Z. TRANSLATION AND THE RIBOSOME

The Ribosome

The ribosome is the enzyme that catalyzes translation; it converts the mRNA sequence into a growing polypeptide chain. In some ways, the chemistry is very simple. The ribosome catalyzes the addition of an amino group to an ester carbonyl, releasing an alcohol (the tRNA molecule) and producing a new peptide bond. On the other hand, this is a chemical process that requires extraordinary fidelity, involves many different substrates, and includes a number of other proteins, "factors", to assist in the process. To facilitate this complexity, the ribosome is an immensely complex molecular assembly, weighing in at 2.5 MDa (2,520,000 g/mol that is) in *E. coli*. Most often, however, the size of the ribosome is measured in Svedberg units (S), which come from ultracentrifugation behavior – how fast does the particle/molecule migrate during centrifugation. The *E. coli* ribosome is thus a 70S ribosome, divided into two subunits – the large subunit (50S, 1.6 MDa) and the small subunit (30S, 0.9 MDa). These are not discrete molecules. Rather, they are ribonucleoprotein assemblies composed of independent proteins and large ribosomal RNA molecules (rRNA's; Table Z.1). The two subunits are associated with different aspects of the translation process. The small subunit mediates interactions between the mRNA and the tRNA's and the large subunit is associated with the transpeptidation (peptide bond formation) reaction. The overall process of translation is complicated, involving many different molecular species in initiating, propagating and terminating the synthesis of the new polypeptide chain. In the following notes, I will sketch out each stage of translation, and provide particular focus on the critical central stage of ribosome action, elongation.

Table Z.1 Composition of the 70S prokaryotic ribosome (*Escherichia coli*)

Large Subunit (50 S)	Small Subunit (30 S)
Two rRNA's	One rRNA
5 S rRNA – 120 bases	16 S rRNA – 1542 bases
23 S rRNA – 2904 bases	
31 ribosomal proteins (L1-L31)	21 ribosomal proteins (S1-S21)

Translation Initiation

To begin translation of an mRNA transcript, several preliminary steps take place in order to prepare the ribosome (Figure Z.1). Important players in this process are the initiation factors, named IF-1, IF-2 and IF-3. These are soluble proteins that interact with the ribosome and the initial substrates of the ribosome, the mRNA molecule and the initiator aminoacyl $tRNA^{fMet}$. The first step in the formation of the initiation complex is the disruption of the 70S ribosome into its two component subunits. This is achieved by IF-1 and IF3. They bind to the 30S, small

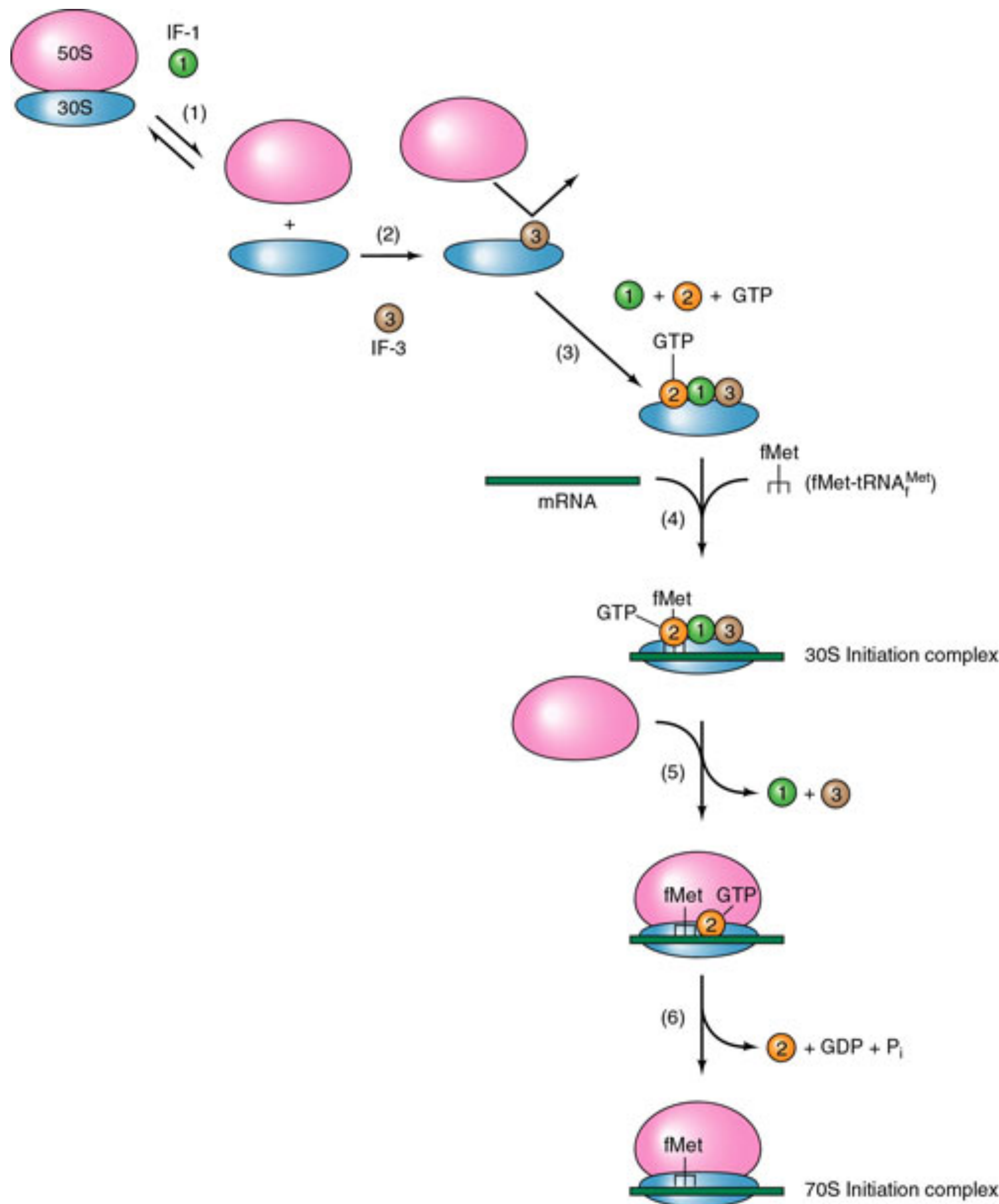

Figure Z.2 Schematic of the events involved in translation initiation.

subunit, and create a stable complex, separate from the 50S, large subunit. Secondly, IF-2 binds as a complex with GTP. IF-2 assists in the recognition and binding of the first aminoacyl tRNA, the initiator tRNA: fMet-tRNA^{fMet} . For most prokaryotic proteins, this tRNA is complementary to the AUG start codon and carries N-formylmethionine (fMet), where the formyl group blocks the α-amino group of the first amino acid (Figure Z.2).

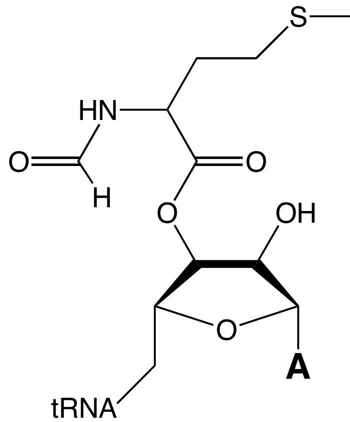

Figure Z.2 Structure of the N-formylmethionyl ester at the 3' end of the initiator tRNA.

After the initiator tRNA has bound, the mRNA binds to the 30S subunit as well. This binding event is mediated by specific Watson-Crick base pairing between the 5' end of the mRNA transcript and the 3' end of 16S rRNA, which is part of the 30S subunit. Transcript mRNA contains a purine (A, G) rich sequence known as the ribosomal binding site (RBS) or Shine-Dalgarno sequence. This is complementary to a pyrimidine rich sequence on the 16S rRNA, allowing for recognition and positioning of the transcript with respect to the ribosome and the anticodon of the initiator tRNA (Figure Z.3). After proper assembly, IF-1 and IF-3 dissociate, allowing the 50S subunit to bind to the remaining species, creating the catalytically functional 70S ribosome, with the initiator tRNA occupying the so-called P (peptidyl) site of the ribosome. At this point, IF-2 hydrolyzes GTP to GDP and inorganic phosphate (P_i) and it too dissociates from the 70S ribosome. Now the complex is prepared for elongation – the synthesis of the polypeptide chain.

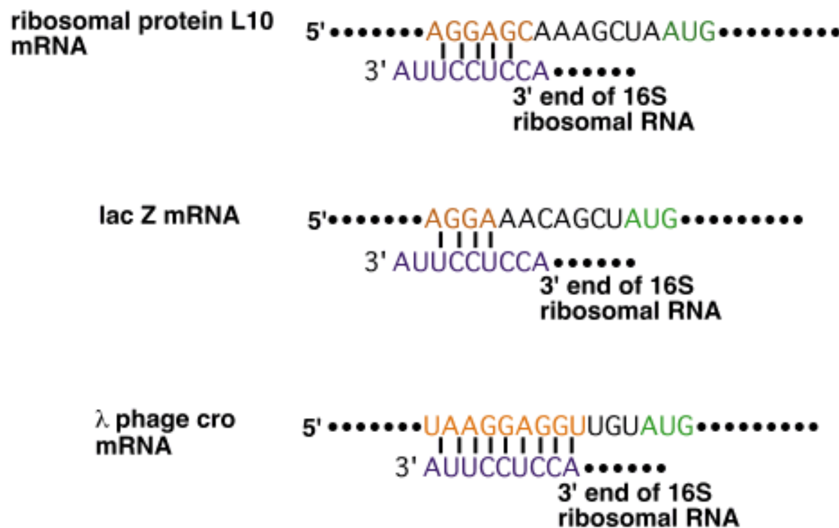

Figure Z.3 Examples of Shine-Dalgarno sequences from mRNA's encoding various proteins. Note that each has regions of complementarity to the 16S rRNA.

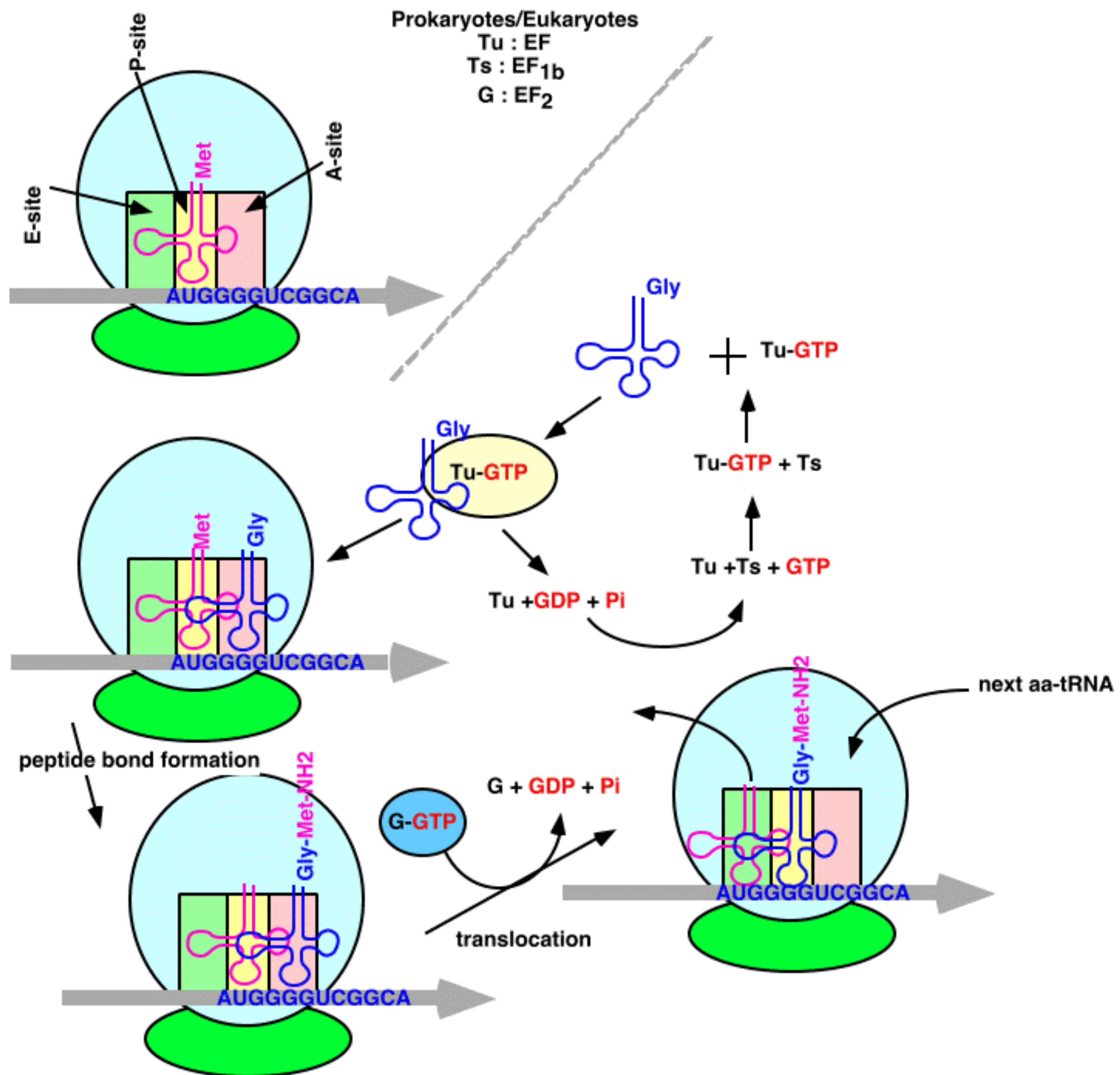

Figure Z.4 Schematic of the elongation cycle, involving EF-Tu, EF-Ts and EF-G.

Elongation: An Overview

Like initiation, elongation takes place through the assistance of three elongation factors, EF-Tu, EF-Ts and EF-G. Each of these proteins participates in the elongation cycle as a discrete entity, and each achieves its function through the hydrolysis of GTP. How GTP hydrolysis has become so important will be addressed below. For starters, though, let's just look at the simplest picture of elongation (Figure Z.4). Each cycle begins with the ribosome complexed to a tRNA in the P site, which is acylated with the growing polypeptide chain. The mRNA remains bound at the interface of the 50S and 30S subunits. The next step is binding of a new aminoacyl tRNA to the A site

(aminoacyl site), as a ternary complex with EF-Tu and GTP. EF-Tu releases the correct aminoacyl tRNA to the A site while hydrolyzing GTP.[1] At this point the "transpeptidation" reaction may take place (Figure Z.5), and the tRNA in the P site is relieved of its peptidyl group, which is now attached to the tRNA in the A site, and is one residue longer thanks to the aminoacyl group that came into the A site with the tRNA. Next, EF-G binds in complex with GTP. GTP is hydrolyzed, the entire ribosomal complex shifts three bases with respect to the mRNA transcript, the P site de-acylated tRNA is ejected into the E (exit) site and the peptidyl-tRNA moves from the A site to the P site, leaving the A site vacant for the next occupant.

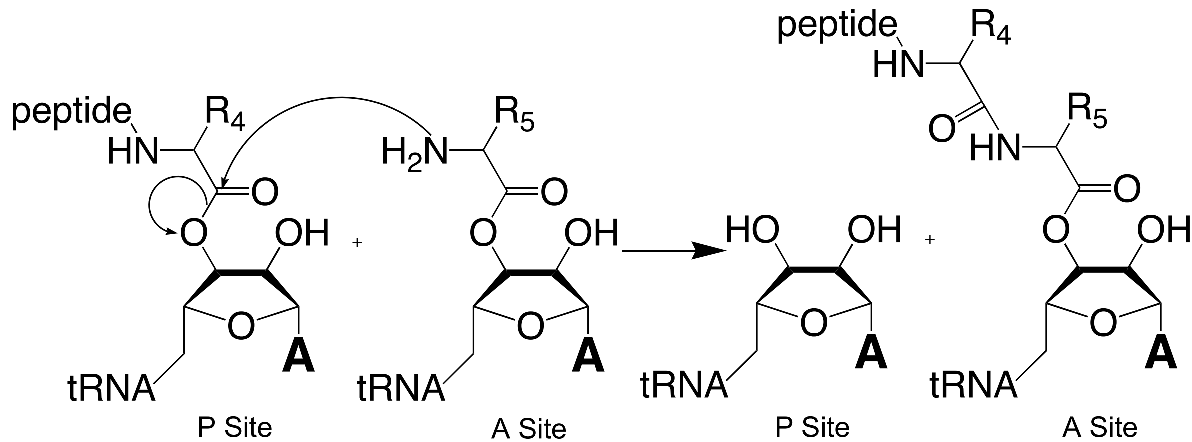

Figure Z.5 The transpeptidation reaction. The α-amino group of the A-site tRNA attacks the ester group of the P-site peptidyl tRNA. An amide bond is formed, transferring the lengthened polypeptide to the A-site tRNA. In this case, the fifth amino acyl residue is attached to a growing tetrapeptide.

The elongation process therefore requires several discrete events to take place in order to add on each additional amino acid. Below, we will concentrate on three issues associated with this cycle: (1) The addition of the correct aminoacyl tRNA to the A site, (2) catalysis of the transpeptidation reaction and (3) advancement of the ribosome complex along the mRNA transcript. But first, a note on the elongation factors, each of which may be classified as a G protein, a class of enzymes that very slowly hydrolyze GTP to GDP and P_i. That they perform this reaction so very slowly (often with k_{cat}'s of less that 1 min^{-1}) indicates that catalytic speed is not their key feature. Instead, these proteins are involved in moderating a variety of cellular processes, and they use the hydrolysis of GTP as a mechanism for inducing a conformational shift in the protein that leads to altered function. We will discuss EF-Tu and EF-G in detail. Both proteins contain a conserved GXP (GXP = GTP or GDP) binding domain with the capacity to catalyze the hydrolysis of GTP. However, their functions are markedly different.

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[1] EF-Tu leaves in complex with very tightly bound GDP. GDP is so tightly bound that it requires a new protein, EF-TS bound to GTP in order to pry the first GDP loose. That again requires the hydrolysis of GTP.

The codon-anticodon interaction on the ribosome

The key interaction involved in delivering the correct aminoacyl tRNA molecule to the correct vacancy in the A site of the ribosome is as simple as a Watson-Crick base triplet between the codon of the mRNA and the anticodon of the tRNA. Note, however, that this is not always a perfect situation. The genetic code is degenerate, meaning that several different codons may correspond to a single amino acid. Often, organisms use fewer tRNA molecules than there are codons to match. The "wobble hypothesis" argues that this can be achieved by allowing the third position of the codon, the so-called wobble position, to make non-canonical base pairing interactions with the 5' base (typically numbered 34) in the tRNA anticodon. For example, in *E. coli* only one $tRNA^{His}$ species exists to pair with both possible His-encoding codons, CAU and CAC. That $tRNA^{His}$ has the anticodon, GUG. The 5' G of the anticodon is able to bind with both cytosine and uracil through the formation of a Watson-Crick base pair, in the case of cytosine, and a "wobble" base pair in the case of uracil (Figure Z.6).

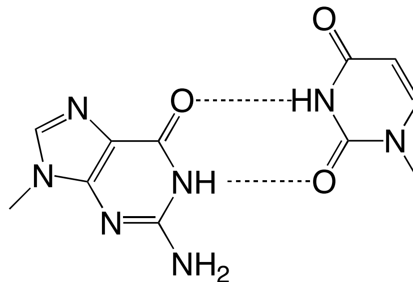

Figure Z.6 The wobble base pair between guanine and uracil.

This phenomenon has been generalized by Crick, to create a list of bases (Table Z.2) that may be used in the wobble position to select for one or more bases at the 3' end of the codon. One argument that has been placed in favor of this method of pairing codons to anticodons is that it allows the more rapid dissociation of the tRNA from the mRNA, because the energetics of attraction between codon-anticodon pairs will be less favorable.

Table Z.2 Possible pairings of bases at the wobble position of the anticodon.

5' anticodon base	3' codon base
A	U
C	G
G	C or U
U	A or G
I	A or C or U

The ribosome's acceptance of this sloppiness in pairing hints at the broader question of how the ribosome is able to assure adequate fidelity of translation based on two standard base pairs and a

third, potentially weaker, base pair. How does the ribosome selectively recognize accurate base pairing between the codon and anticodon? Biochemical studies have shown that two bases, adenines at positions 1492 and 1493 of the 16S rRNA, are protected from chemical modificaton by tRNA binding to the ribosome. Furthermore, mutation of these two adenosine nucleotides to C or G leads to inactive ribosomes and dead *E. coli*.[2] However, the mutation to guanine could be rescued functionally through the use of 2'-deoxyfluororibonucleotides in the mRNA, which replace a hydroxyl group with fluorine on the sugar. It was argued from this observation, that N1 of the adenines is interacting with the 2' OH groups by accepting hydrogen bonds from them. An adenine to guanine mutation replaces a hydrogen bond acceptor with a donor, which will then complement fluorine better than the –OH group.

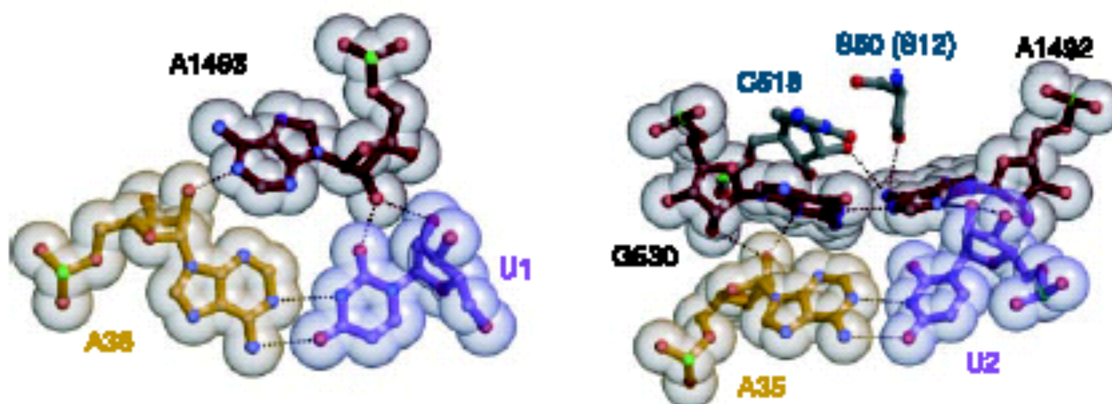

Figure Z.7 Structure of interactions between A1493 and the first codon:anticodon base pair and between A1492 and the second codon:anticodon base pair. Note that G530 also makes an important interaction to the minor groove of the second base pair. (From Ref. 3).

Crystallographic work by Ramakrishnan and co-workers[3] confirms that the 16S rRNA is intimately involved in identifying correct base pairing between the mRNA and the tRNA in the A site. The crystal structure of a 30S-hexauridine-$tRNA^{Phe}$ complex shows that both bases are able to selectively recognize proper Watson-Crick base pairs between ribonucleotides at the first two positions of the codon (Figure Z.7). The N1 acceptor of A1493 hydrogen bonds to the 2' OH of the ribose on the anticodon at position 36 and the 2' OH group of A1493 accepts a hydrogen bond from O2 of the base at position 1 in the codon. This geometry is acceptable for all base pairs, since it only requires ribose sugars in the tRNA and a hydrogen bond acceptor at position O2 of pyrimidines or N3 of purines, which are stereochemically equivalent. At the second base pair, a decidedly non-canonical GA base pair is present in the 16S rRNA that hydrogen bonds to the minor groove of the codon-anticodon interaction. G530 accepts a hydrogen bond from the sugar hydroxyl of the middle nucleotide (A35 here) using its C6 carbonyl, while A1492 accepts a hydrogen bond from the ribose hydroxyl of the mRNA nucleotide at N3 of the adenine base.

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[2] S. Yoshizawa, D. Fourmy and J. D. Puglisi (1999) "Recognition of the Codon-Anticodon Helix by Ribosomal RNA" *Science* **285**, 1722-1725.

[3] J. M. Ogle *et al.* (2001) "Recognition of Cognate Transfer RNA by the 30S Ribosomal Subunit" *Science* **292**, 897-901.

EF-Tu and Fidelity in Translation

Just as in aaRS's there is a problem in specificity associated with the tRNA-mRNA interaction. The inherent differences in hydrogen bonding between a codon and two anticodons, one cognate and the other near-cognate, provides insufficient energetic discrimination between the tRNAs to allow the observed 10^4 fold specificity for correct amino acid insertion in the growing polypeptide chain. The proofreading mechanism to assure that cognate interactions are selected is achieved through the action of EF-Tu (Elongation Factor, Temperature unstable).

EF-Tu, the most abundant protein in *E. coli,* is essentially a chaperone for aminoacyl-tRNA species to the A site of the 70S ribosome. It is a critical factor in assuring that the correct tRNA molecule is paired to the correct codon, though it has no ability to determine whether the correct aminoacyl group is bound or not (that remains the function of the aaRS's). It has three domains, a G-domain that binds GXP (Domain I: residues 1-210), and two domains that bind aa-tRNA (II: 211-310 and III: 311-405). The GTP-bound form of EF-Tu has high affinity for aminoacyl tRNA's, but upon hydrolysis of the GTP to GDP, the affinity drops sharply and EF-Tu releases the aa-tRNA. Thus, EF-Tu delivers its aa-tRNA to the A site of the ribosome, and only upon the hydrolysis of GTP does EF-Tu depart. The following sections are intended to answer four questions related to EF-Tu structure and function:

- How does EF-Tu recognize all aa-tRNAs?

It is important that EF-Tu deliver only aminoacylated tRNA's to the ribosome. Otherwise premature hydrolysis of the peptide chain could result. EF-Tu•GTP binds all aa-tRNA's with roughly the same dissociation constant, about 10 μM, but only binds "naked" tRNA molecules about 1000-fold more weakly. EF-Tu has been crystallized in a complex with GTP, with and without tRNA, and bound to GDP. The aa-tRNA binding site shows high specificity for the L-amino acids, and only those with unblocked amino groups, thus excluding fMet, which reaches the P site during translation initiation. The aminoacyl group must be on the 3' hydroxyl of the tRNA's last nucleotide (A76) and must also be deprotonated at the α-amine, since the latter interacts with two backbone carbonyls and one backbone amide proton to provide three hydrogen bonds (Figure Z.8). There is a large pocket that can nonspecifically accommodate any of the twenty amino acids naturally incorporated by the ribosome. EF-Tu has broad affinity for L-α-amino acids, but will discriminate against D-amino acids due to stereochemical clashes.

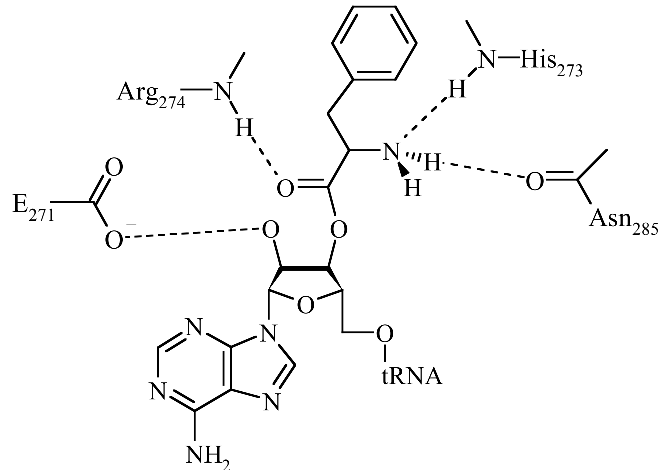

Figure Z.8 Recognition of phenylalanyl-tRNA by EF-Tu.

- How does GTP hydrolysis trigger release of the aa-tRNA to the A site?

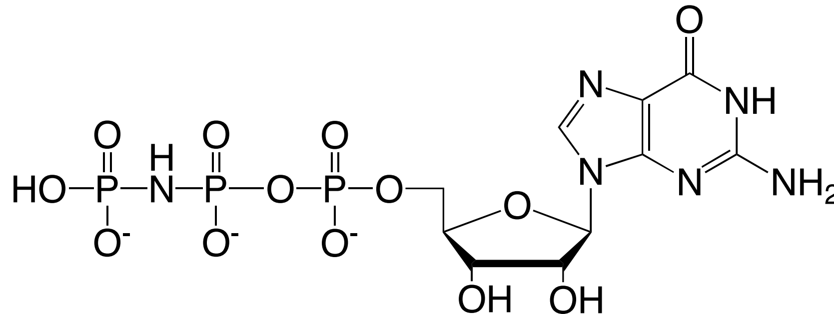

Figure Z.9 GDPNP is a non-hydrolyzable analog of GTP with a phosphoramide linkage between the β and γ phosphorus atoms.

EF-Tu has been crystallized in both GDP bound and GTP bound conformations (GTP is replaced with GDPNP, a non-hydrolysable GTP analog; Figure Z.9). Both bind in domain I (1-210), with differences in two mobile elements regions of the protein: a flexible loop comprising residues 30-64 and a mobile α helix, spanning residues 85-95. In the GDP bound form, these elements are not involved in nucleotide binding, but both are recruited in binding GTP. The γ-phosphate of GTP forms three interactions: ligation to an essential Mg^{2+} ion, and two hydrogen bonds to amide nitrogens of Gly83 and Thr61. When GTP is bound, the two mobile elements are recruited to stabilize the negative charge of the phosphate in the GTP binding site. These shifts in position appear to have a distinct effect on the positions of domains II and III, which are tightly assembled in the GTP bound form (with mobile elements locked in position), but are sprung apart in the GDP bound form. The loss of phosphate is compensated by two water molecules (one bound to magnesium). The protons on the waters repel the amides of Gly83 and Thr61, leading to the gross structural changes. Thr61 is displaced by 17 Å! GTP hydrolysis clearly results in an allosteric change in conformation that disrupts the aa-tRNA binding domains.

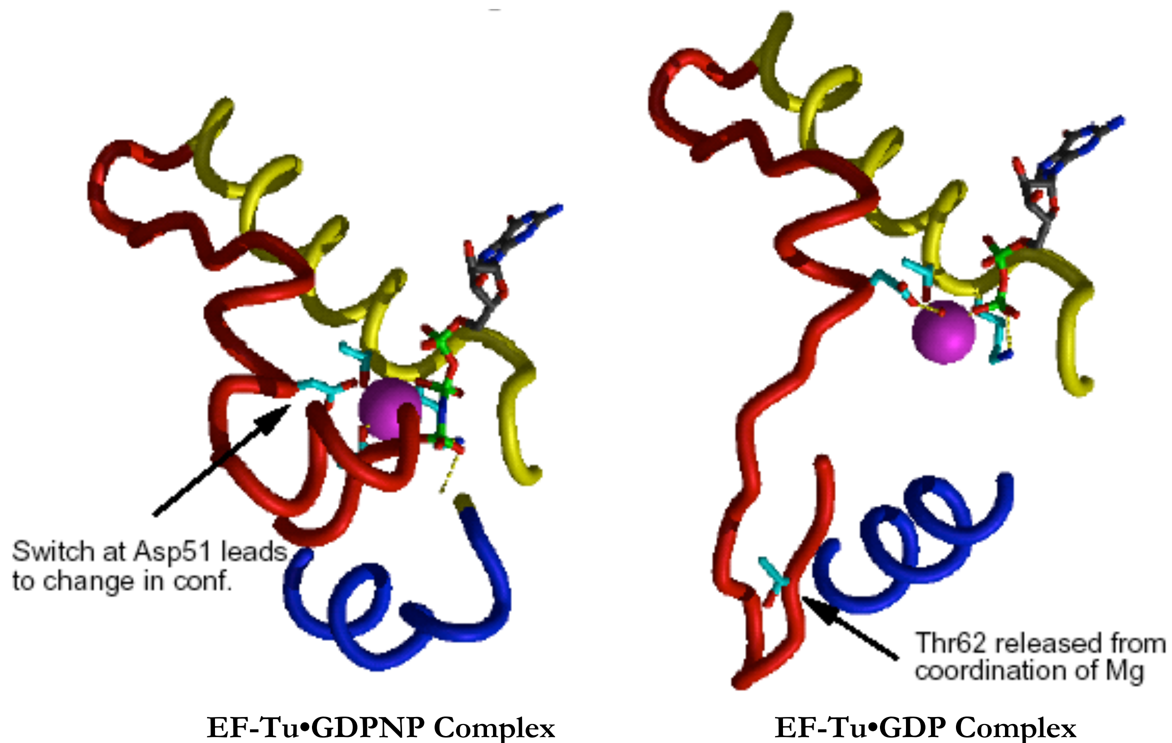

Figure Z.10 Structures of EF-Tu bound to a GTP analog and to GDP. Note the significant motion in the flexible loop (red) and mobile helix (blue). This is prompted by the loss of a phosphate, which affects Mg^{2+} ion coordination and the loss of stabilizing interactions available to the γ phosphate of GTP.

- Why does GTP hydrolysis occur only on the ribosome?

How does GTP hydrolysis occur? This isn't clear, since we don't have atomic resolution data of EF-Tu bound to the ribosome (although there is a cryoelectron microscopy image of this complex[4]). However, there's a tempting argument to assign an incipient "catalytic triad" to the active site of EF-Tu that is only fully assembled when the EF-Tu•GTP•aa-tRNA ternary complex is properly bound in the A site to the correct codon (Figure Z.11). The EF-Tu ternary complex makes extensive interactions with the so-called sarcin-ricin stem loop (SRL) of the 23S rRNA in the 50S subunit of the ribosome.

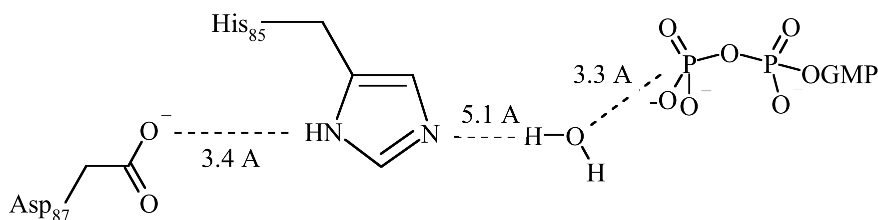

Figure Z.11 Putative active site that forms for GTP hydrolysis upon EF-Tu•GTP•aa-tRNA binding to the ribosome.

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[4] H. Stark *et al.* (2003) "Ribosome interactions of aminoacyl-tRNA and elongation factor Tu in the codon recognition complex" *Nature Structural Biology* **11**, 849-855.

- Why use GTP hydrolysis in executing EF-Tu's function?

EF–Tu·GDPNP Complex　　EF–Tu·GDP Complex

Figure Z.12 Conformational changes associated with GTP hydrolysis. Domain I is in yellow, domain II in green and domain III in turquoise. The flexible loop and mobile helix are red and blue, respectively.

An interesting feature of EF-Tu is that despite binding GTP through more sites than GDP, the K_d of GDP (1.8 nM) is lower than the K_d for GTP (29 nM), reflecting a more energetically favorable interaction with GDP. The weaker binding of GTP to EF-Tu indicates strain in the GTP bound conformation of EF-Tu. Upon GTP hydrolysis, that strain is released and the lower energy conformation (accessible in the GDP bound state) is attained. This conformational shift associated with GTP hydrolysis provides an irreversible release of the aa-tRNA from the clamp formed by domains II and III (Figure Z.12; Scheme Z.1).

Scheme Z.1

Tu + GTP

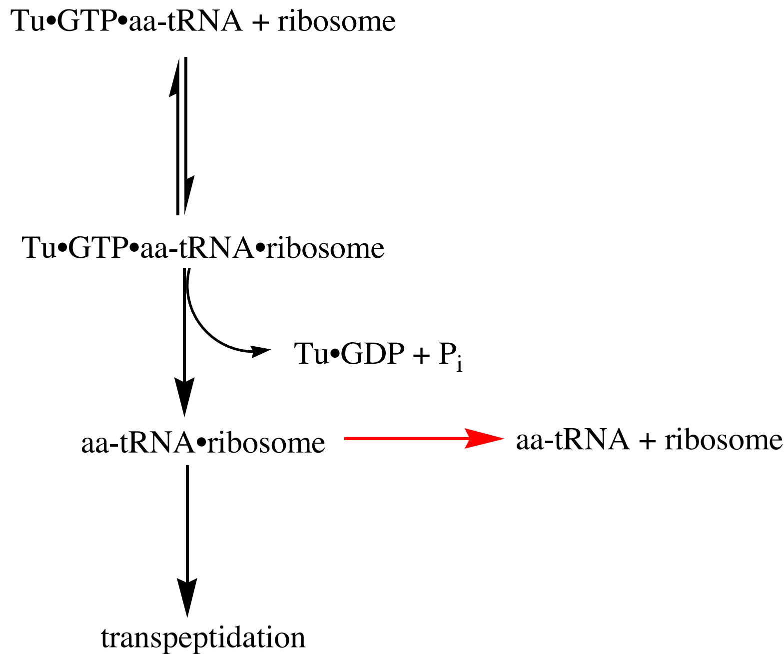
Tu·GTP

Tu + GDP + P_i

Tu·GDP + P_i

However, this only deals with the conformational implications of GTP hydrolysis. There are also good kinetic reasons to include the hydrolysis reaction in bringing an aa-tRNA to the A site. Hopfield, in an insightful 1974 article, anticipated the role of GTP hydrolysis as a proofreading mechanism for codon-anticodon pairing.[5] He proposed a scheme (Z.2) in which there are two stages to an aa-tRNA binding to a codon. In the first stage, wildly non-cognate aa-tRNA's, complexed to EF-Tu•GTP, are rejected efficiently, but "near-cognates" may bind only some small fraction more poorly than the cognate. In Hopfield's mechanism, GTP hydrolysis allows EF-Tu to dissociate and places the aa-tRNA in a new, unstable position for discrimination. The energy of GTP hydrolysis is invested in creating an unstable intermediate, the aa-tRNA•ribosome complex. Now it's a race to the goal. The cognate aa-tRNA should react with the peptidyl-tRNA quickly, while the near non-cognate will dissociate more quickly. Both of those possibilities are essentially irreversible. In the first case, the formation of the peptide bond is thermodynamically favorable, and in the second case, uncomplexed aa-tRNA molecules do not bind well to the ribosome. This second step creates an additional fractional preference for the cognate aa-tRNA that is multiplied times the first discrimination quotient to create enhanced specificity overall.

Scheme Z.2



An important component of this mechanism is the irreversibility of the aa-tRNA dissociation from the ribosome. If the aa-tRNA could reassociate to the 2nd proofreading site (red arrow in Scheme Z.2) then the first level of discrimination would be short-circuited. There could be only one stage of discrimination because the aa-tRNA would bind directly to the ribosome and the role of EF-Tu would be redundant. The thermodynamic drive of GTP hydrolysis permits the formation of a highly unstable aa-tRNA•A site intermediate that either persists long enough to react with the peptidyl-tRNA or dissociates forever. Thus GTP hydrolysis achieves two functions: (1) it acts as a trigger

[5] J. J. Hopfield (1974) "Kinetic Proofreading: A New Mechanism for Reducing Errors in Biosynthetic Process Requiring High Specificity" *PNAS* **71**, 4135-4139.

for the conformational change in EF-Tu that leaves the aa-tRNA at the ribosome and (2) because that trigger is irreversible, it permits a second round of proofreading to take place in matching the anticodon of the tRNA to the codon of the mRNA.

Work by Thompson and Stone[6] confirmed Hopfield's mechanism. They studied the ability of the ribosome to add one of three different amino acids to a growing peptide chain, where the correct amino acid is phenylalanine, encoded by a 5'-UU codon on the mRNA. When Phe-$tRNA^{Phe}$ was placed in the reaction mixture, phenylalanine was added and one molecule of GDP was produced. Conversely, if they added Lys-$tRNA^{Lys}$, which has a UUU anticodon, there was no production of GDP and no addition of lysine. The coup de grace came when they added Leu-$tRNA^{Leu}$, a *near-cognate*, which has a GAG anticodon. In this instance, eight molecules of GDP were formed for every leucine residue added to the polypeptide chain (Figure Z.13). This result is as predicted by Hopfield. The preliminary binding of Phe-$tRNA^{Phe}$ or Leu-$tRNA^{Leu}$ doesn't discriminate adequately against leucine. Therefore, EF-Tu will occasionally hydrolyze GTP and place Leu-$tRNA^{Leu}$ in the proofreading position. For every eight times GTP is hydrolyzed, only once is leucine added to the peptide – the other seven times it is released from the ribosome without allowing the reaction.

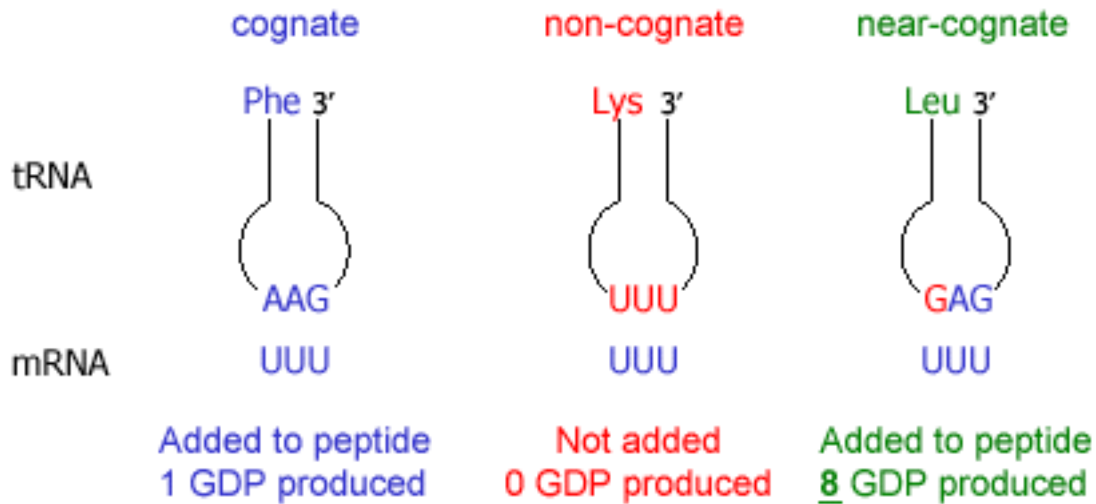


Figure Z.13 Results of the experiment by Thompson and Stone to verify Hopfield's mechanism.

More recently, Wintermeyer has done a thorough kinetic analysis of the editing reaction involving EF-Tu and the ribosome.[7] While the number of discrete steps is greater than anticipated by Hopfield, the results demonstrate the validity of the scheme. In Wintermeyer's kinetic analysis, non-cognate aa-tRNA molecules (in complex with EF-Tu•GTP) do not bind measurably to the ribosome. Near-cognates bind with a K_d of 200 nM and full cognates bind with a K_d of 0.5 nM, reflecting a 400-fold specificity for the cognate aminoacyl-tRNA. GTP hydrolysis leads to a second opportunity to discriminate against near-cognates. Near cognate aa-tRNA molecules dissociate 20-fold more rapidly from the A-site than do cognates, and cognates react with the peptidyl-tRNA 70-

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[6] R. C. Thompson and P. J. Stone (1977) "Proofreading of the codon-anticodon interaction on ribosomes" *PNAS* **74**, 198-202.

[7] Reviewed by M. V. Rodnina and W. Wintermeyer (2001) "Ribosome fidelity: tRNA discrimination, proofreading and induced fit" *Trends in Biochem. Sci.* **26**, 124-130.

fold more rapidly than do near-cognates, yielding an addition 1400-fold (20 times 70) specificity for cognates. Overall, this analysis provides 56,000-fold specificity for cognates vs. near-cognates on the basis of two separate and discrete bound states.

Catalysis of the Transpeptidation Reaction

Among the most exciting results to arise from the crystallographic studies of the ribosome is that the catalytic groups associated with the transpeptidation reaction come from RNA, not protein. In a reaction mechanism that invokes general acid/base catalysis, the attack of the α amino group of the A site aminoacyl tRNA on the acyl carbonyl in the P site is mediated by a binding site that is composed of nucleotides from the 23S rRNA. Steitz and coworkers[8] have crystallized the 50S subunit of the ribosome from *Haloarcula marismortui* in complex with a transition state analog for the transpeptidation reaction (Figure Z.14). As in peptide and ester hydrolysis, the transpeptidation reaction goes through a tetrahedral intermediate that is well modeled by a phosphate linkage. A suitable analog includes a trinucleotide CCdA (two ribonucleotides of cytosine with a 2' deoxyadenosine) linked via a phosphoramidate to the amino group of puromycin, an antibiotic that inhibits ribosome action.

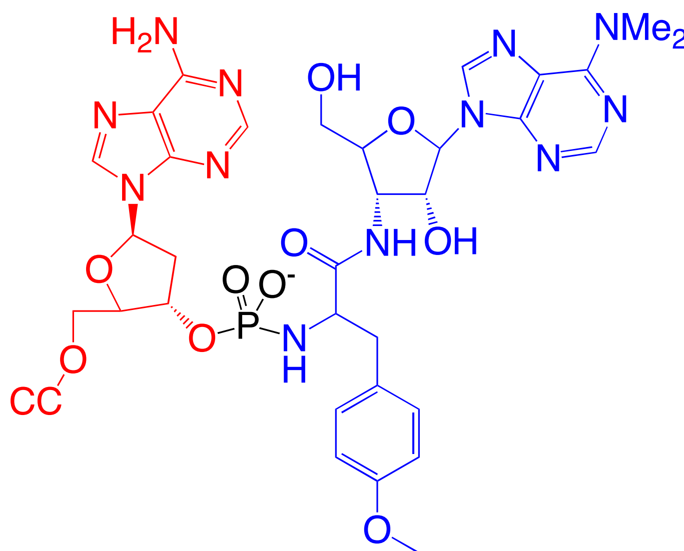

Figure Z.14 Structure of the CCdA-p-Puro inhibitor of the transpeptidation reaction. The structure of puromycin is shown in blue.

The positioning of the CCdA-p-Puro inhibitor reveals a single adenine base (A2541 in *E. coli* numbering) within hydrogen bonding of the phosphate. Steitz has proposed that the N3 of A2541 is an active site general base that cycles between acidic and basic states during catalysis. One difficulty in assigning this role to A2541 is that the pK_a of N3 is roughly 1.5 in solution (N1 has a pK_a of 3.5). However, the close proximity of the phosphonate of the inhibitor (Figure Z.14) to N3

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[8] P. Nissen J. Hanson, N. Ban, P. B. Moore and T. A. Steitz (2000) "The Structural Basis of Ribosome Activity in Peptide Bond Synthesis" *Science* **289** 920-930.

indicates that the base is likely protonated under the crystallization conditions (pH 5.8). How might the active site of the ribosome shift the pK_a of A2541 so significantly? The most likely answer relates to the local interactions made to A2541, which stabilize its cationic conjugate base form, or alternatively stabilize an unusual tautomeric form (Figure Z.15). Hydrogen-bonding from the N6 of A2541 to O6 of G2447, which in turn H-bonds to a backbone phosphate via its N2 amino group, creates a proton/charge relay to assist in deprotonation of the α amino group of the aminoacyl-tRNA in the A site.

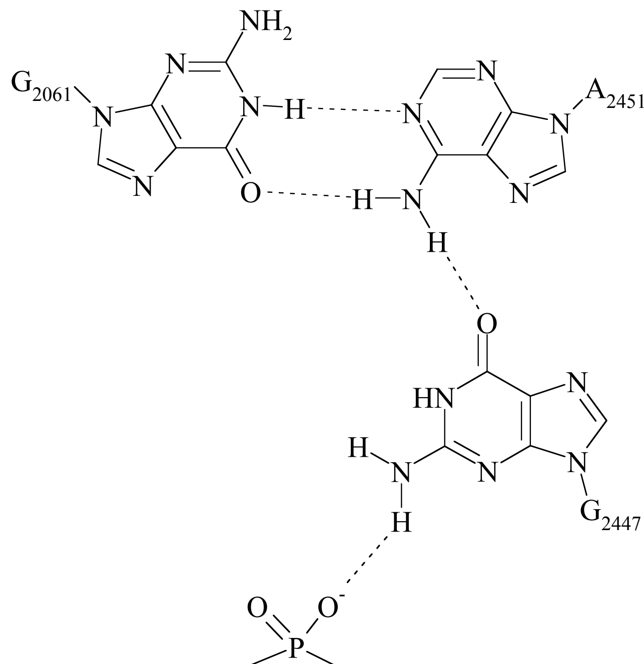

Figure Z.15 General acid base catalysis performed by A2541. (B) Hydrogen bonding environment around A2541.

Solution studies by Strobel's lab[9] support Steitz's hypothesis. Noting that only RNA surrounded the transpeptidation site, they went fishing for a nucleic acid base with a strongly shifted pK_a. After testing the pH dependence of base methylation by dimethyl sulfate (DMS) in the 23S rRNA between bases 2043 to 2625, only one base – A2541 - was found to have its reactivity altered within the region of pH 4.5 to 8.5. If the pK_a were as low as 3.5, the base would remain unprotonated throughout this range. In this instance, the pK_a of A2541 was calculated to be 7.6, roughly six orders of magnitude different from an isolated adenosine! Mutation of A2541 to other nucleotides killed *E. coli* cells that expressed the mutant 23S RNA, and in fact, that position is conserved absolutely among all three kingdoms of life. But…

[9] Muth, G. W., Ortoleva-Donnelly, L. & Strobel, S. A. (2000). A Single Adenosine with a Neutral pK_a in the Ribosomal Peptidyl Transferase Center. *Science* 289, 947-950.

The Ribosome as Entropy Trap

Despite the structural evidence that the ribosome could use an active site adenine as a general base, recent work has shown that the mechanism is not likely to be that simple, and that the role of A2451 has yet to be fully explained. Recently, Rachel Green and Marina Rodnina have independently shown that A2451 is not as important as previously believed. Green's group prepared a series of mutant ribosomes varying at position 2451. Initially, it was found that mutants to 2451 had severely decreased k_{cat} values. However, this assay used puromycin as the A-site nucleophile. When puromycin is replaced with Phe~$tRNA^{Phe}$, close to full activity is seen with the A2451U mutant, which is highly unlikely to be active as an active site base.[10] Rodnina's group has similarly shown that the A2451U mutation retains activity, and, in *Mycoplasma smegmatis* is not necessary for activity. Moreover, the A2451U displays the same pH dependence in activity that the A2451 wild-type ribosome does.[11]

So how does the ribosome catalyze transpeptidation. An interesting hypothesis has been put forward by Richard Wolfenden, working with Rodnina. They hypothesize that the ribosome catalyzes transpeptidation by making the entropy of activation for transpeptidation less negative.[12] The entropy of activation might be made less negative by holding the two substrate acylated tRNA groups fixed in the P- and A-sites, the entropy of the ground state is lowered, requiring less of a loss of entropy to reach the transition state – thus the term "entropy trap". Evidence for this behavior comes from a pair of temperature dependent studies using an uncatalyzed reaction between a primary amine (Tris base) and an amino acyl ester, and the catalyzed reaction between two amino acylated tRNA molecules (Figure Z.16).

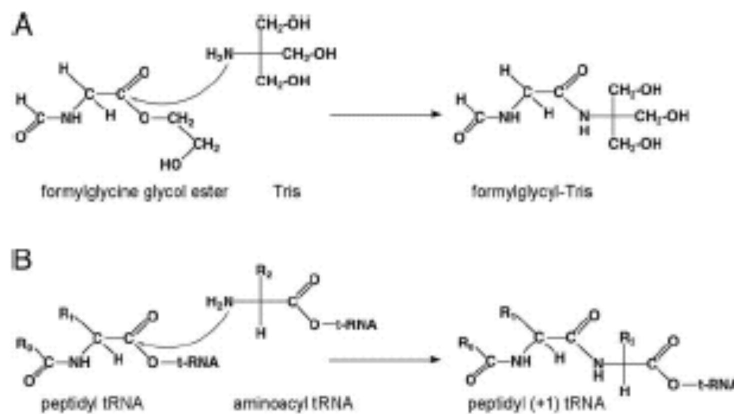

Figure Z.16 (A) Uncatalyzed and (B) catalyzed reactions studied by Wolfenden[12].

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[10] Green, R. (2004) The Active Site of the Ribosome Is Composed of Two Layers of Conserved Nucleotides with Distinct Roles in Peptide Bond Formation and Peptide Release. *Cell* **117**, 589-599.

[11] Rodnina, M. V. (2005) Essential Mechanisms in the Catalysis of Peptide Bond Formation on the Ribosome. *J. Biol. Chem*. **280**, 36065-36072.

[12] Wolfenden, R. (2004) The Ribosome as an Entropy Trap. PNAS 101, 7897-7901.

Aside from noting the immense (2 x 10^7-fold) acceleration afforded by the ribosome for the amide bond forming reaction, the more interesting result is that the rate of each reaction possesses an identical temperature dependence. Using the equation:

$$\ln k = -\Delta H^{\neq}/R(1/T) + C$$

where $C=\ln(\nu^{\neq}\exp^{\Delta S/R})$, the authors demonstrated that both the uncatalyzed reaction with Tris and the ribosome-catalyzed reaction possess enthalpies of interaction of roughly 15 kcal/mol (Figure Z.17). Thus the full 20 million fold increase in rate must be ascribed to a less negative entropy of interaction between the two reaction.

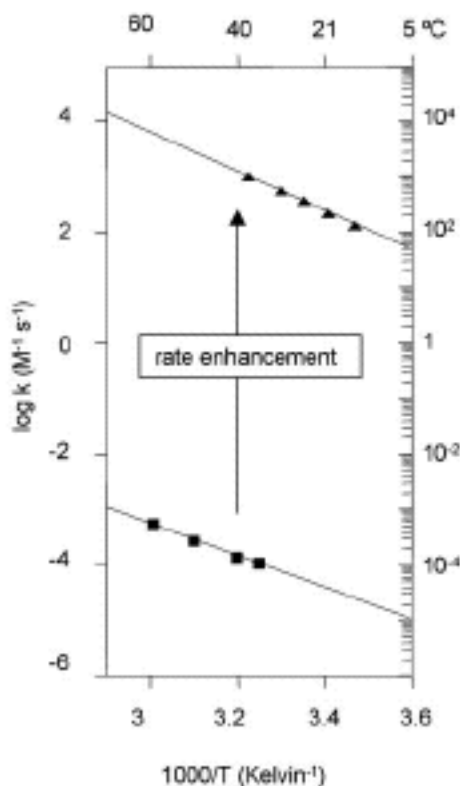

Figure Z.17 Temperature dependence of the rate of amide bond formation in the catalyzed (triangles) and uncatalyzed (squares). The slopes of the plots give $-\Delta H^{\neq}/R$.

Structure and Function in EF-G

After the transpeptidation reaction, the ribosome is in an awkward state. The P-site contains a non-acylated tRNA that should be discarded, and the A-site contains a peptidyl-tRNA that should be in the P-site. To reset the elongation cycle, the ribosome needs to ratchet along the mRNA by three bases, which will move the current P-site and A-site species to a point of exit and the P-site respectively. That *translocation* process is achieved through the action of EF-G, at the expense of one molecule of GTP. EF-G binds after the peptide transfer reaction and promotes the translocation of the new peptidyl-tRNA from the A site into the P site, with the concomitant expulsion of the deacylated tRNA in the P site into the E site. Note that this translocation requires that work must

be done – a physical force must be applied over the distance spanned by three nucleotides in the mRNA. The issue with EF-G is how this G-protein provides that work.

In work by Wintermeyer and colleagues[13] the kinetics of EF-G were observed. EF-G is known to bind to the ribosome in a complex with GTP. Hydrolysis of GTP leads to translocation and subsequent diffusion of EF-G•GDP from the ribosome. These folks worked with the reaction of fMet-$tRNA^{fMet}$ in the P site and Phe-$tRNA^{Phe}$ in the A site, where the $tRNA^{Phe}$ was labeled with proflavin. When the fluorescently labeled $tRNA^{Phe}$ is translocated into the P site, fluorescence intensity increases. When assembled ribosome (containing tRNAs in A and P sites) is subjected to the addition of EF-G•GTP, fluorescence increases, reflecting a rate constant of 25 s^{-1} (for the translocation of peptidyl-$tRNA^{Phe}$ into the P site). However, if EF-G is added in complex with the non-hydrolyzable GTP analog GDPNP, the rate of fluorescence increase is dramatically slower, reflecting a rate constant for translocation of 0.5 s^{-1}.

It was further shown that EF-G•GTP binds at a rate of 120-150 $\mu M^{-1}s^{-1}$ and GTPase activity in the ribosome achieves a rate of 125 s^{-1}. That means that the rate determining step in the process is *not* EF-G binding, *nor* is it GTP hydrolysis – rather it is translocation, which provides the greatest kinetic barrier in the cycle. How does EF-G harness the hydrolysis of GTP to provide energy to surmount that barrier? The answer comes from the structure of the EF-G•GDP complex, which displays a strikingly familiar picture. EF-G has five domains: I, II closely match those of EF-Tu, with I acting as the GTP/GDP binding domain, while domains III, IV and V are spectacularly unoriginal – they mimic tRNA by adopting a "L" shaped fold. In essence EF-G is *mimicking* EF-Tu bound to an aa-tRNA (Figure Z.18). It presumably achieves its function by binding in a fashion similar to the EF-Tu•GTP•aa-tRNA complex.

When domain IV is deleted from the structure, translocation drops to basal rates, identifying this domain as essential for activity. The current hypothesis is that EF-G bound to GTP adopts an extended structure that possesses the same kind of strain as EF-Tu bound to GTP (note that K_d for GTP is slightly higher than for GDP again – 14 μM to 11 μM). Upon GTP hydrolysis, the intermolecular forces applied by the γ phosphate of GTP are presumably lost and the open spring is now ready to shut, but to do so it must translocated the mRNA with the peptidyl-tRNA in the A site by three nucleotides. The free energy of GTP hydrolysis is harnessed as physical work!

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[13] *Nature*, **385**, 37 (1997)

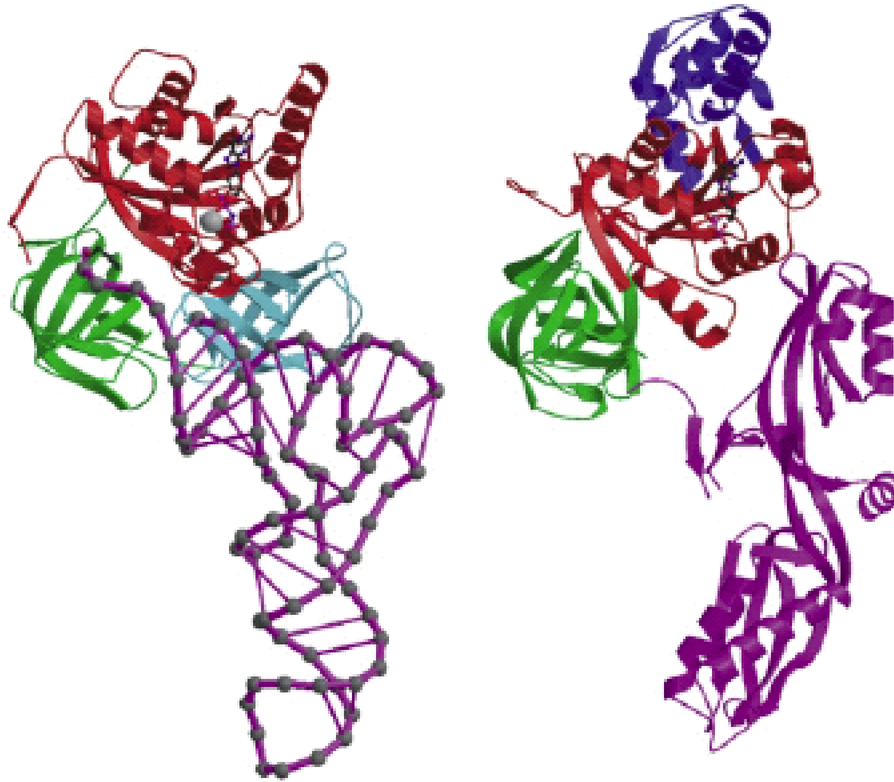

Figure Z.18 Comparison of the structure of EF-Tu•GTP•aa-tRNA on the left and EF-G on the right. The C-terminal domains of EF-G adopt a fold that is strikingly similar to the tRNA structure.

Molecular Mimicry

EF-G is not the only protein involved in translation to exhibit molecular mimicry. New structures indicate that translation termination also utilizes proteins that look like something they're not. Release factors RF1 and RF2 in E. coli bind to stop codons in place of EF-Tu•GTP•aa-tRNA complexes. RF1 binds to UAA and UAG while RF2 binds to UGA. Like EF-Tu, they show enormous selectivity for these sequences, and a helper protein (RF3) assists in generating that specificity through GTP hydrolysis.

The related eukaryotic protein, eRF1 (which replaces both RF1 and 2) from humans looks remarkably like the ternary EF-Tu complex and presumably bind to the ribosome in an analogous fashion. In place of the acceptor stem, in eRF1 (and both bacterial RFs) there is a conserved GGQ sequence that appears in the structurally analogous position. It is hypothesized that the glutamine acts as a general base and activates water for hydrolysis of the peptidyl-tRNA in the P site.

Finally, one more protein – the ribosomal recycling factor – RRF, looks startlingly like a tRNA molecule, and appears to achieve function by binding in an analogous fashion once the peptide has been hydrolyzed from the tRNA in the P site, and the ribosome is ready to be recycled by splitting the 30S and 70S subunits.

A Word on the Peptide Exit Channel

The structure of the 50S subunit of the ribosome bound to the CCdA-p-Puro inhibitor revealed an additional feature of interest. Given the positioning of the groups involved in catalysis, there is only one exit pathway available to the growing polypeptide chain – 100 Å long channel through the 50S subunit.[Steitz ref. #5] The tunnel varies in width between 10 and 20 Å and is lined with hydrophilic groups. Steitz argues that the hydrophilic nature of the tunnel makes strong association of the peptide chain with groups in the tunnel unlikely. Hydrophobic groups will not be able to associate with any hydrophobic surfaces in the tunnel, and full hydration of the tunnel and the growing peptide makes association of polar groups enthalpically neutral. Because the tunnel is narrow, the peptide cannot fold, but its length can shield approximately 40-50 residues, preventing premature folding or association of the peptide chain.