BMB/Ch 178 final Fall, 2016

Instructions

Finals are due Friday, Dec. 9 by 5 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.

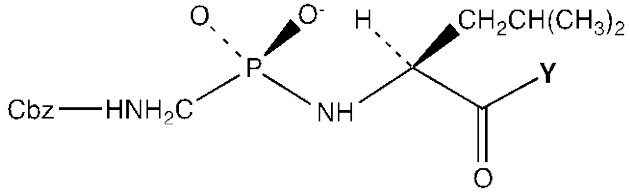
You will be tested based on lecture notes and problem sets. This exam seeks to test that you have a firm and broad grasp of the concepts presented throughout the course, and the ability to make connections between experimental data and molecular mechanisms. Please be concise in your answers. In most 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.

Your 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 out. 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 this. 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.

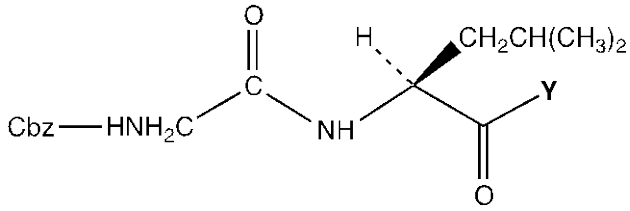
Problem 1. Transition state theory, intrinsic binding energy (14 points)

A series of phosphoamidate di-peptide analogues with different substitutes, Y (**1**-**6**), have been prepared and evaluated as inhibitors of the peptidase thermolysin.



1, Y = D-Ala
2, Y = NH2
3, Y = Gly
4, Y = L-Phe
5, Y = L-Ala
6, Y = L-Leu

Corresponding dipeptide substrates bearing each of the substituents (Cbz-Gly-L-Leu-Y),



were also evaluated for their reaction kinetics with thermolysin. The following data were obtained.

substituent	Inhibitor K_i (nM)	Corresponding substrate reaction data	
		K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
1	1700	16.6	0.3125
2	760	20.6	5.102
3	270	10.8	6.06
4	78	2.4	50.0
5	16.5	10.6	73.5
6	9.1	2.6	142.9

1. Plot the K_m and k_{cat}/K_m values as a function of K_i. Don't forget that free energy analysis applies on the log, instead of linear, scale. Which parameter shows strong correlation with the binding affinity of the phosphoamidate inhibitor? (6 points)

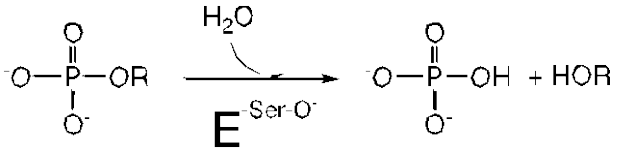
2. Based on this information, what type of inhibitor is the phosphoamidates for thermolysin? List your reasons. (4 points)

3. What principle of enzymatic catalysis do these data illustrate? (2 points)

4. Based on the above information, what is the putative transition state structure of this reaction? (4 points)

Problem 2. Alkaline Phosphatase: catalytic strategies, kinetics 101 (44 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? (4 points)

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

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. (4 points)

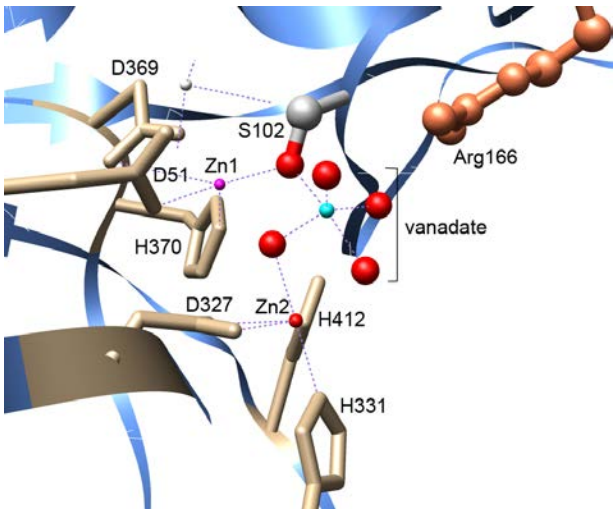


Figure 2 Structure of the AP active site.

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) (6 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. 3):

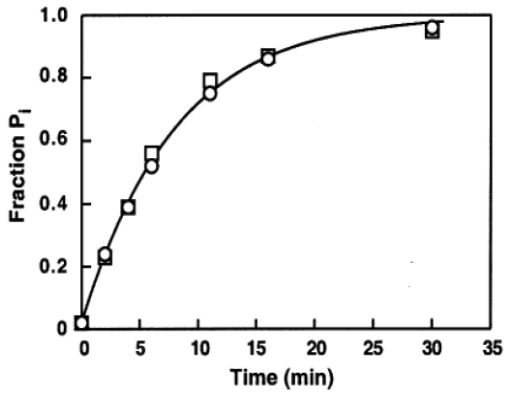


Figure 3 Time course of AP reaction with a radioactive assay.

What are the advantages of this assay over the pNPP based spectroscopic assays? (6 points)

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. 4):

(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)

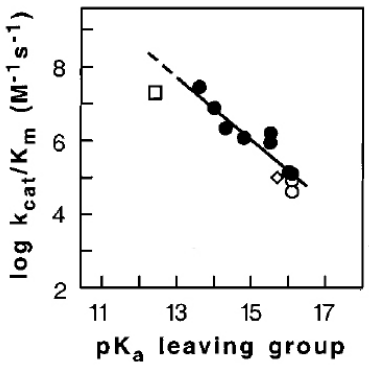


Figure 4 Leaving group dependence for reaction of AP.

5. The k_{cat}/K_m value of the reaction for alkyl phosphates shows a bell-shaped pH dependence (Fig. 5):
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.

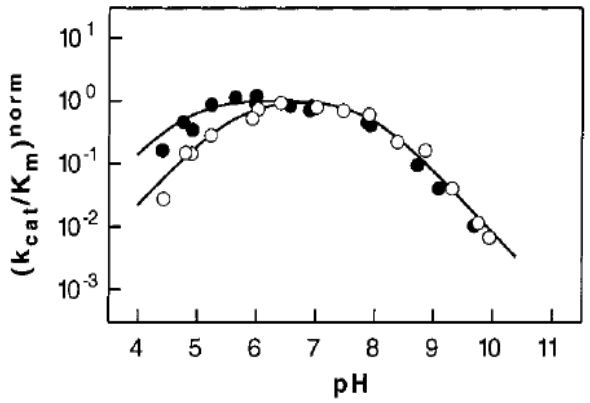


Figure 5. pH dependence of reactions with 2, 2,2-trifluoroethyl phosphate (●) and ethyl phosphate (○).

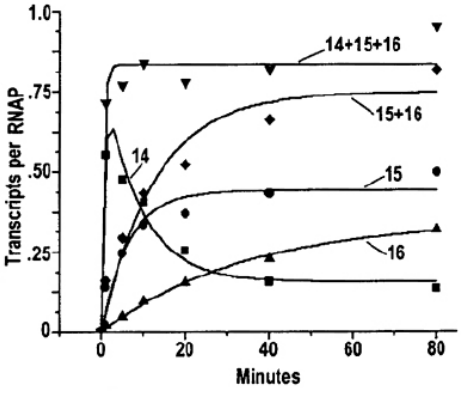
(a) Which deprotonation event is responsible for the acidic limb of the pH dependence in Figure 2? (4 points)

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

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: T7-RNA polymerase – Specificity, pre-steady-state kinetics (22 points)

To test how T7 RNA polymerase (RNAP) handles mismatches that might occur during transcription, the following experiment was carried out. RNAP was allowed to synthesize an RNA chain using the template:
5'-…GGGAGAGGGAGGGATCCCTC…-3' (sense strand)
3'-…CCCTCTCCCTCCCTAGGGAG…-5'



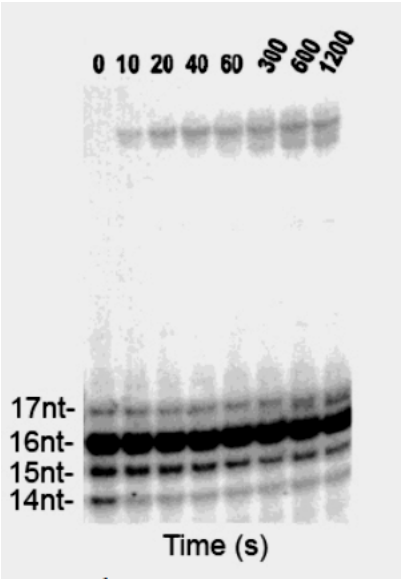
Transcription initiates at the first G on the sense strand (and remember that the anti-sense strand is used as the template during transcription). In this experiment, the transcription reaction was carried out with only GTP and ATP. Therefore, synthesis of a matched RNA product would be expected to stop at 14nt, after which continued RNA chain elongation will produce mismatched products. The distribution of different RNA

chains is shown in the figure on the right, in which the number denotes the length of the transcribed RNA chain.

(a) Why does the amount of 14nt RNA over time show a bi-phasic behavior (2 points)?

(b) Estimate the *half time* for the synthesis of 14nt, 15nt, and 16nt RNA chains. What can you conclude about the rate of RNA synthesis with matched *vs.* mismatched nucleotides (5 points)?

After 80 minutes, CTP and UTP were added to the reaction mixture containing the 14nt, 15nt, and 16nt RNA to allow continued elongation to generate the full-length transcript. The RNA chain distribution is monitored with polyacylamide gels shown in the right. The full-length transcript is the high molecular weight band in the upper part of the gel.



(c) From the disappearance of the bands corresponding to the 14nt, 15nt, and 16nt RNA chain, estimate the half-time for chain elongation starting from each of these intermediates. What does this tell you about the elongation rate of the mismatched RNA chain (5 points)?

(d) Consider the full-length transcription product, for which the halftime for synthesis is about 40 min. Compare this with the half times you estimated from part (c). From which intermediate (the 14, 15, or 16 nt RNA) is the final product primarily synthesized (2 points)?

(e) From the data and conclusions above, describe two mechanisms that the RNA polymerase uses to ensure fidelity of transcription (4 points).

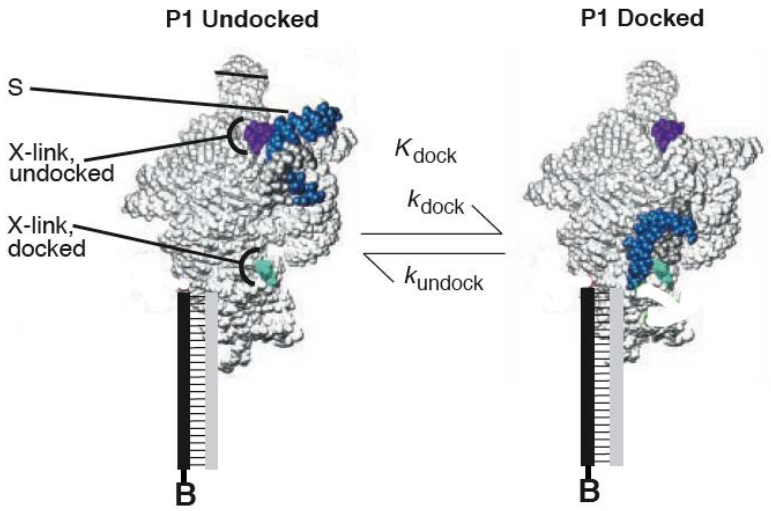
(f) Two classes of mutations were identified that increase misincorporation by RNAP. The H784A mutant enhances extension of the mismatched 15nt and 16nt RNAs and generates a ladder of longer RNA transcripts. In contrast, the G640A, F644A, and G645A mutants increases the production of a mismatched 15nt RNA but the 15mer remains the predominant transcription product after chase with CTP and UTP. What does this tell you about the RNAP active site responsible for preventing misincorporation (4 points)? ***(5')***

Problem 4: Single Molecule Kinetics (20 points)

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

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

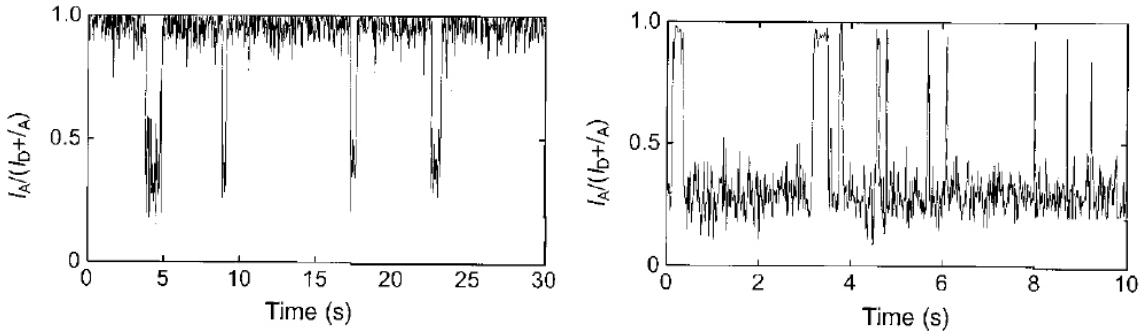
Binding of the oligonucleotide substrate to the enzyme occurs in two-steps, involving (i) base pairing between the substrate and the ribozyme to form a P1 duplex; and (ii) docking of the P1 duplex into the ribozyme active site. A model of the structures of the ribozyme in the docked and undocked states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



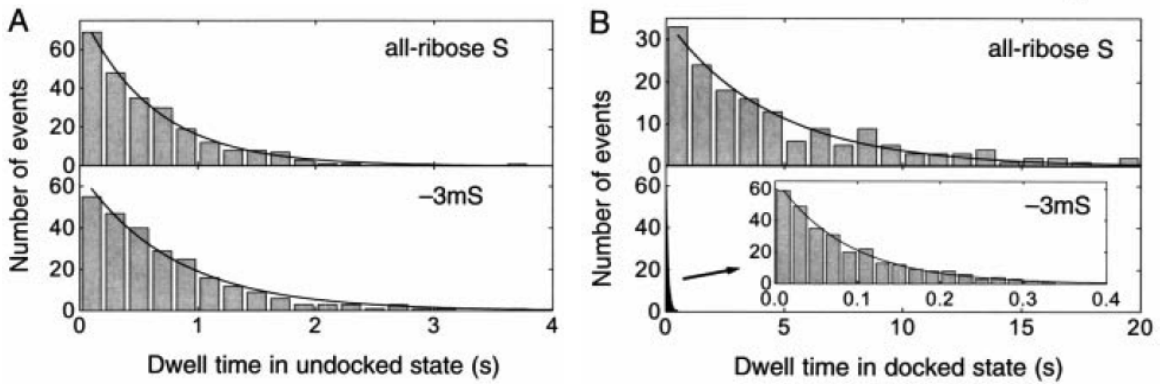
Single molecule fluorescence is used to study the docking of P1 in this ribozyme. To immobilize the ribozyme on a glass surface, a 3'-extension is added to the ribozyme that anneals with a DNA oligo, which is tethered to the glass surface via a biotin tag (Fig. 1).

a) To study the docking of P1 by FRET, where will you place the fluorescent dyes? (2 points) Which pair of fluorescent probes will you use for this FRET study, and why? (2 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? (4 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 constant for docking and undocking, and the equilibrium constants for docking of the all-ribose and the -3mS substrates. (8 points)

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