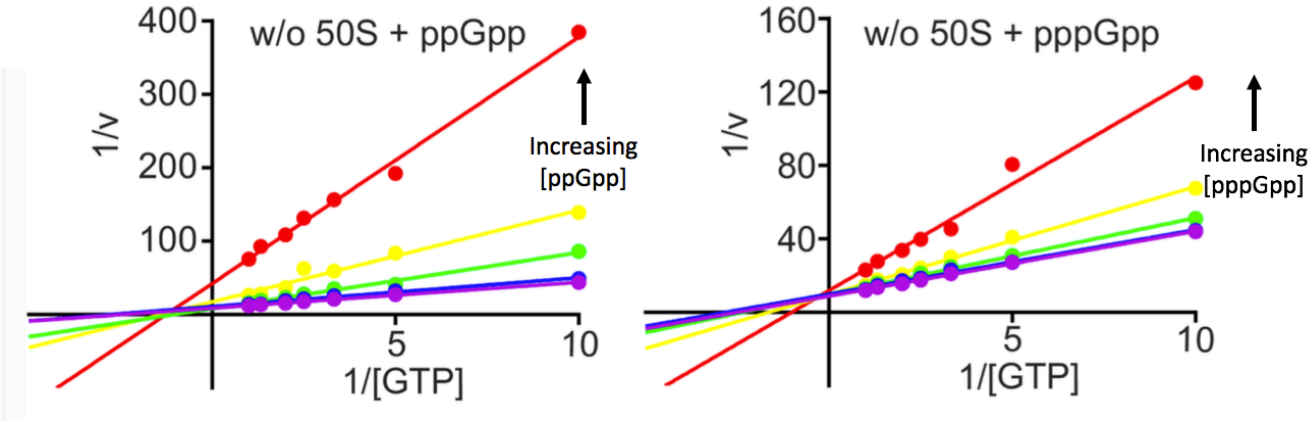
Name: _____ Kinetic Questions

All references to authors or data in this exam are from:

Pausch, P., Steinchen, W., Wieland, T.K., Freibert, S.A., Altegoer, F., Wilson, D.N., and Bange, G. (2018) Structural Basis for (p)ppGpp-mediated inhibition of the GTPase RbgA. **J. Biol. Chem.** [Epub ahead of print].

For this portion of the exam, you will use excel on a lab computer. Accessing any other computer resource (program or internet) is a violation of the academic honesty policy and will result in a zero on this exam and your acute and permanent removal from the course. A grade of "F" will be recorded by the registrar for the course.

The authors test the rate of GTP hydrolysis by RbgA in the absence of the ribosomal 50S subunit but in the presence of various amounts of the inhibitors ppGpp and pppGpp. They conclude that both ppGpp and pppGpp act as competitive inhibitors. The figures below present the authors' kinetic data in Lineweaver-Burk plots for ppGpp (left figure) and pppGpp (right figure).



1. (10 pts.) Looking at the Lineweaver-Buk plots, do you agree with the authors' conclusion that ppGpp and pppGpp both act as competitive inhibitors? Concisely, explain your reasoning [only the first two non-run-on sentences will be read].

2. (20 pts.) As a conscientious reader of the paper, perform an analysis to test the fittest inhibition model (competitive, uncompetitive, or mixed) for ppGpp and pppGpp. They need not be the same. You will find two data files on the computer desktop; one for ppGpp and the other for pppGpp. The critical F-value for all cases here is well approximated at 4.16.

$$\omega = \frac{(SSE_A - SSE_B)(\#\text{ of data points - }\#\text{ of fit parameters in Model B})}{(\#\text{ of fit parameters in Model B - }\#\text{ of fit parameters in Model A})(SSE_B)}$$

Complete the following tables:

ppGpp

	V_{max}	K_M	$K_{I(E)}$	$K_{I(ES)}$	SSE
Null			-	-	
Competitive				-	
Uncompetitive			-		
Mixed					

pppGpp

	V_{max}	K_M	$K_{I(E)}$	$K_{I(ES)}$	SSE
Null			-	-	
Competitive				-	
Uncompetitive			-		
Mixed					

Complete the following tables <u>where applicable</u>:

ppGpp

	ω	F-value	ω - F-value
Null to Competitive		4.16	
Null to Uncompetitive		4.16	
Competitive to Mixed		4.16	
Uncompetitive to Mixed		4.16	

pppGpp

	ω	F-value	ω - F-value
Null to Competitive		4.16	
Null to Uncompetitive		4.16	
Competitive to Mixed		4.16	
Uncompetitive to Mixed		4.16	

3. (5 pts.) Do you agree with the authors' conclusions concerning the mechanism of inhibition for ppGpp and pppGpp?

Name: _____ Protein Purification Questions

All references to authors or data in this exam are from:

Pausch, P., Steinchen, W., Wieland, T.K., Freibert, S.A., Altegoer, F., Wilson, D.N., and Bange, G. (2018) Structural Basis for (p)ppGpp-mediated inhibition of the GTPase RbgA. **J. Biol. Chem.** [Epub ahead of print].

For this portion of the exam, you will need a calculator. You may use a cell phone in "airplane mode" as a calculator. Accessing any function or application other than the calculator on your phone is a violation of the academic honesty policy and will result in a zero on this exam and your acute and permanent removal from the course. A grade of "F" will be recorded by the registrar for the course.

Experimental Procedures *Production and purification of RbgA:*

"Constructs were transformed in *E. coli* BL21(DE3) (Novagen) for overexpression. Cell were growth in two liters of lysogeny-broth (LB) medium, supplemented with 25 g of lactose and kanamycin (50 mg/L). Cells were incubated at 30°C overnight under rigorous shaking (180 revolutions per minute (rpm)). Cells were harvested by centrifugation (3,500 x g, 20 min, 4°C) and resuspended in 20 mL of Buffer A (20 mM HEPES-Na, pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 40 mM imidazole) before lysis in a M-110L Micofluidizer (Microfluidcs). The lysate was cleared at 47,850 x g for 20 minutes at 4°C and the supernatant was applied onto two 1 mL HisTrap FF columns (GE Healthcare) for Ni-NTA affinity chromatography. After a wash step with 15 column volumes (CV) of buffer A, proteins were eluted with three CV of Buffer B (20 mM HEPES-Na, pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 500 mM imidazole). Proteins were concentrated to 1 mL and further purified by size-exclusion chromatography (SEC).

*Sa*RbgA was purified using a HiLoad 26/600 Superdex 75 column (GE Healthcare) equilibrated in Buffer C (20 mM HEPES-Na, pH 7.5, 200 mM NaCl). The main peak fractions were concentrated to 1.5 mL and dialyzed over night at 4°C against 200 mL Buffer C containing 10 g HCl-activated charcoal and 1 mM EDTA to remove Mg^{2+} and co-purified nucleotides. *Sa*RgbA was subsequently subjected to a second SEC step using a HiLoad 26/600 Superdex 75 column (GE Healthcare) equilibrated in Buffer D (20 mM HEPES-Na, pH 7.5, 20 mM KCl, 20 mM $MgCl_2$, 200 mM NaCl).

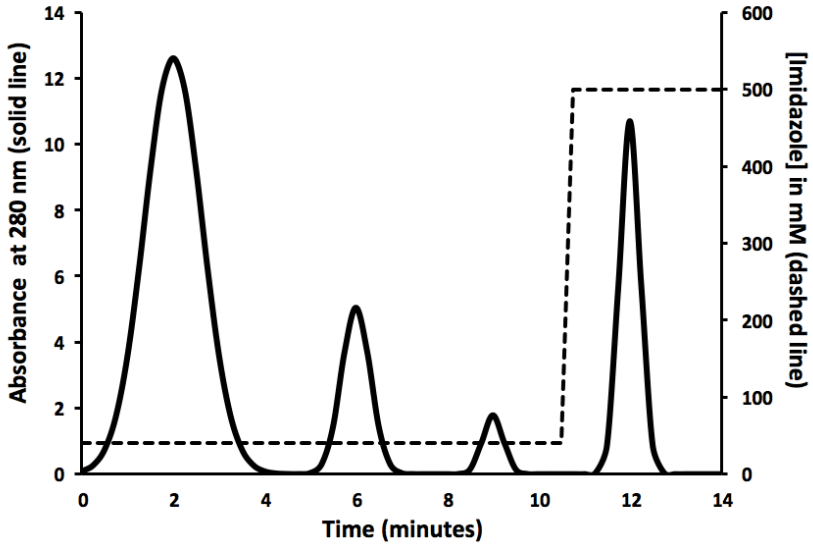
*Bs*RbgA was purified using a HiLoad 16/600 Superdex 200 column (GE Healthcare) equilibrated in Buffer E (50 mM Tris-HCl, pH 7.5, 750 mM KCl, 5 mM $MgCl_2$, 5% glycerol). The main peak fractions were concentrated and concentrations were determined using a Nanodrop Lite Spectrophotometer (Thermo Scientific)"

1. (20 pts.)
   Determine how much of each of the following you would need to prepare 1 L of Buffer A.

   - _____ g HEPES-Na ($C_8H_{17}N_2NaO_4S$)
   - _____ g NaCl
   - _____ g KCl
   - _____ g $MgCl{\bullet}6H_2O$ (Magnesium chloride hexahydrate)

- _____ g Imidazole [sidechain of Histidine without the methylene (-CH_2-) group]

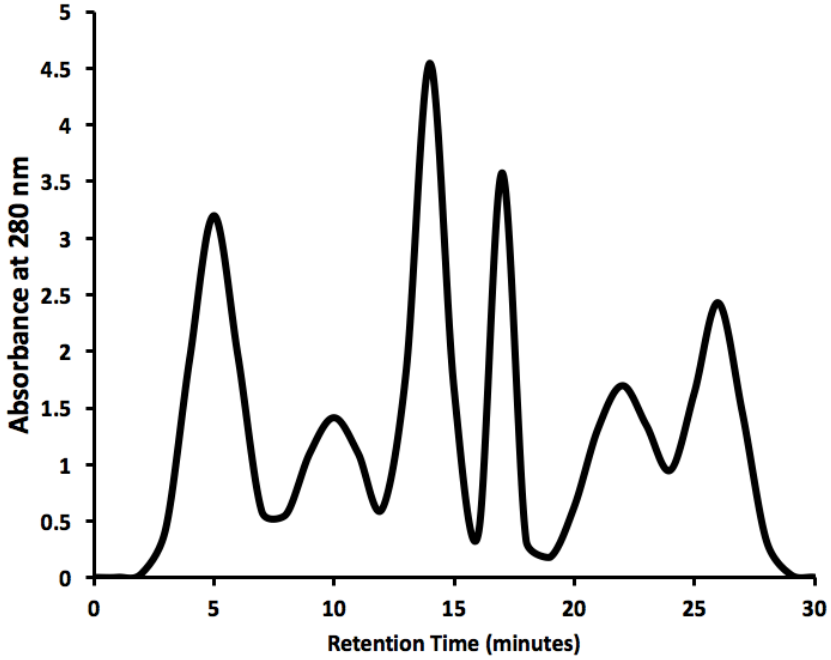
2. (5 pts.) Below is a chromatograph representing the HisTrap FF column result. Label the peak that contains *Sa*RbgA.



3. (5 pts.) Imagine that the peak fraction from the previous column (question #2) contains the following proteins:

Protein	Length (amino acids)	Molecular Weight (Daltons)	pI
SaRbgA	294	33379	9.19
MetE	753	84674	5.61
RlmM	366	41905	7.04
Rs2	241	26744	6.62
Rs6	135	15704	4.94
TorD	199	22473	4.76

Below is a chromatograph representing the HiLoad 26/600 Superdex 75 column result. Label the peak that contains *Sa*RbgA.


Absorbance at 280 nm
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
Retention Time (minutes)

Name: _____ RbgA Structure Questions

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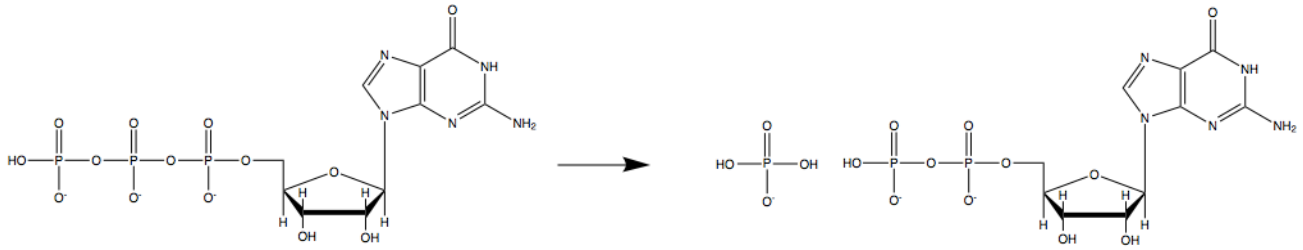
Pausch, P., Steinchen, W., Wieland, T.K., Freibert, S.A., Altegoer, F., Wilson, D.N., and Bange, G. (2018) Structural Basis for (p)ppGpp-mediated inhibition of the GTPase RbgA. **J. Biol. Chem.** [Epub ahead of print].

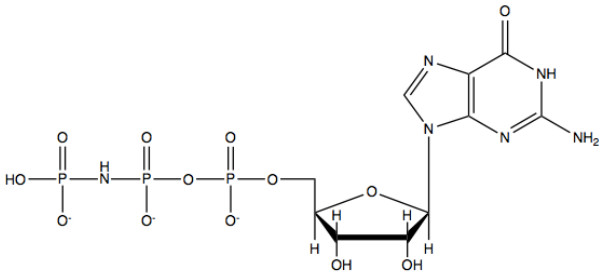
For this portion of the exam, you will use PyMol and the PDB website (www.rcsb.org) on a lab computer. Accessing any other computer resource (program or internet) is a violation of the academic honesty policy and will result in a zero on this exam and your acute and permanent removal from the course. A grade of "F" will be recorded by the registrar for the course.

Search the protein database website (www.rcsb.org) for the structures of RbgA. Use PyMol to visualize the protein structures and answer the following the questions.

1. (9 pts.) Normally, RbgA hydrolyses GTP to GDP and P_i:



The authors were able to crystalize RbgA bound to GMPPNP:

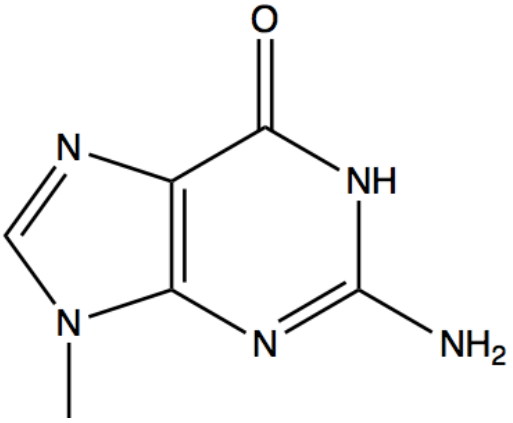


Concisely, explain why the authors were able to capture the structure of the stable complex between RbgA and GMPPNP [only the first two non-run-on sentences will be read].

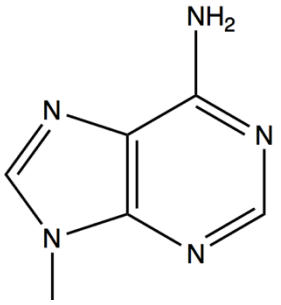
2. (9 pts.) Compare the structures of RbgA bound to GMPPNP, ppGpp, and pppGpp. Concisely, explain whether the structural results are consistent with the kinetic result that ppGpp and pppGpp act as competitive inhibitors and not uncompetitive or mixed [only the first two non-run-on sentences will be read].

3. (9 pts.) RbgA binds to GTP but not ATP. Both molecules are identical except for their attached nucleobases. Using the structure of RbgA bound to GMPPNP, determine the side chain interactions that are formed with the nucleobase of GTP that could not form with the nucleobase of ATP. Indicate the interactions and their distances on the structure of the nucleobase of GTP below. The nucleobase of ATP is provided as a reference.

GTP nucleobase



ATP nucleobase



4. (8 pts.) Part of a proposed catalytic mechanism for GTPases is below. Indicate the type or types of catalysis where appropriate. Place a box around anything you find odd.

-Acid/Base -Covalent -Electrostatic -Metal
-Proximity and orientation effects -Preferential binding of the transition state