ANSWER KEY

5.08 Biological Chemistry II (Spring 2016)
Problem Set #3

This problem set contains one question and three pages.

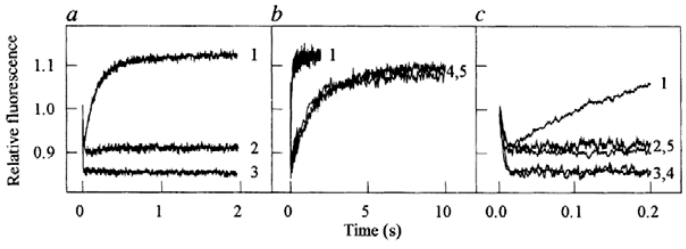
Question 1:

EF-G is involved in the translocation of mRNA during the elongation process. EF-G is a structural mimic of the ternary complex EF-Tu•GTP•aa-tRNA^{aa} (recall from our class discussions that domain IV of EF-G resembles the tRNA). Stopped-flow fluorescence experiments conducted by using fluorescently-labeled aminoacyl-tRNAs and two antibiotics (viomycin and thiostrepton) have been especially effective in providing mechanistic deconvolution of EF-G. These studies are described below. Note: "Stopped-flow fluorescence" means that fluorescence is used for detection (in contrast to absorption or radioactivity in a quenched flow experiment).

Viomycin functions by inhibiting translocation of the mRNA, but has no effect on GTP hydrolysis. Thiostrepton inhibits both GTP hydrolysis and translocation. Prior to the studies described below, the proposed model for the role of EF-G was similar to that proposed for EF-Tu: EF-G was thought to be a GTP dependent switch. Specifically, EF-G•GTP was proposed to bind to the pre-translocational ribosome and induce a conformational change that allowed mRNA translocation and tRNA movement. Following translocation, EF-G•GTP was proposed to hydrolyze GTP and generate EF-G•GDP, which dissociates from the ribosome. The following studies were undertaken to test this model. The results of stopped-flow fluorescence studies are shown in Figure 1. Recall that stopped-flow methods are employed for fast mixing and for studying reactions on a short time scale (pre-steady state).

Figure 1. Note the different scales in Figures 1a, b and c.

FIG. 1 Time course of EF-G-dependent translocation measured by stopped-flow. *a*, Influence of antibiotics. Pre-translocation complex containing deacylated tRNA^{fMet} in the P site and fluorescent fMet-Phe-tRNA^{Phe}(Prf16/17) in the A site (0.2 μM) was mixed with EF-G GTP (1.2 μM) in the absence of antibiotic (trace 1), in the presence of viomycin (trace 2), or of thiostrepton (trace 3). *b*, Translocation with GTP analogues. GTP-free pre-translocation complex (0.2 μM) was mixed with EF-G (4 μM) containing either caged GTP (trace 4) or GDP (trace 5). *c*, EF-G binding in the presence of various guanine nucleotides and antibiotics. Concentrations as for *a*, designation of traces as for *a* and *b*.



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In **experiment 1**, ribosomes that were loaded with fluorescently labeled fMet-F-tRNA^{Phe} in the A site (note: fMet is formylmethionine; fMet-F is the dipeptide fMet-Phe, which is attached to the 3'-end in fMet-F-tRNA^{Phe}) and tRNA^{fMet} (uncharged or deacylated) in the P site were placed in one syringe. [Note that the fluorescently labeled fMet-F-tRNA^{Phe} has a proflavin attached to a single position within the tRNA.] EF-G•GTP was placed in the second syringe. The contents of the two syringes were rapidly mixed (saturating conditions). In **experiment 2**, viomycin was placed in the syringe containing the EF-G•GTP. In **experiment 3**, thiostrepton was placed in the syringe containing EF-G•GTP. The conditions in all three experiments were identical with the exception of the presence or absence of antibiotics. As revealed in **Figure 1a** and the corresponding expanded time scale **1c**, there is a rapid fluorescence decrease followed by an increase in **experiment 1** (no antibiotic). The rapid rate of fluorescence decrease was essentially the same in all three experiments (1, 2, and 3). In the cases of experiments 2 and 3, the fluorescence decrease was not followed by an increase (see Figure 1a and the expanded scale in Figure 1c).

The results from several additional experiments are described in **Figure 1b**. In these experiments, GTP was replaced with either caged GTP or GDP. The results with caged GTP are presented in **experiment 4** and those with GDP in **experiment 5**. These studies revealed that the rapid drop in fluorescence was not affected by substitution of GTP with caged GTP or GDP (Figure 1c traces 4 and 5 which are an expansion of Figure 1b).

In another set of experiments (**Figure 2a**), GTPase rates were measured using [γ-^{32}P]-GTP and a rapid chemical quench apparatus. P_i (inorganic phosphate) release was monitored. The experiment was carried out with no antibiotics (closed circles), with viomycin (open circles) and with no EF-G (open triangles) under conditions identical to those described in Figure 1. The reactions were rapidly quenched by acid. The fluorescence data obtained in Figure 1a and the GTPase data from Figure 2a in the absence of any antibiotics are shown in Figure 2b.

Figure 2.

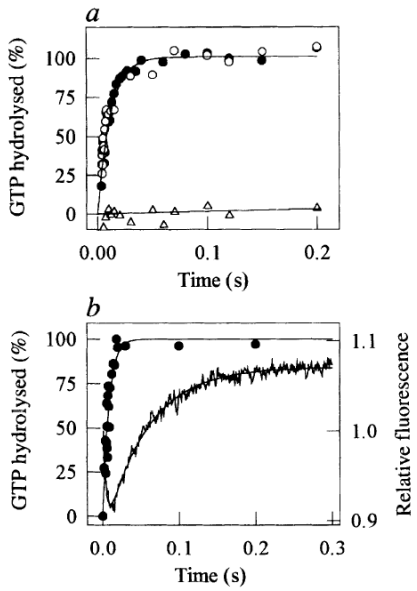

FIG. 2 Kinetics of GTP hydrolysis by EF-G on the ribosome. *a*, Effect of viomycin. Pre-translocation complex (0.1 μM after mixing) was mixed with EF-G[^{32}P]GTP (0.8 μM) without (●) or with (○) viomycin; the control (△) was without EF-G. *b*, GTP hydrolysis and translocation at near-saturating concentration of EF-G (2 μM). The fit of the GTPase (●) yielded a rate of 120 s^{-1}, the fluorescence increase was fitted (smooth curve) with $k_{app} = 25\ s^{-1}$. *c*, Concentration dependence. Rate constants of binding

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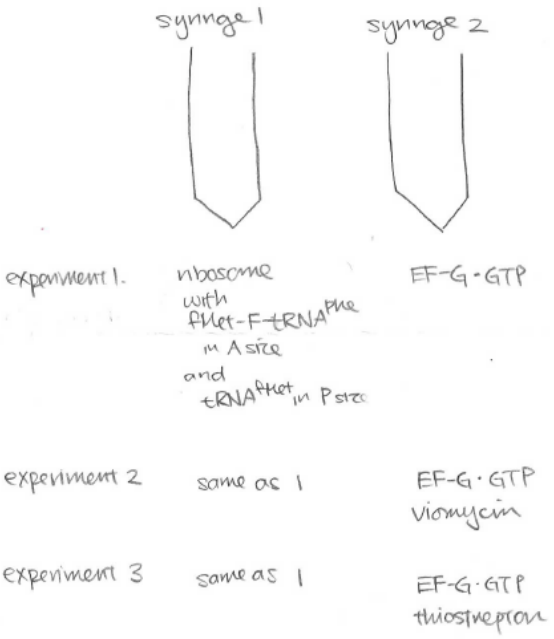
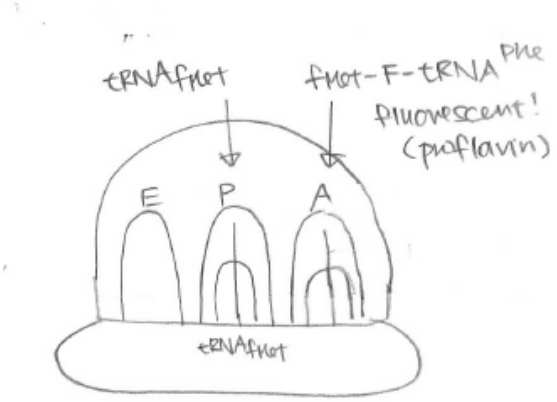
Answer the following questions:

A) Draw a cartoon of a stopped-flow set-up showing the two syringes for experiments 1-3. Label the syringes as "1" and "2" and list the reaction components housed in each syringe for these 3 experiments.



B) Draw a cartoon of the pre-loaded ribosomes placed into syringe 1 in experiments 1-3.



Note that the tRNA is modified by a fluorescent proflavin. A dihydrouracil group was replaced by the proflavin fluorophore (a small molecule). This fluorophore remains attached to the tRNA throughout the process. Experimental observations revealed that such fluorescent tRNAs can be used to monitor the translocation process. In simple terms, the fluorophore provides emission decrease or enhancement at various points during the translocation process.

C) Which step in the elongation process does this pre-loaded ribosome mimic?

It mimics the state right after peptide bond formation where the P and A site are occupied by the deacylated tRNA and peptidyl-tRNA, but before translocation.

D) The dipeptide employed in this work is fMet-F where fMet stands for formylmethionine and F for phenylalanine. Provide an explanation for why this peptide contains fMet.

fMet, or formylmethionine, is the amino acid loaded onto the initiator tRNA. The initiation process begins with a start codon. Since all peptides start with a start codon, there is an fMet at the N-terminus of every growing amino acid chain during translation in prokaryotes. The phenylalanine (F) is the second amino acid on this peptide chain. The fMet-F is meant to mimic the dipeptide which resides in the A site right after peptide bond formation between the fMet-tRNA^{fMet} (initiator tRNA) and a F-tRNA^{Phe} in the A-site.

E) Design an experiment to convince yourself that the ribosome is in fact appropriately loaded to allow investigation of the translocation process. [Hint: think about the use of another antibiotic.]

You could use puromycin or a puromycin analog like CC-puromycin shown in the lecture notes. CC-puromycin has an additional Cytosine-Cytosine (CC) oligonucleotide attachment to better mimic the CCA-3' end of the tRNA and will also bind to the A site. (Recall, puromycin mimics the adenosine and amino acid on the 3' end of a tRNA and binds to the A site) After translocation, the A site is vacated. When puromycin binds, it can form a peptide bond with the Fmet-F-tRNA^{Phe}, which is now at the P site. The product can be isolated and identified, and use of a radiolabeled CC-puromycin would facilitate this process.

F) Given the role of the antibiotics described above (viomycin, thiostrepton), provide a hypothesis for the increase in fluorescence observed in experiment 1 and not in experiments 2 and 3 of Figure 1, following the initial rapid decrease in fluorescence observed in all three experiments.

Rapid mixing using a stopped flow apparatus allows fast reactions to be monitored. Rate constants up to 500 s^{-1} can be measured by this technique! We see a rapid drop in fluorescence in the data shown in Figure 1a. In fact, we see this drop in fluorescence for all experiments 1 – 3 (look back to what is in your syringes for each experiment – question A of this section). In other words, we observe that this drop in fluorescence occurs in both the absence and presence of antibiotics (viomycin and thiostrepton). See Figures 1a and 1c.

Recall that the tRNA is labeled with proflavin, which is fluorescent. After the initial rapid drop in fluorescence, only the inhibitor-free experiment (experiment 1) shows an increase

in the observed fluorescence. Because this increase in fluorescence was suppressed with viomycin (inhibits translocation, but not GTP hydrolysis) and by thiostrepton (inhibits translocation and GTP hydrolysis), the increase in fluorescence in the inhibitor-free experiment can be interpreted to be associated with translocation.

Also note (not required for answer): There is an initial rapid decrease in fluorescence observed in all three experiments. This was proposed to be associated with the binding of EF-G to the pre-translocation complex. This proposal makes sense because EF-G can bind to the ribosome under all experimental conditions (neither antibiotic prevents EF-G binding). To confirm this proposal, one can measure the effect of varying EF-G concentration on the initial drop in fluorescence or doing site-directed mutagenesis on EF-G and creating mutants with a fluorescent probe that are incapable of binding.

G) What do the data in Figure 2a tell you?

See part H. Both G and H are answered below.

H) What do the data and the fit to the data in Figure 2b tell you?

The data shows that GTP hydrolysis, measured by release of inorganic phosphate, occurs at a rate constant of 120 s^{-1}. Furthermore, we observe the same rate constant with or without viomycin, indicating that viomycin does not have an effect on GTP hydrolysis. The control, where EF-G is absent, shows that GTP hydrolysis does not take place. This control indicates that EF-G is required for GTP hydrolysis.

Figure 2b indicates that the rate of GTP hydrolysis measured by phosphate release is 120 s^{-1}, whereas the rate of the overall reaction measured by fluorescence is 25 s^{-1}. This comparison indicates that GTP hydrolysis is faster than translocation.

I) Propose a model for the role of EF-G in translocation based on these data. Explain how the data support your model.

GTP is hydrolyzed before translocation. We can propose that EF-G hydrolyzes GTP and harnesses the chemical energy that is released into mechanical energy to translocate the tRNAs and mRNA relative to the ribosome.

If you have trouble thinking about this problem you can go to the literature and read the paper from which this data was taken: Nature 385 37-41 (1997).

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