Accuracy of protein synthesis and its tuning by mRNA modifications

GABRIELE INDRISIUNAITE
The ribosome is a large macromolecular complex that synthesizes all proteins in the cell in all kingdoms of life. Proteins perform many vital functions, ranging from catalysis of biochemical reactions to muscle movement. It is essential for cells and organisms that proteins are synthesized rapidly and accurately. This thesis addresses two questions regarding the accuracy of protein synthesis. How do bacterial and eukaryotic release factors ensure accurate termination? How do mRNA modifications affect the accuracy of bacterial protein synthesis?

Bacterial release factors 1 (RF1) and 2 (RF2) are proteins that recognize the stop codons of mRNA and catalyze the release of a synthesized protein chain from the ribosome. It has been proposed that RFs ensure accurate termination by binding to the ribosome in an inactive, compact conformation and acquiring a catalytically active, extended conformation only after recognizing a correct stop codon. However, the native compact conformation was too short-lived to be captured by conventional structural methods. We have developed a fast-kinetics approach for determining when the RFs are in a compact conformation on the ribosome and then used time-resolved cryogenic electron microscopy to capture the compact conformations of native RF1 and RF2 bound to a stop codon. We have also measured the effect of eukaryotic release factor 3 (eRF3) on the rate and accuracy of peptide release by eukaryotic release factor 1 (eRF1) in a yeast (Saccharomyces cerevisiae) in vitro translation system.

Modifications of mRNA nucleotides are post-transcriptional regulators of gene expression, but little is known about their role in protein synthesis. We have studied the effect on accuracy of protein synthesis by two of these modifications: 2'-O-methylation and N6-methylation of adenine. 2'-O-methylation greatly reduced the maximal rate (kcat) and efficiency (kcat/Km) of cognate (correct) codon reading by decreasing the initial GTPase activity in elongation factor Tu and enhancing proofreading losses of cognate aminoacyl-tRNAs. Remarkably, N6-methylation reduced the efficiency of codon reading by cognate aminoacyl-tRNAs and release factors, leaving the efficiency of the corresponding non-cognate reactions much less affected.

Keywords: Ribosome; Protein synthesis; Translation; Accuracy; Release factor; Termination; mRNA modifications

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ISSN 1651-6214
ISBN 978-91-513-0667-4
urn:nbn:se:uu:diva-382490 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-382490)

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*First authors with equal contribution

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<tr>
<td>PrmC</td>
<td>release factor glutamine methyltransferase</td>
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<td>RC</td>
<td>ribosomal release complex</td>
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<td>RF</td>
<td>release factor</td>
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<td>Tyr</td>
<td>tyrosine</td>
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**Note:** The table contains abbreviations and their full forms related to molecular biology, specifically focusing on RNA processing and translation initiation.
Introduction

Each cell and organism store the information about how to be a cell or an organism in their chromosomes, made of deoxyribonucleic acid (DNA). DNA is used for storing genetic information and for passing it on to the next generation. Before each cell division its DNA is duplicated by DNA polymerases in a process called replication. After cell division, each of the daughter cells inherits a copy of their parent cell’s DNA. The DNA is used as a template for transcription into messenger ribonucleic acid (mRNA). Each nucleotide triplet in mRNA encodes one amino acid. During protein synthesis (translation) the mRNA sequence is used as a template to synthesize a chain of amino acids linked together by peptide bonds to form a polypeptide. A functional protein can be made of one or more polypeptides. The genetic code that defines which codon corresponds to which amino acid is universal to all living beings. The flow of information from DNA through RNA to proteins is called the central dogma of molecular biology (Figure 1), formulated by Francis Crick (Crick, 1970) and occurs in all kingdoms of life. Also, there are some special cases of information transfer, usually employed by viruses, when RNA is synthesized from RNA or DNA from RNA.

Figure 1. The central dogma of molecular biology describes the flow of information in biological systems. Solid lines – flows general to all organisms, dotted lines – special cases.

It is essential for cells and organisms that proteins are synthesized rapidly and accurately. Proteins perform many vital functions, ranging from catalysis of biochemical reactions to muscle movement. They work as regulators of gene expression, molecule transporters, receptors and structural components, such as keratin in skin and hair. Proteins also participate in making other proteins. Together with ribosomal RNA (rRNA) they form ribosomes that synthesize all proteins in the cell.

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The ribosome

The ribosome is a large macromolecular complex made of proteins and rRNA. Essentially, it is an enzyme that converts its substrate (aminoacyl-tRNAs) to a product (polypeptide). The sequence of a polypeptide is determined by mRNA template. Amino acids are delivered to the ribosome by transfer RNAs (tRNAs). Each aminoacyl-tRNA has an amino acid covalently attached to its acceptor stem and a trinucleotide anticodon, complementary to the mRNA codon of that amino acid. The ribosome recognizes correct tRNAs by checking the geometry of codon-anticodon interaction. Aminoacyl-tRNAs are charged with appropriate amino acids by specific enzymes, aminoacyl-tRNA synthetases.

There are two types of mRNA codons—sense codons that encode amino acids and are recognized by their respective tRNAs and stop codons that signal the end of a polypeptide chain and are recognized by release factors (RF). The RFs then release the finished peptide from the P-site tRNA. The codon-anticodon interaction can be either correct (cognate) or incorrect (non-cognate). If non-cognate interaction is mistakenly recognized as cognate, wrong amino acid is incorporated or peptide is released on a sense codon. Such translation errors can produce inactive or even toxic proteins. A special case of non-cognate interaction is near-cognate: when a codon differs from the correct codon by only one nucleotide.

The ribosome is made of two subunits: the small and the large. Ribosomes and their subunits are named after their sedimentation coefficients in Svedberg units (S). For example, 70S bacterial ribosome is made of 50S and 30S subunits, while a larger 80S eukaryotic ribosome is made of 60S and 40S subunits. The size and composition of the subunits slightly vary between different kingdoms of life, but all ribosomes have a conserved common core that performs the key processes of protein synthesis (Yusupova and Yusupov, 2014). The small subunit contains the decoding center (DC), where the codon-anticodon interaction is monitored. The large subunit contains the peptidyl transferase center (PTC), where the chemical catalysis of peptide bond formation or peptide release from the P-site tRNA occurs. Also, the ribosome has three binding sites that span both subunits. The A site is the binding site for the incoming aminoacyl-tRNA, the P site harbors the peptidyl-tRNA and the E site is where decylated tRNA binds before dissociating from the ribosome.

Protein synthesis

Protein synthesis can be divided into four phases: initiation, elongation, termination and recycling. In each phase the ribosome is assisted by a different set of protein factors. In short, during initiation the ribosome is assembled on the mRNA, and the ribosome has three binding sites that span both subunits. The A site is the binding site for the incoming aminoacyl-tRNA, the P site harbors the peptidyl-tRNA and the E site is where decylated tRNA binds before dissociating from the ribosome.

The ribosome

The ribosome is a large macromolecular complex made of proteins and rRNA. Essentially, it is an enzyme that converts its substrate (aminoacyl-tRNAs) to a product (polypeptide). The sequence of a polypeptide is determined by mRNA template. Amino acids are delivered to the ribosome by transfer RNAs (tRNAs). Each aminoacyl-tRNA has an amino acid covalently attached to its acceptor stem and a trinucleotide anticodon, complementary to the mRNA codon of that amino acid. The ribosome recognizes correct tRNAs by checking the geometry of codon-anticodon interaction. Aminoacyl-tRNAs are charged with appropriate amino acids by specific enzymes, aminoacyl-tRNA synthetases.

There are two types of mRNA codons—sense codons that encode amino acids and are recognized by their respective tRNAs and stop codons that signal the end of a polypeptide chain and are recognized by release factors (RF). The RFs then release the finished peptide from the P-site tRNA. The codon-anticodon interaction can be either correct (cognate) or incorrect (non-cognate). If non-cognate interaction is mistakenly recognized as cognate, wrong amino acid is incorporated or peptide is released on a sense codon. Such translation errors can produce inactive or even toxic proteins. A special case of non-cognate interaction is near-cognate: when a codon differs from the correct codon by only one nucleotide.

The ribosome is made of two subunits: the small and the large. Ribosomes and their subunits are named after their sedimentation coefficients in Svedberg units (S). For example, 70S bacterial ribosome is made of 50S and 30S subunits, while a larger 80S eukaryotic ribosome is made of 60S and 40S subunits. The size and composition of the subunits slightly vary between different kingdoms of life, but all ribosomes have a conserved common core that performs the key processes of protein synthesis (Yusupova and Yusupov, 2014). The small subunit contains the decoding center (DC), where the codon-anticodon interaction is monitored. The large subunit contains the peptidyl transferase center (PTC), where the chemical catalysis of peptide bond formation or peptide release from the P-site tRNA occurs. Also, the ribosome has three binding sites that span both subunits. The A site is the binding site for the incoming aminoacyl-tRNA, the P site harbors the peptidyl-tRNA and the E site is where decylated tRNA binds before dissociating from the ribosome.

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mRNA from its small and large subunits, a start codon (AUG) on mRNA is located and a specialized initiator tRNA, charged with amino acid methionine, is delivered to the P site. During elongation, the ribosome moves along mRNA, decoding each codon and incorporating a corresponding amino acid into a growing polypeptide chain. When the ribosome encounters one of the stop codons on mRNA (UAA, UAG or UGA), a protein release factor binds to the A site and cleaves the finished polypeptide from the P-site tRNA. During recycling phase the ribosome is split by recycling factors and the subunits can be used for the next round of initiation. The phases of protein synthesis most relevant to the work presented in this thesis (elongation and termination) are discussed in more detail in the following chapters.

**Elongation**

Bacterial elongation starts with a 70S initiation complex, formed during initiation phase: initiator Met-tRNA^fMet is bound to the start codon (AUG) in the P site of the ribosome and the second codon is displayed in the A site. Elongation is a cyclic process consisting of two main events. Incorporation of each amino acid into a growing peptide chain is followed by translocation: movement of the ribosome along the mRNA by one codon. The steps of one amino acid incorporation are shown in Figure 2. Each codon is read by a corresponding tRNA that enters the A site as part of a tRNA-ribosome complex (T) consisting of aminoacyl-tRNA (aa-tRNA), elongation factor Tu (EF-Tu) and guanosine triphosphate (GTP). At this stage the aa-tRNA is bound to the ribosome in A/T conformation: its anticodon is base-paired with mRNA codon in the A site, but the acceptor stem is bound to EF-Tu and cannot interact with the PTC (Schmeing et al., 2009; Valle et al., 2003). Codon-anticodon interaction is checked by monitoring bases: A1492, A1493 and G530 of 16S mRNA (Ogle et al., 2001). They sense the geometry of the first two base pairs of the codon-anticodon helix. If base pairing is incorrect, there is a high probability for this aa-tRNA to dissociate from the ribosome. If base pairing is correct, they form stable interactions with the minor groove of the codon-anticodon helix (Ogle et al., 2001). This sends a signal for EF-Tu to hydrolyze GTP. After GTP hydrolysis, EF-Tu dissociates from the ribosome and the aa-tRNA can accommodate in the A site. Its acceptor stem moves to interact with the PTC. When the aa-tRNA is accommodated, the amino group of its amino acid performs a nucleophilic attack on the carbonyl carbon of the ester bond connecting the nascent peptide to the P-site tRNA. Thus the P-site tRNA is deacylated, the peptide is lengthened by one amino acid and transferred to the A-site tRNA. Deacylation of the P-site tRNA causes the ribosome to enter a ratcheted state, meaning that its subunits are rotated in relation to each other (Frank and Agrawal, 2000). This relative rotation brings the tRNAs into a hybrid state (A/P or P/E). The anticodon stem of the A-site tRNA remains in the A site on the 30S subunit, but the anticodon interaction is checked by monitoring bases: A1492, A1493 and G530 of 16S mRNA (Ogle et al., 2001). They sense the geometry of the first two base pairs of the codon-anticodon helix. If base pairing is incorrect, there is a high probability for this aa-tRNA to dissociate from the ribosome. If base pairing is correct, they form stable interactions with the minor groove of the codon-anticodon helix (Ogle et al., 2001). This sends a signal for EF-Tu to hydrolyze GTP. After GTP hydrolysis, EF-Tu dissociates from the ribosome and the aa-tRNA can accommodate in the A site. Its acceptor stem moves to interact with the PTC. When the aa-tRNA is accommodated, the amino group of its amino acid performs a nucleophilic attack on the carbonyl carbon of the ester bond connecting the nascent peptide to the P-site tRNA. Thus the P-site tRNA is deacylated, the peptide is lengthened by one amino acid and transferred to the A-site tRNA. Deacylation of the P-site tRNA causes the ribosome to enter a ratcheted state, meaning that its subunits are rotated in relation to each other (Frank and Agrawal, 2000). This relative rotation brings the tRNAs into a hybrid state (A/P or P/E). The anticodon stem of the A-site tRNA remains in the A site on the 30S subunit, but the anticodon interaction is checked by monitoring bases: A1492, A1493 and G530 of 16S mRNA (Ogle et al., 2001). They sense the geometry of the first two base pairs of the codon-anticodon helix. If base pairing is incorrect, there is a high probability for this aa-tRNA to dissociate from the ribosome. 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ratcheted ribosome is bound by elongation factor G (EF-G) and GTP. GTP
hydrolisis by EF-G facilitates the movement of the mRNA by one codon in
relation to the ribosome, the tRNAs enter P/P and E/E states and the ribo-
some returns to a non-ratcheted state. EF-G-GDP dissociates, leaving the
ribosome ready for the next ternary complex.

Elongation is the most conserved phase of protein synthesis and proceeds
essentially in the same way in eukaryotes, as described above for bacteria.
Eukaryotic elongation factor 1A (eEF1A) and elongation factor 2 (eEF2) are
structurally and functionally similar to their bacterial counterparts EF-Tu and
EF-G, respectively (reviewed in Dever et al., 2016). Fungi have an addi-
tional elongation factor 3 (eEF3) (Skogerson and Wakatama, 1976). Its func-
tion is not completely clear, but eEF3 seems to facilitate the release of decay-
ylated tRNA from the E site (Triana-Alonso et al., 1995).

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Termination of protein synthesis occurs when a translating ribosome encoun-
ters one of the three stop codons (UAA, UAG, UGA) on mRNA. These co-
dons do not have corresponding tRNAs, but are recognized by protein re-
lease factors (RFs). Prokaryotes have two release factors with overlapping
stop codon specificity: release factor 1 (RF1) recognizes UAA and UAG
codons, while release factor 2 (RF2) recognizes UAA and UGA (Scollnick et
al., 1968). Unlike bacteria, eukaryotes have one class I release factor, eRF1,
which recognizes all three stop codons (Konecki et al., 1977). RF1 and RF2
share high sequence (Shin et al., 2004) and structural (Petry et al., 2005)
similarity, but have different codon recognition motifs: PA/V/T (proline-
adenine-valine-threonine) in RF1 and SPF (serine-proline-phenylalanine) in
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coding center of the 3OS subunit of the ribosome. Eukaryotic eRF1 has a different structure from prokaryotic RFs (Song et al., 2000) with N-terminal domain harboring several conserved codon recognition motifs: Asn-Ile-Lys-Ser (NlKS), YxGxGdyF (Tyr, Cys, Phe, x- amino acid) and Gly-Thr-Ser (GTS) (Bulynin et al., 2010; Chavatte et al., 2002; Frolova et al., 2002). The stop codon adopts a compact U-turn conformation within a pocket formed by eRF1 and the ribosome which also pulls the 4th nucleotide into the A site (Brown et al., 2015; Mathiesi et al., 2015).

Remarkably, class I release factors in all kingdoms of life share a conserved catalytic glycine-glycine-glutamine (GGQ) motif (Frolova et al., 1999). During peptide release the GGQ is placed in the peptidyl transferase center of the large ribosomal subunit (Perry et al., 2005; Rawat et al., 2003). The glutamine residue of the GGQ motif coordinates a nucleophile that attacks the ester bond connecting the nascent peptide to the P-site tRNA (Song et al., 2000). The nature of the nucleophile is not completely clarified. A water molecule (Song et al., 2000) or OH- ion (Kahlenkoetter et al., 2011) has been suggested to perform this function. The glutamine residue of the GGQ is N-methylated in bacterium (Dinches-Brenqvist et al., 2000) and eukaryotic (Heurgue-Hamard et al., 2005) class I RFs. In bacteria it is methylated by methyltransferase pmcF (Heurgue-Hamard et al., 2002). This modification moderately (around 2-fold) increases termination rate (Dinches-Brenqvist et al., 2000).

An overview of termination is presented in Figure 3. After peptide release, RF1 and RF2 are recycled by a class II release factor, the GTP-ase RF3 (Freistroffer et al., 1997; Goldstein and Caskey, 1970). RF3 accelerates the dissociation of RF1 and RF2 from the ribosome in a GTP-dependent manner, but has no effect on the rate of peptide release (Freistroffer et al., 1997). In contrast, eukaryotic class II release factor 3 (eRF3) significantly increases the rate of peptide release by eRF1 (Alkalaeva et al., 2006; Eyler et al., 2013). RF3 is not required for cell viability (Grentzmann et al., 1994), while eRF3 is an essential protein (Chao et al., 2003; Ter-Avanesyan et al., 1993). The catalytic rate of eukaryotic termination is also accelerated by ribosome recycling factor ABCE1 (Rli1 in yeast) (Pisarev et al., 2010; Shoemaker and Green, 2011).

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The role of eRF3 in eukaryotic termination

Termination of eukaryotic protein synthesis is more complex than the corresponding process in bacteria and employs more protein factors. The role of each factor and the sequence of termination events in eukaryotes have been extensively studied in recent years (reviewed in Hellen, 2018; Jackson et al., 2012), but major questions remain unanswered. One of them is how eRF1 recognizes all three stop codons (UAA, UAG, UGA), but effectively discriminates against the tryptophan-encoding UGG codon.

It has been proposed that stop codon recognition and peptide release by eRF1 is coupled by GTPase activity of eRF3 (Salas-Marcos and Bedwell, 2004). eRF3 forms a complex with eRF1 in solution (Stansfield et al., 1995; Zhouravleva et al., 1995) and has been proposed to enter the ribosomal A site as a part of the eRF1-eRF3-GTP complex (Alkalaeva et al., 2006). However, in their recent paper Beissel and coworkers suggest that helicase Dbp5, and not eRF3, delivers eRF1 to the already eRF3-bound ribosome (Beissel et al., 2019). eRF3 accelerates peptide release by eRF1 (Alkalaeva et al., 2006; Shoemaker and Green, 2011; Zhouravleva et al., 1995), especially when eRF1 is in sub-stoichiometric concentrations to the ribosomes (Eyler et al., 2013). When bound on the ribosome in a pre-termination complex with eRF3 and non-hydrolysable GTP analog, eRF1 is in catalytically inactive conformation with the GGQ motif away from the PTC (Preis et al., 2014). However, eRF1 is in catalytically active conformation when bound on the ribosome in a post-termination complex with the eukaryotic ribosome recycling factor ABCE1 (Brown et al., 2015; Preis et al., 2014). These findings are compatible with a model where GTP hydrolysis by eRF3 induces a conformational change to catalytically active conformation in eRF1 (Figure 4). This change is followed by A-site binding of ABCE1 and probably dissociation of eRF3 (reviewed in Hellen, 2018). ABCE1 then stimulates the catalytic step of peptide release (Shoemaker and Green, 2011) and after peptide release splits the ribosome into subunits (Pisarev et al., 2010; Shoemaker and Green, 2011).

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The present work

The aim of this thesis is to answer questions regarding the accuracy of protein synthesis with main focus on bacterial termination. We discuss the hypothesis that a conformational change in RF1 and RF2 increases the accuracy of termination in bacteria. We introduce a rapid kinetics method to determine the rate of such a conformational change (Paper I). This method is combined with time-resolved cryogenic electron microscopy (time-resolved cryo-EM) to obtain the real-time distribution of native structures of release factors on the ribosome, prior and post to the stop-codon dependent conformational change (Paper II). We also discuss the effect of eRF3 on the rate and accuracy of termination by eRF1. In addition, we describe the effect of different mRNA modifications on the rate of elongation (Paper III) and on the accuracy of elongation and termination (Paper IV) in bacteria.

Methods

*Escherichia coli in vitro translation system*

For all experiments on bacterial protein synthesis we used an *in vitro* translation system with purified components from the translation machinery of *Escherichia coli* (E. coli). Since every component is individually purified, each can be added at specific concentrations or omitted altogether, thus tailoring the translation system for solving each experimental problem. All *in vitro* experiments were performed in polymix buffer, which has been chosen for high translation speed and optimal accuracy, as first demonstrated in poly-phenylalanine peptide synthesis (Jelenc and Kurland, 1979) and later refined for a more varied set of peptides (Zhang et al., 2016). For the measurements of GTP hydrolysis, the polymix was supplemented with an energy regeneration system consisting of ATP, GTP, phosphoenolpyruvate, pyruvate kinase and myokinase. This system increases the rate and accuracy of translation, likely by shifting the steady-state concentrations towards ATP and GTP and reducing the concentrations of their hydrolytic products to near-undetectable levels (Jelenc and Kurland, 1979). Ribosomal release complexes (RCs), used for termination experiments (Papers I, II and IV), were assembled *in vitro* and purified from other components. Since every component is individually purified, each can be added at specific concentrations or omitted altogether, thus tailoring the translation system for solving each experimental problem. All *in vitro* experiments were performed in polymix buffer, which has been chosen for high translation speed and optimal accuracy, as first demonstrated in poly-phenylalanine peptide synthesis (Jelenc and Kurland, 1979) and later refined for a more varied set of peptides (Zhang et al., 2016). For the measurements of GTP hydrolysis, the polymix was supplemented with an energy regeneration system consisting of ATP, GTP, phosphoenolpyruvate, pyruvate kinase and myokinase. This system increases the rate and accuracy of translation, likely by shifting the steady-state concentrations towards ATP and GTP and reducing the concentrations of their hydrolytic products to near-undetectable levels (Jelenc and Kurland, 1979). Ribosomal release complexes (RCs), used for termination experiments (Papers I, II and IV), were assembled *in vitro* and purified from other components. Since every component is individually purified, each can be added at specific concentrations or omitted altogether, thus tailoring the translation system for solving each experimental problem. All *in vitro* experiments were performed in polymix buffer, which has been chosen for high translation speed and optimal accuracy, as first demonstrated in poly-phenylalanine peptide synthesis (Jelenc and Kurland, 1979) and later refined for a more varied set of peptides (Zhang et al., 2016). For the measurements of GTP hydrolysis, the polymix was supplemented with an energy regeneration system consisting of ATP, GTP, phosphoenolpyruvate, pyruvate kinase and myokinase. This system increases the rate and accuracy of translation, likely by shifting the steady-state concentrations towards ATP and GTP and reducing the concentrations of their hydrolytic products to near-undetectable levels (Jelenc and Kurland, 1979). Ribosomal release complexes (RCs), used for termination experiments (Papers I, II and IV), were assembled *in vitro* and purified from other components.
ponents of the translation system by ultracentrifugation on sucrose cushions. Because of their high molecular weight, the ribosomes are pelleted at the bottom of centrifugation tubes. Unbound or loosely bound components with smaller molecular weight, such as protein factors, tRNAs and mRNAs, remain suspended and are removed together with the cushion. Peptidyl-tRNAs were detected. The presence of formyl group on the methionine did not affect peptide synthesis.

Saccharomyces cerevisiae in vitro translation system

80S ribosomes were purified by ultracentrifugation on sucrose cushions from Saccharomyces cerevisiae (S. cerevisiae) strain FY116, deficient in several vacuolar protease genes and a mitochondrial RNAse NUC1 (Gerik et al., 1998). The yeast cells were harvested in an exponential phase of growth. Since we do not study initiation, this stage was simplified by using leaderless mRNA that can initiate on non-split 80S ribosomes and without initiation factors (Andreev et al., 2006). S. cerevisiae ribosomes can accurately initiate on leaderless mRNAs in vivo, although leaderless mRNAs are translated less efficiently than mRNAs with full-length 5' leader sequences (Maicas et al., 1990). The only heterogeneous component used in this study was E. coli initiator tRNA fMet-tRNAfMet labelled with tritium (3H) on the methionine. The products were analyzed by high pressure liquid chromatography (HPLC) and around 50% of the synthesized peptide was the expected fMet-Phe-Tyr, with lower amounts of fMet-Phe and fMet. No other peptides were detected. The presence of formyl group on the methionine did not affect peptide synthesis.

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Quench-flow technique

The quench-flow technique was used to study pre-steady state kinetics of elongation and termination reactions that were too fast to perform by hand. The time resolution of the quench-flow quench-instrument is 2 ms. Equal volumes of two reactants are rapidly mixed together and allowed to react for a pre-selected time while being pushed along a reaction loop (this is the ‘flow’ part of the process). The reaction time depends on the length of the loop and the rate at which the reaction mixture is pushed through it. After a set amount of time the reaction mixture reaches the end of the reaction loop and the reaction is stopped by mixing with a quenching solution (this is the ‘quench’ part of the process). Subsequently, the reaction products at different reaction times are analyzed and quantified by other methods. In the experiments presented here, the quenching solution was formic acid which, in addition to stopping the reaction, also precipitates large RNA molecules. In order to quantify the reaction products, initiator tRNA, Met-tRNA\textsubscript{Met} used for these experiments was labelled with tritium (\textsuperscript{3}H) on the methionine. For termination studies (Papers I, II and IV), precipitated peptide-tRNA was separated from soluble released peptide by centrifugation and the amounts of released and tRNA-bound peptides were determined by liquid scintillation counting of \textsuperscript{3}H radiation. Scintillation liquid absorbs the energy emitted by radioisotopes and re-emits it as a detectable light signal. Through this experimental setup, the average time for all the steps from ternary complex binding to the ribosome up to and including hydrolysis of the ester bond connecting peptide with P-site tRNA can be estimated at high precision. At the same time, the rate of released peptide dissociation from the ribosome is not measured. The method allows for validation of those stopped-flow results, to be described below, in which the rate of peptide dissociation can be rate limiting. For protein elongation studies (Paper III), the reaction products were analyzed by HPLC, equipped with an on line radiation detector, that can separate different peptides with single amino acid resolution. For the measurements of GTP hydrolysis (Paper III), the amounts of \textsuperscript{3}H-GTP and \textsuperscript{3}H-GDP were also determined by HPLC.

Stopped-flow technique

The initial phase of a stopped-flow experiment is similar to that of a quench-flow experiment – equal volumes of two reactants are rapidly mixed, but instead of quenching the reaction at each time point separately, the reaction is continuously monitored in real time in an optical cell. Depending on the setup of the instrument and the reaction products, a change in fluorescence, light absorption or light scattering intensity can be recorded. Since there is no need to take each time point separately, much more points per one reaction curve can be monitored to improve precision. Also, since there is no limit.
washing step between time points, these experiments are performed much faster than the quench-flow experiments, which is advantageous when using easily degradable components or reaction conditions that might cause the components to lose activity (such as high pH). Since most reactions with native components do not cause directly observable changes, reaction components for stopped-flow experiments are often labelled with fluorescent molecules. These molecules are often quite bulky, making it important to validate that the presence of a fluorescent label does not significantly affect the kinetics of a reaction. In the experiments presented here stopped-flow was used for measuring the rate of coumarin-labelled peptide release at different pH values (Paper I) and validated by comparison with quench-flow data.

Accuracy of protein synthesis

The accuracy (A) of an enzymatic reaction is defined as the efficiency (kcat/Km) by which an enzyme forms a reaction product using a correct (cognate) substrate divided by the efficiency by which it forms an incorrect (non-cognate) substrate. Accordingly, the termination accuracy and the total accuracy of elongation are defined as a ratio of the efficiencies of peptide bond formation or peptide release between cognate (c) and non-cognate (nc) reactions:

\[
A = \frac{(k_{\text{cat}}/K_m)_c}{(k_{\text{cat}}/K_m)_nc}
\]

The total accuracy of elongation can be further divided into the accuracy of initial selection (I) and proofreading (F):

\[
A = I \times F
\]

These two steps are separated by an irreversible step of GTP hydrolysis on EF-Tu. I is the accuracy of steps leading to GTP hydrolysis and F is the accuracy amplification in steps following GTP hydrolysis where non-cognate aminoacyl-tRNA dissociates with much higher probability than cognate aminoacyl-tRNA. Initial selection is defined as the ratio of the efficiencies of GTP hydrolysis on EF-Tu, (kcat/Km)GTP , during incorporation of cognate and non-cognate amino acids:

\[
I = \frac{(k_{\text{cat}}/K_m)_{c,GTP}}{(k_{\text{cat}}/K_m)_{nc,GTP}}
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Then the proofreading factor F can be written as:

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The concept of kinetic proofreading was first introduced by Hopfield and Ninio (Hopfield, 1974; Ninio, 1975) and later shown to occur in protein elongation (Ruusala et al., 1982; Thompson and Stone, 1977). It was noticed that the accuracy that results only from the energy difference between cognate and non-cognate codon-anticodon duplexes was too low to account for the observed elongation accuracy. When using kinetic proofreading, the ribosome can employ the same energy difference in several steps, provided that they are separated by an irreversible step, such as GTP hydrolysis. Then the total accuracy of the whole process follows as the product of the accuracies in each step. In the case of elongation, the codon-anticodon interaction is first monitored during initial selection. There is a probability close to one that a cognate aa-tRNA in ternary complex with EF-Tu and GTP passes the initial selection step leading to the triggering of GTP hydrolysis on EF-Tu. At the same time the probability that a non-cognate ternary complex passes the initial selection step to GTP hydrolysis is very low. Furthermore, it was early recognized that the number of GTPs hydrolyzed per non-cognate peptide bond is much larger than the number of GTPs hydrolyzed per cognate peptide bond, leading to the conclusion that non-cognate tRNAs are discarded from the ribosome after GTP hydrolysis with high probability (Ruusala et al., 1982; Thompson and Stone, 1977). Then the accuracy amplification by proofreading, or the proofreading factor F, is the probability that a non-cognate aa-tRNA is discarded from the ribosome after GTP hydrolysis on EF-Tu divided by the corresponding probability for a cognate aa-tRNA.

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Accuracy of termination in bacteria and yeast
Termination of protein synthesis has to be fast and accurate for optimal cell growth. Here, in Papers I and II, we present biochemical and structural evidence that bacterial RF1 and RF2 ensure high termination accuracy by undergoing a conformational change from catalytically inactive to active conformation after recognizing a stop codon. We have also looked into the accuracy of eukaryotic termination and show here that eRF3 increases the accuracy of stop codon recognition by eRF1.

Conformational change in bacterial release factors

Accuracy of bacterial termination
Termination of protein synthesis has to be very accurate, because frequent termination on sense codons would cause accumulation of non-functional or even toxic truncated proteins, harmful to a cell. Unsurprisingly, both in vitro (Jorgensen et al., 1993) and in vivo (Freistroffer et al., 2000) experiments have confirmed that the termination error frequency is indeed very low: around 1 error per 10^5 termination events. However, determining the mechanism used to achieve such high accuracy has not been as straightforward. Identification of RF3, a GTPase participating in termination, lead to the suggestion that the ribosome might increase termination accuracy by employing kinetic proofreading, a mechanism already confirmed for recognition of sense codons by aminoacyl-tRNAs (Ruusala et al., 1982; Thompson and Stone, 1977). However, subsequent experiments demonstrated that this hypothesis is not correct – the presence of RF3 not only does not increase termination accuracy, but even slightly reduces it (Freistroffer et al., 2000). According to molecular dynamics simulations performed by Sund and coworkers, RF-mRNA interactions themselves provide sufficient discrimination power to ensure high termination accuracy (Sund et al., 2010). According to another hypothesis, described in the following chapters, RF1 and RF2 undergo a stop codon-induced conformational change as a mechanism to ensure high termination accuracy (Rawat et al., 2003).

Structure of bacterial release factors 1 and 2
RF1 and RF2 have similar structures and sequences (Youngman et al., 2007). Each one consists of four domains, of which domains II and III are most important for peptide release. Domain I contains a recognition motif (SPF in RF1 and PA(A/V)T in RF2, see above) which interacts with the stop codon on mRNA, placed in the decoding center (DC) of the 30S subunit. The tip of domain III contains the catalytic GGQ motif. In order to release the peptide from the P-site tRNA, the GGQ motif has to reach into the peptidyl-transfer center (PTC) in the 50S subunit of the ribosome.

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The first X-ray structures of RF1 (Shin et al., 2004) and RF2 (Vestergaard et al., 2001) showed the conformations of these factors free in solution. The RFs were in a compact conformation, with the stop codon recognition motif and the GGQ motif just 25 Å apart. However, in early cryo-EM structures of RF1 (Petry et al., 2005) and RF2 (Klaholz et al., 2003; Rawat et al., 2003) on the ribosome both factors were in an extended conformation, with the stop codon recognition and the GGQ motifs 75 Å apart and thus spanning the distance between the DC in the 30S subunit and the PTC in the 50S subunit (Klaholz et al., 2003; Petry et al., 2005). A small-angle X-ray scattering (SAXS) study of E. coli RF1 in solution concluded that RF was most likely in an extended conformation (Vestergaard et al., 2005), same as on the ribosome and contrary to the earlier X-ray structures. It was unclear, whether the compact form was biologically relevant or just an artifact caused by non-native crystallization conditions. However, an X-ray structure of a compact RF in complex with methyltransferase prmt6 demonstrated that the compact form had at least one physiological function (Graille et al., 2005). The Graille et al. study was followed by another SAXS study showing that Thermus thermophilus RF2 was predominantly compact, similar to the X-ray structures in crystal (Zoldak et al., 2007).

Back in 2003, Rawat and coworkers offered a solution to the apparently different structures of free and ribosome-bound RFs that could also be relevant to high accuracy of bacterial termination. They proposed that RF1 and RF2 enter their ribosome in compact conformation and then, in response to a stop codon in the A site, undergo a conformational change to an extended, catalytically active conformation (Rawat et al., 2003). Strict validation of this hypothesis lingered, because at the time there was no experimental method for capturing such short-lived states with sufficient resolution.

Biochemical evidence of the conformational change in RFs (Paper I)

The first evidence from rapid kinetics experiments that there is a conformational change in class I RFs is described in Paper I. We used stopped-flow technique to measure the effect of pH on the maximal rate of termination (kcat) in an E. coli in vitro translation system. Ribosomes containing I Met-Phe-Phe-tRNA75 in the P site and UAA stop codon in the A site (RC0 in Fig. 5a) were rapidly mixed with saturating amounts of releasing factors RF1 or RF2. The methionine in peptide-tRNA (Figure 5a) was labelled with a fluorescing coumarin derivative and the amount of released peptide at different OH ion concentrations was monitored as a change of fluorescence intensity over time. At rate-saturating RF concentration, the association time of RF and the ribosome, 1/(koff[RF]), is very short and thus negligible to the total reaction time. Then the total reaction time is 1/kcat which is the sum of times of the conformational change of the factor, 1/kcat, hydrolysis of the ester bond in peptide-tRNA, 1/kkoff, and the time for peptide dissociation from the ribo-

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some, 1/k_{diss} where k_{diss} is strongly pH-dependent (Kuhlenkoetter et al., 2011; Shaw et al., 2012).

**Figure 5.** Termination in bacteria as described in Paper I. (a) A compact class I RF binds the A site of the ribosomal release complex RC_{0}, forming complex RC·RF_{comp} with association rate constant k_{a} and compounded rate constant k_{a}[RF]. RF undergoes conformational change from compact to extended form with pH-independent rate constant k_{hydr} transforming complex RC·RF_{hydr} to RC·RF_{rel} where the pH-dependent ester bond hydrolysis in peptidyl-tRNA is activated by movement of the universal GQG motif into the PTC. Hydrolysis occurs with pH-dependent rate constant k_{hydr}, leading to complex RC·RF_{post}. Finally, the released peptide dissociates with rate constant k_{diss}. It leads to post-termination complex RC·RF_{post}. The label L is 3H in quench-flow or a fluorescent coumarin derivative in stopped-flow experiments. (b) Maximal rate constant (k_{cat}[y-axis]) as function of OH concentration (x-axis), estimated with 0.04 µM RC_{0} reacting to rate saturating concentrations of unmethylated RF1 at 37⁰C, as measured in stopped-flow. (c) Quench-flow measurement of the same reaction.

At low pH, k_{cat} increased with OH concentration, as observed before (Kuhlenkoetter et al., 2011; Shaw et al., 2012). The linear dependence can be explained either by titration of a catalytically essential group, or by the OH ion itself acting as a nucleophile (Kuhlenkoetter et al., 2011). These two cases cannot be distinguished in our experimental conditions (Paper I). At high OH concentrations, the k_{cat} value became saturated and no longer increased with pH (Figure 5b). This saturation was not observed before (Kuhlenkoetter et al., 2011; Shaw et al., 2012) and we took it to identify the reaction step other than the ester bond hydrolysis becoming rate-limiting. We

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could exclude that $k_c$ [RF] is rate-limiting and tested the possibility of a rate limiting $k_{	ext{cat}}$ at high OH concentrations. Since the main change of fluorescence in stopped-flow experiments occurs when the peptide dissociates from the ribosome into solution, we also applied the quench-flow method to the termination reaction. This method is blind for peptide dissociation from the ribosome after ester bond hydrolysis. Since the saturated $k_{	ext{cat}}$ value at high OH concentration was the same (around 600 s⁻¹; Figure 5c), we concluded that (i) at low pH the rate-limiting step is the ester bond hydrolysis and (ii) at high pH the reaction is limited by a previously unobserved pH-independent step. In principle, another possible explanation could have been a titration of a catalytically essential group in the PTC, but no such groups have been identified. Therefore we suggested that the rate-limiting step at high pH ($k_{	ext{cat}}$) is the previously proposed conformational change in class I release factors upon stop codon recognition (Rawat et al., 2003). Indirect evidence that rate constant $k_{	ext{cat}}$ corresponded to a reaction step, not occurring in the PTC, came from comparing pH dependence of methylated and unmethylated release factors (Paper I). It had previously been shown that methylation of glutamine in the GGQ motif increases the catalytic rate of ester bond hydrolysis around two-fold (Dinpas-Renqvist et al., 2000). In our experiments the $k_{	ext{cat}}$ value was increased by RF-methylation at pH 7.5 (Table 2 in Paper I), but not at high pH (Table 1 in Paper I). This indicated that the saturated rate corresponded to a process not occurring in the PTC.

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**Structural evidence of the conformational change in RFs (Paper II)**

With these results in mind, we set out to capture the structures of compact release factors on the ribosome. The field of structural biology has been rapidly developing since the idea of this conformational change was first proposed and a method capable of capturing the short-lived compact state of ribosome-bound release factors at high resolution has become available. We used time-resolved cryogenic electron microscopy (time-resolved cryo-EM) developed by our coworkers in prof. Joachim Frank’s laboratory (Colombia University, New York, USA).

**Time-resolved cryo-EM combines kinetic and structural studies by capturing biological complexes at different time points of a reaction.**

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These predictions were confirmed by the time-resolved cryo-EM results, even though the fractions of compact RFs were smaller than anticipated from the modelling (Figure 7 d-e). Compact forms of RF1 and RF2 on the ribosome were captured with 3.5 - 4 Å resolution. In the compact structure, the GGQ motif is around 60 Å away from the PTC and positioned next to domain II of the RF and the anticodon stem of the P-site tRNA (Figure 8 a-b). The overall position of domain III is similar to X-ray structures of RFs in solution.

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Figure 6. The maximal rate ($k_{\text{conf}}$) of peptide release by RF2 at different DHF concentrations in 25°C. (a) Extent of RF2-dependent Met-Phe-Phe peptide release from P-site bound Met-Phe-Phe-tRNA (y-axis) versus time (x-axis) at different DHF concentrations. 0.02 µM release complexes were reacted with saturating (0.8 µM) RF2 concentrations. $k_{\text{conf}}$ increased to a plateau value ($k_{\text{conf}} = 10.8 \pm 0.9$ s⁻¹) with increasing DHF (x-axis).

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Figure 7. Time evolution of ribosome ensembles in termination of translation (a) Visualization of the pathway from RF-free release complex (RC0) to peptide release. Compact release factor (RF) binds to RC0 and forms the RC·RF_{compact} complex with compounded rate constant $k_a[R_{RF}]$. Stop codon recognition induces conformational change in the RF which brings the complex RC·RF_{compact} to RC·RF_{extended} with rate constant $k_{conf}$. The ester bond between the peptide and the P-site tRNA is hydrolyzed with rate constant $k_{hydr}$ (b–c) Predicted fractions of ribosomes in RC0, RC·RF_{compact} and RC·RF_{extended} forms (y-axis) as functions of time (x-axis). (d–e) Populations of RC·RF_{compact} (red) and RC·RF_{extended} (blue) at 20 ms, 60 ms and long incubation time points as obtained by time-resolved cryo-EM.
At 60 ms, the GGQ motif is placed in the PTC (Figure 8 d-e). The position of domain III in relation to domain II is changed by a rearrangement of a switch loop region, connecting the domains II and III (Korostelev et al., 2008; Laurberg et al., 2008). In the extended structure the switch loop region is stabilized by interactions with flipped out 18S rRNA nucleotides A1492 and A1493 and ribosomal protein S12 (Figure 8). No interaction with S12 was observed for the compact structures (Figure 8c).

While these results were being prepared for publication, an X-ray structure of a compact RF1 bound to a stop codon was published (Svidritskiy and Korostelev, 2018). Svidritskiy and Korostelev had mutated the switch loop region to slow down the conformational change and used the antibiotic blasticidin S to block the GGQ motif from docking into the PTC. In their structure the decoding region is in a conformation more similar to that of extended RFs in our structures, but the domain III is in a position similar to that in the compact RFs (Supplementary Figure 4b in Paper II). It is possible, that their structure could resemble an intermediate on the pathway from the compact to extended RF (Paper II).
The accuracy of yeast termination

The role of eRF3 in the accuracy of termination by eRF1

We have measured the accuracy of termination by eRF1 as a ratio of efficiencies (kcat/Km) on stop and sense codons in a yeast (S. cerevisiae) in vitro translation system (see Methods). Our preliminary results show that eRF1 alone could not efficiently discriminate between stop codon UAA and sense codon UGG, but terminated with very high accuracy in the presence of eRF3.

0.04 µM of purified 80S release complexes, containing Met-Phe-Tyr-tRNA\(^{29}\) in the P site and UAA or UGG codon in the A site were reacted to increasing amounts of eRF1, either in presence or absence of saturating amounts of eRF3 (10 µM). The reactions were performed in polyimix-HEPES buffer, supplemented with energy regeneration system at 30°C. The experiments were performed either in quench-flow or by hand and stopped at different time points by quenching with formic acid. Precipitated peptidyl-tRNA was separated from soluble released peptide by centrifugation and the amount of released and non-released peptide determined by scintillation counting of \(^{3}H\) radiation.

The plots of released peptide over time were biphasic with the fast phase comprising around 50% of total amplitude (a-b in Figures 9 and 10). Since 50% of release complexes contained Met-Phe-Tyr-tRNA the rest had Met-Phe-of Met-tRNA in the P site and (Methods), we treated the rate constant of the fast phase as corresponding to tripeptide release in our preliminary analysis presented here. The slow phase most likely corresponded to the release of the shorter peptides.

The results showed a strong effect of eRF3 on the accuracy of termination by eRF1. The accuracy was expressed as the ratio of efficiencies (kcat/Km) of peptide release on UAA and UGG codons. On its own, eRF1 discriminated between UAA and UGG with the accuracy of only 4 (Table 1; Figure 9). It means that, assuming equal concentrations of ribosomes containing these two codons, eRF1 would be only 4 times less likely to terminate on UGG than on UAA codon. However, with eRF3 and GTP present, eRF1 could discriminate against UGG with the accuracy of 10,400 (Table 1; Figure 10).

Table 1. Accuracy of termination by eRF1 with and without eRF3.

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Fold increase (+) or decrease (−)

390% | 21% | 2600%

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The accuracy of termination on UGG versus UAA with eRF3 present (10,400; Table 1) was comparable to that measured in an in vitro system with E. coli components: accuracy of 13,000 by RF1 and 2400 by RF2 (Freistroffer et al., 2000). This effect was mainly caused by almost 400-fold increase of k_{cat}/K_m on UAA, but also by moderate reduction of k_{cat}/K_m on UGG (Table 1). This finding is in line with molecular dynamics simulations performed by Lind and coworkers that predict the energetic penalty of read-

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eRF3 also increased the $k_{\text{on}}$ of termination 2-fold: from 0.35 s$^{-1}$ to 0.77 s$^{-1}$ (Table 1), comparable to 4-fold $k_{\text{on}}$ increase reported by Eyler and coworkers for Met-Lys peptide release (Eyler et al., 2013). One would expect the in vivo $k_{\text{on}}$ values to be higher than observed in our experiments, because our in vitro translation system did not include Rli1 and eIF5A, both reported to increase the $k_{\text{on}}$ of peptide release (Schuller et al., 2017; Shoemaker and Green, 2011).

Figure 10. Peptide release by eRF1 in complex with eRF3 and GTP. (a-b) Percentage of released fMet-Phe-Tyr peptide (y axis) over time (x axis, log10 display) at different eRF1 concentrations. (c-d) The rate constant of peptide release ($k_{\text{rel}}$) (y axis) over eRF1 concentration (x axis) on UAA and UGG codons. All reactions were performed with saturating amounts (10 µM) eRF3, 0.04 µM release complexes containing Met-Phc-Tyr-tRNATyr in the P site and indicated codon in the A site in Polymix-HEPES buffer with 2.2 mM GTP (energy system) at 30°C.

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The effect of mRNA modifications on elongation and termination phases of protein synthesis

Modified RNA nucleotides are abundant and functionally important in transfer RNA and ribosomal RNA (reviewed in Ontiveros et al., 2019). Recently, some of these modifications have also been identified in messenger RNA (Table 1 in Nachtergaele and He, 2018) and there is a growing body of evidence for their importance in regulation of gene expression. Less is known about the effect these modifications have on protein synthesis. In this thesis work, we have tested the effect of 2'-O-methylation on elongation (Paper III) and the influence of N6'-methylation on termination (Paper IV) of protein synthesis. The modifications are shown in Figure 7.

![Figure 11. Modifications on mRNA. (a) 2'-O-methylation, (b) N6'-methylation on adenosine. Added methyl groups are shown in red.](Image)

2'-O'-methylation of mRNA in bacterial elongation (Paper III)

Recently, many 2'-O'-methylation sites have been identified in human mRNA: around 2000 sites in HeLa and around 700 in HEK293 cell lines (Dai et al., 2017). 2'-O'-methylation sites are the most common in 5' untranslated and protein coding regions, while the most frequently modified nucleoside is adenine. Added methyl groups are shown in red.

The effect of mRNA modifications on elongation and termination phases of protein synthesis

Modified RNA nucleotides are abundant and functionally important in transfer RNA and ribosomal RNA (reviewed in Ontiveros et al., 2019). Recently, some of these modifications have also been identified in messenger RNA (Table 1 in Nachtergaele and He, 2018) and there is a growing body of evidence for their importance in regulation of gene expression. Less is known about the effect these modifications have on protein synthesis. In this thesis work, we have tested the effect of 2'-O-methylation on elongation (Paper III) and the influence of N6'-methylation on termination (Paper IV) of protein synthesis. The modifications are shown in Figure 7.

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tRNA accommodation are shown in Figure 12. The function of this modification is not completely clarified, but it has been shown to help eukaryotic cells to discriminate between their own and viral mRNAs (Dafnis et al., 2010; Zust et al., 2011) and to mark correctly capped mRNAs, thus protecting them from degradation (Picard-Jean et al., 2018). 2'-O-methylation reduces peptide yield and induce ribosome stalling when the modification is in the second position of the codon (Hoernes et al., 2016) through an unknown mechanism.

In order to obtain a clear picture of how 2'-O-methylation affects different elongation steps, we have used *E. coli* in vitro translation system for measuring initial selection and proofreading values (see Methods) on modified and unmodified codons. Initial selection and proofreading stages of amino acid incorporation are separated by irreversible GTP hydrolysis on EF-Tu. Most non-cognate tRNAs are rejected at the initial selection stage. During the proofreading stage, codon-anticodon interaction is monitored again, so that only cognate aa-tRNAs proceed with high probability to A-site accommodation and peptide bond formation, while the small fraction of remaining non-cognate tRNAs is further culled by dissociation through the proofreading pathway. The steps from binding of the ternary complex, to tRNA accommodation are shown in Figure 12.

We used ribosomes programmed with *H*-labelled initiator Met-tRNA^fMet^ in the P-site and lysine-encoding AAA codon in the A site. This codon was either unmodified (AAA) or 2'-O-methylated at the second position (AAmA). The ribosomes were reacted to a ternary complex (T₃), consisting of Lys-tRNA^Lys^, EF-Tu and GTP. The amounts of peptides formed and GTPs hydrolyzed over time were determined by HPLC.

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The efficiency ($K_{m}/V_{max}$) of initial codon selection was reduced 300-fold on AA,A codon, compared to unmodified AAA (Figure 4 in Paper III). The proofreading factor was increased 5-fold, compared to that of the unmodified codon (Figure 5 in Paper III). These results show that with the AA,A codon in the A site, the cognate Lys-tRNA$^{\text{AAA}}$ was 300 times more likely to be rejected in the initial selection stage and 5 times more likely to be rejected in the proofreading stage. These numbers in combination estimate a total modification-induced efficiency loss in AAA codon reading by its cognate tRNA to a factor of 1500.

Since the modified codon could not be efficiently read by its cognate tRNA, its presence most likely would cause the ribosome to stall. Indeed, single-molecule FRET measurements performed by our co-workers from the Puglisi group (Stanford University, Palo Alto, USA) showed long stalling times on modified codons and multiple unproductive Lys-tRNA$^{\text{AAA}}$ binding events that did not result in peptide bond formation (Paper III). This effect was most likely caused by the modification disrupting the interaction of 18S rRNA monitoring bases with the codon-anticodon helix in the A site (Ogle et al., 2001). Both bulk kinetics and single molecule FRET results showed that the effect of 2’-O-methylation can be reversed by the introduction of error-inducing antibiotics like paromomycin and neomycin (Figure 6 in Paper III). These antibiotics induce translation errors by hyper-activating the monitoring bases (Ogle and Ramakrishnan, 2005).

2’-O-methylated sites present in the untranslated region near the 5’ cap serve as markers indicating that mRNA is correctly capped and native to the cell and thus should not be degraded or induce an immune response (Daffis et al., 2010; Picard-Jean et al., 2018; Zust et al., 2011). Since this region is not translated by the ribosome, the profound reduction of translation rate caused by 2’-O-methylation is of no consequence in this case. It is rather more puzzling that such a modification would also be present in the coding sequences, as has been recently shown (Dai et al., 2017). It is possible, that 2’-O-methylation is used to modulate the translation rate, for example by inducing a pause that would allow a nascent peptide to fold correctly. Alternatively, ribosome stalling at 2’-O-methylated sites might recruit specialized protein factors that enhance decoding of the modified codons, similar to translation of polyproline stretches in bacteria by elongation factor P (EF-P) (Doezter et al., 2013; Ude et al., 2013).

The effect of N6-methylated adenosine in mRNA on bacterial termination (Paper IV)

$N^{6}$-methyladenosine ($m6A$) is the most abundant mRNA modification, present in all three kingdoms of life (reviewed in (Roignant and Soller, 2017)). It is of a particular interest, because abnormal changes in $N^{6}$-methyladenosine

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patterns were shown to play part in the development of human diseases, such as leukemia (Li et al., 2017) and human immunodeficiency virus infection (Kennedy et al., 2016; Lichinchi et al., 2016; Tirumuru et al., 2016). The main function of N⁶-methylation in mRNA seems to be the fine-tuning of gene expression by affecting mRNA stability, alternative splicing and translation in response to changing environment or developmental status (reviewed in Nachtergaele and He, 2018; Roignant and Soiller, 2017). The m⁶A modification induced cognate tRNA rejection during initial selection and proofreading stages of elongation (Choi et al., 2016), but to a much lesser degree than 2'-O-methylation described above. The presence of m⁶A perturbs the canonical A-U base pairing in the codon-anticodon duplex (Choi et al., 2016), an earlier step than the interaction of monitoring bases, disrupted by 2'-O-methylation. Our decision to look into the effect of m⁶A on termination was influenced by studies, showing that m⁶A is enriched around stop codons (Meyer et al., 2012). Here, we used a bacterial in vitro translation system to test the effect of m⁶A on termination by RF2 (Paper IV). Purified ribosomal release complexes containing Met-Phe-Tyr-tRNA⁶⁰ in the P site were reacted with RF2 manually or in a quench-flow instrument, in order to determine the termination rate constants on modified and unmodified codons.

RF2, which normally terminates at UAA and UGA codons, strongly selected against termination at UAG, as shown before (Freistroffer et al., 2000). Indeed, RF2 preferred termination at its cognate UAA codon with an accuracy of 14,000 (Table 2). When the m⁶A modification is present in the 2⁰ position of the non-cognate UAG codon (Um⁶AG), both kcat and kcat/Km values were reduced about 2-fold (Table 2). These results show that the modification has no effect on the rate constant for hydrolysis of the ester bond, connecting the peptide to the P-site tRNA, but affects the efficiency of RF2 binding to the A site and decoding the stop codon. When the m⁶A modification was introduced into the 2⁰ position of the non-cognate UAG codon (Um⁶AG), both kcat and kcat/Km values were reduced about 2-fold (Table 2).

Table 2. Kinetic parameters of peptide release by RF2 on N⁶-methylated codons.

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These results are in agreement with the effect of m6A on the accuracy of translation elongation. N6-methylation reduced the $k_{cat}/K_{m}$ value for cognate peptidyl transfer reaction, leaving the $k_{cat}/K_{m}$ of non-cognate reaction virtually unaltered (Paper IV). These results suggest that m6A affect elongation and termination stages through a similar mechanism, presumably by disrupting the interaction between the mRNA codon and either tRNA anticodon (Choi et al., 2016) or recognition motif of a release factor.
The aim of the work presented in this thesis has been to answer several questions regarding the accuracy of protein synthesis and its tuning by mRNA modifications. The main conclusions from this work are:

1. Both bacterial release factors RF1 and RF2 undergo a conformational change upon binding to the stop programmed ribosome, presumable as a mechanism to ensure accurate termination.

2. Yeast class II release factor eRF3 strongly increases the accuracy of stop codon recognition by eRF1.

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The time-resolved cryo-EM structures of ribosomal complexes with RF1 and RF2 in contact with a stop codon in a compact conformation likely provide the definitive proof that these class I RFs undergo a conformational transition from compact to open form on the native pathway from free factor and ribosome to factor-induced ester bond hydrolysis on the terminating ribosome. In contrast, there are still many unanswered questions regarding the exact order of events in eukaryotic termination and individual contributions of participating protein factors.

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The data on mRNA modifications have been rapidly accumulating in the recent years and it is very likely that more modifications will be identified in the near future. Their function both in translation and in the general regulation of gene expression in the cell have become a new exciting field of research. In due time, it might offer therapeutic solutions to diseases caused by aberrant mRNA modifications.

Conclusions and future outlook
Ribosomen är ett stort makromolekylläkomp lex som syntetisera alla proteiner i alla levande varelserns celler. Proteinerna utför merparten av cellernas livsuppehållande funktioner från katalysering av biokemiska reaktioner till muskelrörelser. En förutsättning för allt liv på jorden är att proteinaenaet sammanfogas snabbt och noggrant från aminosyror.

Denna avhandling fokuserar på två frågor angående proteinaenes noggranhet. Hur kan ribosomens termineringsfaktorer i bakterier (RF1 och RF2) och eukaryoter (RF1/2) avläsas proteinaen med hög precision? Hur påverkar kemiska modifieringar av budbärar-RNA (mRNA) hastighet och noggranhet vid översättning av kodord för aminosyror av trNA och stopsignaler av termineringsfaktorer?

RF1/2 känner igen stopp-kodord i mRNA och frigör syntetiserade proteiner som peptid-kedjor. RF1/2 har ännu ett hög noggrant genom att inaktiv, kompakt form binda till ribosomen och sedan övergå i en aktiv, utsträckt form när termingsfaktorn läser ett stopp-kodon. I naturliga ribosomkomplex termineringsfaktornernas kompakt form kortlivad och har tidigare bara observäts i långlivat tillstånd i närvaro av ribosom-mutationer och inhibitor.

Vi har utvecklat en snabb-kinetikmetod för att estimera tiden RF1/2 förblir kompakt efter ribosom-bindning i närvaro av stopp-kodon och använde sedan tidstasplott kryo-elktronmikroskopi för att visualisera termineringsfaktornernas kompaktuform.

Vi har studerat effekterna av två kemiska modifieringar av mRNA: 2'-O-metylering och N6-metylering av adenosin. 2'-O-metylering minskar kraftigt den maximala hastigheten (kcat) och effektiviteten (kcat/Km) vid kognat (korr.) kodon-läsning genom drastisk reduktion av den GTPaseaktiviteten som är första delen av koden-läsningen och en kraftig ökning av färstutan av kognata trNA genom överdriven korrekturnslutning. Annämningsvärt neg N6-metylering felet vid kodon-läsning genom att minska effektiviteten för kognat kodon-läsning med termineringsfaktorer och trNA, medan de motsvarande icke-kognata reaktionerna påverkas mycket mindre.

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Acknowledgements

My supervisor, Måns, thank you for your time and your science.
My co-supervisor, Suparna, for helping with things big and small.
Zi-ao Jack Fu, Sandip Kaledhonkar and prof. Joachim Frank for the RF conformation project and for teaching me about time-resolved cryo-EM.
Valérie Heurgué-Hamard and Emmeline Huvelle, for having me in their lab and teaching me some things about proteins.
Prof. Erik Johansson and Pia Osterman, for their help with yeast cultures.
Junhong Choi and prof. Jody Puglisi, for the 2'-O-methylation project.
Ka-Weng: for joint projects and answering my questions.
Anneli, Chandu, Jingji, Mikael, for small things that add up.
Ray, for components.
Mamai, tėčiui, Rimai, Simona, Aurelijai, Gedre, Rūtai ir Simonai, ačiū už viską!

Acknowledgements

My supervisor, Måns, thank you for your time and your science.
My co-supervisor, Suparna, for helping with things big and small.
Zi-ao Jack Fu, Sandip Kaledhonkar and prof. Joachim Frank for the RF conformation project and for teaching me about time-resolved cryo-EM.
Valérie Heurgué-Hamard and Emmeline Huvelle, for having me in their lab and teaching me some things about proteins.
Prof. Erik Johansson and Pia Osterman, for their help with large scale yeast cultures.
Junhong Choi and prof. Jody Puglisi, for the 2'-O-methylation project.
Ka-Weng: for joint projects and answering my questions.
Anneli, Chandu, Jingji, Mikael, for small things that add up.
Ray, for components.
Mamai, tėčiui, Rimai, Simona, Aurelijai, Gedre, Rūtai ir Simonai, ačiū už viską!
References


References


References


Zoldak, G., Redecke, L., Svergun, D.I., Konarev, P.V., Voertler, C.S., Dobbek, H.,
Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S.,
is governed by two interacting poly peptide chain release factors, eRF1 and
eRF3. The EMBO journal 14, 4065-4072.
Zoldak, G., Redelecke, L., Svergun, D.I., Konarev, P.V., Voertier, C.S., Dobbek, H.,
Thermus thermophilus: structural, spectroscopic and microcalorimetric studies.
Nucleic acids research 35, 1345-1353.

Vestergaard, B., Van, L.B., Andersen, G.R., Nyborg, J., Buckingham, R.H., and
Kjeldgaard, M. (2001). Bacterial polypeptide release factor RF2 is structurally
distinct from eukaryotic eRF1. Molecular cell 8, 1375-1382.
tion by release factors induces structural rearrangement of the ribosomal decod-
ing center that is productive for peptide release. Molecular cell 28, 533-543.
neutralizes potential error hotspots in genetic code translation by transfer RNAs.
Rna 22, 896-904.
Zhoureleva, G., Frolova, L., Le Geoff, X., Le Guelc, R., Inge-Vechtomov, S.,
is governed by two interacting poly peptide chain release factors, eRF1 and
eRF3. The EMBO journal 14, 4065-4072.
Zoldak, G., Redelecke, L., Svergun, D.I., Konarev, P.V., Voertier, C.S., Dobbek, H.,
Thermus thermophilus: structural, spectroscopic and microcalorimetric studies.
Nucleic acids research 35, 1345-1353.

Vestergaard, B., Van, L.B., Andersen, G.R., Nyborg, J., Buckingham, R.H., and
Kjeldgaard, M. (2001). Bacterial polypeptide release factor RF2 is structurally
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eRF3. The EMBO journal 14, 4065-4072.
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Thermus thermophilus: structural, spectroscopic and microcalorimetric studies.
Nucleic acids research 35, 1345-1353.
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