Name: _____

5.08 Exam #1

This exam is worth **100 points**.

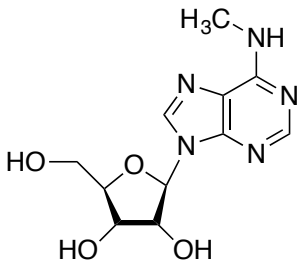
This exam contains **13 pages**.
Check that you have all pages before commencing the exam.
Read all questions, figure captions, and figures carefully.
Write neatly – what cannot be read or deciphered will not be graded.

Question 1: _____ / **57 pts**

Question 2: _____ / **43 pts**

TOTAL: _____ / 100 pts

1. (**57 pts total**) In class and in problem set #1, we saw that many nucleobases in tRNAs are post-transcriptionally modified. Post-transcriptional modification also occurs for the nucleobases in mRNA. Recently, studies have demonstrated that N^6-methylation of adenosine (forming m^6A) is the most abundant post-transcriptional modification in the coding regions of mRNA. This observation begs a fundamental question: *what is the role of m^6A in mRNA decoding and polypeptide synthesis by the ribosome?* This problem is based on recent studies performed to address this question (*Nat. Struc. Mol. Biol.* **2016**, *23*, 110-115).



N^6-methyladenosine (m^6A)

These studies examined the effect of m^6A in the context of the kinetic model for tRNA selection and peptide bond formation originally described by Rodnina and co-workers. We discussed this model in class, and in recitations #2 and #3, and the model is given in the Appendix.

In **experiment #1**, the researchers sought structural information about the codon-anticodon interaction in the ribosome A-site when a codon containing m^6A is in the A-site. Prior studies showed that m^6A forms a canonical Watson-Crick base-pair with uridine (U). The researchers crystallized 30S ribosomal subunits from *Thermus thermophilius* with an oligonucleotide corresponding to the anticodon stem loop of $tRNA^{Lys}$ (anticodon UUU) and one of four different short mRNAs bound. Each structure indicated canonical Watson-Crick base-pairing of A–U and m^6A–U in the A-site of the decoding center. The four short mRNAs employed are listed below.

5'-(m^6A)AA–UUU-3'
5'-A(m^6A)A–UUU-3'
5'-AA(m^6A)–UUU-3'
5'-AAA–UUU-3'

In **experiment #2**, the researchers performed quench-flow kinetic experiments to evaluate the effect of m^6A on the ribosome-dependent rate of GTP hydrolysis by EF-Tu ($\boldsymbol{k_{GTP}}$). In this experiment, 70S *E. coli* ribosomes with $fMet\text{-}tRNA^{fMet}$ in the P-site and a mRNA with either AAA or (m^6A)AA in the A-site were rapidly mixed with the EF-Tu•[^{3}H]-GTP•$Lys\text{-}tRNA^{Lys}$ ternary complex. The reactions were quenched with formic acid at varying time points and the amount of GTP hydrolyzed was quantified. The resulting data are shown in **Figure 1.**

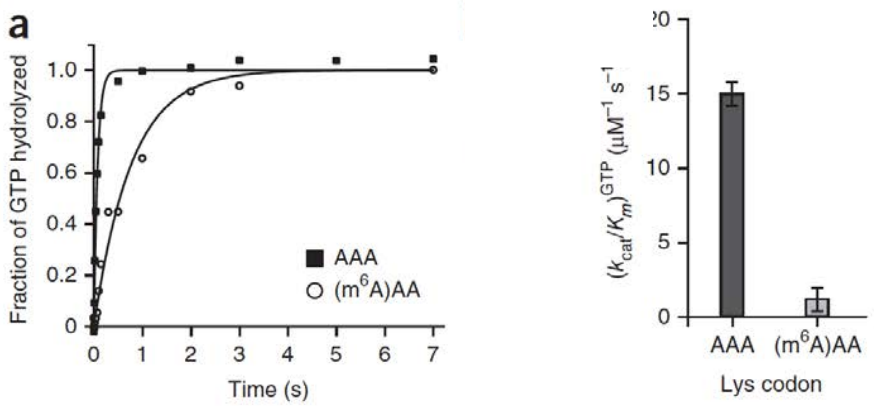


Figure 1. GTP hydrolysis by EF-Tu with AAA or (m^{6}A)AA in the A-site.
(a) Fraction of GTP hydrolyzed over time. [initiation complex] = 1 μM. [ternary complex] = 0.5 μM.
(b) Comparison of the k_{cat}/K_M for GTP hydrolysis by EF-Tu for decoding of AAA or (m^{6}A)AA. Additional experiments were conducted to obtain data for determining k_{cat}/K_M (details and data not shown).

Notes:
(i) The ribosome and ternary concentrations listed are the concentrations after mixing in the quench-flow.
(ii) The buffer contained $MgCl_2$ (5 mM total concentration).

In **experiment #3**, the researchers performed the same quench-flow experiments except that they monitored formation of fMet-Lys dipeptide. They used [^{3}H]-fMet-tRNA^{fMet} and unlabeled GTP in these assays. Using the data for the kinetics of GTP hydrolysis and the data for fMet-Lys dipeptide formation, the researchers performed a mathematical analysis that allowed them to determine the rate constant for tRNA accommodation plus peptidyl transfer. Thus, this rate constant includes all steps after GTP hydrolysis and includes peptidyl transfer. We call this rate constant $\boldsymbol{k_{acc\text{-}pep}}$. Note: This rate constant includes all steps after GTP hydrolysis and includes peptidyl transfer and thus is not given in the kinetic model of Rodnina. The data from these experiments and the mathematical analysis are given in **Figure 2.**

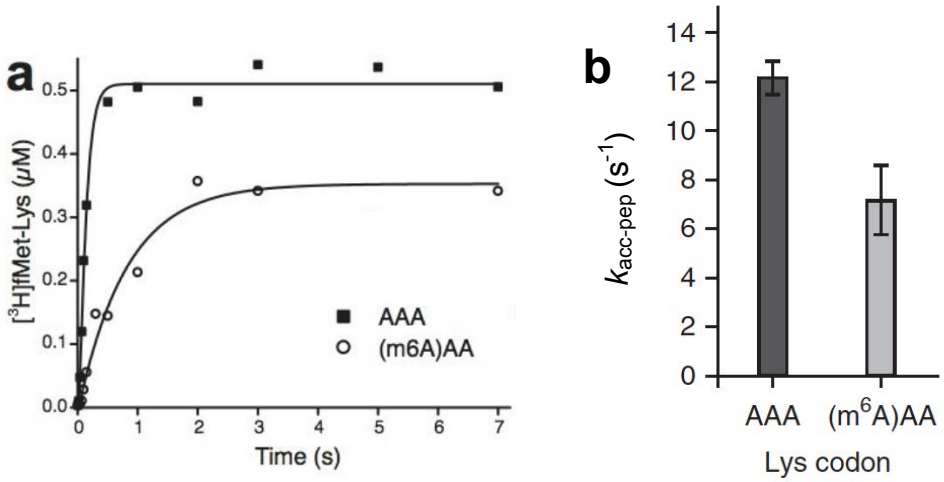


Figure 2. Formation of [^{3}H]-fMet-Lys by the ribosome with AAA or (m^{6}A)AA in the A-site.
(a) Dipeptide formation versus time. [initiation complex] = 1 μM. [ternary complex] = 0.5 μM.
(b) Calculated compound rate constants $k_{acc\text{-}pep}$.

Note: the ribosome and ternary concentrations listed are the concentrations after mixing in the quench-flow.

Answer the following questions:

1. (**5 pts**) Draw the Watson-Crick base-pair for A–U.

2. (**5 pts**) The resolution of each crystal structures obtained in **experiment #1** was between 3.35-3.45 Å. Will the structural determination provide you with information about the different conformation of the 6-methyl group of m^6A? Briefly explain your reasoning.

3. (**2 pts**) Why was it important for the crystallographic work to be performed?

4. (**5 pts**) Draw the ribosome employed in **experiments #2 and #3** prior to mixing. Be sure to label all components and include the relevant mRNA codons.

5. (**5 pts**) Draw the quench flow system employed for **experiments #2 and #3.** Indicate the components of each syringe.

6. (**5 pts**) Using the chemical structures for reactants and products, provide the equation for [^{3}H]-GTP hydrolysis by EF-Tu and indicate how you will monitor this reaction.

7. (**10 pts**) What do we learn from the data in **Figure 1a (experiment #2)** about the effect of a codon containing m^{6}A on ribosome-dependent GTP hydrolysis by EF-Tu? Be sure to use all of the data in the figure to answer this question.

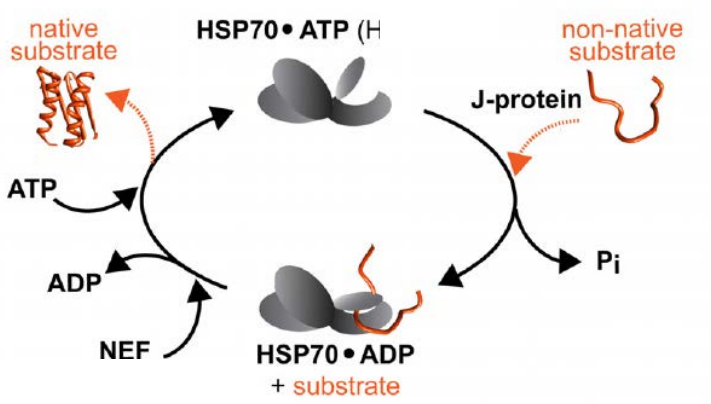
8. (**5 pts**) What do we learn from a comparison of the k_{cat} / K_M values shown in **Figure 1b (experiment #2)**? Be sure to include a definition of this parameter in your answer.

9. (**10 pts**) What do we learn from the data presented in **Figure 2a (experiment #3)**? Be sure to use all of the data in the figure to answer this question.

10. **(5 pts**) This work was motivated by a fundamental question: *what is the role of m^6A in mRNA decoding and polypeptide synthesis by the ribosome?* Using the data presented in this problem as well as class discussions and your knowledge of the kinetic model presented by Rodnina and co-workers, provide a reasonable answer to this question in the context of the kinetic model.

2. (43 pts total) Protein misfolding and aggregation is associated with many human diseases, including neurological disorders. As a result, there is significant interest in understanding the pathological processes that occur in diseased cells and result in misfolding as well as how healthy cells prevent protein aggregation and, when necessary, eliminate intracellular aggregates. This problem is based on recent studies that addressed the latter phenomenon and specifically interrogated the disaggregase activity of the HSP70 (HSP = heat shock protein) chaperone of human cells (*Nature* **2015**, *524*, 247-251).

The HSP70 cycle is shown below where an unfolded polypeptide is the substrate. By a generally accepted extrapolation, this cycle can be extended to occur on the surfaces of protein aggregates. In class, we discussed the DnaK/DnaJ cycle. Recall that DnaK (chaperone) is a Hsp70 and DnaJ (co-chaperone) is a J-protein. We learned that J-proteins have structural and functional diversity.



In *E. coli* and other bacterial systems, there is a ”protein disaggregase” named Hsp100 that can work with DnaK/DnaJ to disaggregate and refold insoluble aggregated protein. In the current model, the action of DnaK/DnaJ exposes some polypeptide segments from the aggregate. Hsp100 can bind to these exposed segments and unfold the bound polypeptide. As a result, the polypeptide becomes soluble and is released from the aggregate. Subsequently, DnaK/DnaJ or other chaperone machinery like GroEL/GroES can fold the polypeptide to its native form. If this process happens many times, we can imagine that an aggregate can be completely eliminated.

In humans, there is no HSP100, and HSP70 itself (with its nucleotide exchange factor HSP110) exhibits very poor disaggregase activity *in vitro*. These observations beg a fundamental question: *how does the human cell eliminate protein aggregates? Do select co-chaperones work with HSP70 to disentangle aggregated protein in human cells?*

[Note: in bacteria, the abbreviations for proteins only have the first letter capitalized (e.g. Hsp) whereas for eukaryotes all three letters are capitalized (e.g. HSP).]

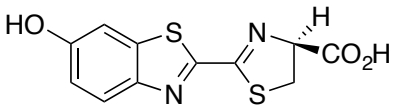
Humans have >50 J-proteins that can be divided into three classes named A, B and C. In prior work, class A and B J-proteins have been implicated in protein quality control. Thus, the researchers hypothesized that certain class A and B J-proteins may cooperate with HSP70 to afford robust disaggregation activity. They designed a series of assays to test this notion.

The majority of these assays utilize an enzyme named firefly luciferase. This enzyme catalyzes oxygenation of luciferin by the following two-step reaction:

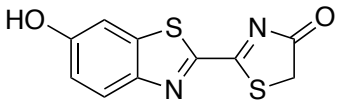
luciferin + ATP → luciferyl adenylate + PP_i

luciferyl adenylate + O_2 → oxyluciferin + AMP + light

The structure of firefly luciferin and oxyluciferin is shown here:



Firefly luciferin



Oxyluciferin

Light is emitted because the reaction forms oxyluciferin in an electronically excited state, which emits a photon of light upon its return to the ground state. These reactions provide fireflies with their luminescence!

The researchers found conditions where **thermal denaturation of luciferase results in formation of insoluble aggregates**. These aggregates were used as a model substrate for the studies described below.

In **experiment #1**, pre-formed luciferase aggregates were incubated with HSP70-HSP110 (HSP110 is the nucleotide-exchange factor for HSP70) in the absence or presence of J-proteins JA2 (class A J-protein) and JB1 (class B J-protein). These mixtures contained 2 mM ATP and an ATP regenerating system. Reactivation of aggregated luciferase was monitored over time. To monitor for luciferase reactivation, a 1-μL aliquot of the mixture described above was taken and transferred to a buffer containing luciferin (125 μM), ATP (2.5 mM), and $MgCl_2$ (7.5 mM). Because light is emitted as a result of luciferase activity, luminescence was monitored. The resulting data are shown in **Figure 1**.

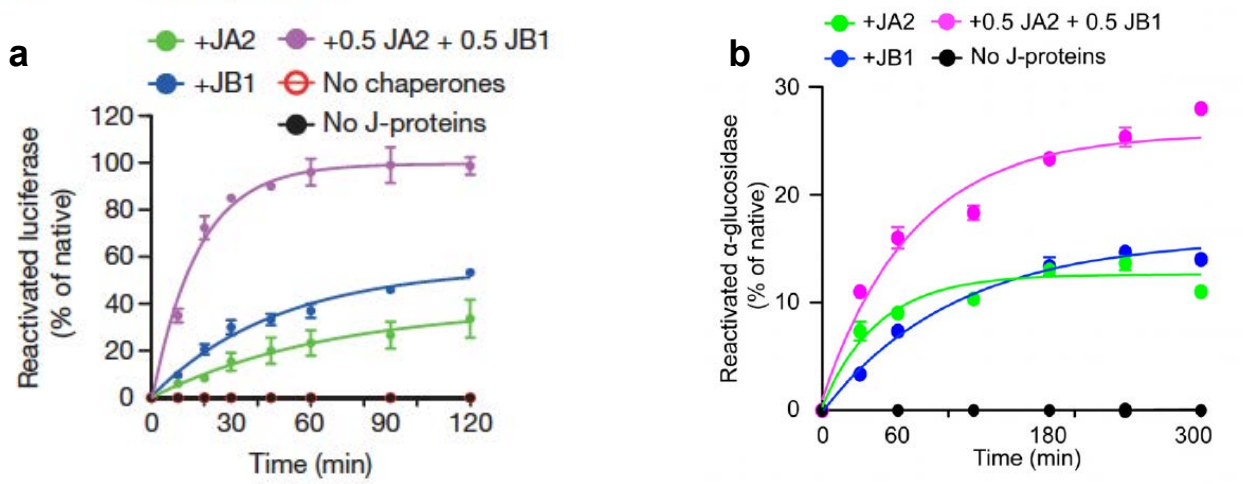


Figure 1. Reactivation of enzymes by HSP70-HSP110 in the absence and presence of J-proteins.
(a) Reactivation of luciferase. The experimental details are described above.
(b) Reactivation of a different enzyme, α-glucosidase. Details for this assay are not needed.

In **experiment #2**, the authors incubated aggregated [^{3}H]-luciferase with HSP70-HSP110 and the J-proteins JA2 and JB1 in the presence of a GroEL variant named GroEL(D87K). This GroEL variant has a single point mutation at position 87 in the amino acid sequence (Asp → Lys). Prior characterization of GroEL(D87K) showed that it is deficient in ATP hydrolysis and, as a result, it can bind and trap non-native substrates. In this

experiment, the researchers performed two analyses: (i) they determined how much [^{3}H]-luciferase was trapped by GroEL(D98K) and (ii) they quantified how much luciferase was reactivated in the presence of GroEL(D87K). The assay set-up was the same as for **experiment #1** except that the luciferase was radiolabeled and GroEL(D87K) was included in the assay. GroEL(D87K) was in excess over the luciferase concentration. The resulting data are shown in **Figure 2.**

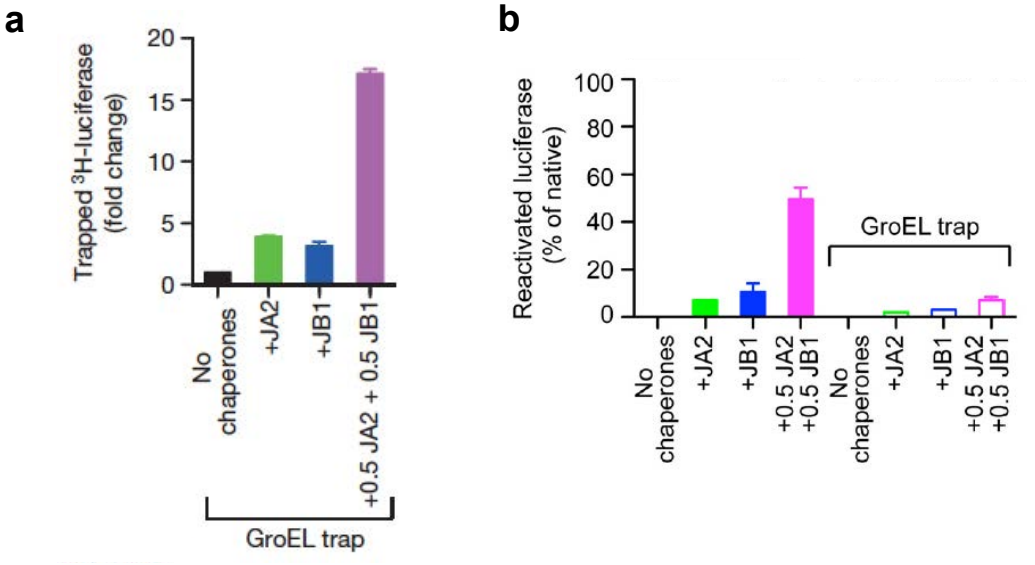


Figure 2. Experiments with GroEL added to mixtures containing aggregated [^{3}H]-luciferase, HSP70-HSP110 and the J-proteins JA2 and JB1.

(a) Amount of [^{3}H]-luciferase trapped in GroEL.
(b) Reactivation of luciferase in the absence and presence of the GroEL trap. This data was recorded 40 minutes after initiation of the assay.

In **experiment #3**, the researchers again incubated [^{3}H]-luciferase with HSP70-HSP110 and J-proteins JA2 and JB1. After 120 minutes, the samples were analyzed by size-exclusion chromatography (SEC). This method separates biomolecules on the basis of size, and larger biomolecules come off the column at lower elution volumes. In this case, the SEC column was attached to a scintillation counter. The resulting data are shown in **Figure 3**.

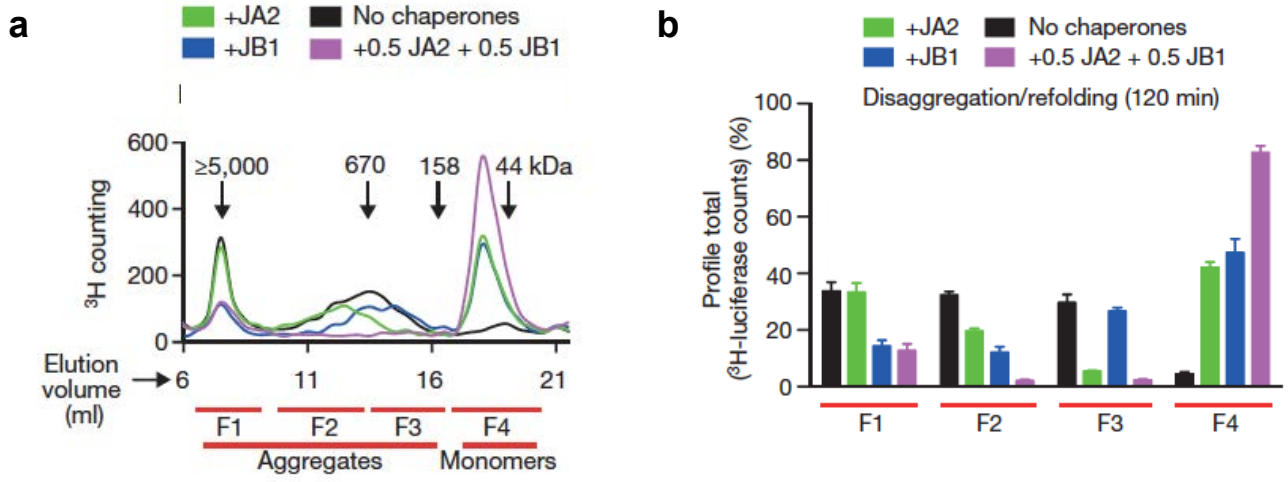


Figure 3. Analysis of samples of aggregated [^{3}H]-luciferase treated with HSP70-HSP110 and J-proteins.

(a) SEC chromatograms. F1–F4 are fractions 1 through 4. The kDa labels indicate molecular weight.
(b) Quantification of total radioactivity in fractions F1–F4 for each experimental condition.

Answer the following questions:

1. (**2 pts**) The luciferase-catalyzed reaction is familiar. Where have we seen this chemistry before?

2. (**2 pts**) In experiment #1, the authors monitor reactivation of luciferase. What steps must occur for luciferase to be reactivated?

3. (**5 pts**) What do we learn from the data in **Figure 1a (experiment #1)** about the action of J-proteins JA2 and JB1? Be sure to use all of the data in the figure to answer this question.

4. (**4 pts**) The data in **Figure 1b (experiment #1)** were obtained for a different protein aggregate. Briefly comment on why this experiment was performed (mostly likely reason) and what the data show.

5. (**5 pts**) Draw a cartoon of GroEL/GroES indicating its subunit composition.

6. (**5 pts**) Briefly explain what happens in the assays where GroEL was added (**experiment #2, Figure 2)**.

7. (**10 pts**) What do we learn from the data presented in **Figure 3 (experiment #3)** about the function of JA2 and JB1 in protein disaggregation?

8. (**5 pts**) How do these experiments inform our understanding of protein disaggregation in human cells?

9. (**5 pts**) It is always important to consider how the conditions of *in vitro* studies and the results relate to the physiological environment. For this set of experiments, how might the *in vitro* assays differ from disaggregation in the cell? (Full credit for any reasonable answer that is clearly articulated and justified.)

END OF EXAM

5.08J Biological Chemistry II

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