

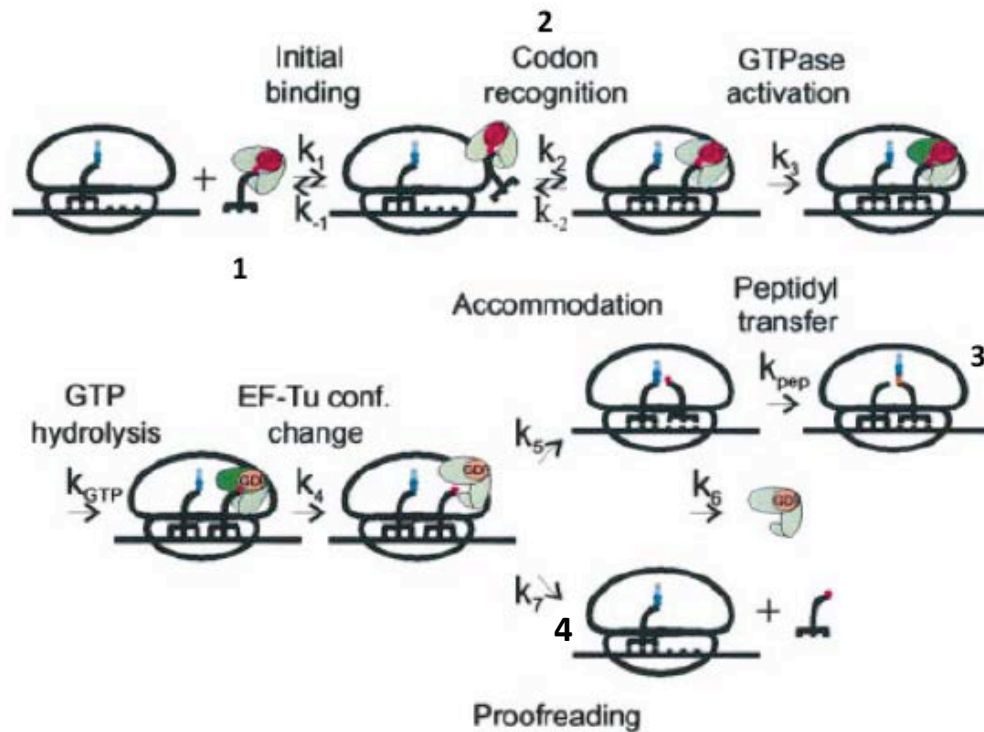
ANSWER KEY

5.08 Biological Chemistry II (Spring 2016) Problem Set #2

This problem set contains two questions and four pages.

Question 1:

The following cartoon depicts the model for EF-Tu-dependent aa-tRNA^{aa} binding to the ribosome. The E site is not shown in this depiction. This model is based on numerous experiments. You will examine a number of these experiments during recitation 3. The information that will be presented in recitation 3 is not required for answering this question.



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Answer the following questions:

A) In the cartoon above, what is depicted by the molecules labeled as “1”? What is the process defined by k_1 and k_{-1} ?

The molecule depicts an aminoacylated-tRNA bound to EF-Tu•GTP. It is the EF-Tu•GTP•aa-tRNA^{aa} ternary complex.

The process defined by k_1 and k_{-1} is the initial binding process. EF-Tu•GTP•aa-tRNA^{aa} ternary complexes bind to the ribosome weakly. They are recruited by the ribosomal proteins L7 and L12. This step is independent of the mRNA.

B) Describe the process labeled “2.” Provide an explanation for the forward and reverse reactions (k_2 and k_{-2}).

This step depicts the codon-anticodon recognition process, also known as decoding. The anticodon on the aminoacyl-tRNA should match (cognate) that of the mRNA codon in the A-site of the PTC. If EF-Tu•GTP•aa-tRNA^{aa} delivers the cognate tRNA to the A site, then the aminoacyl-tRNA will be bound to the A site. Conformational change occurs that allows for GTPase activation (next step, k_3) and the EF-Tu hydrolyzes GTP to GDP and P_i (subsequent chemical step, k_{GTP}). If the codon-anticodon does not match (e.g. near-cognate), the aminoacyl-tRNA leaves the A site (k_{-2} , reverse reaction). Note that Rodnina and co-workers measured these rate constants and determined that k_2 (forward reaction of process labeled 2) is nearly invariant for all cognate and near-cognate ternary complexes. In contrast, k_{-2} varies tremendously and is 10^2 - 10^3 faster for near-cognate than cognate! Also note there is a peptidyl-tRNA in the P site.

C) What is depicted in the ribosome cartoon shown as “3”

Peptide bond formation occurs in cartoon 3. The growing polypeptide chain on the P-site tRNA is loaded onto the incoming amino acid on the A-site tRNA. The alpha amino group of the A-site aa-tRNA^{aa} is the nucleophile (nucleophilic attack on the ester linkage of the P-site peptidyl-tRNA). Think about pK_a values – the ribosome must have a means to tune the pK_a of the alpha-amino group such that it is lower than we expect (R-NH₂ instead of R-NH₃⁺).

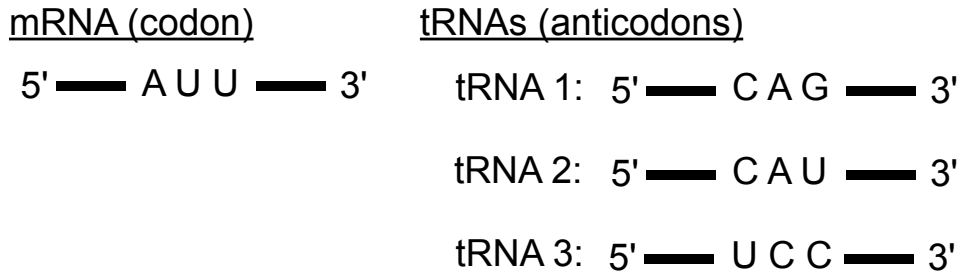
D) In the cartoon above, what steps are important for fidelity? Why?

Steps labeled as 2 and 4 are important for fidelity.

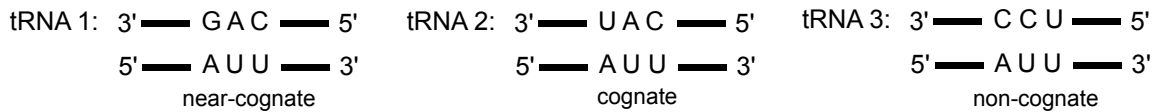
Step 2 – as described above, this is the codon/anticodon recognition step. The correct or cognate codon/anticodon match is needed for the genetic code to be translated accurately and avoid incorporation of the wrong amino acid into the growing polypeptide chain. In this step, the k_{-2} varies tremendously and is 10^2 - 10^3 faster for near-cognate than cognate, which allows for near-cognate to be rejected by the ribosome. In contrast, the cognate tRNA will remain bound.

Step 4 – this step (defined by k_7) is a final proof-reading step. It occurs after GTP hydrolysis by EF-Tu. If a near-cognate tRNA ended up in the A site after GTP hydrolysis, this proof-reading step can result in rejection of the near-cognate tRNA from the A-site to maintain fidelity. Note that this proof-reading occurs at the expense of GTP.

E) Consider the following tRNAs and mRNA:



i) Draw the codon / anticodon interactions for each mRNA / tRNA pair and indicate whether the pair is a cognate, near-cognate or non-cognate match.



ii) What is the consequence of each mRNA/tRNA pair on the process labeled “2”?

mRNA + tRNA 1 – because it is a near-cognate pair, step 2 will proceed in the reverse direction; k_{-2} will increase by 10^2 - 10^3 fold and thereby allow the near-cognate EF-Tu•GTP•aatRNA^{aa} to be rejected by the ribosome.

mRNA + tRNA 2 – this is a cognate pair. Step 2 will proceed in the forward direction such that GTPase activation occurs.

mRNA + tRNA 3 – this is a non-cognate pair. It is rejected by the ribosome.

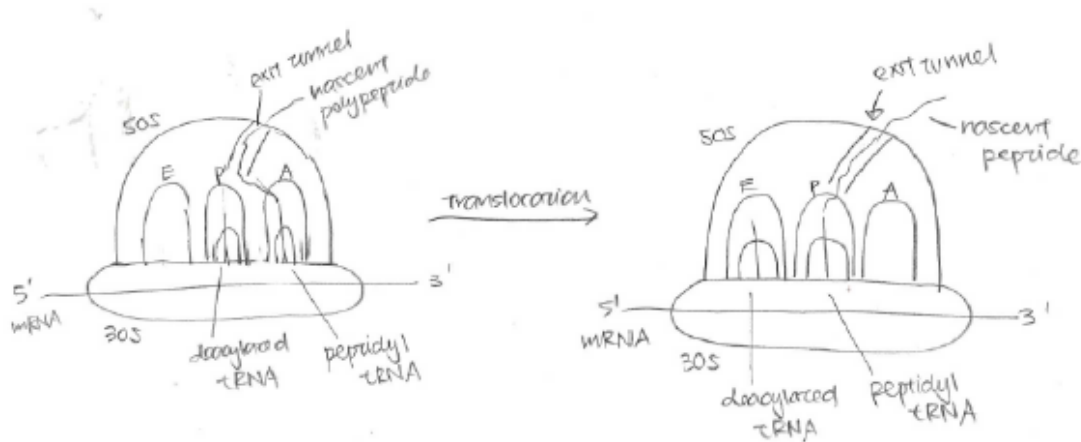
iii) What is the consequence of each mRNA/tRNA pair on the process labeled “4”?

mRNA + tRNA 1 – if the near-cognate tRNA were to remain bound to the ribosome to this stage, proof-reading in step 4 would result in its rejection from the A-site.

mRNA + tRNA 2 – none; proof-reading would not occur because this pair is cognate.

mRNA + tRNA 3 – this is a non-cognate pair. It is rejected by the ribosome.

F) What occurs immediately after peptidyl transfer? Draw a cartoon of the ribosome in this state. Include and label (i) the mRNA (labeling 5' and 3' end), (ii) the three sites of the PTC, and (iii) nascent polypeptide emerging from the exit tunnel in your cartoon.



Translocation where the deacylated tRNA in the P site moves to the E site, and the peptidyl tRNA in the A site moves to the P site. The mRNA moves as well. Recall that the “hybrid states” form immediately after peptide bond formation. The 3' end of the deacylated tRNA enters the E site while the anticodon end remains in the P-site. The 3' end (with growing peptidyl chain) of the new peptidyl-tRNA enters the P-site and the anticodon end remains in the A-site. Subsequently, ratcheting of the ribosome occurs (conformational change) and EF-G is the elongation factor required for translocation.

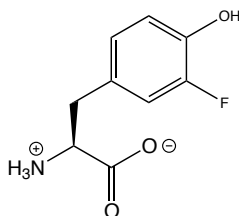
Question 2:

Nascent polypeptide chains must fold into native conformations, and we will address protein folding in module 2. In many labs, protein folding is performed and studied in aqueous solution, hence in the “test tube.” These studies provide useful information, but the folding environment provided by simple aqueous buffer is very different from what the polypeptide encounters in the crowded environment of the cell. Imagine forgetting the test tube for folding experiments and venturing into the cell. Professor Gary Pielak’s lab at University of North Carolina Chapel Hill is pioneering studies of protein folding inside living cells – wow! One approach that the Pielak Lab uses is to incorporate ^{19}F labels into the protein of interest and employ ^{19}F NMR spectroscopy to study protein folding in live *E. coli* cells (see *J. Am. Chem. Soc.* **2010**, *132*, 321-327 if you are curious; you need not read the paper to answer this question). In these experiments, the *E. coli* cells produce the ^{19}F -labeled protein in the cytosol, and these *E. coli* are placed in a NMR tube and the ^{19}F signal(s) recorded.

Why the choice of the ^{19}F label? In brief, ^{19}F NMR is a useful technique because ^{19}F is a spin $\frac{1}{2}$ nucleus and has 100% natural abundance. The ^{19}F nucleus gives a wide range of chemical shifts (400 ppm versus 13 ppm for ^1H) and the chemical shifts are very sensitive to the local environment (consider that a fluorine nucleus is surrounded by 9 electrons compared to 1 electron for a proton). Moreover, taking a ^{19}F NMR spectrum is simple, very much like recording a ^1H NMR. Various fluorinated amino acids, including 3-fluorotyrosine, have been employed in ^{19}F NMR and in the cell-based experiments reported by the Pielak Lab. Note: ^{19}F is not a radioisotope.)

Imagine you wish to study the folding landscape of your protein of interest in living *E. coli* cells by ^{19}F NMR. To do so, you will have *E. coli* site-specifically incorporate the unnatural amino acid (UAA) 3-fluorotyrosine (3-FTyr) into your protein using its endogenous ribosome. Your protein is 100-aa in length.

A) Draw the chemical structure of 3-fluorotyrosine.



B) You will need an evolved or “new” tRNA/aaRS pair in order to incorporate 3-fluorotyrosine into your protein. Based on class discussion, describe the essential features of this tRNA/aaRS pair.

Background: The Shultz method for UAA incorporation requires a unique codon that uniquely designates the UAA (reassignment of the Amber STOP codon), a evolved or “new” tRNA that is specific for the unique codon, and a corresponding aaRS that loads the UAA onto the unique tRNA.

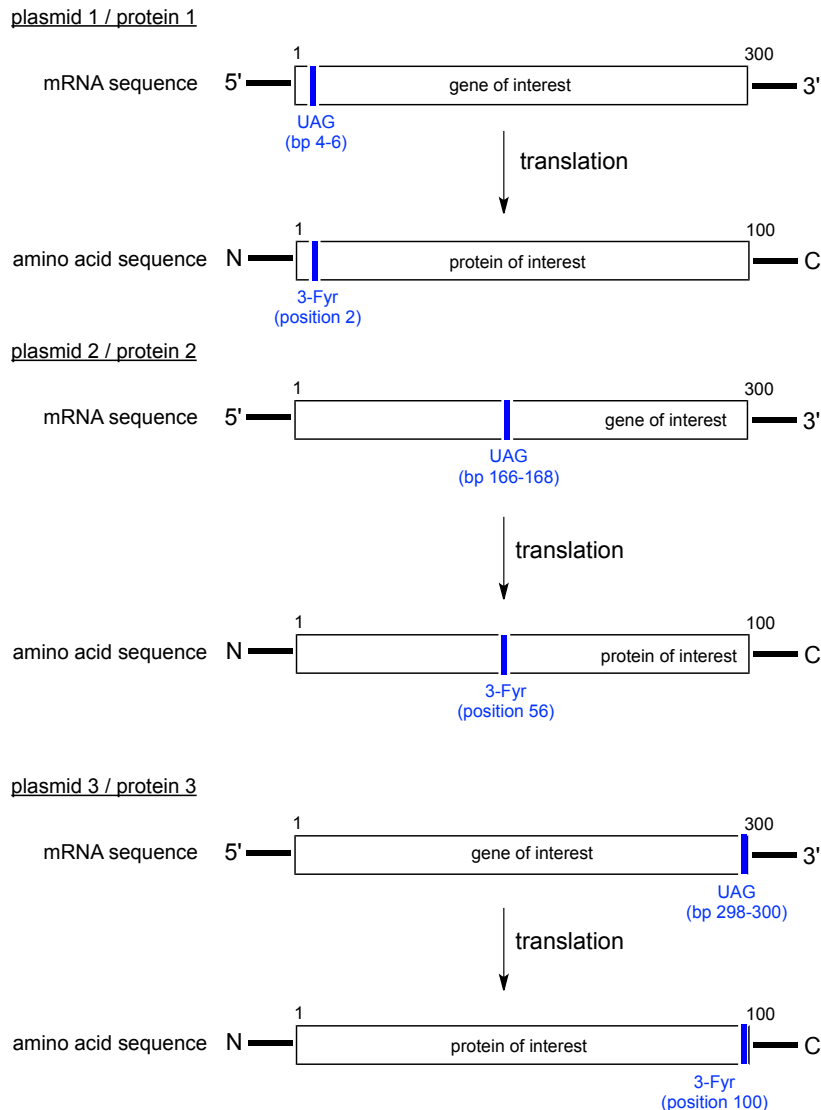
The tRNA/aaRS pair must be orthogonal to the endogenous components of *E. coli*. In other words, there cannot be cross-reactivity between the endogenous protein biosynthetic machinery and the machinery introduced to allow for site-specific incorporation of the UAA of interest into the protein being expressed in *E. coli*.

In particular:

- 1) The new tRNA must only translate the codon for the UAA
- 2) The new tRNA cannot be a substrate for any endogenous aaRS
- 3) The new aaRS must only recognize the new tRNA
- 4) The new aaRS must only aminoacylate the new tRNA with the UAA

Also note that the UAA, in this case 3-fluorotyrosine, cannot be a substrate for endogenous aaRS. If it were a substrate and transferred to endogenous tRNAs, then 3-fluorotyrosine incorporation into endogenous proteins would occur (think: why would this be a problem for your ^{19}F NMR experiment?). Lastly, another factor to keep in mind is that the UAA must be able to enter the cell. Based on prior work by the Pielak Lab and others, you can assume that sufficient 3-fluorotyrosine is taken up into *E. coli* for your experiment.

C) You perform site-directed mutagenesis to place the Amber STOP codon at various positions in your plasmid that harbors the gene for your 100-aa protein. In plasmid 1 (encoding protein 1), the Amber STOP codon is at position 2 (the bp triplet encoding residue 2). In plasmid 2 (encoding protein 2), the STOP codon is at position 56. In plasmid 3 (encoding protein 3), the STOP codon is at position 100 (C-terminal residue). Using the diagram for the plasmid/protein 1 mutant as a guide, sketch the corresponding mRNAs that arise from transcription of the gene of interest and the full-length polypeptides that arise from translation for the position 56 and 100 variants.



D) In class, we learned about release factor 1 (RF1). This release factor is a problem for UAA incorporation by the endogenous ribosome because it recognizes the Amber STOP codon and hydrolyzes the peptidyl-tRNA, causing formation of truncated proteins. In designing your experiments further, you consider the potential effect of RF1 on the synthesis of proteins 1-3 described above. Describe the expected outcomes for translation of the mRNAs encoding proteins 1-3 taking RF1 action into account.

Protein 1: In this case, the UAA is at position 2 of a 100-aa protein.

If the $3^{\text{FTyr}}\text{-tRNA}_{\text{CUA}}$ enters the A-site, then 3FTyr will be incorporated and there will be one ^{19}F label near the N-terminus of the polypeptide chain.

If RF1 enters the A-site, translation will be terminated. There will be no synthesis of a polypeptide chain because only $\text{fMet-tRNA}^{\text{fMet}}$ will be in the A-site and RF1-catalyzed hydrolysis will result in release of fMet.

Protein 2: In this case, the UAA is at position 56 of a 100-aa protein.

If the $3^{\text{FTyr}}\text{-tRNA}_{\text{CUA}}$ enters the A-site, then 3FTyr will be incorporated and there will be one ^{19}F label in the middle (position 56) of the polypeptide chain.

If RF1 enters the A-site, translation will be terminated. In this case, premature termination will result in formation of a truncated polypeptide that is approximately one-half of the size (55 residues) of the full-length 100-aa peptide of interest.

Protein 3: In this case, the UAA is at position 100 of a 100-aa protein (C-terminus)

If the $3^{\text{FTyr}}\text{-tRNA}_{\text{CUA}}$ enters the A-site, then 3FTyr will be incorporated and there will be one ^{19}F label at the C-terminus of the 100-aa polypeptide chain.

If RF1 enters the A-site, translation will be terminated. In this case, premature termination will result in formation of a 99-aa peptide that only lacks the C-terminal residue.

Provided that both processes occur in the cell, a mixture of the desired full-length ^{19}F -labeled protein and the truncated 99-aa protein lacking a ^{19}F label.

E) Based on your answers to C and D, what mutant protein you will select to employ in your first in-cell ^{19}F NMR experiments? Briefly explain your reasoning. Hint: to answer this question, ask what are the possible consequences of your answer in D on your planned ^{19}F NMR experiment.

Background: Recall we learned in class that the incorporation of UAA by the Schultz method is low, ~20-30% for incorporation of 1 UAA, because of competition between the tRNA_{CUA} and RF-1 is a major limitation for this method. As a result, it is expected that both the desired full-length and ¹⁹F-labeled product and the product of premature termination will exist in the cell. Moreover, we expect that less than one-half of polypeptide translated will be full-length and include the ¹⁹F label.

As a result, we must consider how the truncation products and mixtures of truncated/full-length protein will influence the experiment we aim to perform. In this case, we want to watch polypeptide folding in the cell by monitoring the ¹⁹F label. The truncated polypeptide do not have the ¹⁹F label, and so we will not be able to detect them by NMR. However, we must consider whether the presence of the truncated protein influences the ¹⁹F NMR signal of the full-length polypeptide or the state of the cell. In other words, will the folding trajectory of the 100-aa polypeptide be perturbed if there is a high concentration of a truncated product? What if the truncated product form aggregates that have deleterious consequences for the *E. coli* cell and, hence, the experiment? And are the other possible pitfalls to consider?

Protein 1: In this case, a mixture of products will have the desired 100-aa ¹⁹F-labeled polypeptide and fMet (position 1). It is reasonable to predict that the fMet resulting from premature termination will be a minimal perturbation.

Protein 2: In this case, a mixture of products will have the desired full-length ¹⁹F-labeled protein and the truncated and unlabeled 55-aa polypeptide. The presence of the 55-aa truncation product may be a serious issue for the experiments (see background notes above).

Protein 3: In this case, a mixture of products will have the desired full-length ¹⁹F-labeled protein and the truncated and unlabeled 99-aa polypeptide. The 99-aa polypeptide is very similar to the full-length polypeptide of interest. It is probable that the 99-aa polypeptide will fold similarly to the 100-aa form because it only lacks the C-terminal residue. However, the ¹⁹F label would be reporting on <50% of the total protein of interest. Will this be an issue for the experiment? With the current information, it is difficult to predict. If the polypeptide forms oligomers and the resulting oligomers are mixtures (for instance, imagine that homodimers form and result in a mixed population with no, one or two ¹⁹F labels), there could be complications for the analysis.

Based on these considerations, I would select protein 1 for my first experiments.

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