturally occurring peptides (Kramer et al. 2001). However, there is data indicating, that while most nascent peptides exit through the expected tunnel, giving entirely expected cross-links to the components of the 50S subunit in the process, some portion of the nascent chains of some tested proteins give cross-links to the 30S instead, consistent with an alternative exit pathway through the intersubunit space (Choi et al. 1998).

There is growing evidence that the presence of the nascent peptide *per se* is important for the accuracy and correct programming of the ribosomal working cycle, most likely by inducing a conformational change in the ribosome. Translocation of the peptide moiety (or even N-acetyl moiety of N-acetyl-aa-tRNA) to the P site is required for the accurately coupled translocation of mRNA and tRNAs (Fredrick and Noller 2002). Removal of peptidyl moiety from the P site is a prerequisite for binding of EF-G, IF2 and RF3, but not EF-Tu (Zavialov et al. 2002, Zavialov and Ehrenberg 2003).

The p-tRNA has a natural propensity to dissociate from the ribosomes co-translationally, thus constituting an error of processivity (Menninger 1976). Dong and Kurland (1995) found a correlation between the p-tRNA drop-off and translational accuracy. By testing various hyper-accurate S12 mutants, they found that the level of reduction of the suppression of the UGA stop codon correlates with the increase in the drop-off of the p-tRNA from the ribosome. In addition, the hyperaccurate 23S rRNA mutant G2583C (Saarma and Remme 1992), exhibits increased rates of p-tRNA drop-off (Maiväli et al. 2001). It is possible that both the increased accuracy and the decreased processivity of translation are caused by a decrease in the affinity of tRNA towards mutant ribosomes. Unfortunately, the aforementioned correlation does not hold for the ribosome mutants with greatly larger-than-wild-type suppression rates, which still exhibit increased drop-off of the p-tRNA (Dong and Kurland 1995). Still, relA strains, which have increased translational error frequencies, exhibit reduced rates of p-tRNA drop-off (Menninger et al. 1983).

The dissociation rates of peptidyl-tRNA are much higher from the A site than from the P site of the ribosome (Karimi and Ehrenberg 1994, 1996). In the case of the error-prone ribosomes, carrying mutations in the 30S protein S4, the rates of p-tRNA dissociation are reduced in the A site, but increased in the P site. Hyperaccurate mutations in S12 reduce p-tRNA affinity to the P site, but do not greatly change its affinity to the A site. Addition of the error-inducing antibiotic streptomycin to wild-type ribosomes enhances the p-tRNA dissociation from the P site but reduces dissociation from the A site (Karimi and Ehrenberg 1994, 1996).

The rates of the p-tRNA drop-off are far from uniform across different tRNAs (Menninger 1978, Cruz-Vera et al. 2003, Olivares-Trejo et al. 2003) and are differently increased in response to starvation for different amino acids (Caplan and Menninger 1979). The p-tRNA drop-off rates can also be increased by mutations in the PTC of the ribosome (Maiväli et al. 2001). Also, the p-tRNAs with peptidyl moieties shorter than 7 amino acids are much more prone to the drop-off than longer p-tRNAs (Heurgue-Hamard et al. 2000). The enzyme peptidyl-tRNA hydrolase or Pth (Schmitt et al. 1997) catalyses the hydrolysis of peptidyl moieties and, to a lesser extent, N-acyl-amino acids (but not N-formyl-Met) from tRNA (Menninger et al. 1970, Heurgue-Hamard et al. 2000). Pth is necessary for viability of *E. coli* (Menninger 1979), but not of *S.*

cerevisiae (Rosas-Sandoval et al. 2002). Conditional lethality of temperature-sensitive Pth in *E. coli* can be rescued by over-expression of $tRNA^{Lys}$, indicating that sequestering of this tRNA leads to lethalty (Heurgue-Hamard et al. 1996). Peptidyl-$tRNA^{Lys}$ happens to be the dominant species of tRNA, which accumulates under normal growth conditions as p-tRNA (Menninger 1978). Similar rescue experiments work with $tRNA^{Arg}$ and $tRNA^{Ile}$ when these tRNAs are preferentially sequestered as p-tRNAs by the over-expression of artificial growth-inhibitory minigenes, ending with Arg or Ile codons (Tenson et al. 1999).

Although the frequency of processivity errors in translation is comparable to that of missense errors (Menninger 1976) the processivity errors may be more destructive. This, because of some of the prematurely dissociated peptides can be poisonous, or because of the energetically expensive need to degrade the half-made dysfunctional proteins. In contrast, most missense errors result in wholly or almost functional full-length proteins (Kimura 1985, Kurland et al 1996). Indeed, because both the processivity of translation and the accuracy of amino acid incorporation apparently depend on tRNA-mRNA interactions with the ribosome, it may be that the need for reducing the more dangerous processivity errors may, as a side effect, drive the frequency of missense-errors (Kurland et al. 1990). Of course, it may also be, that the reduction in the speed of hyper-accurate translation is the limiting factor that keeps the processivity levels where they are. Thus, the processivity errors may offer insight into the nature of the translational cycle where several factors compete for overlapping binding sites and delays in the process lead to abortive branches leading off the productive pathway. The translational "cycle" really consists of alternative branchings, some that lead to product formation, some that lead to discarding of incorrect substrates (see Chapter 4), and some that lead to abortive drop-off of half-made products. It is instructive, that rather than reduce the clearly harmful processivity errors to a tolerable minimum, there is a special mechanism (that is Pth) to hydrolyse the p-tRNAs in the cytoplasm. In fact, the frequency of the p-tRNA drop-off (about 10^{-4} *per* codon; Menninger 1976) is an order of magnitude higher than that of the other types of processsivity errors (termination on sense codons and spontaneous frame-shifts; Jorgensen et al. 1993, Dong and Kurland 1995, Freistoffer et al. 2000). One may surmise that the unproductive off-pathway of the p-tRNA dissociation might be a necessary consequence of the competition between the productive on-pathway and various regulatory or the-need-for-accuracy-derived branchings. In other words, competition among different pathways requires time (remember Buridan's ass) and apparently has an error rate of its own, exemplified by the p-tRNA drop-off. Alternatively, the observable p-tRNA drop-off rate may reflect trade-off between the conflicting needs to bind tRNAs tightly for maximal processivity and lightly for maximal speed of translocation.

Chapter 8. The dynamic ribosome and translocation

Translocation is the process by which, after peptide transfer to the A site tRNA, the p-tRNA moves from the A to the P site and the deacylated tRNA moves from the P to the E site. All this happens concomitantly with the movement of the mRNA (Studer et al. 2003). Although a crystal structure of the ribosome, with all three tRNA binding sites filled, is available (Yusupov et al. 2001), the actual path from here to there and the molecular mechanisms of movement remain unclear. There are two reasons, which contribute to the hardness of the translocation problem.

The trivial reason is the topological complexity of the required movements of the tRNAs. Because of the topological necessity of accommodating two tRNAs on consecutive codons, the A and P site codons are kinked by 45° and rotated from a single plane by 26° (Fig 7). The planes of the P and E site tRNAs form an angle of 46° (Yusupov et al. 2001, Noller et al. 2002). While the A and P site tRNA anticodon stems point apart (the closest approach of the backbones of the anticodon stems being 10 Å), the acceptor stems come together (to 5 Å). In contrast, anticodon stems of the P and E site bound tRNAs are close together (closest approach is 6 Å), but acceptor stems point apart (3'-ends are 50 Å apart). All this means, that while the anticodon loop must move 28 Å from the A to the P site and 20 Å from the P to the E site, the elbow of the tRNA must move with rotation of 40 Å and 55 Å from the A to the P to the E site (Yusupov et al. 2001). In reality, this movement probably goes through the A/P and the P/E hybrid states (Moazed and Noller 1989b). Interestingly, Noller et al. (2002) could not model the relatively short tRNA replacement from the A/A to the A/P hybrid state (a 14 Å movement of the acceptor arm, a 26 Å movement of the tRNA elbow) without resorting to hypothetical conformational changes of either tRNA and/or the ribosome.

The less trivial problem is the large number of contacts between all three tRNAs and the ribosome, that must put strong constraints on the aforementioned molecular ballet. In order for tRNAs to move, these contacts (described in Chapters 4 and 5) must be broken and remade; not all-at-once at the beginning and at the end of the journey, but very likely piecemeal, and also in between the destinations. Otherwise, the translocation process would need to climb a single very high activation energy barrier, instead of multiple lower barriers that would guarantee a much faster process. The GTP-binding protein EF-G catalyzes this process by lowering the activation energies from 96 kJ/mol (factor-free translocation), to 67 kJ/mol (Wintermeyer et al. 2001). Yet, it is by no mean feat, that the ribosome can (although with much reduced speed) accomplish translocation all by itself (Pestka 1974, Gavrilova et al. 1976, Chetverin and Spirin 1982). This leaves the energy in the aminoacyl ester bond, which is released upon the peptide bond formation as the only possible energy-source for translocation (except thermal energy). As a consequence of peptide transfer, the A site bound p-tRNA has higher affinity for the P site and the P site bound

deacylated tRNA has higher affinity for the E site (Holschuh and Gassen 1980, Holschuh et al. 1981). This difference in affinities should be sufficient to induce the falling of tRNAs to hybrid states (Moazed and Noller 1989b). However, there are indications that EF-G-GTP may catalyse the hybrid state formation (Valle et al. 2003c). Furthermore, the peptidyl transferase inhibitor sparsomycin, that stabilizes the p-tRNA in the P site and changes conformation of the 23S rRNA nucleotide A2602 (that lies in the path of the A to P site movement of the tRNA 3'-CCA end), can stimulate the factor-free translocation by at least 900-fold (Fredrick and Noller 2003).

Interestingly, the modification of ribosomes with a cystein-specific reagent leads to the weakening of intersubunit association (if measured in the absence of tRNAs and mRNA), and at the same time greatly increases the speed of accurate factor-free translocation (Gavrilova and Spirin 1971, Southworth et al. 2002). This result emphasizes the need to reorganize the intersubunit contacts during translocation. Therefore, although p-tRNA has higher affinity to the P site than to the A site, and deacylated tRNA has higher affinity to the E site than to the P site, efficient translocation cannot be achieved by simply dissociating the tRNA-mRNA complex from one ribosomal site and rebinding it to the other. Instead, the process takes place in several steps, occurs to a certain degree independently in both subunits and may, in fact, be actively propagated by the relative movements of the two subunits. Translocation must go through several intermediates in order to reduce the energetic barriers (that would otherwise disallow the process) to manageable proportions (Spirin 2002). The point is to subdivide one great barrier into several smaller sub-barriers. By this view, the role of EF-G-GTP is to catalyse translocation by stabilizing conformational transition state(s), thus increasing the speed of crossing the highest sub-barrier(s). The hydrolysis of GTP will then release the transition state and allow translocation to go to the completion (Chetverin and Spirin 1982, Spirin 2002). This point of view allows us to explain otherwise confusing biochemical data by which, although in the fast kinetic analysis the hydrolysis of GTP precedes translocation (Rodnina et al. 1997), the translocation rate can also be substantially enhanced by EF-G in complex with non-hydrolysable GTP analogues (Belitsina et al. 1975, 1976). EF-G in its GTP form will catalyze the climbing of the lowered barrier, thus increasing the rate of translocation. Upon GTP hydrolysis EF-G changes conformation into its GDP-form and dissociates, therefore releasing the conformational transition state and allowing translocation to go to completion. This means, that in the presence of EF-G with non-hydrolysable GTP analogue the ribosomal transition state could be relatively stable and therefore analyzable by structural methods. This seems indeed to be the case. Cryo-EM studies give surprising insights into the nature of this transition state (Frank and Agrawal 2000, Gao et al. 2003, Valle et al. 2003c). When EF-G with non-hydrolysable GTP analogue is stably bound to pre-translocation ribosomes (Phe-tRNA in the A site and deacylated tRNA in the P site), a 6° counterclockwise rotation of the 30S subunit in relation to the 50S

takes place (if seen from solvent side of the 30S; Frank and Agrawal 2000). In a recent re-interpretation, based on an improved resolution cryo-EM study, the magnitude of rotation was reduced to 4° (Gao et al. 2003, Spahn et al. 2004). This rotation is not dependent on the presence of tRNAs, it is accompanied by defined movements of the L7/L12 stalk, a rotational movement of the 30S head and a relative opening of the mRNA channel of the 30S subunit (Frank and Agrawal 2000). In the presence of EF-G in its GTP-state, little translocation (20%) was seen (Agrawal et al. 1999a). Yet, the rearrangements in the conformation of the 30S subunit led to the loosening of the A site tRNA anti-codon stem-loop contacts with the 30S, apparently in preparation of translation (Agrawal et al. 1999a, Frank and Agrawal 2000). Both the movement of the 30S in respect to the 50S and the direction of rotation of the head of the 30S are in accordance with the direction of tRNA movements (Frank and Agrawal 2000, Spahn et al. 2004). Therefore, the ribosomal rearrangements observed upon the EF-G-GTP binding may actively transport tRNAs some distance along the translocation trajectory.

The EF-G-GTP-state of the ribosome may well correspond to the conformational transition state that is frozen by the stable EF-G binding (Spirin 2002). Supporting this conjecture is the complex, where tRNAs are located in the P and the E sites but EF-G-GDP is still stably bound in a complex with fusidic acid (Frank and Agrawal 2000). Here the 30S rotates backwards (but apparently not fully), again independently of the presence of tRNAs/mRNA. The fusidic acid (fus) is supposed to freeze the EF-G in its GTP binding conformation after GTP hydrolysis (Burns et al. 1974). This is, however, not quite true. EF-G-GDP-fus has a different substrate specificity towards the ribosome, if compared with EF-G-GTP (Zavialov and Ehrenberg 2003). Also, its conformation in the ribosome (although heterogeneous, especially in portions that contact the A site of the 30S subunit) differs from that of the EF-G-GTP (Valle et al. 2003c). Therefore, it seems that the EF-G-GDP-fus complex with the ribosome is actually another frozen intermediate (or a series of intermediates) between the GTP-state and the true post-translocational state, where EF-G-GDP has dissociated from the ribosome. The pre-translocational state (before EF-G-GTP binding) and the post-translocational state (after EF-G-GDP dissociation) are in fact identical, as far as the ribosome conformation in the cryo-EM resolution range is concerned (Agrawal et al. 2000). If the binding of the EF-G-GTP leads to the rotated conformational intermediate, how does the GTP hydrolysis reverse the rotation of the 30S subunit? Coupled mRNA/tRNA translocation in the 30S seems to coincide with this reverse rotation. One possibility would be the spontaneous reversal of rotation upon the EF-G-GDP dissociation. The stable 70S-EF-G-GDP-fus intermediate with its half-reversed rotation does not support this hypothesis. When bound to the ribosome in the GTP-form, the tip of domain IV of EF-G is displaced by 37 Å from its position in the free GDP-form EF-G (Valle et al. 2003c). This domain forms a part of the tRNA-mimic and is thrust into the A site of the 30S subunit. It is therefore

possible that its large movement after GTP hydrolysis actively rotates the 30S subunit backwards and/or actively translocates tRNAs in the 30S. Indeed, constraining the movement of EF-G domains by cross-linking of domains I and V led to a 10^4-fold inhibition of translation (Peske et al. 2000). This hypothesis is further supported by fast kinetic measurements, according to which the P_i release (30 s^{-1}) is much slower than the GTP hydrolysis (170 s^{-1}) and occurs with comparable rates to translocation (Wintermeyer et al. 2001). The implication is that the P_i release is limited by the slow change in the EF-G conformation that actively drives translocation (25 s^{-1}). If EF-G domains IV and V are deleted, the rate of the P_i release is inhibited to the same extent as translocation, suggesting functional coupling (Savelsbergh et al. 2000).

How does translocation proceed in the 50S subunit (in the context of the 70S ribosome)? To get a glimpse of the answer, another cryo-EM study must be looked into. Valle *et al.* (2003c) bound EF-G with a non-hydrolysable GTP analogue or with GDP and fusidic acid to 70S ribosomes with the empty A site and a deacylated tRNA in the P site. In this study, the addition of EF-G (in both forms) led to the falling of the P site tRNA to the P/E hybrid state and the full rotation of the 30S subunit. In addition, the L1 stalk of the 50S subunit counter-rotated towards intersubunit space and apparently contacted the P/E site tRNA. This implies an active role for the L1 stalk in translocation. The EF-G-dependence of the hybrid states formation was confirmed by EF-G-GDPNP binding in biochemical assays, where a deacylated tRNA was bound to the P site and a dipeptidyl-tRNA to the A site (Zavialov and Ehrenberg 2003). These results are in variance with biochemical studies of Moazed and Noller (1989b) and Sharma *et al.* (2004), that show EF-G-independent hybrid state formation after peptidyl transfer to the A site bound tRNA. This discrepancy may be caused by the binding of an unwanted tRNA to the E site in the system of Zavialov and Ehrenberg (2003) and Valle *et al.* (2003c), and anyhow does not change the conclusion that EF-G in its GTP-form stabilizes the hybrid state. In other words, the ribosome with tRNAs in the hybrid states is a good substrate for the EF-G-GTP binding. The importance of the hybrid states for translocation is also implied by the finding that deletion of two 2' OH groups of the P site tRNA (at positions 71 and 76), which do not make contact with the ribosome in the P site, but do so in the E site, disrupt the EF-G dependent translocation (Feinberg and Joseph 2001).

Another problem with the system described by Valle *et al*. (2003c) is that EF-G-GDP-fus fails to induce the partial backwards rotation of the 30S, which is observed with both the complexes with two tRNAs (in the E and P sites) and without tRNAs/mRNA (Frank and Agrawal 2000). In addition, unlike in the complexes of Frank and Agrawal (2000), the L7/L12 stalk was not seen by Valle *et al.* (2003c), implying conformational heterogeneity in their ribosome preparation.

How are the ribosomal subunits connected in the 70S ribosome? How do these contacts change upon EF-G-GTP binding? In view of the need to

reorganize the relative positions of ribosomal subunits during translocation and to separate the subunits altogether in termination of protein synthesis (see Chapter 3), it is perhaps surprising that a substantial number of direct subunit-subunit contacts augments the two tRNAs, that bridge the subunits. In fact, tRNAs are not needed for subunit association *in vitro* (Blaha et al. 2002). The presence of various rRNA regions in intersubunit contacts was first determined by chemical footprinting (Chapman and Noller 1977, Herr and Noller 1979) and modification interference methods (Herr et al. 1979). These heroic efforts pre-date the technology for accurately placing the modified bases into the primary sequences of ribosomal RNAs. The first unambiguously located intersubunit contact was placed by chemical cross-linking between the 23S rRNA helix 69 and the 16S rRNA helices 44 and 45 (Mitchell et al. 1992). More recent chemical footprinting studies determined the identities of a number of rRNA positions, which are protected by the formation of 70S ribosomes (Merryman et al. 1999 a, b). These are generally in good correspondence with the intersubunit bridges, derived from the crystallographic model of *Thermus thermophilus* ribosome (Yusupov et al. 2001) and cryo-EM models of the *Escherichia coli* ribosome (Gabashvili et al. 2000, Gao et al. 2003). The structural studies define, in addition to RNA-RNA contacts, several protein-RNA and protein-protein interactions. The 30S proteins S13, S15, S19 and the 50S proteins L2, L5, L14 and L19 are involved in intersubunit contacts. The model of Yusupov *et al.* (2001) incorporates 12 intersubunit bridges, which translate into more than 30 individual interactions between the 30S and the 50S subunits. The bridges seem to be largely conserved between the three kingdoms of life (Spahn et al. 2001a, Gao et al. 2003). The more central intersubunit areas (that is, closer to the PTC in the 50S and to the decoding center in the 30S) are occupied by bridges, consisting entirely of RNA, while protein-containing bridges are more peripheral. Centrally located bridges contribute more than 80% of the intersubunit contacts (Gao et al. 2003). Gao *et al.* (2003) present a close study of changes in *E. coli* intersubunit bridges upon the EF-G-GTP binding. By using real space refinement of a 12 Å-resolution cryo-EM map they apparently claim to see changes in the conformation of intersubunit bridges that exceed 3Å. As the 30S rotates, two bridges (B1a and B7b) are completely broken and the contacts, that form the B1b, are substantially reorganized. Bridges B1a and B7b consist of protein-RNA contacts. In each case it is the protein component that moves (S13/S19 and L2, respectively) and RNA component that does not. Bridge B1b is the only protein-only bridge in *E. coli*, consisting of proteins S13 and L5. S13 is a part of the head of the 30S subunit and contacts the central protuberance of the 50S. S13 is the ribosomal component that moves the most (12 Å) during the EF-G-GTP binding. On the 50S side L5 answers with a 13° rotation thus maintaining the B1b, albeit with a different set of contacts. So it appears, that all the bridges that change conformation are peripheral (that is, away from the rotational center, which is located centrally in the decoding region of the 30S subunit). This observation has recently been reproduced in *S. cerevisiae* (Spahn

et al. 2004). Central RNA-only bridges apparently remain unchanged at this step of translocation. This includes the bridge B2a, whose principal component, 23S rRNA helix 69, is located between the A and the P site bound tRNAs, and therefore must move when tRNAs are translocated. Therefore, the greatest changes in the structure of the conformational intermediate, that precedes translocation, are away from the actual tRNA-ribosome contacts and result in loosening of the intersubunit contact. In addition, the 30S subunit experiences a general loosening of its structure. The head of the 30S moves 4° resulting in a more open conformation of the 30S. A tight cluster of proteins S6, S8 and S11 in the body of the 30S subunit loosens up in the EF-G-GTP complex. S12 that is the only protein close to the decoding center and the only 30S protein that interacts with EF-G (Agrawal et al. 1999a) goes through a 19° rotation towards intersubunit space, a move which could enable it to contact EF-G (Gao et al. 2003). The 50S subunit does not experience comparable general loosening of structure. The observed movements occur in the central protuberance (consisting of mostly 5S rRNA), the base of the L1 stalk (23S rRNA helix 76) swings by 15° towards the central protuberance, and the base of the L7/L12 stalk (23S rRNA helices 43, 44) also moves towards the central protuberance.

In conclusion, the 4° rotation of the subunits and the head of the 30S, concomitant with the EF-G-GTP binding, leads to the loosening of the contacts between the mRNA-2xtRNA complex and the ribosome. This is achieved by the displacement of the tRNAs from their respective binding sites in the ribosome (Spahn et al. 2004) and/or by loosening of the ribosomal structure around the tRNAs (Gao et al. 2003). Subsequent GTP hydrolysis on EF-G leads to the backward rotation of the subunits, in concert with a large movement of the EF-G tRNA-mimicking domains in the decoding center. Assumingly, the backward rotation must somehow help to disrupt the rest of the contacts of the mRNA-2xtRNA complex with the ribosome and yet allow the mRNA-2xtRNA complex to stay together for accurate translocation into the P and E sites. At least the helix 69 of 23S rRNA must additionally move during translocation and is a candidate for an active participant in the process. Perhaps the helix 69 interacts simultaneously with both tRNAs, keeping the mRNA-2xtRNA complex together, and moving it like a molecular crane? This inference is supported by the inherent flexibility of the helix 69 (Harms et al. 2001), and by the observation that the presence of mRNA is not necessary for the translocation of tRNAs *in vitro* (Belitsina et al. 1981). This implies, that when the ribosome/EF-G actively moves its substrates, it is achieved by interactions with the tRNAs rather than with the mRNA.

Chapter 9. A summary of ribosomal complexes as described by structural methods.

The last chapter of the literature overview presents in concise form most ribosomal complexes, which are available in print. My intention is, by collating this information, to put the references to the structural studies into context, and to provide a snapshot of the state of the art in the ribosomal structure; as of spring, 2004.

Table 3. Descripition of a selection of cryo-EM ribosome reconstitution studies of ribosome-tRNA complexes.

complex	res	comments	references
EC 70S	25Å	Extensive system of unrealistic channels, overly porous particles.	Frank et al. 1995
	23Å	More recent reconstitutions of similar resolution that use high-resolution crystallographic data in interpretation look quite different.	Stark et al. 1995
EC 70S with point mutations in the 16S rRNA "Dahlberg switch"	19 Å	Global rearrangements in both subunits.	Gabashvili et al. 1999b
EC 30S	37Å	Structural change of the 30S upon incorporation into the 70S.	Lata et al. 1996
	23Å	Independent movements of the 30S head, platform and body upon the 70S formation.	Gabashvili et al. 1999a
EC 70S, p(U) A:OH-tRNA^{Phe} P: OH-tRNA^{Phe} E: OH-tRNA^{Phe}	25Å 22Å	Localization of the decoding and the PT sites in the ribosome.	Agrawal et al. 1996 Agrawal et al. 1999
EC 70S, MF-mRNA P: fMet-tRNA$_f^{Met}$	15Å	Fitting of the L1 and the tRNA. A useful discussion on the determination of resolution	Malhotra et al. 1998
	11,5Å	The RNA helixes, peripheral r-proteins and intersubunit bridges appear. Conformational change in the GAC upon the 70S association. Gao et al. (2003) re-interpret this map using real space refinement.	Gabashvili et al. 2000
EC 70S, MF-mRNA P: tetrapeptidyl-tRNA$_f^{Met}$	12,8Å		Rawat et al. 2003

Table 3 (continuation)

complex	res	comments	references
EC 70S P: 3 different p-tRNAs E: tRNA	13,2–16Å	Discussion of nascent peptide-ribosomal tunnel interactions.	Gilbert et al. 2004
EC 70S, MF-mRNA P: OH-$tRNA_f^{Met}$	17Å	In high $MgCl_2$ (15 mM), tRNA is perhaps partially in the P/E hybrid state.	Agrawal et al. 1999b
BT mitochondrial 55S P: tRNA	13,5Å	Mt r-proteins generally do not appear to directly substitute for the "missing" rRNA helixes.	Sharma et al. 2003
SC 80S P: tRNA	15,4Å	Intersubunit bridges revealed.	Spahn et al. 2001a
Rabbit 80S	21Å (3σ)	Locating the expansion segments.	Dube et al. 1998a
Rat 80S	25Å (3σ)	Locating the expansion segments and the peptide channel.	Dube et al. 1998b

All resolutions of cryo-EM complexes are given by the FSC 0.5 cut-off criterion, unless only the 3σ criterion was published (see Frank 2002 for discussion on resolution determination). EC – *Escherichia coli*, BT – *Bos taurus*, SC – *Saccharomyces cerevisiae*.

Table 4. Cryo-EM studies of the ribosome with bound ternary complex.

complex	res	comments	references
EC 70S, MF-mRNA P: fMet-$tRNA_f^{Met}$ A/T: Phe-$tRNA^{Phe}$/	18Å (3σ)	The general location of the ternary complex on the ribosome. The tRNA is fitted as a rigid body, EF-Tu conformation is slightly changed.	Stark et al. 1997b
EF-Tu/GDP/ kirromycin	16Å	Deformation of the A-site tRNA anticodon region is modelled into the anticodon loop.	Stark et al. 2002
Same as previous, except addition of E: OH-tRNA	11Å	tRNA deformation is modelled as a kink in the anticodon stem. The interactions of the tRNA with the GAC are discussed.	Valle et al. 2002
	10Å	Separation of electron densities of EF-Tu and tRNA. tRNA shifts in relation to EF-Tu upon binding to the 70S.	Valle et al. 2003b

Table 5. Cryo-EM studies of translocation.

complex	res	comments	references
EC 70S, mRNA A:Met-Phe-tRNA^{Phe} P: OH-tRNA$_f^{Met}$	20Å (3σ)	Modelling of tRNAs on the ribosome. Hybrid states are undeterminable. (PRE)	Stark et al. 1997a
	17 Å	Hybrid states are not seen.	Agrawal et al. 2000
	10,2Å	tRNAs at the A/A (by inference from P/P), P/P and E/E sites.	Valle et al. 2003c
EC 70S, mRNA P: Met-Phe-tRNA^{Phe} E: OH-tRNA$_f^{Met}$	20Å (3σ)	Modelling of tRNAs on the ribosome. (POST)	Stark et al. 1997a
	17 Å	Codon-anticodon ineraction at the E site appears feasible.	Agrawal et al. 2000
EC 70S, MF-mRNA P: OH-tRNA^{Ile} E: tRNA (unknown)	12,8Å	Conformation of the tRNA/70S does not change upon puromycin reaction that releases MFTI tetrapeptide.	Valle et al. 2003c
EC 70S, MF-mRNA P: MFTI-tRNA^{Ile} E: tRNA (unknown)	11,2Å	70S locked by peptide: EF-GTP does not bind, EF-G-GDP-fus does not lead to rotation/70S conformational change.	Valle et al. 2003c
EC 70S , p(U) F: EF-G/ GMPP(CH2)P A: Phe-tRNA^{Phe} P: OH-tRNA$_f^{Met}$	17,5Å	Low EF-G occupancy. Conformational change in the 30S (head moves towards L1) and the 50S (L7/L12) upon factor binding. 30S rotates 6º in relation to 50S.	Agrawal et al. 1999a Frank and Agrawal. 2000
	12,3Å	30S rotation reduced to 4°. A detailed study of changes in intersubunit bridges.	Gao et al. 2003
EC 70S, MF-mRNA A/F: EF-G/GDPNP P/E: OH-tRNA^{Ile}	10,8Å	Subunit rotation, even in the absence of the A site tRNA. PRE state, absence of A site tRNA blocks formation of the POST site.	Valle et al. 2003c
EC 70S A/F: EF-G/ GMPP(CH$_2$)P	17,9Å	Rotation between subunits still observed, although no tRNAs/mRNA is present.	Agrawal et al. 1999a Frank and Agrawal 2000
EC 70S, p(U) A/F: EF-G-GDP-fus P: Phe-tRNA^{Phe} E: OH-tRNA$_f^{Met}$	18,4Å	Part of the 50S conformational change appears to reverse after GTP hydrolysis.	Agrawal et al. 1999a Frank and Agrawal 2000

Table 5 (continuation)

complex	res	comments	references
EC 70S, MF-mRNA A/F: EF-G/GDP/fus P: MFTI-tRNA^{Ile} E: tRNA (unknown)	13,1Å	EF-G-GDP-fus does not lead to rotation and the 70S conformational change.	Valle et al. 2003c
EC 70S, MF-mRNA A/F: EF-G/GDP/fus P/E: OH-tRNA^{Ile}	11,7Å	Subunit rotation, even in the absence of the A site tRNA. PRE state, absence of the A site tRNA blocks formation of the post site.	Valle et al. 2003c
EC 70S A/F: EF-G/GDP/fus	20Å (3σ) 18Å	The conformation of the EF-G domain IV between the PRE and POST states. Strong connection of the EF-G to the L7/L12 stalk is largely lost.	Stark et al. 2000 Frank and Agrawal 2000
EC 70S, MF-mRNA F: EF-G/GTP/thio A: fMet-Phe-tRNA^{Phe} P: OH-tRNA$_f^{Met}$	18Å (3σ)	No subunit rotation observed, as seen in the EF-G-GMPP(CH2)P complexes (Frank and Agrawal 2000).	Stark et al. 2000
EC 70S, MF-mRNA F: EF-G/GDP/thio P: fMet-Phe-tRNA^{Phe} E: OH-tRNA$_f^{Met}$	17Å (3σ)	Large conformational change of the EF-G domain IV upon translocation, the tip of domain IV reaches into the 30S A site.	Stark et al. 2000
SC 80S EF2/ sordarin P: tRNA (not directly seen)	17,5Å 11.7Å	EF2-L7/L12 stalk interactions are more extensive that in EC, domain IV may contact P site tRNA. Large rotation of the 30S head that is not seen in EC. Domain IV of eEF2 contacts 23S rRNA H69.	Gomez-Lorenzo et al. 2000 Spahn et al. 2004

Table 6. Various ribosomal complexes obtained by cryo-EM.

complex	res	comments	references
TT 30S IF3	27Å	Consistent with the IF3 binding to the 30S subunit interface.	McCutcheon et al. 1999
EC 70S A: RF2 P: OH-tRNA	21Å 11Å	RF2 conformational change in 70S. RF2 contacts 23S rRNA helix 69.	Klaholz et al. 2003 Rawat et al. 2003
EC 70S MFTI-mRNA F: RF3/GDPNP P or E: OH-tRNA	25Å	Two (sequential?) binding modes for RF3. 6° rotation of 30S upon tRNA movement from the P to the E site (sic!). C-terminal domain contacts tip of the 23S rRNA helix 69.	Klaholz et al. 2004
EC 70S RRF	12Å	RRF binding site overlaps with both the 50S A and P site, RRF contacts bridges B2a (helix 69) and B3.	Agrawal et al. 2004
Wheat 80S SRP P: p-tRNA (90 AAs, incl. signal sequence)	12Å	S domain of the SRP contacts the exit point of the peptide channel and Alu domain contacts the GAC.	Hallic et al. 2004
SC 80S, mRNA P: p-tRNA (incl. signal sequence) Sec61 complex	26Å 15,4Å	 Description of the protein channel, and of the Sec61-80S complex. A model of co-translational protein translocation is presented.	Beckmann et al. 1997 Beckmann et al. 2001
Dog 80S Sec61 complex E: tRNA (?)	17,5Å	The L1 stalk in its "closed" conformation, gating the E site. The ribosome-channel connections relatively porous, neascent chain might slip through to cytoplasm.	Morgan et al. 2002
EC 70S, MF-mRNA P: fMet-tRNA$_f^{Met}$ Tet(O)/GTPγS	16Å	Unlike for the EF-G, the tip of Tet(O) domain IV does not go into A site but locates near tetracycline binding site.	Spahn et al. 2001b
rabbit 40S HCV IRES RNA	~20Å	The IRES binding changes the global conformation of the 40S into a more closed form.	Spahn et al. 2001c
TT 70S, mRNA that ends with AUG in the P site. A/F: Ala-tmRNA/ SmpB/EF-Tu/GDP/ kirromycin P: fMet-tRNA$_f^{Met}$	13Å	The SmpB goes into A site, interacts with the 23S rRNA helix 69.	Valle et al. 2003a

Table 7. X-ray structures of various ribosomal complexes.

complex	res	comments	references
EC 70S	10Å	The 30S head adopts closed conformation, the body remains in its open conformation. The L1 stalk is rotated away from the 50S.	Vila-Sanjurjo et al. 2003
EC 70S, S12 SmD, mRNA A: OH-$tRNA_f^{Met}$ (non-cognate, 50% occupancy) P: OH-$tRNA_f^{Met}$	9Å	Hyper-accurate. The 30S head adopts a closed conformation, the body remains in the open conformation. L1 stalk rotated away from the 50S. The "Dahlberg switch" is still in error-prone conformation.	Vila-Sanjurjo et al. 2003
TT 70S, mRNA (Met-Phe-Lys)	7,8Å	Culver et al. (1999) elaborate on the intersubunit bridge B4.	Cate et al. 1999
A: tRNA (low occupancy)	7Å	Description of the mRNA path in the 70S.	Yusupova et al. 2001
P: OH-$tRNA_f^{Met}$ E: tRNA (low occupancy)	5,5Å	The most important work on the ribosome since anyone should remember.	Yusupov et al. 2001
TT 70S, mRNA (Met-Phe-Lys)	7,8Å		Cate et al. 1999
P: ASL^{Phe} E: tRNA (low occupancy)	5,5Å		Yusupov et al. 2001
TT 70S, mRNA (Met-Phe-Lys)	7,8Å		Cate et al. 1999
A: OH-$tRNA^{Lys}$ P: ASL^{Phe}	7Å		Yusupov et al. 2001
TT 30S	5,5Å	Phosphate backbone, protein α-helices visible. dsRNA identifiable.	Clemons et al. 1999.
	4,5Å	Phosphates of RNA backbone appear as bulges, individual bases not well resolved. Prominent protein folds appear.	Tocilj et al.1999
	3,3Å	Many purines, pyrimidines and protein side chains are separable.	Schluenzen et al. 2000
	3Å	Localization of 180 metal ions.	Wimberly et al. 2000
	3Å	Detailed description of the 30S proteins.	Brodersen et al. 2002
TT 30S, mRNA U_6 A: ASL^{Phe} P: spur mimics tRNA	3,3Å	Induced fit (A1492, A1493, G530 interact with codon-anticodon duplex).	Ogle et al. 2001
TT 30S, mRNA U_6 A: ASL^{Phe} P: spur mimics tRNA paromomycin	3,1Å	Paromomycin does not change conformation of cognate tRNA-30S complex.	Ogle et al. 2001

Table 7 (continuation)

complex	res	comments	references
TT 30S, mRNA U_6 ASL^{Leu2}	3,8Å	No electron density for ASL^{Leu2}-disordered binding.	Ogle et al. 2002
TT 30S mRNA U_6 ASL^{Ser}	3,7Å	No electron density for ASL^{Ser}-disordered binding.	Ogle et al. 2002
TT 30S mRNA U_6 A: ASL^{Leu2} P: spur mimics tRNA paromomycin	3Å	Near-cognate ASL binding leads to induced fit. The codon-anticodon pairing geometry differs.	Ogle et al. 2002
TT 30S mRNA U_6 A: ASL^{Ser} P: spur mimics tRNA paromomycin	3,4Å	The near-cognate ASL binds: induced fit. Codon-anticodon pairing geometry differs. G:U pair probably in two alternative conformations.	Ogle et al. 2002
TT 30S A: IF1	3,2Å	Binds to the A site precluding tRNA binding, leads to the induced fit (both local and global).	Carter et al. 2001
TT 30S IF3	4,2Å	The IF3 lies away from 50S contact areas. The relevance of this interaction was questioned on the basis of hydroxyl radical probing and crystal packing interactions, which would preclude binding of the soaked in IF3 to its more likely binding site in the subunit interface (Dallas and Noller 2001).	Pioletti et al. 2001
HM 50S	9Å	Long dsRNA regions are recognizable.	Ban et al. 1998
	5Å	>300 bp of A form dsRNA fitted, ssRNA difficult to trace. Protein α-helices (but not β-sheets) easily identifiable.	Ban et al. 1999
	2,4Å	Bases and protein side chains usually unequivocally identifiable. Many water molecules and metal ions evident in electron density. Klein et al. (2004) present further refinement of the 50S model and a detailed discussion of the LSU proteins.	Ban et al. 2000
HM 50S A: CCA P: CCA (low occupancy) E: CCA	2,9Å	Description of the 50S E site.	Schmeing et al. 2003
HM 50S E: minihelix-CCA	3,1Å	Description of the 50S E site.	Schmeing et al. 2003

Table 7 (continuation)

complex	res	comments	references
HM 50S A/P: CCdA-p-puro	3,2Å 3Å	The "Yarus inhibitor" – relevance as a model of PT –reaction intermediate subsequently challenged (Hansen et al. 2002 b).	Nissen et al. 2000a Hansen et al. 2002 b
HM 50S A: Minihelix-CC-puro	3,2Å 3Å		Nissen et al. 2000a Hansen et al. 2002 b
HM 50S A:, P: CC-puro-biotin	3Å	Both sites with ca 50% occupancy.	Hansen et al. 2002 b
HM 50S P: CC-puro-biotin sparsomycin	3Å		Hansen et al. 2002 b
HM 50S A:, P: CC-puro-Phe-biotin	3Å	Both sites with ca 50% occupancy.	Hansen et al. 2002 b
HM 50S P: CC-puro-Phe-biotin sparsomycin	3Å		Hansen et al. 2002 b
DR 50S	3,1Å	Structure is more complete than HM 50S structure, incl. helices involved in intersubunit bridges.	Harms et al. 2001.
DR 50S A: acceptor stem analogue or ACC-Puromycin	3,5Å 3,7Å	Definition of a 2-fold axis in the A-P sites	Bashan et al. 2003 a

TT — *Themrus thermophilus*, HM — *Haloarcula marismortui*, DR — *Deinococcus radiodurans.* ASL – anticodon stem-loop.

Table 8. Ribosome — antibiotic co-crystal studies.

complex	res	comments	ref
DR 50S Chloramphenicol	3,6Å	Binds to the PT cavity, interacts exclusively with the 23S rRNA. A completely different binding site is described in Hansen et al. (2003).	Schlünzen et al. 2001
DR 50S A: acceptor stem analogue sparsomycin	3,6Å	Sparsomycin triggers conformational changes in the PTC.	Bashan et al. 2003 a.
DR 50S clindamycin	3,2Å	Binds to the PT cavity, interacts exclusively with the 23S rRNA.	Schlünzen et al. 2001
DR 50S erythromycin	3,6Å	Binds to the entrance of peptide tunnel.	Schlünzen et al. 2001
DR 50S clarithromycin	3,6Å	Binds to the entrance of peptide tunnel.	Schlünzen et al. 2001
DR 50S roxithromycin	3,9Å	Binds to the entrance of peptide tunnel.	Schlünzen et al. 2001
DR 50S telithromycin	3,4Å	Blocks the peptide tunnel, interacts exclusively with the 23S rRNA.	Berisio et al. 2003a
DR 50S troleandomycin	3,4Å	Blocks the peptide tunnel, induces conformational change in the tunnel component L22.	Berisio et al. 2003b
DR 50S ketolde ABT-733	3,5Å	Blocks the peptide tunnel.	Schlünzen et al. 2003
DR 50S azithromycin	3,2Å	Blocks the peptide tunnel, two binding sites in the tunnel.	Schlünzen et al. 2003
DR 50S dalfopristin quinupristin	3.4Å	Dalfopristin binds binds directly to PTC, quinupristin binds to the peptide tunnel. Cooperative binding changes conformation of the PTC.	Harms et al. 2004
HM 50S virginiamycin M	3Å	Binds to portions of both the A and P sites, causes conformational change in the PT region.	Hansen et al. 2003
HM 50S tylosin	3Å	Binds near the entrance of peptide tunnel.	Hansen et al. 2002 a
HM 50S carbomycin	3Å	Binds near the entrance of peptide tunnel.	Hansen et al. 2002 a
HM 50S spiramycin	3Å	Binds near the entrance of peptide tunnel.	Hansen et al. 2002 a
HM 50S azithromycin	3Å	Binds near the entrance of peptide tunnel.	Hansen et al. 2002 a
HM 50S anisomycin	3Å	Binds to the PT region in the A site.	Hansen et al. 2003
HM 50S chloramphenicol	3Å	Binds to the entrance of the peptide tunnel. Completely different binding site was described in DR (Schlünzen et al. 2001).	Hansen et al. 2003

Table 8 (continuation)

complex	res	comments	ref
HM 50S blacticidin S	3Å	Binds to 23S rRNA P loop, excludes binding of the C75 of the P site tRNA.	Hansen et al. 2003
HM 50S P: CCA-phe-cap-biotin sparsomycin	2,8Å	Interacts directly with the P site bound CCA.	Hansen et al. 2003
TT 30S spectinomycin	3Å	Binds to the helix 34. Blocks the 30S movement during translocation?	Carter et al. 2000
TT 30S streptomycin	3Å		Carter et al. 2000
TT 30S paromomycin	3Å	Binds to the helix 44, flips out the A1492 and A1493.	Carter et al. 2000
TT 30S pactamycin	3,4Å	Binds to the E site and displaces the mRNA.	Brodersen et al. 2000
TT 30S hygromycin B	3,3Å	Binds to the helix 44. May tamper with the 30S conformational flexibility during translocation.	Brodersen et al. 2000
TT 30S tetracycline	4,5Å 3,4Å	Altogether six binding sites, one in the A site. Two binding sites, one in the A site.	Pioletti et al. 2001 Brodersen et al. 2000
TT 30S edeine	4,5Å	Possible interaction with the P site tRNA. May hinder the 30S conformational plasticity.	Pioletti et al. 2001

PRESENT INVESTIGATIONS

Mutagenesis of the peptidyl transfer region of *E. coli* 23S rRNA (paper I)

The mutagenesis approach has been instrumental in illustrating the ways in which the peptidyl transferase region of 23S rRNA can influence various aspects of translation. Mutations in the PTC have been shown to reduce rates of peptide transfer to aa-tRNA, increase or decrease the levels of amino acid mis-incorporation into protein, increase ribosomal frame-shifting, and stop codon read-through (Saarma and Remme 1992, Gegory et al. 1994, O'Connor and Dahlberg 1995, Porse and Garrett 1995, Porse et al. 1996, Spahn et al. 1996 a, b, Saarma et al. 1998, Gregory et al. 2000). Also, tRNAs harboring mutations in their 3'CCA ends (which interacts with the PTC) can promote ribosomal frameshifting and stop codon read-through (O'Connor et al. 1993b). We have extended the repertoire of mutations of the PTC by showing that 23S rRNA mutations at positions 2582 and 2583 increase peptidyl-tRNA dissociation from translating ribosomes *in vivo*. This was shown in two independent experimental systems. Firstly, we expressed the 23S rRNA mutants in an *E. coli* strain that is temperature sensitive towards the peptidyl-tRNA hydrolase (Pth). Because the accumulation of peptidyl-tRNA is lethal in *E. coli* (Menninger 1979), we could assess the levels of the p-tRNA drop-off from the ribosomes by measuring the colony forming ability of the cells grown in liquid media at 42°C. Secondly, we directly measured the accumulation of the p-tRNA. We isolated total RNA from the pth^{TS} strain expressing mutant 23S rRNA, treated it with Pth or water, and subsequently aminoacylated it with ^{14}C-labelled leucine. Both methods of study agree in that the expression of 23S rRNA harboring mutations G2582A or G2583C leads to an increase in p-tRNA accumulation. At the permissive temperature the same mutations lead to markedly reduced translation levels *in vivo* (paper I) and in poly(U)-directed poly(Phe) synthesis *in vitro* (Ü.M., unpublished data, Saarma and Remme 1992). In addition, they have a pronounced bacteriostatic effect when induced at the permissive temperature (30°C) in pth^{TS} strain (paper I) or at 37°C in a wt *pth* context (unpublished data). All aforementioned experiments were performed in the chromosomally-encoded wt 23S rRNA background, where only about 30% of the translationally competent ribosomes contained plasmid-encoded 23S rRNA (estimated by a phenotypically silent marker-mutation in the plasmid-encoded 23S rRNA). The large growth effects and the reduction of the *in vivo* translation levels should be therefore labelled as pseudo-dominant over the background of wt ribosomes. The presence of wt 23S rRNA background also allowed us to measure the fractions of mutant 23S rRNAs in polysomes, 70S ribosomes and 50S subunits. Mutations at positions 2582 and 2583 led to the reduction of the plasmid-encoded 23S rRNA fraction in the polysomes and to the concomitant increase in

the mutant rRNA fraction in the 70S ribosomes and 50S subunits. This result (in concert with defects in poly(U)-translation) strongly ties the effects of these mutations with the elongation phase of the protein synthesis and is consistent with the increased p-tRNA drop-off causing the observed phenotypes (growth inhibition and reduction in levels of translation).

The neatness of the above discourse on the molecular basis of the phenotypic effects of 23S rRNA mutations 2582 and 2583 is shattered, when one considers that the same mutations are defective in the puromycin model-reaction of the peptidyl transferase (Saarma and Remme 1992, Porse et al. 1996, Saarma et al. 1998) and, more importantly, that the mutations at position 2583 increase the accuracy of protein synthesis both *in vitro* (Saarma and Remme 1992) and *in vivo* (Saarma et al. 1993). The effects on the puromycin reaction may result from the weakening of the interactions of the substrates with the PTC, from a direct effect of the mutations on catalysis or less directly, from the mutations changing the general shape of the PTC (thus influencing substrate specificity and/or catalysis). Taken together with the data on increasing the p-tRNA drop-off and decreasing the incorporation of incorrect amino acids into protein, the simplest explanation is that each effect is a consequence of the weakened tRNA ribosome interaction in the ribosomal A site.

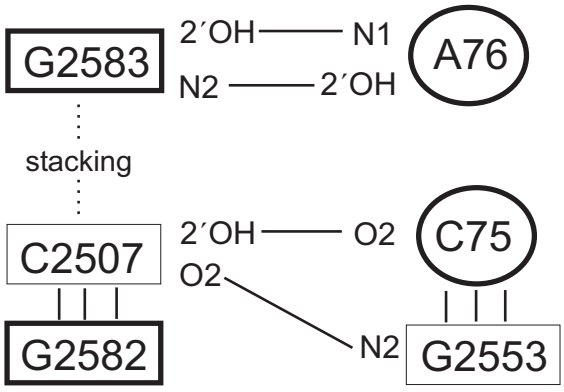


Figure 11. Scheme of interactions of 23S rRNA nucleotides G2582 and G2583 with A site bound tRNA and 23S rRNA. Solid lines denote potential hydrogen bonds, dotted line denotes potential stacking interaction. 23S rRNA nucleotides are in boxes, tRNA nucleotides are encircled.

Indeed, crystallographic studies of the *Haloarcula marismortui* (Ban et al. 2000, Hansen et al. 2002) and *Deinococcus radiodurans* (Harms et al. 2001) 50S subunits in complexes with tRNA 3'-end analogues place 23S rRNA positions G2582 and G2583 squarely within the A site (Figure 11). G2583 (N2) forms a hydrogen bond with the 2'-OH of the ribose of A76 of the A site bound tRNA.

In addition, the 2'-OH of the ribose of G2583 forms a hydrogen bond with N1 of A76. N2 of G2583 also hydrogen-bonds with O2 of U2506, which in turn forms a hydrogen bond between its 2'-OH and the 2'-OH of the A76 of tRNA. Also, G2583 may form a stacking interaction with C2507, which contacts the base (O2) of C75 of the A site tRNA. G2582 forms a W-C pair with C2507, which contacts the C75 of the A site tRNA. In addition, C2507 gives a base-base (O2-N2) hydrogen bond to G2553, which base-pairs with C75 of the A site tRNA. This makes it likely that mutations in G2582 and G2583 cause weakening or reorganizing of the tRNA CCA-end interactions with the 50S A site.

Such a weakening of the tRNA contact with the A site is suggestive of the explanation for the observed increase in p-tRNA drop-off (i.e. from the pretranslocational state of the ribosome). Nevertheless, we cannot exclude that the p-tRNA drop-off occurs during translocation. The drop-off from the P site is less likely because the 2582 and 2583 mutations should directly influence the A site, but not the P site.

How could the weakening of tRNA interaction with the ribosomal A site lead to increasing translational accuracy? It could increase translational accuracy in the proofreading step by differentially affecting rejection rates and/or accommodation rates of correct and incorrect tRNAs (Rodnina and Wintermeyer 2001a). An effect on the initial selection seems less likely, because in the ternary complex the CCA-end of tRNA is shielded by EF-Tu and is quite far from the PTC and, therefore, G2582 and G2583 (Andersen et al. 2003, Valle et al. 2003b). To explain the increased accuracy through initial selection one must postulate a direct, tRNA-independent, signalling between the unoccupied PTC and the decoding centre of the 30S subunit. A good candidate for a ribosomal component, capable of connecting the PTC and the decoding centre, is helix 69 of 23S rRNA. Helix 69 not only contacts the 30S decoding centre (Yusupov et al. 2001, Gao et al. 2003), but also interacts with the A site tRNA both in the ternary complex (Valle et al. 2003b) and in its accommodated form (Yusupov et al. 2001). Mutations in helix 69 can indeed reduce the accuracy of translation (O'Connor and Dahlberg 1995). In addition, DMS-modification of two adenosines in helix 69 can cause an *in vitro* defect in 70S association in the absence of tRNA (paper II), consistent with the proposed independent role of helix 69 in signaling between the PTC and the decoding centre (Bashan et al. 2003b).

Determination of functional interactions between ribosomal subunits in the 70S ribosomes (paper II).

Crystallographic and cryo-EM studies have defined contact areas between the ribosomal subunits that are classified as 12 intersubunit bridges (Yusupov et al. 2001, Gao et al. 2003). These translate into more than 30 individual contacts between the subunits. The available resolution of crystal structure determination (5.5 Å) and the nature of the crystallographic structure determination itself make it impossible to verify the actual existence of individual hydrogen bonds (Allewell and Trikha 1995). Indeed, even if individually verified, the relatively large number of intersubunit contacts makes it unlikely that all of them exhibit equal functional significance. Recent cryo-EM experiments with ribosome-EF-G-GTP complexes show, that the central RNA-only bridges are not disrupted by the EF-G binding and the ensuing ratchet-like movement of the ribosomal subunits. On the other hand, the more peripheral protein-containing bridges tend to change conformation or to be broken altogether (Gao et al. 2003, Valle et al. 2003c, Spahn et al. 2004, Chapter 8).

We have used the damage selection strategy to determine on a "micro-level" the 23S rRNA contacts with the 30S ribosomal subunit. We modified *E. coli* 50S subunits with low concentrations of DMS or CMCT, re-associated the 70S ribosomes *in vitro* in the absence of tRNA by using modified 50S subunits, and purified the 70S and 50S fractions by sucrose-gradient ultracentrifugation. The modification patterns were analyzed by a primer extension procedure. DMS methylates adenosines at N1 and guanosines at N7. It also modifies, albeit to a lesser extent, N3 of cytosines (Brunel and Romby 2000). CMCT modifies N1 of guanosines and N3 of uridines (Brunel and Romby 2000). Modified positions that were reduced in the 70S fractions were designated as interfering with association of the 70S ribosome. To increase the sensitivity of the assay, subunit re-association experiments were conducted in suboptimal re-association conditions (6 mM $MgCl_2$) where about half of the 50S subunits associated to form 70S ribosomes.

We discovered that DMS-modifications of three adenosines (at 23S rRNA positions 715, 1912 and 1918) interfere very strongly with 70S formation when 6 mM $MgCl_2$ was used in the re-association reaction. Much less interference with 70S re-association was observed when optimal (13 mM) $MgCl_2$ concentration was used. 13 mM $MgCl_2$ guarantees strong subunit association and maximal levels of poly(U) translation when polyamines are not included in the buffer (Ü.M. and Aivar Liiv, unpublished results). No CMCT-specific interferences with 70S ribosome formation were detected in 23S rRNA. All three DMS-specific interfering adenosines are located in known intersubunit bridges: A1912 and A1918 of the helix 69 are components of the bridge B2a,

and A715 of the helix 34 is a part of the bridge B4 (Yusupov et al. 2001, Gao et al. 2003).

Due to our experimental design we can find 23S rRNA contact sites with the 30S subunits that are accessible for the modification in the native 50S subunits, and where any given modification causes a functional defect in subunit association. This means, that our assay is a direct measure for the functionality of the observed contacts between the ribosomal subunits. However, although the observed interferences are highly suggestive of the functionality (and therefore the existence) of the proposed intermolecular contacts, they should not be interpreted as implying similar effects by mutations of A715, A1912 or A1918 *in vivo*. Indeed, expression of the plasmid-encoded A715G mutant 23S rRNA in the background of 70% chromosomally encoded wt 23S rRNA leads to a modest reduction in plasmid-encoded 23S rRNA in polysomes and in the poly(U)-directed *in vitro* protein synthesis (at 13 mM $MgCl_2$). A1912G mutant has a large effect and A1918G mutant has no effect in the aforementioned assays (Ü.M., Diana Karitkina, manuscript in preparation).

Another possibility, that must be addressed, is whether the observed interferences are caused by larger rearrangements in the ribosomal structure upon methylation of the three adenosines, rather than disruption of the direct contacts of modified positions with the 30S subunit? At least for A715 and A1918 this does not seem likely, because mutations in these positions have modest phenotypes *in vivo* (see above). In addition, helices 34 and 69 are relatively independent structural elements of the 50S subunit (Fig 12), making it unlikely that any methylation in their outlying loops could lead to large rearrangements in the structure of the 50S subunit.

What are the inter-subunit interactions that are disrupted by the interfering DMS-modifications? The commonest long-range RNA-RNA interaction in the ribosome is the A-minor motif, which involves hydrogen bonding of N1, N3 and the 2'-OH of an adenosine with the minor groove of a long-range (preferably G:C) base-pair (Nissen et al. 2001). DMS-methylation of the N1 positions of adenosines would disrupt such an interaction. Current structural work exhibits insufficient resolution to verify potential inter-subunit A-minor interactions by direct observation, but the existing lower-resolution placement of bridges B2a and B4 into the 30S structure provide ample possibilities for A-minor interactions for all three adenosines. A715 could form an A-minor interaction with a base pair of 16S rRNA helix 20 and A1912 and/or A1918 could form A-minor interactions with a base-pair in the proximal part of 16S rRNA helix 44 (Yusupov et al. 2001, Gao et al. 2001).

N1 of adenosines is also involved in WC base-pairing. So, any defect in the subunit association resulting from adenosine N1 methylation could, in principle, be caused by the disruption of a long-range A:U base-pair. Suitable uridines for possible intersubunit WC-pairs are, however, harder to find in the 16S rRNA side of bridges B2a and B4. The portion of 16S rRNA helix 20, which participates in B4, has one internal A:U base-pair (A579:U761). For the U761

to become accessible for potential intermolecular base pairing, the disruption of the intramolecular helix 20 would be required. Bridge B2a portion of the 16S rRNA helix 44 has an internal U1406:U1495 loop which, however, while not forming a base pair, still fits very well into the geometry of the A-form RNA helix (Wimberly et al. 2000, PDB accession number 1J5E). Possible intermolecular base-pairing with A1912 or A1918 would therefore require the disruption of the A-form RNA helix. The U1406:U1495 pair in 16S rRNA is a part of the aminoglycoside antibiotic binding site and adopts different sheared geometries in the 30S co-crystals with different aminoglycosides (Vicens and Westhof 2003). Mutants harboring base-substitutions in the U1406:U1495 pair are viable and confer aminoglycoside resistance to the mutant ribosomes (Pfister et al. 2003). Consistent with the flexibility of the 16S rRNA helix 44 around U1406:U1495, N3 position of the U1495 is accessible to modification by CMCT in the 30S ribosomal subunits (Arto Pulk, unpublished data). Furthermore, CMCT-modification of the U1495 strongly interferes with 70S re-association under similar conditions as used in paper II (Arto Pulk, unpublished data). This is, of course, suggestive of an intermolecular base pair between the U1495 of 16S rRNA and the A1912 or the A1918 of 23S rRNA.

Construction and testing of ribosomes that contain phosphorothioate substitutions in the backbone of their 23S rRNA (paper III)

Traditional methods of RNA structure probing include modification of bases with low-molecular weight chemical probes (DMS, CMCT, kethoxal, DEPC) or structure-specific RNases (T1, T2, U2, V1), attacking backbone phosphates with ENU, and cleaving ribose rings with Fe^{2+}/EDTA/H_2O_2 (Moine and Ehresmann 1997, Brunel and Romby 2000). Base-specific chemical probes can be used in ribosome studies in three basic experimental settings: probing of rRNA secondary/tertiary structure and structure dynamics (Moazed et al. 1986, Noller et al. 1987), footprinting different ligands on the ribosome (Moazed and Noller 1986, 1989a, b, Moazed et al. 1988) and modification interference (Herr et al. 1979, von Ahsen and Noller 1995, Bocchetta et al. 1998, Bocchetta et al. 2001). Limitations of base-specific reagents in footprinting and modification interference experiments include low accessibility of rRNA bases in ribosomal particles: less than 10% of 23S rRNA bases can be modified in functional 50S subunits (Ü.M. unpublished data, Egjeberg et al. 1989). Hydroxyl-radical probing of the sugar-phosphate backbone is more effective in chemical footprinting of ribosomal complexes (Brunel and Romby 2000, Joseph and Noller 2000), but since it leads to RNA backbone cuts, has, to my knowledge, not been used in modification interference experiments.

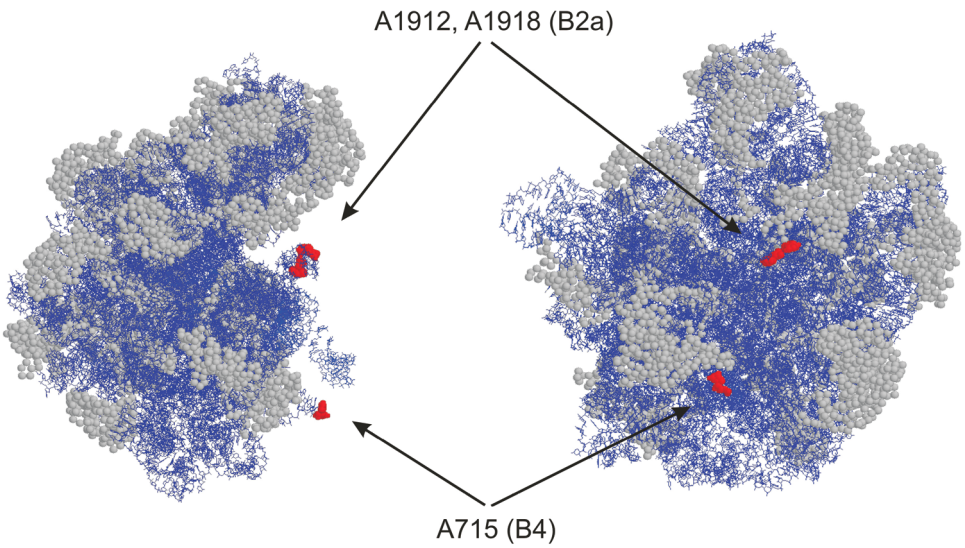


Figure 12 is reproduced from paper II. Modelling of interfering positions into the crystal structure of *Deinococcus radiodurans* 50S subunits (Harms et al. 2001, PDB accession code 1KC9). The left view is from the L1-side and the right view is from the 30S-side of the 50S subunit. Arrows indicate interfering positions (*E. coli* numbering) and corresponding bridges. RasWin molecular graphics was used to highlight interfering positions in red spacefill. Rest of the RNA is blue and á-carbons of r-proteins are in grey spacefill.

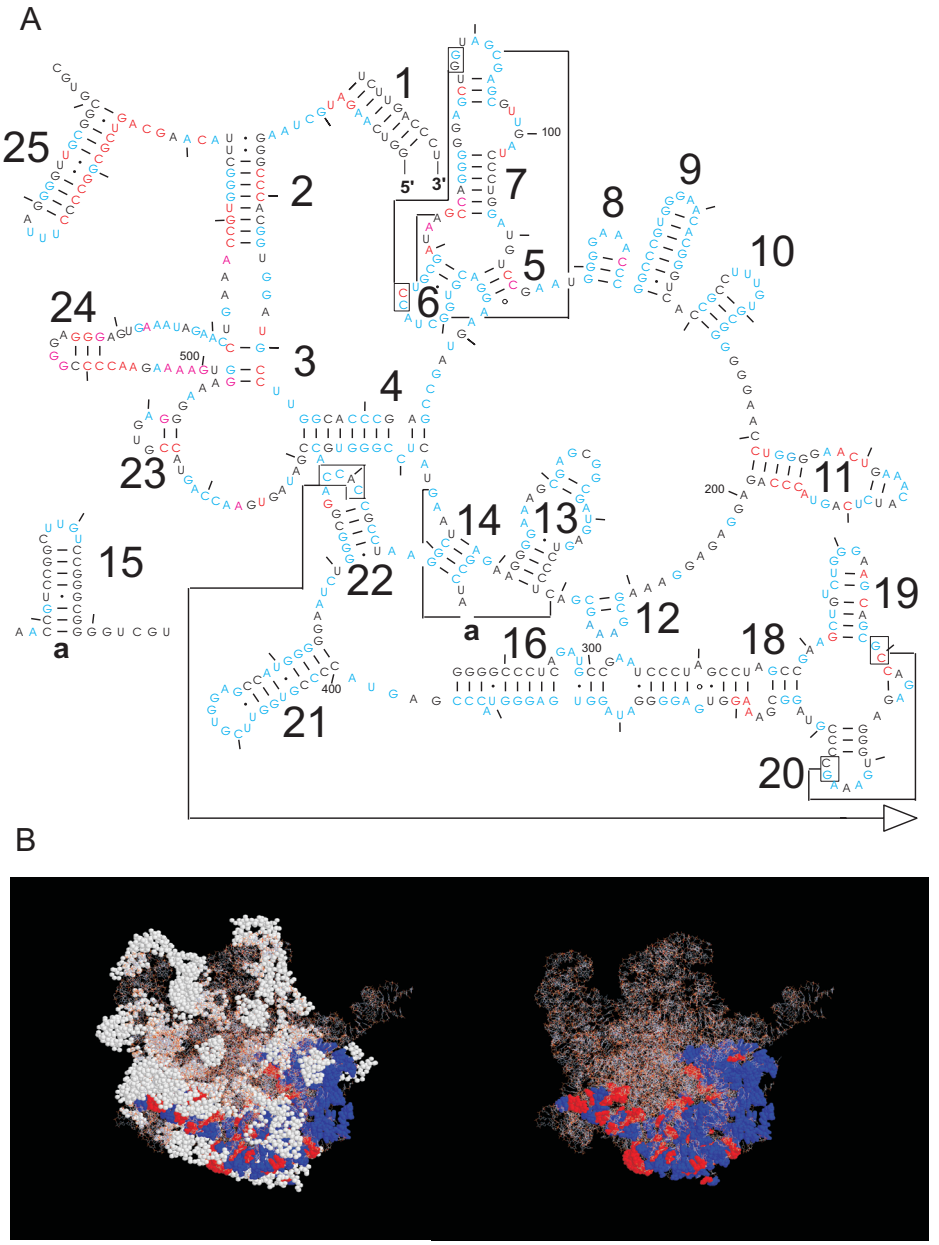


Figure 13 is adapted from paper III.
A. Summary of protected and accessible positions in domain I of *T. aquaticus* 23 S rRNA as determined by iodine cleavage of reconstituted transcripts. Protected positions are red and accessible positions blue. Positions whose accessibility could not be determined are shown in black.
B. Protection data, modelled into the structure of *Deinococcus radiodurans* 50 S. A solvent-side view of the 50 S is presented using RasWin Molecular Graphics. Protected nucleotides are shown in red space fill and accessible nucleotides in blue space fill. Left: -carbons of r-proteins are shown in white space fill. Right: r-proteins are omitted.

A complementary strategy to base modifications is to incorporate α-phosphorothioates into RNA *in vitro*, often in conjuction with various nucleotide analogues (Vortler and Eckstein 2000). Phosphorothioates can be detected by iodine-cleavage of RNA backbone in thioated positions. This method has been very useful in modification-interference studies of small catalytic RNAs (Strobel 1999). We have made an effort to extend the use of the phosphorothioate-incorporation strategy to the studies of 23S rRNA. This requires two modifications in the experimental procedures, as compared to the studies of small RNAs.

First, reconstitution of the active 50S subunits from *in vitro* synthesized phosphorothioate-containing 23S rRNA and 5S rRNA with native 50S proteins (TP50) is needed. For this, we used the *Thermus aquaticus* 50S reconstitution system of Khaitovich *et al.* (1999). Subunits were reconstituted from TP50 and from 23S rRNA and 5S rRNA, which were transcribed by T7 RNA polymerase. They sedimented during sucrose gradient centrifugation as 50S and exhibited *ca* 15% of the puromycin reactivity of the native 50S subunits. Incorporation of 5% of one of each rNTPαS did not change the sedimentation rate or the peptidyl transferase activity of the reconstituted particles.

Second, unlike the small RNAs, where cuts in the backbone can be studied directly by PAAG-electophoresis of labeled molecules, iodine-induced cleavages in large RNAs need to be studied by reverse-transcriptase directed primer extension (Stern et al. 1988). *T. aquaticus* 23S rRNA is rich in secondary structure and in G:C pairs, which have a propensity to cause unwanted stop-signals in the primer-extension reaction. To determine the effectiveness of our primer extension procedure and assess the structural homogeneity of the reconstituted 50S, we chose to footprint the phosphorothioate-containing 23S rRNA domain I in reconstituted 50S subunits. The comparison of iodine-cleavage patterns of naked phosphorothioate-containing 23S rRNA with the iodine-cleavage patterns of reconstituted 50S subunits should define the r-protein binding sites in the 23S rRNA domain I. Crystal structures of the *H. marismortui* (Ban et al. 2000) and *D. radiodurans* 50S subunits (Harms et al. 2001) can serve as arbiters of the accuracy of protein footprints in 23S rRNA. Strong and accurate protein footprints would indicate the homogeneity of the phosphorothioate-containing reconstituted 50S population. Domain I of the 23S rRNA is located largely on the surface of the solvent side of the 50S subunit, away from the intersubunit space and near the exit of the nascent-protein conducting channel (Ban et al. 2000, Harms et al. 2001). Therefore, the observable protections from iodine should be more likely caused by the shielding by specific r-proteins than from the placement of protected rRNA region in the generally non-accessible subunit interior.

From the ~580 23S rRNA positions analyzed, iodine-cleavages at about 370 positions were detected in naked 23S rRNA. Remaining positions are either masked from analysis by secondary-structure-derived iodine-independent universal primer extension stops or (to a lesser extent) by lacking a sufficiently

strong iodine-dependent stop signal. In the reconstituted 50S subunits, out of the 360 consistently analyzable positions, 280 remained as in naked 23S rRNA and 80 were less accessible to iodine (Fig 13a).

A small number of positions (around a dozen), that were accessible in naked 23S rRNA, failed to give a consistent footprint in the reconstituted 50S particles and were therefore excluded from the analysis. Their presence may be indicative of certain heterogeneity in the reconstituted 50S population, or of the inherent difficulties in the primer extension protocol. However, the vast majority of the observed protections were consistent in successive experiments (see fig 2, 3 of paper III). Modelling of the protection data into the *H. marismortui* and *D. radiodurans* 50S crystal structures shows that most of the iodine protections in 23S rRNA domain I cluster near the r-protein binding sites (Ü.M., unpublished data, Fig 13b). Much fewer protections are tentatively associated with the shielding by rRNA, and the presence of some protections in two helices could not be rationalized using available crystal structures (see paper III for more detailed discussion). The data collectively suggest a reasonable structural homogeneity for the reconstituted 50S subunit and a reasonable efficiency of the primer extension procedure for the detection of iodine-cleavages in a highly structured RNA template.

We interpreted the results described in paper III, as encouraging for commencing more demanding experiments in the modification interference idiom. To construct an experimental system for the modification interference studies, we successfully incorporated deoxy-phosphorothioates into the *T. aquaticus* 23S rRNA using mutant T7 RNA polymerase (Y369F), which exhibits reduced substrate selectivity (Bonner et al. 1992). The placement of 20–25% of the corresponding deoxy-phosphorothioate into the *in vitro* transcription reaction resulted in a comparable incorporation efficiency of 3–5% ribo-phosphorothioate incorporation and active reconstituted 50S subunits (Ü.M., Arto Pulk, unpublished data). The exceptions were incorporation of TTPαS, deoxy-UαS and 5-methyl-UTPαS into 23S rRNA, which strongly inhibited subsequent 50S reconstruction.

Next, we developed a *T. aquaticus* 50S reconstitution system using fragmented 23S rRNA *in vitro* transcripts (Ü.M. and Arto Pulk, unpublished data). Such a reconstitution system could be useful in reducing the number of incorporated modified nucleotides *per* 23S rRNA molecule, since each of the fragments can be synthesized without the addition of unnatural nucleotide analogues.

In our first attempt at 23S rRNA fragmentation the plasmid-encoded 23S rRNA was cloned apart at the loop of helix 25 of 23S rRNA (at position 568, *T. aquaticus* nomenclature). The two fragments were transcribed separately and mixed together at equimolar concentrations in the reconstitution reaction. The ensuing reconstituted 50S retained about 1/3 of the puromycin reactivity as compared to the reconstituted 50S that carried its 23S rRNA in the single piece.

Our second attempt was made to fragment 23S rRNA at the loop of helix 45 (at position 1000). This effort turned out to be entirely successful: 50S that were reconstituted with fragmented 23S rRNA retained full activity in the puromycin reaction.

We also made a serious effort to use deoxy-phosphorothioates-containing reconstituted 50S in a modification interference study. We reassociated modified 50S subunits with native *T. aquaticus* 30S subunits in the presence of tRNA (Ü.M., Arto Pulk, unpublised experiments). Resulting 70S and 50S fractions were purified by sucrose gradient ultracentrifugation, RNA was further purified from ribosomal proteins and subjected to iodine-treatment. In the absence of tRNA, *T. aquaticus* 70S ribosomes were unattainable by re-association of the subunits (Ü.M., unpublished data). Modified 50S, which were re-associated in the presence of tRNA, however, failed to exhibit any promising candidates for interferences with 70S re-association (Arto Pulk, Ü.M., unpublished data). Such a disappointing result can be discussed in two ways.

First, it is possible that no 23S rRNA 2'OH is important in intersubunit contacts. The author does not think so. Although the intersubunit interactions are currently not understood in atomic detail, analogy with the long-range intramolecular interactions suggests, that the A-minor motifs could contribute in much larger numbers to intersubunit interactions, than do the long-range base-pairings (Nissen et al. 2001). Unlike WC base-pairs, which do not use the 2'OH in hydrogen bonding, all types of A-minor interactions do so (Nissen et al. 2001). Therefore, it is likely that 2'-hydroxyls play a functionally important role in ribosomal subunit association.

Secondly, it is possible that the 70S re-association conditions used in our study do not allow for the potential interferences to occur. In other words, loss of every single 2'OH interaction in re-associated modified 70S is compensated by the remaining interactions in the experimental conditions used. This is entirely possible because, due to variability of the efficiency of reconstituted 50S re-association ability, we could not find consistent experimental conditions for suboptimal 70S re-association. Although replications of the reconstitution experiments produced 50S subunits, which exhibited quite consistent puromycin reaction activities, their ability to form 70S ribosomes (when incubated with 30S subunits and tRNA) was much more variable (Ü.M., A.P., unpublished data). It may therefore well be, that the *T. aquaticus* reconstitution system is more suitable for studying events occurring in the PTC (for example, see Polacek et al. 2001, 2003) than for more dynamic aspects of translation (like 70S association or translocation). Indeed, native *T. aquaticus* ribosomes perform rather poorly in existing *in vitro* translation systems (Tanel Tenson, personal communication).

CONCLUSIONS

1. Mutations of 23S rRNA in the acceptor site of the large ribosomal subunit (at positions 2582 and 2583) increase peptidyl-tRNA dissociation rates from the ribosomes *in vivo*. They also lead to strong inhibition of the cell growth, and of the protein synthesis in the elongation phase of translation. We believe that abnormal tRNA 3'CCA-end binding to the mutated ribosomal A site leads to defective elongation phase of translation, which is manifested in increased peptidyl-tRNA drop-off, and may in its turn lead to the observed growth defects.
2. The intactness of the N1 positions of 23S rRNA adenosines at positions 715, 1912 and 1918 is essential for the ability of the 50S ribosomal subunits to associate with the 30S subunits *in vitro*. We propose that adenosines 715, 1912 and 1918 directly interact with 16S rRNA via A-minor interactions with specific base-pairs.
3. We developed a method for incorporation of nucleoside phosphorothioates into the functionally active *Thermus aquatiqus* 23S rRNA. The presence of phosphorothioates can be determined by specific iodine-cleavages at the the sugar-phosphate backbone positions, where non-bridging oxygens are substituted with sulfur ions. Phosphorothioate-containing *in vitro* synthesized 23S rRNAs were reconstituted into functional 50S subunits, which are reasonably active in the peptidyl transferase model reaction with puromycin. We further tested the feasibility of the assay system by determining, which RNA positions in the domain I of the phosphorothioate-containing 23S rRNA become protected from iodine-cleavage in the reconstituted 50S subunits. We found 80 specific protections, the majority of which were within known protein binding areas, as determined by the crystallographic studies. Therefore, the phosphorothioate-containing reconstituted 50S subunits are functionally active and structurally homogeneous, making them suitable for footprinting various ligand-ribosome complexes and for functional studies in the modification interference idiom.

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SUMMARY IN ESTONIAN

Uurimus bakteri ribosoomi struktuuri-funktsiooni seostest

Valgusünteesi läbi viiv makromolekulaarne kompleks, ribosoom, on ensüümide maailmas tõeline hiiglane. Kui tüüpilse valgulise ensüümi molekulmass jääb paarikümne ja paarisaja kilodaltoni vahele siis 200–250 Å diameetriga bakteriaalne ribosoom kaalub u. 2,6 MDa. Ribosoom koosneb kahest alamühikust, millest mõlemal on funktsionaalselt oluliseks komponendiks suure molekulmassiga RNA molekul. Lisaks kuulub mõlema alamühiku koosseisu paarkümmend erinevat valgu molekuli, millest enamiku funktsioon piirdub suurte ribosoomi RNAde kompaktse kolmemõõtmelise struktuuri stabiliseerimisega. Ribosoomi suure alamühiku põhifunktsioon on peptiidsideme moodustumise katalüüs. Lisaks paikneb suures alamühikus põhiline translatsioonifaktorite sidumiskoht, mis on ka suuteline aktiveerima GTP hüdrolüüsi translatsioonifaktoritel. Ribosoomi väike alamühik tegeleb mRNA nukleotiidsesse järjestusse kodeeritud geneetilise info tõlkimisega valgu aminohappejärjestuseks. Täpne tõlge saavutatakse tRNA vahendusel, mille antikoodoni paardumine väikeses alamühikus paikneva mRNA koodoniga viib tRNA külge aheldatud aminohappe suure alamühiku katalüütilisse keskusesse. Peptiidsideme moodustumise tagajärjel tekkiv produkt on ühe aminohappe võrra pikenenud peptidüül-tRNA. Seega, et saavutada pika polüpeptiidahela sünteesi, peab ribosoom töötama protsessiivselt ja tagama suunatud substraadivoo läbi ensüümi (aminoatsüül-tRNA siseneb ribosoomi akseptor-saiti, liigub sealt peale peptiidijäägi ülekannet peptidüül-tRNA kujul doonor-saiti ning peale järmist peptiidi ülekannet deatsüleeritud kujul exit-saiti). Arvatavasti on oluline põhjus, miks ribosoom koosneb kahest alamühikust, just substraadivoo ja sellega seotud protsessiivsuse tagamine.

Suurem osa eksperimentaalsest tööst, millel põhineb käesolev dissertatsioon, tegeleb ribosoomi alamühikute vaheliste kontaktide ning translatsiooni protsessiivsuse uurimisega. Lisaks kirjeldan eksperimentaalset süsteemi, mis võimaldab lülitada kunstlikke nukleotiidianalooge funktsionaalselt aktiivsetesse ribosoomi RNA-desse ja nende edukat kasutamist ribosomaalsete valkude seondumiskohtade määramisel 23S ribosomaalse RNA esimeses struktuurses domäänis. Minu poolt saavutatud põhitulemused on järgmised:

1. 23S rRNA mutatsioonid, mis asuvad ribosoomi suure alamühiku akseptor-saidis (sinna seondub aminoatsüül-tRNA) põhjustavad peptidüül-tRNA dissotsatsiooni valku sünteesivalt ribosoomilt. See tähendab, et muutused suure alamühiku katalüütilises keskuses paikneva tRNA aminoatsüleeritud otsa kontaktides ribosoomiga viivad koodon-antikoodon interaktsiooni kadumisele *ca* 75 Å kaugusel asuvas ribosoomi väikses alamühiku dekodeerivas keskuses ja seega valgusünteesi protsessiivsuse langusele.

Ribosoomi kristallograafilised ja elektronmikroskoopilised uuringud on viinud keskmise lahutusega (5.5–10 Å) mudeliteni kus funktsionaalse ribosoomi alamühikute vahel on postuleeritud tublisti üle 30 molekulaarse kontakti. Kristallograafial ja elektronmikroskoopial põhinevate uuringute abil on raske teada saada, kas mõni postuleeritud kontaktidest on funktsionaalselt olulisem kui teised.

2. Käesolev töö näitab, et ribosoomi suure alamühiku RNA kolme adenosiini metüleerimine N1 positsioonist muudab modifiteeritud alamühikud võimetuks kasutatud katsetingimuste juures väikese alamühikuga seonduma. Seega osalevad need kolm adenosiini funktsionaalselt olulistes interaktsioonides ribosoomi väikese alamühikuga. Olemasolevatele struktuursetele andmetele toetudes püstitasime hüpoteesi, mille kohaselt kolm 23S rRNA adenosiini (A715, A1912 ja A 1918), mille modifitseerimine segab 70S ribosoomi assotsatsiooni, interakteeruvad 16S rRNAga A-minor tüüpi interaktsioonide läbi. Kõik kolm adenosiini asuvad eelnevalt väljapakutud alamühikute kontaktalades. Neist kaks asuvad 23S rRNA 69. juuksenõelas, mis lisaks väikesele alamühikule kontakteerub nii ribosoomi akseptor-saiti kui doonor-saiti seotud tRNAga. Seega on 69. juuksenõel tugev kandidaat osalemaks valgusünteesi käigus ribosoomi suure ja väikse alamühiku töö koordineerimisel.

Natiivsete ribosoomide töötlemine erinevaid nukleotiide osi modifitseerivate keemikaalidega on läbi ribosoomiuuringute ajaloo andnud tähtsat teavet ribosoomi struktuuri ja erinevate substraatide seondumiskohtade kohta ribosomaalsel RNAl. Selle lähenemise puudusteks on vähene erinevate modifikatsioonide hulk, mida on võimalik rRNAsse viia. Samuti on probleemiks paljude nukleotiidide kättesaamatus modifikaatoritele, mis tuleneb ribosoomi kompaktsest ehitusest. Selle tõttu ei ole paljud funktsionaalselt olulised positsioonid rRNAs keemilise modifitseerimise teel analüüsitavad.

3. Käesolevas töös kirjeldatakse eksperimentaalset süsteemi, mis põhimõtteliselt võimaldab lülitada funktsionaalsete ribosoomide koosseisu laia valikut erinevaid nukleotiidianalooge ning neid 23S rRNAs detekteerida. Detektsioonisüsteem põhineb modifitseeritud nukleotiidi α-fosfaadi asendamisel fosforotioaadiga, mis viib katseklaasis sünteesitud RNAs modifitseeritud nukleotiidi kohal suhkur-fosfaat selgroos mittesildava hapniku asendusele väävliga. Jooditöötlusega on võimalik mittesildava väävli juurest RNA selgroog katkestada ja RNAst sõltuva DNA polümeraasi (pöördtranskriptaasi) abil ka katkestused lokaliseerida. Me sünteesisime *in vitro Thermus aquaticus*e 23S rRNA lülitades sinna 3–5% ulatuses ühte neljast αS-NTPst ning ühte neljast αS-desoksü-NTPst. Välja arvatud tümidiini või selle derivaate sisaldavate transkriptide korral, suutsime me natiivseid *T. aquaticuse* ribosomaalseid valke kasutades kõigist modifit-

seeritud RNAdest rekonstrueerida aktiivsed 50S ribosomaalsed alamühikud. Rekonstrueeritud 50S alamühikuis joodiga töödeldes osutusid joodi eest kaitstuks eelkõige 23S rRNA 1. struktuurse domääni osad, millele seonduvad ribosomaalsed valgud. Seega on modifitseeritud ja rekonstrueeritud 50S alamühikud piisavalt homogeensed ning funktsionaalselt aktiivsed, et neid saab kasutada substraatide sidumiskohtade kaardistamisel ribosoomil, samuti üksikute nukleotiidimodifikatsioonide poolt erinevatele valgusünteesi etappidele avaldatava mõju kindlakstegemisel.

ACKNOWLEDGMENTS

I gratefully acknowledge the co-authors of the experimental work presented in this dissertation for their many and varied contributions. Merike Leego, Eva-Liis Loogväli, Arto Pulk, Jaanus Remme, Urmas Saarma, your heroism in putting up with me will never be forgotten.

Jaanus Remme has been a supervisor always available for help and discussion.

Rya Ero, Silja Kuusk, Eva-Liis Loogväli, Jaanus Remme, Juhan Sedman and Tanel Tenson have all made a brave effort of critically reading the manuscript. It helped.

Over the years, I have been fortunate to use both mental and material resources, not only of Jaanus's lab, but also of the labs of Juhan Sedman, Tanel Tenson, Maia Kivisaar, Mart Ustav, Andres Metspalu, and Richard Villems. Members of these labs, and others, make up the friendly bustle that is our institute.

I also thank Aivar Liiv for the music, Ivar Ilves for the poetry, Tõnu Margus for the good times, Kai Virumäe for her stern but motherly love and Tanel Tenson for being mostly harmless.

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CURRICULUM VITAE

Ülo Maiväli

Date of birth:	10.26.1972
Address:	Institute of Molecular and Cell Biology, Tartu University, Vanemuise st. 46–113, Tartu, Estonia
E-mail:	ymaivali@ebc.ee
Phone:	+372 7 375 032

Education

1991	Tallinn 37th secondary school
1995	B.Sc Tartu University, Institute of Molecular and Cell Biology
1997	M.Sc Tartu University, Institute of Molecular and Cell Biology
1997–2003	Graduate studies in Tartu University, Institute of Molecular and Cell Biology

Professional employment

2003– …	Tartu University, Institute of Molecular and Cell Biology, researcher.

CURRICULUM VITAE

Ülo Maiväli

Sünniaeg ja koht:	26.10.1972, Tallinn, Eesti
Kodakondsus:	Eesti
Perekonnaseis:	vallaline
Aadress:	TÜ MRI, molekulaarbioloogia õppetool, Vanemuise 46–113, Tartu, Eesti
Telefon:	+372 7 375 032
E-mail:	ymaivali@ebc.ee

Haridus

1991	Jaan Kreuksi nimeline Tallinna 37. keskkool
1995	Bakalaureus TÜ bioloogia-geograafia teaduskonna molekulaarbioloogia-geneetika erialal.
1997	Magister TÜ bioloogia-geograafia teaduskonna molekulaarbioloogia erialal.
1997–2003	TÜ MRI doktorant

Erialane teenistuskäik

2003–...	TÜ MRI teadur

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