4 Discussion

In the thesis we improved and broadened the application of existing *in vitro* protein systems in order to yield higher productivity and accuracy of translation. We applied these systems for the study of selected antibiotics and to deepen our insights into their inhibition mechanisms.

4.1 Optimization of a simple *in vitro* system for poly(Phe) synthesis with near *in vivo* features

Two poly(U)-dependent poly(Phe) synthesis systems have been described with comparable efficiency and accuracy, the polymix system of the Uppsala group (Jelenc and Kurland, 1979) and a system developed in our laboratory: a more simple Mg^{2+}/NH_4^+ polyamine system (Bartetzko and Nierhaus, 1988). In the latter system the posttranslocational state (POST state) is better defined as indicated by a stable E-site occupation in contrast to a spontaneous release of the E-tRNA within minutes in the polymix system (Semenkov et al., 1996). A stable binding of the E-tRNA is also a feature of native polysomes (Remme et al., 1989).

The poly(U)-dependent poly(Phe) synthesis system depends strongly on the supply of energy-rich components and their regeneration. Important limiting factors are GTP, ATP and the molecules participating in energy regeneration.

A frequently used energy regeneration system is based on phospho*enol*pyruvate (PEP) containing one of the most energy-rich bonds in the cell ($\Delta G°'$ = -14.8 kcal/mol, see chapter 16.1.7 in Ref. Berg et al., 2002). PEP is not only one of the most costly molecules of a translation system, but also gives - as a by-product - inorganic phosphate complexing magnesium (Kim and Swartz, 2000). Moreover PEP requires the addition pyruvate kinase and we found in some lots of commercially available pyruvate kinase a high content of RNases, a serious disadvantage for *in vitro* systems. We replaced PEP by the cheaper and more efficient acetyl phosphate (Ac-P) as suggested by Spirin and coworkers (Ryabova et al., 1995). Ac-P is easily dissolvable in water and has a neutral pH. Ac-P regenerates ATP by acetate kinase (E.C.1.2.3.3), which is present in the cell lysate.

GTP (mM)	ATP (mM)	Acetyl-Phosphate (mM)				
		0	1	2	3	4
		(%)	(%)	(%)	(%)	(%)
0	0	2.6	1.1	2.2	2.2	0.7
	1	32.9	32.4	36.1	52.9	72.8
	2	49	43.6	49.7	81.4	105.9
	3	56.8	58	66.7	114.5	121.3
	4	107.1	76.6	81.4	89.1	50.7
1	0	2.6	2.7	3.3	3.6	3.7
	1		62.8	69.9	102.9	144.9
	2	94.2	77.7	83.6	124.6	150
	3	136.1	60.1	97.8	134.8	150
	4	91	80.3	26.2	47.8	23.5
2	0	3.2	4.3	2.2		2.9
	1	94.8	79.8	90.7		139.7
	2	114.2	87.2	100	131.9	137.5
	3	127.1	95.2	106	139.1	105.9
	4	116.8	41	7.7	5.8	8.8
3	0	3.2	3.2	2.7	2.9	2.9
	1	115.5	81.9	95.1		133.1
	2	134.8	99.5	98.9	121.7	95.6
	3	141.3	100	68.3	87.7	23.5
	4	53.5	9.6	71	4.3	6.6
4	0	3.2	2.1	2.2	1.4	2.2
	1	60	33	7.1	5.1	6.6
	2	31.6	4.8	3.8	3.6	5.9
	3	7.7	2.1	1.1	2.2	4.4
	4	3.2	1.6	1.6	3.6	2.2

Table 2: Optimization of the energy source. The concentrations of three different energy components (GTP, ATP and Ac-P) have been analyzed according to optimization of poly(Phe) synthesis. The synthesis obtained in the reaction mixture, where all three components are equal to 2 mM, is tentatively set 100%. Colors where used to indicate reactions above 100%, i.e. blue 100-120%, yellow 120-140%, red >140%

Table 2 presents the optimisation of three compounds, ATP, GTP and acetyl-phosphate for a poly(U)-dependent poly(Phe) synthesis system, which was developed in the Nierhaus group and where I also participated. These results are the part of a publication, which is now in preparation. In Table 2 the observed Phe incorporation per ribosome at 2 mM of each acetyl phosphate, GTP and ATP was about 190 Phe incorporations per ribosome and tentatively set to 100%, a color-code facilitates to identify the highest Phe incorporations per ribosome. Two red islands with the highest Phe incorporations were observed, one with 3 mM of each GTP and ATP but without acetyl phosphate, and a second one with 1 mM GTP, 1-3 mM ATP and 4 mM acetyl phosphate. A fine-tuning optimization was performed within and around the second island, which was more stable for slight concentration changes as the first island, and led to a set of the following optimal concentrations: 1.5 mM GTP, 3 mM ATP and 5 mM acetyl phosphate.

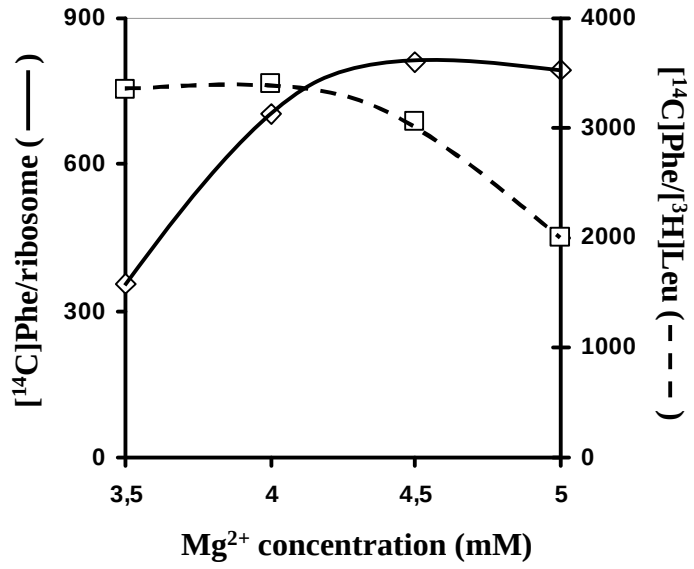

Figure 28: Incorporation of miscoded Leu in the presence of various Mg^{2+} concentrations measured for optimization of poly(U)- dependent poly(Phe) synthesis.

Figure 28 demonstrates that our current system with 4.5 mM Mg^{2+} is in an optimal region between 4 and 4.5 mM considering simultaneously the yield of Phe incorporation per ribosome and the accuracy as defined a the incorporation of one Leu per n Phe residues.

4.2 Different definitions of near- and non-cognate aa-tRNAs

During translation reaction three different types of errors can be distinguished: (1) processivity errors, (2) frameshifts, i.e. a loss of the correct reading frame, and (3) incorporation of a wrong amino acid concerning the A-site codon.

Processivity errors are defined as premature release of peptidyl-tRNA from the ribosome. The frequency of these errors in the cell is estimated to be around $4x10^{-4}$ (four in 10,000 amino acids incorporations) (Menninger, 1976). For the cell this type of errors is dangerous due to strong ester bond that links the peptidyl residue to the tRNA and which is more stable then bond between aminoacyl moiety and tRNA. Is means that peptidyl-tRNAs are accumulated in the cell, sequestering the tRNAs and prohibitively restricting protein synthesis. To prevent this, a cytoplasmic enzyme termed peptidyl-tRNA hydrolase, which is essential for cell viability, cleaves the relevant ester bond of free peptidyl-tRNA, thus recycling the tRNAs (Schmitt et al., 1997).

The most dangerous type of errors for the cell is the loss of reading frame (frameshift), because it leads immediately to the loss of the genetic information, so maintaining the reading frame is one of the essential tasks of ribosome. In the cell, a loss in the reading frame occurs only once in 30,000 elongation cycles (Jorgensen and Kurland, 1990).

The most frequent error is the misincorporation of an amino acid not related to the codon at the A-site with a frequency of about one per 3000 elongation cycles (Bouadloun et al., 1983). Concerning the A-site codon the competing ternary complexes (in *E. coli* 42 different can be distinguished by differences at the anticodon) can be assigned to three different classes: (i) The cognate one is that aminoacyl-tRNA, the anticodon of which has the best complementarity to the codon at the A site. (ii) The anticodon of the near-cognate type is similar to the cognate one, and (iii) the non-cognate one (90% of the ternary complexes) which carries a dissimilar anticodon. A deeper understanding of these three classes concerns the GTP consumption, which is strikingly different from class to class. The cognate amino acid is incorporated consuming one, not more than two GTPs, the near-cognate consumes six or more GTPs (Weijland and Parmeggiani, 1993) and the non-cognate one no GTP at all. This functional definition indicates at the

same time, that the near-cognate can be mis-incorporated, whereas the incorporation of a non-cognate one was until now not yet observed. It follows that there are not more than three of four near-cognate tRNAs with respect to a distinct A-site codon (Nierhaus, 1990). A further important aspect of these three classes are their relationship to the logic of the genetic code: near-cognate codons code for amino acids, which are chemically similar to the cognate one, for example, UUU/C codes for the hydrophobic Phe, the near-cognate UUA/G for the hydrophobic Leu, or GUU/C for the hydrophilic acidic Asp, the near-cognate GUA/G for the hydrophilic acidic Glu. This is the reason that a misincorporation of a near-cognate amino acid is usually not harmful for folding, structure and/or function of a protein. In fact, only one in 400 decoding errors cuases a problem for the corresponding protein (Kurland et al., 1990).

An alternative definition of near-cognate aa-tRNA compiles all tRNAs with a single mismatch in the codon-anticodon interaction (Rodnina et al., 2005). This definition compromises the functional differences, since for example a mismatch in the middle codon position is never misread.

We analyzed incorporation of non-Phe amino acids into the poly-Phe chain analyzed by our highly efficient poly(Phe) synthesis system, where we can incorporate about 150 to 200 Phe on average pre ribosome allowing a very precise assessment of the mis-incorporation. Since the specific activity of Phe *versus* the “wrong” amino acid was 10 cpm *versus* at least 3,000 cpm, an incorporation of a wrong amino acid has a resolution limit of about one per 10,000 Phe. We amplified the misincorporation by adding either streptomycin or increasing the Mg^{2+} concentration (4.5 to 14.5 mM), which increases the error by a factor of about 30 and 21, respectively (see Figure 13, page 82). Leu is heavily misread (mismatch of the wobble position) a mismatch in the firs position is just detectable (Ile), whereas a mismatch in the middle position does not lead to an incorporation. It follow, that a mismatch in the middle position represents a non-cognate tRNA rather than a near-cognate one.

4.3 Cell-free coupled transcription-translation systems

The most comprehensive *in vitro* system is the coupled transcription-translation system, where one adds a plasmid carrying the gene to be expressed,

usually after a T7 promoter. The gene is then transcribed with added T7 polymerase and subsequently translated. We mainly based on the Rapid Translation System (RTS) purchased from Roche (See Material and Methods, page 35). Other system can be supplied by RiNA GmbH (Berlin, Germany), which gives comparable expression level. The RTS 500 consists of two chambers, one for the reaction and another for the feeding mix, separated by a semi-permeable membrane. The reaction chamber houses the machinery for mRNA and protein production, together with the DNA template. The chamber with the feeding mix is ten times larger than that of translation and supplies NTPs and amino acids, and allows removing by-products. The final product is accumulated in the reaction chamber. We achieved routinely an expression of about 4 mg per ml of the expressed protein, usually the Green Fluorescent Protein (GFP).

4.3.1 The folding of GFP and its usage in RTS 100/500 as a reporter protein

As mentioned above Green Fluorescent Protein (GFP) was used as a reporter protein. GFP is a fluorescent molecule found in the jellyfish *Aequorea Victoria*. Isolated in 1961 (Shimomura et al., 1962) and cloned in 1992 (Prasher et al., 1992), GFP has become a popular reporter molecule for gene expression.

The biochemistry of GFP has been studied extensively. It exists as a 238-amino-acid protein; its crystal structure illustrates its conformation as a rigid β can enclosing an α helical region that contains the chromophore (Figure 1c, page 21; Ormo et al., 1996; Yang et al., 1996). The chromophore itself is a cyclic tripeptide (Ser65-Tyr66-Gly67), and this region is required for fluorescence. Because of its compact structure, and because the chromophore is protected from the external environment inside the β structure, GFP is extremely stable. Folding and the oxidation process forming the fluorophore is a slow process, the maturation process in *E. coli* requires an overnight incubation at 4°C. Folding of GFP denatured by acidic pH or 6 M guanidine hydrochloride occurs within 1 to 5 min (Makino et al., 1997; Ward and Bokman, 1982) suggesting that most of the maturation time is used for the fluorophore formation. A mutant also used here carries three mutations at positions 100, 154 und 164. The mutations improve the folding efficiency, reduce the maturation times to 3 to 4 h and improve the yield of

stable, active recombinant GFP not only in *E. coli* but also in eukaryotic Chinese Hamster Ovary (CHO) cells (Crameri et al., 1996; Tsien, 1998). The latter observation indicates that the active fraction of GFP can be improved by accelerating the intrinsically slow folding of this protein *via* mutations. The active fraction of GFP can also be influenced by other factors. (i) Temperature: wild-type GFP showed a sharply decreased active fraction when synthesized at 37°C, a temperature much higher than that of the habitat of *Aequorea victoria* in cold pacific water (Ogawa et al., 1995). (ii) Chaperon concentrations: Another possibility was demonstrated with proteins essential for the *E. coli* cell and dependent on GroEL/ES for a successful folding. When these proteins were overexpressed in *E. coli* up to 70% were insoluble, upon an ~5-fold increase of GroEL/ES *in vivo* the solubility increased 2- to 3-fold (Kerner et al., 2005), however, a direct involvement of chaperones in the folding of GFP has not yet been shown. (iii) Differences in modes of folding and kinds of chaperones in bacteria and eukaryotes: Here the observation is pertinent that GFP expression showed a significant higher yield of soluble GFP in *Saccharomyces cerevisiae* (about 90% of the totally synthesized GFP) than in *E. coli* (about 60%; ref. (Chang et al., 2005) similar to the amount seen in our experiments). The authors put forward the explanation that this difference reflects the co-translational folding process in eukaryotes in contrast to the prevailing post-translational folding mode in bacteria (see below). Another not necessarily alternative explanation is the fact that the different chaperone equipment of the eukaryotic cell is superior in supporting the folding of the eukaryotic GFP protein as compared to the bacterial chaperones. In agreement, Sacchetti suggested that the different expression of GFP variants in *E. coli* and mammalian cells was caused by different chaperon sets (Sacchetti et al., 2001). (iv) Different relation of transcription and translation in bacteria and eukaryotes: Here in this thesis we presented evidence that also the loss of the bacterial coupling of transcription and translation will impair the output of active and stable GFP.

For our experiments we have used the commercially available the 100% active GFP from Roche (rGFP). The assumption that this GFP is 100% active requires a more detailed consideration. GFP synthesized *in vivo* shows strikingly different active fractions depending on the organism, *viz*. whether GFP has been

synthesized in *E. coli* or yeast (Chang et al., 2005). The low active fraction in *E. coli* have been explained with constrains on *de novo* folding consistent with the assumption of a largely post-translational folding mechanism in bacteria. The authors postulated an “interference of GFP with adjacent domains during folding due to the particular topology of the β-barrel GFP structure”. In the same paper evidence was presented that the solubility nicely corresponds to the active fraction of GFP regardless whether GFP has been synthesized in yeast or *E. coli* (Figure 3 in Chang et al., 2005). The fact that the commercially available GFP is perfectly soluble therefore justifies our assumption of its 100% activity. Furthermore, under optimized conditions reported here the activity of the synthesized GFP could be raised to 100% but never significantly above this value. This observation adds further credit to the assumption of 100% activity of the reference GFP and indicates that the low active fraction observed in *E. coli* is not an intrinsic feature of bacterial systems, but rather might have reasons additional to those mentioned by (Chang et al., 2005), namely the unfavorable usage of T7 polymerase routinely used for induced gene expression in *E. coli*.

4.3.2 Coupling of transcription and translation in cell-free systems

The cell-free coupled transcription-translation systems like RTS utilize artificial T7 polymerase, which is about five- to eight-fold faster than the endogenous *E. coli* RNAP (Iost and Dreyfus, 1995) what generates some disadvantageous for quality of protein synthesis.

A tight coupling of the transcription and translation processes exists in bacteria: The *E. coli* RNAP proceeds with a speed of ~60 nucleotides (nt) per second, and a ribosomes initiating translation on the nascent chain of mRNA proceeds with a speed of ~20 amino acids per second (Bremer and Dennis, 1996) corresponding to ~20 codons (or equally to 60 nt) per second. It follows that the first ribosome pursues directly the transcriptase causing the tight coupling of transcription and translation and leaving no room for a significant gap between the transcriptase and the following ribosome. Therefore, the nascent mRNA chain cannot form secondary structures and thus complicate or even block translation elongation or transcription *via* R-loop formation (Gowrishankar and Harinarayanan,

2004). Moreover, the presence of ribosomes also protects the mRNA against endonucleolytic degradation (Iost and Dreyfus, 1995).

One of the general problems of *in vitro* transcription-translation in bacterial cell-free systems is the uncoupling of the naturally coupled processes of transcription and translation. This results from the use of bacteriophage T7 RNA polymerase (RNAP) at 37°C instead of *E. coli* RNAP (Chamberlin and Ring, 1973). The T7 RNAP is five to eight times faster than *E. coli* RNAP, thus breaking the tight coupling between transcription and translation/ribosome assembly with two unfavorable consequences: (i) Strong secondary RNA structures can form that hinder the path of the translating ribosome over the mRNA probably impairing the co-translational folding and thus the yield of active proteins. (ii) R-loops can be formed, where nascent RNAs emerging from the RNAP exit channel can form heteroduplices with the upstream region of the template DNA strand. These heteroduplices can interfere not only with translation/assembly, but also with the next round of transcription or even with replication (for review see (Gowrishankar and Harinarayanan, 2004). The result is that only a tiny fraction - within a few percent of the mRNA transcripts - are used for translation (Iost and Dreyfus, 1995). Likewise, only a minor fraction of T7 RNAP transcripts of rRNA is used for the assembly of 50S subunits (Lewicki et al., 1993). The use of *E. coli* RNAP together with *E. coli* promoters is not an easy way to overcome this drawback, since most of the *E. coli* transcriptases are removed with the membranes, and the addition of the multi-subunit *E. coli* RNAP is a demanding task in terms of isolation and maintaining activity. Therefore, using the monomeric T7 polymerase with its high processivity is the decision of choice.

In our experiments we tested slow T7 RNAP. The utilization the slow T7 RNAP caused decreasing of total GFP production but contrary to total expression the active amount significantly increased (Figure 8, page 74). In spite of the low yield we can conclude that a reduction in the rate of transcription significantly improves the active fraction of the synthesized protein probably by re-establishing the coupling of transcription and translation.

Similar effect to slow mutant of polymerase T7 could be observed when the temperature of coupled reaction is decreased. In these conditions T7 polymerase is slower. We performed RTS reaction at various temperatures. The results strictly

indicated that lower temperature of the reaction result in the higher yield of the active amount with simultaneously reduce of total protein production. The experiment was confirmed by expression of different protein - luciferase, which give similar results to these obtained from GFP.

Moreover, the results suggest that the rate of T7 polymerase is more affected by the lower temperature than the translation rate of ribosomes, thus re-establishing the coupling of transcription and translation and causing the beneficial effects observed. Furthermore, lowering the incubation temperature and thus the elongation rate has two beneficial effects on the active fraction: (i) The aggregation of synthesized GFP is prevented thus increasing the active fraction, as has been demonstrated at lower temperatures *in vivo* (Schlieker et al., 2002). The reason is that overexpression of proteins at 37 °C *in vivo* might allow contacts of unfolded proteins, and these contacts of hydrophobic patches of unfolded proteins lead to aggregations and inclusion bodies. (ii) We are dealing with a second effect not previously seen with *in vivo* studies: Slowing down the elongation rate at 20°C improves the accuracy, since with the higher elongation rates at 30°C we observe a low active fraction of about 50% right from the very beginning of kinetic measurements (30 min; Figure 7, a to c, page 73), where aggregation has not yet occurred.

4.3.3 How to increase the total protein yield in *in vitro* expression systems?

We showed that the efficiency of *in vitro* protein synthesis in a coupled transcription-translation system can be significantly increased to several mg per ml by incubating the reaction mix in a semi-continuous system at 20°C for about 15 h and adding an amino-acid mix after about 10 h of the incubation. The protein synthesized is virtually 100% active, and therefore the low-cost bacterial system can be used under these conditions (i) for structural analysis such as crystallography or NMR after incorporation of *e.g.* ^{13}C and ^{15}N isotopes during the incubation, and (ii) for folding and functional studies.

The optimization of the coupled system as it stands after these analyses can still be pushed forward. Let us compare the efficiency of the RTS (Roche) used here with that of an *E. coli* cell. The reaction mix before synthesis contains about

40 mg per ml total protein, and after 10 h the amount of GFP synthesized approaches about 10% of the total protein (4 mg/ml; Figure 7c, page 73). A continuation of this synthesis rate would lead to a doubling of the protein content in the reaction mixture (total proteins + synthesized GFP) after 100 h. *E. coli* has a doubling time of 20 min under rich medium conditions, where it doubles the total protein synthesized and distributes it over the two daughter cells. It follows that the system used here is still 300-fold less efficient than protein synthesis *in vivo* in agreement with a recent report showing that the bulk protein synthesis rate in an optimized *E. coli*-based cell-free system is 200-fold slower than the *in vivo* rate (Underwood et al., 2005).

4.3.4 Recently discovered Elongation Factor 4 (EF-4 or LepA) increases accuracy of translation

The previous name of EF-4 is LepA protein (Qin et al., 2006). This protein is present in all prokaryotic cells and mitochondria being characterized as a one of most conservative protein on our planet with an amino acid identity of about 55-68%. For a long time scientists could not find a phenotype for LepA (Dibb and Wolfe, 1986). Using novel techniques of molecular biology our colleague has demonstrated the function of LepA as a back-translocator recognizing defective translocated state and by back-translocating it giving EF-G a second chance for a proper translocation reaction. The assumption is that a defectively translocated ribosome does not proper display its A-codon thus increasing the probability for an incorrect aminoacyl-tRNA selection.

Qin and coworkers have used a coupled transcription-translation cell-free system (RTS 100) and analyzed how LepA affects GFP expression (Qin et al., 2006). In the presence of increasing amounts LepA, the total GFP amount increases and peaks at a ratio of 0.1 molecules LepA added per 70S. Further addition of LepA leads to a rapid reduction in the GFP production, eventually blocking the total synthesis completely at a molar ratio of LepA:70S = (0.5):1, in agreement with the toxic effects of overproduced LepA *in vivo*. In contrast, the native gel reveals that the active GFP amount increases to attain the same levels as the total GFP amount at LepA stoichiometries of more then two LepA per 70S. In other words, addition of LepA promotes the synthesis of fully active proteins.

We conclude that LepA not only increases the total protein yield, but more importantly improves the activity of the produced protein.

Interesting results with LepA were obtained, when misreading factors were added to the translation reaction prior to LepA. Two factors were used, magnesium ions and paromomycin. Interestingly, LepA could counterpart only the misreading effect of increased magnesium concentrations in contrast to that of the antibiotic. This finding is in agreement with the observation that LepA is essential for growth at low pHs in the presence of higher ionic strength (Z. Karim and K. H, Nierhaus, unpublished observation) and explains, why a systematic knock-out analysis of the genes of Helicobacter pylori has identified LepA as one of the genes essential for the survival of this species at the acidic milieu in the stomach (Bijlsma et al., 2000). There is also an application potential for LepA in *in vitro* systems.

4.4 RTS system as a tool for overall determination of the accuracy of protein synthesis

The fluorescence of GFP allowed us to develop a new and comprehensive misreading-detection system, where the overall misreading events can be observed by a decrease of GFP fluorescence. The synthesized GFP was measured both, in SDS and native gels. The former assesses the total amount of synthesized protein, whereas the latter determines the fluorescence in the gel and thus reflects the fully folded, active GFP. Our expectation was that a drug inducing misincorporations will reduce the active fraction more strongly than the total synthesis, so that the ratio active fraction to total synthesis will decrease with increasing drug concentration.

We first analyzed streptomycin (Str), a well-known inducer of misincorporation, and we compared results with the control drug thiostrepton known not to interfere with the accuracy and with an antibiotic of non-determined inhibition mechanism (Novel Ribosomal Inhibitor, NRI). The results were as expected demonstrating the suitability of GFP synthesis for an overall estimate of the accuracy. The control drug, thiostrepton did not affect accuracy of translation what was in the agreement with literature, where it was characterized as translocation inhibitor (Hausner et al., 1988).

Interesting results were obtained when GFP was synthesized with NRI. This antibiotic reduced accuracy of translation to the same extent as Str. NRI blocks protein synthesis also in the RTS system. An interesting result was observed in the poly(Phe) system, where NRI did not induce an increased error (leu incorporation). For the first time we report here a drug being a strong induces of misreading but showing no effect in the standard poly(Phe) synthesis system. One explanation is that NRI might induce selection problems at the A site only with specific tRNA, but not with $tRNA^{Leu}$ and $tRNA^{Phe}$.

4.4.1 Edeine as misincorporation inducer from the ribosomal E-site

Another surprising finding was that Edeine (Ede) induced strong misincorporation paralleling those of the classic misreading antibiotic Str. This finding is of particular interest since, to date, misreading antibiotics have been discovered that bind either to the decoding center of the A site, like aminoglycosides, or within the vicinity of h27 near to the decoding center, such as Str, but not in proximity to the E site. It was recently reported that chloramphenicol, as well as the oxazolidinone class of antibiotics, which bind within the PTF center of the large subunit, induce miscoding events, such as frameshifting and nonsense suppression (Thompson et al., 2002), supporting the notion that certain antibiotics can exert long-range effects on the fidelity of translation. Indeed, a rational explanation can be put forward for Ede-induced misreading, since it has been demonstrated that the presence of the E-site tRNA enhances the fidelity of decoding at the A site (reviewed by Blaha and Nierhaus, 2001): the G693-C795 base pair induced by Ede binding disrupts the path of the mRNA through the E site (Pioletti et al., 2001); therefore, it is conceivable that the allosteric effect that the E-site tRNA normally conveys to fidelity of A-site decoding is impaired. In this case, the post state ribosome in the presence of Ede may not have fully converted from the closed to the open 30S conformation (Ogle et al., 2003), thus increasing the probability that near-cognate aa-tRNAs are accepted at the A site, in a situation where they would normally be rejected. The idea that occupation of the E site affects binding results from breaking the G693:C795 base pair formed upon Ede binding. The implication being that tRNA binding to the P site of the 30S and 70S

requires a non-base-paired conformation of G693 and C795, but upon binding to the P site the base pair is formed again. This is supported by the observation that binding of tRNA to the P site of 70S ribosomes led to protection of both bases (Moazed and Noller, 1990). Therefore, we propose that Ede, by inducing the G693:C795 base pair, mimics a ribosome state where the P site tRNA is already bound and that Pct restores the conformation to the unbound state. However, although in the presence of Pct P site tRNA binding can occur, the lack of the G693:C795 base pair and/or the presence of Pct obviously result in preventing the later translocation events.

4.5 The prevention of tRNA binding and inhibition of canonical translation initiation caused by Ksg

The crystal structure of the complex of Ksg bound to the 30S subunit of *T. thermophilus* unexpectedly reveals two antibiotic binding sites, both located within the path of translated mRNA. At the primary binding site Ksg forms interaction with A794 and G926 , which is consistent with the protections observed by Ksg on the 30S subunit (Woodcock et al., 1991), as well as the fact that mutations at these positions give rise to Ksg resistance (Vila-Sanjurjo et al., 1999).

The similarity in the protections of Ksg and P-tRNA on the 30S subunit, coupled with the observation that Ksg prevents binding of initiator tRNA, led to the suggestion that the binding site of Ksg overlaps with the P-tRNA. Surprisingly, in the structure determined by Fucini and co-workers (Schluenzen et al., 2006) Ksg is not seen to overlap with the position of the P-tRNA, but rather binds in two separate positions within the path of the mRNA, being localised at the kink between P and E site codons (Ksg1) and at the first nucleotide position of the E site codon (Ksg2) (Figure 29). The clear overlap between Ksg and the mRNA suggests a direct competition between the two molecules. The effect of Ksg will therefore depend on the affinity for the ribosome of the specific mRNAs with which Ksg has to compete.

Although many studies addressing the action of Ksg have clearly demonstrated an inhibition at the level of translation initiation, differential inhibitory activities were observed depending on the type of functional complexes tested. In the light of a model whereby Ksg action operates through the mRNA, many of the

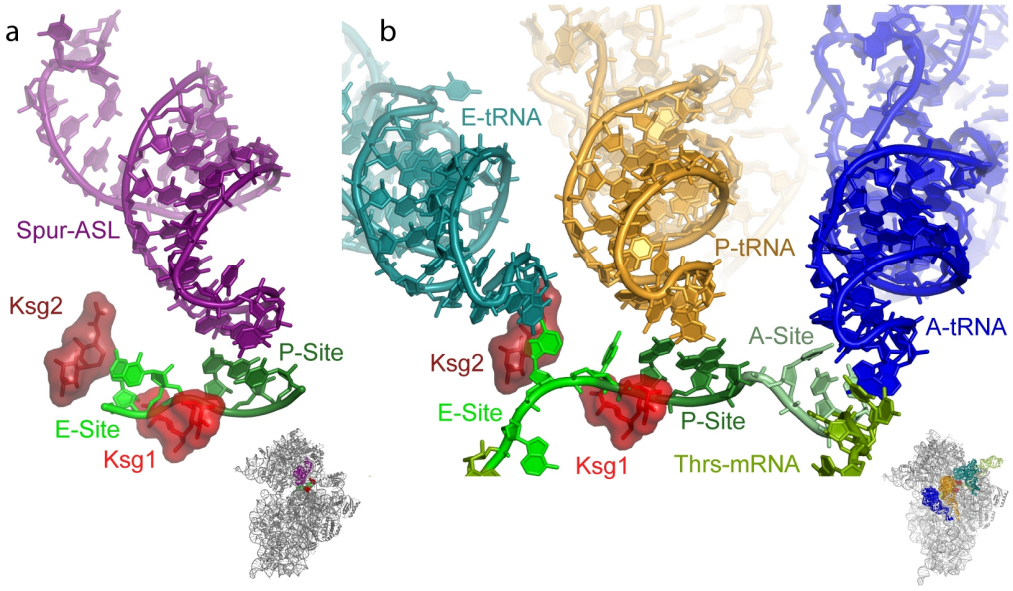

Figure 29: Ksg overlaps with P-site mRNA, but not P-tRNA (taken from Schluenzen et al., 2006)

(**a**) The primary (Ksg1, dark red) and secondary (Ksg, light red) kasugamycin binding sites are shown relative to the spur-anticodon stem loop (spur-ASL, purple) and a mRNA mimic (green) taken from PDB: 1IBL or 1IBM. Three nucleotides of the P- and two nucleotides of the E-site codons are shown in dark and light green, respectively.

(**b**) The primary (Ksg1, dark red) and secondary (Ksg, light red) kasugamycin binding sites are shown relative to the A- (blue), P- (orange) and E-tRNAs (cyan) and ThrS-mRNA (green), taken from PDB: 1YL3 or 1YL4. The respective A-, P- and E-site codons are shown in different shades of green.

puzzling and often contradictory results obtained when using this antibiotic can be rationalized. For example, it has been observed *in vivo* that Ksg exhibits differential inhibition on the translation in *Escherichia coli* of mRNAs encoding different envelope proteins (Hirashima et al., 1973). Even stronger is the discrepancy observed in the translation inhibition of more artificial mRNAs, which vary from a very poor inhibition, namely 2-12% for the Poly(C)-dependent poly(Pro) synthesis, to a modest one, ~50-60% for Poly(U)-dependent poly(Phe) synthesis, and a very strong ~90% inhibition on the Poly(A)-dependent poly(Lys) synthesis (Tanaka et al., 1966a; Tanaka et al., 1966b). These studies are complemented by the observation that *in vitro* the translation of natural mRNAs such as the viral proteins from the f2 and MS2 phages is inhibited to different extents by Ksg, ranging from 30% to 80% for the coat and maturation protein, respectively (Kozak and Nathans, 1972; Kubota et al., 1972).

Despite a clear influence of the mRNA on Ksg inhibition, direct competition between Ksg and the mRNA has not been previously reported, suggesting that the effect of Ksg does not operate by completely preventing the binding of the mRNA to the ribosome. Indeed, multiple regions of the ribosome, such as the antiSD or

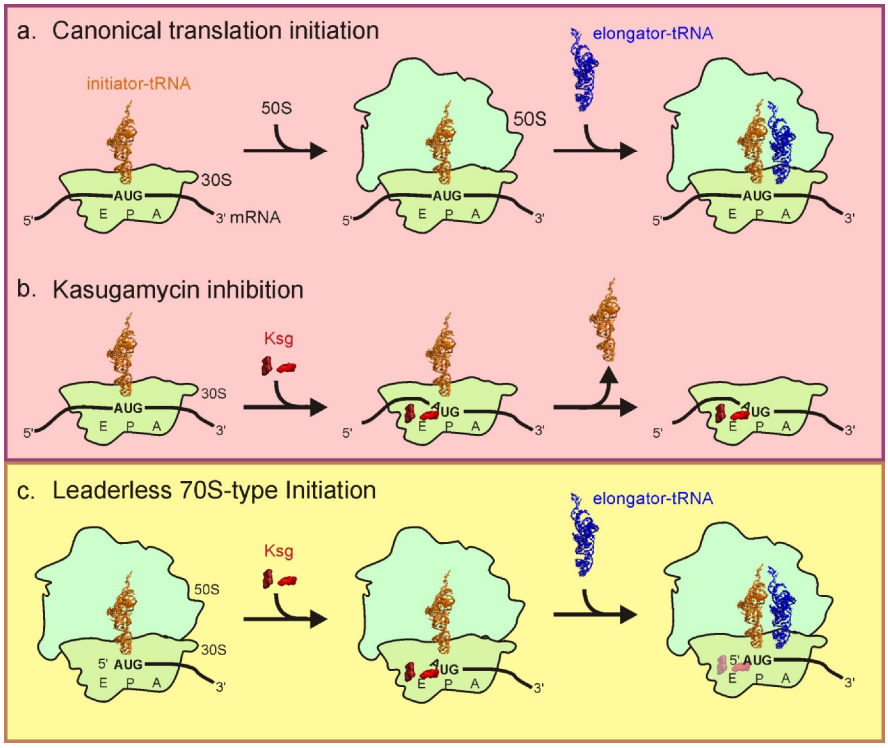

Figure 30: Model for Ksg action during translation initiation

(**a**) Canonical translation initiation involves binding of the initiator-tRNA (orange) and mRNA to the small ribosomal subunit (30S) with initiation factors (not shown) to form a 30S-preinitiation complex, where the initiator tRNA is positioned at the P site. Subsequently, the large subunit (50S) binds to form a 70S ribosome, after which the elongator tRNA (blue) can bind at the ribosomal A site.
(**b**) The presence of kasugamycin (Ksg, red) is proposed to bind within the path of the mRNA, specifically overlapping the first position of the P site codon (in this case, the A of the AUG) as well as the E site codon. This in turn perturbs the mRNA-tRNA codon-anticodon complex, leading to destabilization and release of the initiator-tRNA form the P site of the 30S subunit.
(**c**) Translation of leaderless mRNAs is proposed to proceed through a 70S–type initiation where the initiator-tRNA binds directly to the P site of a 70S ribosome. Kasugamycin (Ksg, red) does not prevent translation initiation of leaderless mRNAs suggesting that if Ksg does bind to a 70S initiation complex, then the additional contacts that the 50S subunits establishes with the initiator tRNA stabilize the tRNA against the dissociative effect of Ksg.

the mRNA channel, are likely to contribute to the stability of mRNA binding such that overlap with the P and E site positions of an mRNA would be enough to destabilize mRNA binding only in extreme cases. Consistent with this idea, we observe a modest influence (up to 25%) of Ksg on the binding of mRNA to the 30S

subunit or 70S ribosome (Figure 17 a and c, page 87). Ksg is slightly better at inhibiting the binding of MVF-mRNA when the SD sequence is lacking, consistent with the observation that the strength of the SD sequence significantly reduces the K_d of mRNA on a 30S subunit (Studer and Joseph, 2006).

The most easily observed effect of Ksg is on the inhibition of tRNA binding to the ribosome, specifically, for natural mRNAs, the binding of the initiator fMet-tRNA to programmed 30S subunits rather than to 70S ribosomes. On the basis of our results, we conclude that the effect of Ksg on the binding of the tRNA operates indirectly by influencing the binding and/or positioning of the mRNA through the P and E site within the mRNA pathway. Since the sequence and secondary structure of different mRNAs influences the affinity of the mRNA for the ribosome (de Smit and van Duin, 1994; Studer and Joseph, 2006), the inhibitory activity of Ksg depends on how well the drug can compete with the mRNA. Strong competition will displace and/or perturb the mRNA, which in turn impairs the binding of the fMet-tRNA (Figure 30a and b). In this respect, it is interesting to note that as early as 1972, it was recognized that a link existed between the degree of inhibition of Ksg and the structure of the translated mRNA, specifically in the region around the AUG codon (Okuyama and Tanaka, 1972). Okuyama and co-workers observed that coat proteins, which are less inhibited by Ksg, form a hairpin loop structure encompassing the AUG start codon. Conversely, translation of maturation proteins, which do not form such hairpin loop structures, is more sensitive to Ksg action. In contrast to unstructured mRNAs, the binding of hairpin loop containing mRNAs has been shown to occur in two distinct steps, a rapid initial binding of the structured mRNA followed by a slower unfolding step (Studer and Joseph, 2006). The increased thermal stability of the structured mRNA during the initial binding step is likely to prevent Ksg from perturbing the conformation of the mRNA on the ribosome and may even lead to displacement of Ksg from the 30S subunit. Such a scenario would explain the reduced ability of Ksg to inhibit translation of structured mRNAs.

Regardless of whether the mRNA is structured or unstructured in the pre-initiation complex, the interaction of the P-tRNA on a 30S subunit predominantly encompasses the codon-anticodon interaction, whereas on a 70S ribosome 85% of the interactions of a P-tRNA are with the 50S subunit (Moazed and Noller, 1989;

Schäfer et al., 2002; Yusupov et al., 2001). This suggests that for the same mRNA, the effect of Ksg should be stronger on 30S subunits than on 70S ribosome (Figure 30c). Accordingly, Polderman *et al.* (Poldermans et al., 1979) have shown that Ksg can efficiently remove fMet-tRNA from a 30S subunit while its action is less efficient on the 70S ribosome. In our study, the difference is even more dramatic, such that Ksg is completely ineffective at preventing the binding of P-tRNA to 70S ribosomes (Figure 17b, page 87).

In contrast to the canonical 30S initiation, translation initiation of leaderless mRNAs is not inhibited by Ksg *in vivo* (Chin et al., 1993; Moll and Bläsi, 2002). This can be rationalized at a structural level since the overlap between the binding position of Ksg and canonical mRNAs is quite extensive in the P and E sites (Figure 30b), whereas for initiation of leaderless mRNA, the overlap is reduced to only one nucleotide position (as illustrated in Figure 30c). Another factor that could explain the differential effect of Ksg on canonical and leaderless mRNAs is their distinct mode of translation initiation: Translation of canonical mRNAs involves the initial formation of a 30S pre-initiation complex, before joining of the 50S subunit to form a 70S ribosome (Gualerzi and Pon, 1990). Because Ksg action is stronger on 30S subunits than on 70S ribosomes, canonical translation initiation, by passing through a 30S complex, is much more susceptible to the action of Ksg. In contrast, our results support the proposal that translation initiation of leaderless mRNAs operates through a 70S-type initiation pathway. The binding of leaderless mRNA in the presence of fMet-tRNA is very stable to 70S ribosomes (0.8 per 70S), but very poor to 30S subunits (0.2 per 30S) as shown in the Figure 17c, page 87. Additionally, we find that Ksg is much less effective at destabilizing fMet-tRNA from 70S ribosomes, than from 30S subunits using canonical or leaderless mRNAs (Figure 17 b and c, page 87).

Therefore, we conclude that leaderless mRNAs operate through a 70S-type initiation pathway and that the contribution of the 50S subunit to stabilize the initiator-tRNA on a 70S ribosome plays a primary role in the observed resistance of translation of leaderless mRNAs to Ksg action (Figure 30c). In contrast, the susceptibility of translation initiation of canonical mRNAs to Ksg action is defined by their primary and secondary structure and the fact that the pathway involves the formation of a 30S complex.

4.5.1 Old and new studies on aminoglycosides

The first indication about how the aminoglycosides block protein synthesis came from Hausner and coworkers (Hausner et al., 1988). The approach based on the allosteric three-site model for the ribosomal elongation cycle (Rheinberger and Nierhaus, 1986), where two types of A-site occupation exist: the A-site occupation after initiation (i-type), with only the P-site is occupied by a tRNA (initiator tRNA); and the A-site occupation after an elongation cycle (e-type), with two filled sites, namely the P and E sites containing peptidyl-tRNA and deacylated tRNA, respectively. Hausner and coworkers analyzed individual reactions of the elongation cycle, including both types of A-site occupation in the presence of various antibiotics. The essential conclusion from their work was that aminoglycosides severely inhibit A-site occupation of the e-type in contrast to that of i-type. They used a system allowing the functional studies to be made with quantitative tRNA binding at 6 mM Mg^{2+}.

We were repeated and extended the experiments done by Hausner and coworkers. We could fully reproduce the Hausner results with poly(U), but above that a number of differences appeared. Our results suggest in accordance with Frederick and colleagues (Shoji et al., 2006) that results of Hausner were not caused by a freezing of the POST state thus preventing A-site occupation, but rather by an induced back-translocation, which refilled the A site. However, we could extend the findings of Frederick in that we show that these back-translocation events do not play a role under more physiological conditions for two reasons: (i) by using the Hausner system in the presence of heteropolymeric mRNAs and $tRNA_f^{Met}$ in the E site only a moderate inhibition could be observed (Figure 26b, page98). (ii) In the presence of EF-G and GTP the aminoglycoside effects on back-translocation were not relevant anymore (Figure 27, page 97).

The synthesis of poly(Phe) in the presence of aminoglycosides brought one unexpected observation, which we described for the first time. All aminoglycosides stimulated rather than inhibited synthesis of poly(Phe). Poly(Phe) was synthesized on two types of mRNA, long poly(U) composed of more then 100 nucleotides and short mRNA composed of twelve UUC codons. Simultaneous stimulation for both mRNAs suggests that aminoglycosides do not induce frameshifts, otherwise

inhibition would have been observed in the case of oligo(UUC). Moreover, we analysed the effects of aminoglycosides on the synthesis of poly(Lys) in the presence of poly(A) mRNA. Also with this homopolymeric mRNA a stimulation of poly(Lys) synthesis was observed; neomycin, kanamycin and paromomycin could even double the Lys incorporation. This observation suggests that aminoglycosides stimulate expression *via* enhancing ribosome recycling. Since protein synthesis on the natural mRNA is strongly inhibited, it might well be that the stimulation of poly(Phe) and poly(Lys) synthesis is specific to the tRNAs involved. We observed a similar case with pactamycin: This drug inhibited the translocation of some tRNAs but not of others (Dinos et al., 2004).

We consider the work presented here as a small contribution towards a solution of the huge problem of the ever increasing antibiotic resistance and for a better understanding of drug mechanisms and drug binding-sites in order to improve our tools so important for medical treatment.