BMB/Ch 178 final Fall, 2017

Instructions

Finals are due Friday, Dec. 8 by 6 pm. Please drop off the completed finals in a box outside of Shu-ou's office (109 Braun).

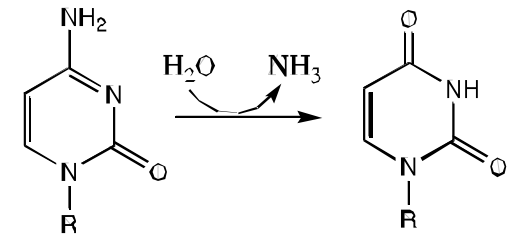
The final exam is **3 hours** and you will have use of lecture notes and course material. You are not allowed to discuss the exam with others and all the work should be your own. You are not allowed to use online resources to aid in the completion of final. You cannot consult exams from previous years.

This exam seeks to test that you have a firm and broad grasp of the concepts presented throughout the course, and the ability to make connections between experimental data and molecular mechanisms. Please be concise in your answers. In most cases the answer is fairly specific, and either you know it or you do not. You won't be expected to describe minutiae, and writing long paragraphs in the hope that you will chance upon the right key words will not help you.

You are encouraged to use a word processor for the final but it is not a requirement. If you type your exam on a computer, you don't have to count time for printing. If you write your answers by hand, please make sure that they are legible! You won't get credit for answers that can't be read. Where figures are required, you can either draw them or make them using a data analysis software (such as excel).

Please set a timer when you start and stop immediately when the time limit is reached. You can do work after the time limit is up but you cannot expect full credit for work past the time limit. In such cases, please note explicitly when the time is up. Please note that this is done purely on the honor system, which Caltech takes seriously.

Problem 1. Transition state theory, binding energy (15+4 points)
Cytidine deaminase catalyzes the hydrolytic deamination of cytosine nucleotides to uracil nucleotides and ammonia (Eq 1):

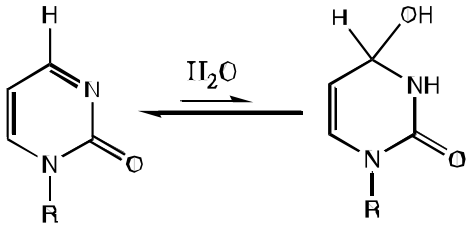


Cytidine $K_m = 120\ \mu M$ **uradine** $K_m = 800\ \mu M$ (1)

1. The reaction proceeds through a tetrahedral-like intermediate, with the help of a Zn ion that coordinates the nucleophilic water. Based on this information, write the sequence of chemical conversions during the reaction. Don't forget electron pushing and the movement of protons. (4 points)

2. Draw the putative transition state structures for both steps of the reaction (Reminder: in a transition state, partial bonds are formed to the nucleophile, the leaving group, and protons). (4 points)

3. Zebularine (**1**) acts as a potent inhibitor of the enzyme. The apparent inhibition constant determined for **1** is $K_{d,app}$ = 0.46 µM. However, NMR and UV spectra data showed that this compound binds the enzyme in the hydrate form (**2**). The equilibrium for conversion of **1** to **2** is 4.8×10^{-6}.

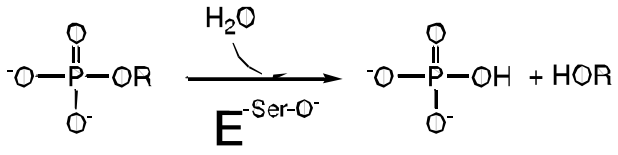


1 **2**

Based on this information, calculate the equilibrium dissociation constant for binding of **2** (3 points). Is this compound a substrate or a transition state analogue? List your two reasons. (4 points)

4. (**Bonus**) The same reaction proceeds in solution with a half-time of 73 years. Based on transition state theory and the information above, estimate a lower limit for the rate constant of the enzymatic reaction (k_{cat}). (4 points)

Problem 2. Alkaline Phosphatase: catalytic strategies, kinetics 101 (36 points)
Alkaline phosphatase (AP) catalyzes the hydrolysis of phosphate monoesters using an active site serine as a covalent nucleophile (Eq 2).



(2)

1. Why is it advantageous for the enzyme to use serine 102, instead of an active site-bound water, as the nucleophile? (2 points)

2. Figure 1 depicts the active site structure with an inhibitor, vanadate, bound.

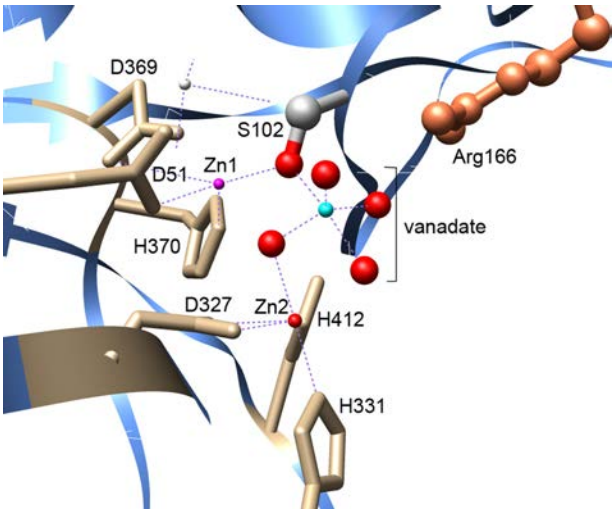


Figure 1 Structure of the AP active site.

(a) What are the catalytic roles of each Zn^{2+} ion? (4 points)

(b) What is the catalytic role of Arg166? (2 points)

(c) What kind of inhibitor is vanadate likely to be (e.g., competitive, noncompetitive, or uncompetitive)? List your reasons. (2 points)

(d) Suppose that you have an assay to measure the hydrolysis of a substrate, p-nitrophenol phosphate (pNPP), by AP. What experiments will you do to test the mode of inhibition by vanadate proposed above? Draw the predicted results from your model (don't worry about numbers, only the patterns are important) (4 points)

3. In traditional assays for the AP reaction, the reaction is monitored by absorbance changes. Multiple turnover reactions are followed and reasonable data can only be obtained with at least 0.5 µM pNPP substrate because of the low absorption coefficients of pNPP. However, one of the products of the reaction, P_i, binds to AP with K_d < 0.1 µM.

(a) Will this assay accurately measure the reaction rates? What are the problem(s)? (4 points)

(b) To measure reaction rate constant more accurately, a radioactive assay is developed in which ^{32}P-labeled substrates, at concentrations < 1 nM, is used. The concentration of AP is varied and in excess to substrate. The following reaction time course can be observed (Fig. 2):
What are the advantages of this assay over the pNPP based spectroscopic assays? (6 points)

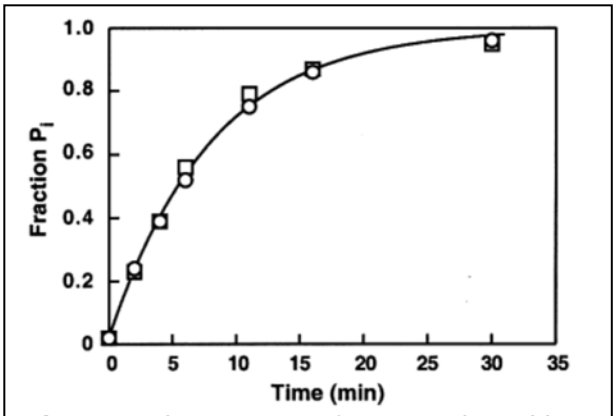


Figure 2 Time course of AP reaction with a radioactive assay.

4. AP is non-specific with respective to its leaving group. This allows a Brønsted relationship between reaction rate and leaving group ability to be constructed for a series of alkyl phosphates (Fig. 3):

(a) Estimate the leaving group Brønsted slope of this reaction (2 points)

(b) What does this result suggest about the nature of the transition state for the AP reaction? (2 points)

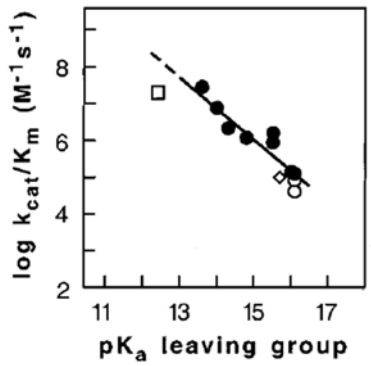


Figure 3 Leaving group dependence for reaction of AP.

5. The k_{cat}/K_m of the reaction for alkyl phosphates shows a bell-shaped pH dependence (Fig. 4): The acidic limbs of the pH dependences for trifluoroethyl phosphate (●) and ethyl phosphate (○) give pK_a values of 5.6±0.1 and 6.4±0.1, respectively. The solution pK_a values for deprotonation of 2,2,2-trifluoroethyl phosphate monoanion is 5.62±0.02, and that for ethyl phosphate monoanion is 6.36±0.02.

(a) Which deprotonation event is responsible for the acidic limb of the pH dependence in Figure 2? (2 points)

(b) Which is the reactive species for the AP reaction, the phosphate monoanion or the phosphate dianion? (2 points)

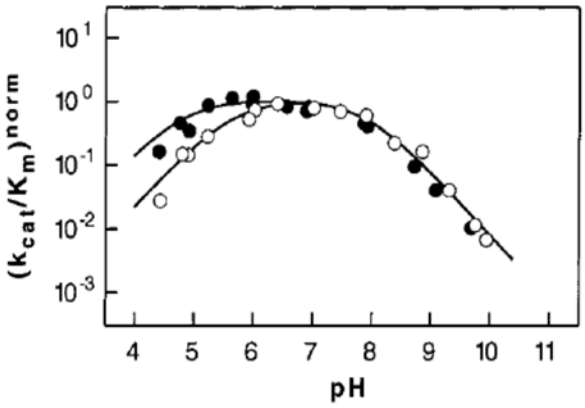


Figure 4. pH dependence of reactions with 2, 2,2-trifluoroethyl phosphate (●) and ethyl phosphate (○).

6. Being one of the most ancient phosphomonoesterases, alkaline phosphatase also harbors promiscuous activity towards phosphate diesters and sulfate esters. Suggest how this catalytic promiscuity could have contributed to the evolution of phosphodiesterases and sulfatases. (4 points)

Problem 3. Cooperativity and allostery (16 points)

Ire1 is a sensor of unfolded proteins in the endoplasmic reticulum. In response to unfolded proteins detected by its luminal domain, Ire1 oligomerizes, and activates its endonuclease activity in the cytoplasmic domain (Figure 5). Ire1's RNase activity is also allosterically regulated by the binding of nucleotides (ATP or ADP) and by phosphorylation of its activation loop.

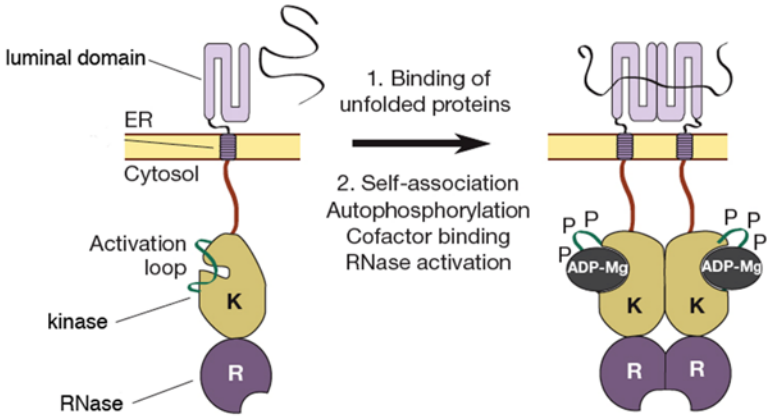


Figure 5 Ire1 activity controlled by multiple allosteric factors.

1. Figure 6 shows the activation profiles for Ire1. What does this data suggest about the role of nucleotides in Ire1 activation? (2 points).

In which nucleotide-bound state is Ire1 more active, ADP or ATP? (2 points)

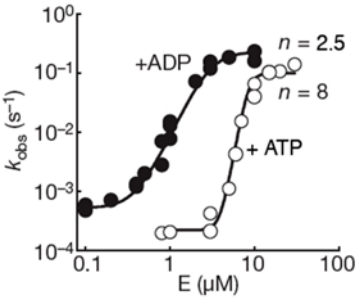


Figure 6 Activation profile of Ire1 with different nucleotides. E denotes Ire1.

2. Explain why the cooperativity of Ire1 activation is reduced in the presence of ADP than ATP. (4 points)

3. Phosphorylation of Ire1 also activates its RNase activity. In the simplest model, how would the cooperativity of Ire1 activation change if it is phosphorylated, and why? (4 points)

4. Substitution of the β-phosphoryl oxygen of ADP (ADPβS) significantly reduces the activation of Ire1, an effect that is rescued by using metal ions alternative to Mg^{2+}, such as Mn^{2+} or Cd^{2+}. Oligomerization of Ire1 is affected by these modifications in the same manner. What does this suggest about the active site interactions of ADP and its role in Ire1 activation? (4 points)

Problem 4: Pre-steady state kinetics (15 points)

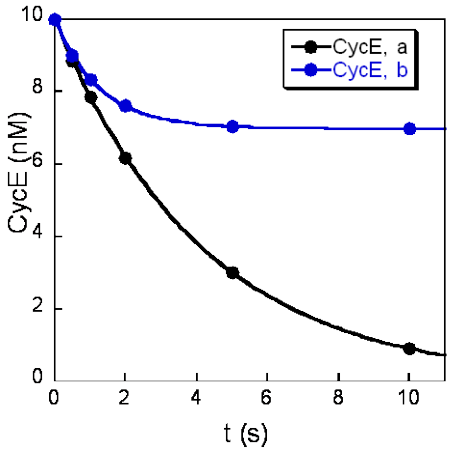
Ubiquitination of proteins is an important process in regulated protein degradation. During the last step of the ubiquitination reaction, a ubiquitin ligase (E3 or SCF) binds an ubiquitin conjugating enzyme loaded with ubiquitin (E2-Ub) and the substrate (CycE), and catalyzes the transfer of ubiquitin from E2 to a lysine residue on the substrate. The binding between the substrate CycE and SCF is not tight, so that the substrate may fall off prior to the reaction.

1. Briefly describe the difference of the molecular events that can occur between the following two experimental setups (6 points):

In setup (a), the following mixtures are combined to initiate the reaction: (i) saturating concentration (10 µM) of E2-Ub, and (ii) 150 nM SCF was pre-incubated with 10 nM CycE, which was radiolabeled to visualize the reaction substrate and product. The reaction was quenched at different time points, and the extent of the reaction is quantified using SDS-PAGE.

In setup (b), the following two pre-incubated mixtures are combined to initiate the reaction: (i) saturating concentration (10 µM) of E2-Ub pre-incubated with an 1000-fold excess of unlabeled substrate CycE (10 µM), (ii) 150 nM SCF pre-incubated with 10 nM radiolabeled CycE as above. The reactions were analyzed as in (a).

2. To simplify analysis, an engineered ubiquitin molecule is used to allow only one ubiquitin to be transferred onto the substrate. Two reactions were carried out using the setups (a) and (b) described in part (1). The reaction time courses under the two conditions are given below.



The equations that describe the data for setup (a) is: [CycE] = $10 \times e^{-0.24t}$ and for setup (b) is: [CycE] = $3 \times e^{-0.8t} + 7$.

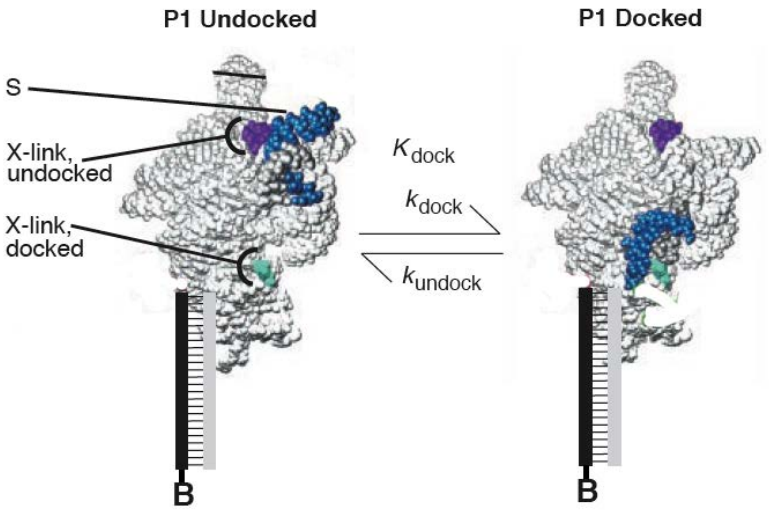
Calculate the rate constants for the chemical step and for dissociation of CycE from SCF (i.e., values of k_c and k_{off}) using these data (9 points).

Problem 5: Single Molecule Kinetics (18 points)

The *Tetrahymena* group I ribozyme catalyzes the cleavage of an oligonucleotide substrate:

$$CCCUCUA_5 \rightarrow CCCUCU_{OH} + pA_5$$

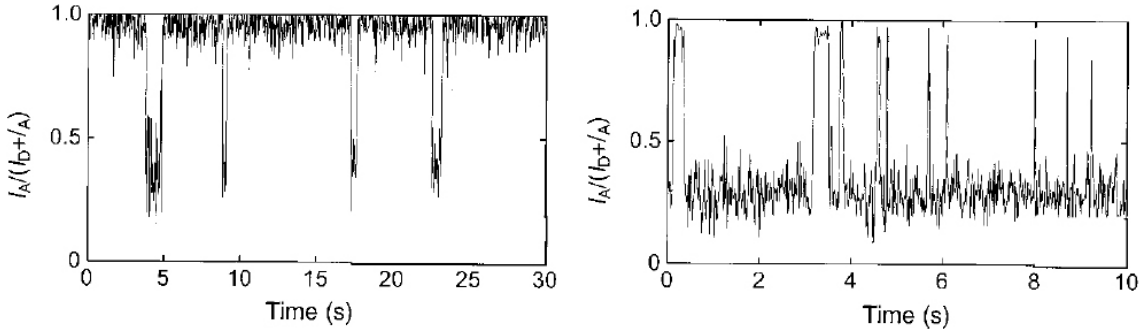
Binding of the oligonucleotide substrate to the enzyme occurs in two-steps, involving (i) base pairing between the substrate and the ribozyme to form a P1 duplex; and (ii) docking of the P1 duplex into the ribozyme active site. A model of the structures of the ribozyme in the docked and undocked states based on crosslinking data (substrate highlighted in *blue*) is shown below



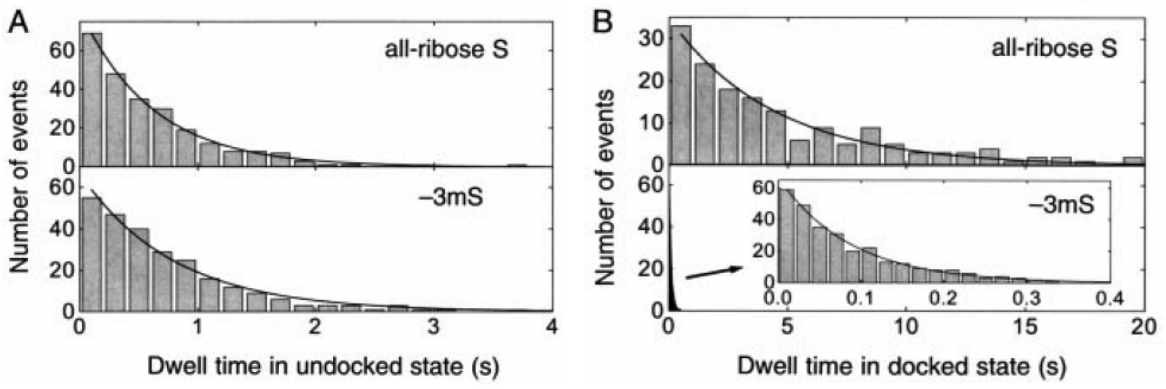
Single molecule fluorescence is used to study the docking of P1 in this ribozyme. To immobilize the ribozyme on a glass surface, a 3'-extension is added to the ribozyme that anneals with a DNA oligo, which is tethered to the glass surface via a biotin tag (Fig. 1).

a) To study the docking of P1 by FRET, where will you place the fluorescent dyes, and which pair of fluorescent probes will you use? Explain why. (3 points)

b) The following fluorescence time traces were observed for the wildtype all-ribose substrate (rS) and for a modified substrate, -3mS, in which docking of P1 is substantially destabilized by a methoxy substitution of a 2'-hydroxyl group. Which one of these time traces belongs to rS, and which one belongs to -3mS? (2 points)



c) The dwell times of molecules in the docked and undocked states are analyzed and their population distributions are shown below:



Based on these data, estimate the rate constants for docking and undocking, and the equilibrium constant for docking of the all-ribose and the -3mS substrates. (9 points)

(4) Which kinetic parameter does the -3m substitution primarily affect? (2 points)
Based on this information, can you suggest how the -3m substitution disfavors docking of the P1 duplex? (2 points)