

ID number _____

Name _____

DUE: November 28, 2005

CH461/561 Exam 3

This is a take-home exam. You may use any notes, textbook or reference material as you work on it. You may also use a computer, and will be required to use one for certain problems. The only restriction is that you must work on this alone; you are not allowed to discuss the exam with anyone until after you have turned it in. You may consult the instructor if you do not understand a question.

For the problems involving calculations, you will need to show your work to receive full credit. I must be able to see how you arrived at your conclusions. If you use a computer for calculations, then you must print out your spreadsheet, graphs, etc. You must label them clearly, so that I can see what you are doing. If this is difficult for me to do, you will receive only partial credit. Be sure to use the correct units and indicate them clearly at all stages.

Protein structures may be downloaded directly from the Protein Database or from my website by option-clicking (right-clicking) on the protein names (<http://www.bama.ua.edu/~kredding/CH461/PDBcodes.html>).

If you download a protein structure, you may give the unmodified file to someone else who is unable to download it.

The exam is due by midnight on Monday, November 28.

The problems are each worth **24** points each; there are **5** of them.

If you are a CH461 student, you must answer **4** for full credit.

(If you answer all, I will give you 1/2-credit for the one on which you scored lowest.)

If you are a CH561 student, you must answer all **5** for full credit.

The maximum amount of points is thus **96** (CH461) or **120** (CH561).

Problems:	1	
	2	
	3	
	4	
	5	
Total		

PLEASE SIGN: I have not discussed this exam with anyone.

1. You are investigating a “wasting disease” in the local cow herd, manifested as worsening loss of energy and well-being, until the cows become near catatonic before dying. You are able to trace it to the inhibition of the enzyme adenylyl cyclase in a certain class of neurons. This enzyme catalyzes the following reaction:



Cyclic AMP (cAMP) is a second messenger used in several signal transduction cascades. However, it is a very specific isoform of the enzyme that is inhibited in this disease, and this isoform is expressed only in a specific class of neurons. Eventually, you are able to track down the source of the problem: a low-grade infection by an opportunistic bacterium called *Microbacterium umusicus*. Normally this bacterium is not pathogenic, but the version causing the problem appears to have picked up a plasmid that gives it the ability synthesize a toxin.

- a) You wish to start by understanding this isoform of adenylyl cyclase (called AC-3 α), so you purify the enzyme and perform a kinetic analysis. The enzyme is a membrane protein and appears to be 57 kDa by SDS-PAGE. You initiate each reaction by adding **5 μL** of a solution of 0.1 $\mu\text{g/mL}$ enzyme to each assay tube, which already contains buffer and various amounts of ATP in a total volume of **45 μL** . After each reaction has proceeded for **40 seconds**, during which time it should still be in the initial phase, you terminate it by adding 50 μL of 0.2 M HCl. You then assay the reaction mixture by adsorbing it to plastic wells and using an ELISA assay (with anti-cAMP antibodies) to estimate the amount of cAMP present, by comparison to a standard. (There is an important detail: in order to get this assay to work, you have to add a second protein to activate AC-3 α , called G_{s α} (GTP). For more information, see Chapter 12. This should not affect your analysis.)

[ATP] in tube before reaction starts (mM)	amount of cAMP after reaction (nmol)
3	17
10	50
30	113
100	190
300	244

From these data, calculate k_{cat} and K_m of activated adenylyl cyclase AC-3 α for ATP.

What is the specificity constant?

Comment on the efficiency of the enzyme – is it “catalytically perfect”?

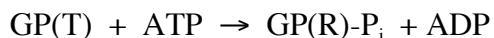
- b) Now you start the analysis of the inhibitor. After a lot of work, you are able to purify the molecule from the pathogenic strain of *M. umusicus* that seems to be responsible for the wasting disease, and call it MUI-1. You repeat the assays exactly as in part (a), except that the tubes also contain MUI-1. Here is the relevant data:

[ATP] in tube before reaction starts (mM)	amount of cAMP (nmol) after reaction when [MUI-1] = (nM)					
	1	3	10	30	100	300
3	17	16	13	8.9	4	1.5
10	47	43	34	20	8.4	3.2
30	105	90	62	33	13	4.4
100	173	137	85	42	15	5.0
300	209	170	98	46	15	5.4

What sort of inhibitor is MUI-1: competitive, uncompetitive, or mixed?

What is the K_i and/or K_i' of MUI-1?

2. Kinases are commonly used to regulate the activities of other enzymes by phosphorylating them. For example, glycogen phosphorylase (GP) is an enzyme used to break down glycogen, so its activity must be tightly controlled so that it is active only when the cell needs a rapid increase in glucose. **Phosphorylase kinase** phosphorylates GP on a crucial Ser residue (Ser14), causing it to shift into the R state:



You wish to characterize the phosphorylase kinase enzyme in order to understand what sort of mechanism it might use to carry out this reaction. You have at your disposal purified GP (as substrate) and purified phosphorylase kinase. You make 20 tubes containing buffer, ions (to mimic the intracellular environment), and various combinations of GP and ATP. You initiate each reaction by adding phosphorylase kinase (final concentration = 0.2 nM), and you measure the initial rate of the reaction in each tube, which is tabulated below:

[ATP] (mM)	initial velocity (nM/s) when [GP] (μM) =			
	0.2	1	5	25
0.3	0.52	2.44	8.49	19.1
1	1.52	6.79	25.2	47.8
3	3.38	14.8	47.3	91.4
10	5.90	25.6	81.8	139
30	6.99	32.0	92.6	153

Which mechanism are these data most consistent with?

- "Ping-Pong" mechanism
- ternary complex with ordered pathway (which one binds first?)
- ternary complex with random pathway

Give the reasons for your conclusion.

Determine the following parameters for phosphorylase kinase:

K_m and/or K_d for GP

K_m and/or K_d for ATP

k_{cat} of the enzyme

3. This problem is a self-driven exercise to appreciate the variety of protein structures available to enzymes. There are 6 types of enzymes (review Table 6-3). The Enzyme Commission (E.C.) has assigned every enzyme a unique 4-part number (*e.g.* 4.3.17.8) based on the reaction it catalyzes. The first part is 1-6, based on the major class. The other parts depend on the kind of reaction and substrates. The Protein database now has a new search feature, with which one can search the protein structures based upon the E.C. number.

1. Go to the RCSB site = <http://www.rcsb.org/pdb/>. From there, you should link to "SearchFields"
2. At the bottom of the search form is a section called "Customize the search fields on this query form." Check the box for "EC Number and Classification." (You can select or deselect any other ones you want, but it is not necessary.) Then hit the button for a "New form."
3. You can input the enzyme number or class/name, but I think the best way is to use the browse feature ("Browse and Select from Enzyme Classification"). Using this, you can browse through each class of enzyme; at each step, you are given new choices until you have a 4-digit number that defines a unique enzyme. (Click the EC number link, not the link in "# of structures".) The browser will tell you how many structures they have of that enzyme. The different structures may be slightly different crystals from the same protein, or they may represent the same kind of enzyme from very different species. You have to examine them more closely to see this.

Your goal:

- 1) Identify an enzyme.
- 2) For your enzyme, get and examine the structures of proteins from two very different species.

The restrictions:

- 1) You may **not** use any enzyme that we have covered in class (*i.e.* no serine proteases, metalloproteases, ribonucleases, hexokinases, lysozymes, enolases, *etc.*)
- 2) The 2 structures should be from species that are phylogenetically distant. Different domains (Eubacteria, Archaeobacteria, and Eukaryota) would be best, but different kingdoms would be acceptable. For example, comparing the same enzyme from yeast and rats would be good. Comparing the same enzyme from dogs and rats would not be good. (If you are unsure, ask me.)

For each enzyme, examine the structures of the 2 proteins. Describe each protein **in as much detail as possible**:

of subunits (4° structure), # of domains, domain arrangement, folding motif of each domain, *etc.* If possible, describe the active site of the enzyme and any allosteric sites. Compare and contrast the 2 structures: Are the 2 proteins similar in structure? How similar? How are they different? **In your opinion, is it the same fold or not?**

[This is a case of looking beyond the trees to see the forest. I don't want to see lots of lists of how many helices, strands, turns, *etc.* each protein has. This is *not* helpful to visualize the protein structure, and it's lazy. Do the work of *seeing* the protein structure and describing it *succinctly*.]

ID number _____

Enzyme name _____

E.C. number _____

What reaction does this enzyme catalyze?

The two examples you chose to examine:

	Choice #1	Choice #2
PDB code		
species of origin		

Analysis of the structures:

(In your discussion, use the PDB codes to refer to the different structures. Your grade will be based on how thoroughly you demonstrate your knowledge.)

(1) Residues important for binding isocitrate

Residue (amino acid & #)	Type of secondary structure element	Likely role	Distance to nearest atom of substrate

(2) Residues important for binding NADP

Residue (amino acid & #)	Type of secondary structure element	Likely role	Distance to nearest atom of substrate

(3) Residues important for binding Mg^{2+}

Residue (amino acid & #)	Type of secondary structure element	Likely role	Distance to nearest atom of substrate

- c) Write below a proposed mechanism for the ICDH-catalyzed reaction. Include at least 3 residues that would be involved in such a mechanism, and briefly explain what each is doing (*e.g.* acting as acid/base at a specific step, stabilizing a specific intermediate state, *etc.*)

- d) Examine the structure of the phosphorylated enzyme (4ICD). It might also be helpful to look at 2 mutants that were made at position 113: Ser to Asp (6ICD) or Glu (7ICD). Such mutations are often made to mimic the phosphorylated Ser residue. Briefly speculate about how phosphorylation of Ser113 inhibits activity of ICDH. Also explain briefly why the mutants (Ser113→Asp/Glu) would mimic the phosphorylated enzyme.

5. You have found a novel biochemical phenomenon while studying the regulation of an enzyme in some medicinal plants. There are several enzymes that are involved in the pathways to synthesize secondary metabolites with antibiotic properties. These secondary metabolites are constantly being made, but their synthesis is rapidly increased when the plant tissues become infected with certain kinds of bacteria, and they probably serve a defensive role. Most of the enzymes in the pathway exist in 2 forms: A-form and B-form. In the pre-stimulated state, the A-forms predominate, and these are all free, soluble enzymes. After stimulation, the B-forms predominate, and these are all found associated with a high-molecular-weight “scaffold” protein. If the B-forms are dissociated from the scaffold by salt washes, they are found to be only slightly more active than the A-forms. The thinking is that, because the enzymes and the substrates/products are present in very low abundance, their clustering together makes synthesis much more efficient and quick as products from one enzyme have a much shorter distance to go to become substrates of the next enzyme.

The real surprise is that all the B-form enzymes have the same N-termini – 25 residues are exactly the same in all: MQEGYDTRSLDAHNEIEGCDTMTRY... The A-forms are the same, but they all begin MTRY... When the genes of these enzymes are sequenced, they encode the A-forms; no genes for the B-forms are found. Thus, it is as if the 21-residue peptide, MQEGYDTRSLDAHNEIEGCDT, were added to the amino termini of the A-form enzymes to convert them to the B-forms. If one synthesizes a 25-residue peptide (MQEGYDTRSLDAHNEIEGCDTMTRY-amide), it has high affinity to the scaffold, but the MTRY-amide peptide does not bind to the scaffold at all. 25-residue peptides lacking one or more of the Asp and Glu residues have lowered affinity. Someone has the bright idea of searching for a gene for the 21-residue peptide, and they find it – a short gene encoding MQEGYDTRSLDAHNEIEGCDT. People also make antibodies against the synthetic 21-residue peptide to measure its abundance, and they find that the 21-residue peptide exists in a free form in the plant cells. During stimulation of the plant cells, its abundance drops quickly as the A-form enzymes are converted to the B-form. Thus, this represents an unusual case of enzyme regulation by irreversible covalent modification.

Your project is to understand *how* this 21-residue peptide (which you’ve dubbed BFP-21, for “B-form peptide”) is added to the A-form enzymes. After long months of effort, you have isolated an enzyme with the following properties:

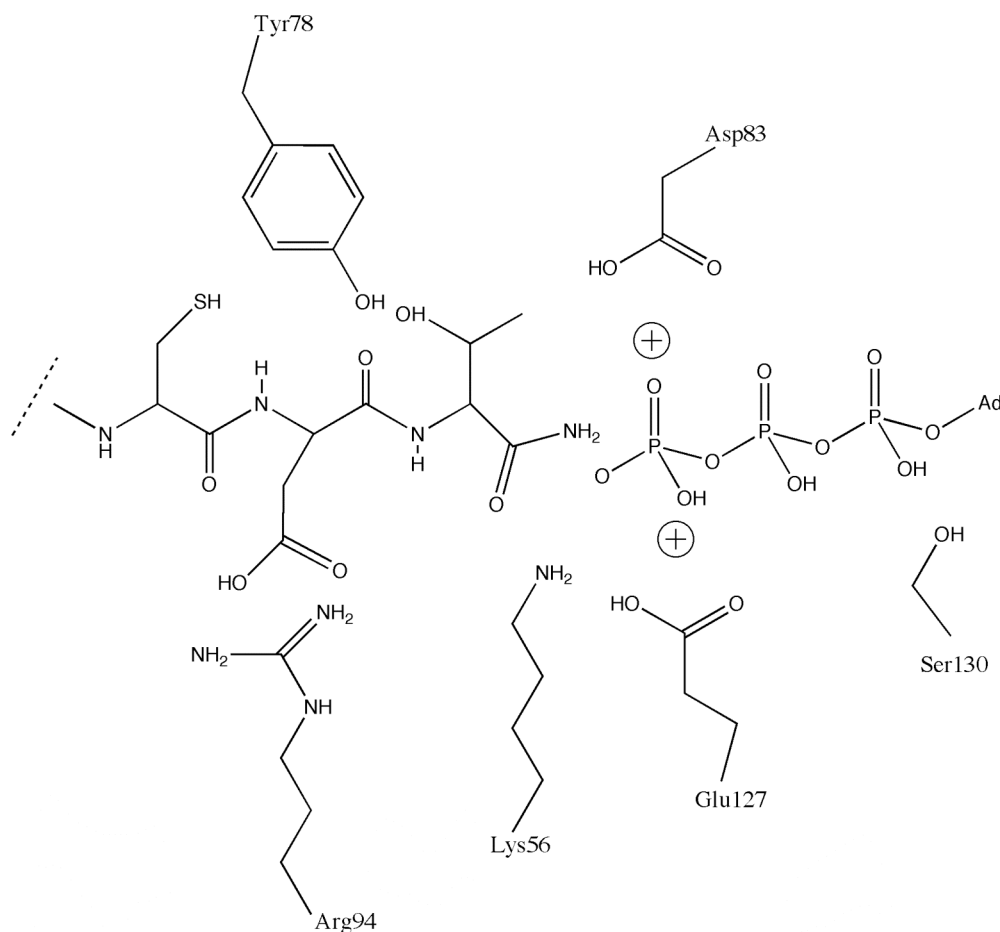
1. It is a homodimer (2 copies of a 42-kDa subunit)
2. It catalyzes the addition of BFP-21 to A-form enzymes in an ATP-dependent manner.
3. The activity is absent in unstimulated plant cells, but becomes abundant after stimulation.

You name the enzyme **BFP ligase**, since it appears to catalyze the following reaction:



In order to understand how BFP ligase works, your research team has initiated an intensive characterization of the enzyme, using kinetic analysis, site-directed mutagenesis, and X-ray crystallography. After several years of work, you know the following:

- a) If you add ATP and BFP-21 to the enzyme, and then denature the mix, you find an intermediate product: BFP-21-P_i, a phosphorylated peptide, where the phosphate is linked to the peptide by a mixed acid anhydride to the carboxy-terminus. BFP-21-P_i is bound very tightly to the enzyme, which makes it difficult to do a kinetic analysis of this reaction. You do know that you can inhibit it if you remove Mg²⁺ (by chelating agents).
- b) You make a version of the BFP-21 peptide with an amide at the C-terminus (BFP-21-amide). This molecule makes a good competitive inhibitor, and you can also do an analysis of the binding of this molecule to BFP ligase without having to worry about catalysis. In the absence of ATP, the enzyme has low affinity for BFP-21-amide (K_d ≈ 1 mM), but affinity increases dramatically in the presence of ATP (K_d ≈ 0.1 μM).
- c) You crystallize the enzyme complexes with BFP-21-amide and ATP and determine the structure by X-ray crystallography. A schematic model is shown below. (The circled + charges stand for 2 Mg²⁺ ions seen in the structure. “Ad” refers to adenosine (not shown).

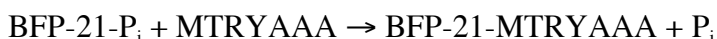


d) You find that you can add BFP-21- P_i to the enzyme in the absence of ATP, and it will catalyze the ligation of BFP-21 to an A-form enzyme. Thus, you know that the enzyme catalyzes two reactions:

- Activation: $\text{BFP-21} + \text{ATP} \rightarrow \text{BFP-21-}P_i + \text{ADP}$
- Ligation: $\text{BFP-21-}P_i + \text{MTRY-Enz} \rightarrow \text{BFP-21-MTRY-Enz} + P_i$

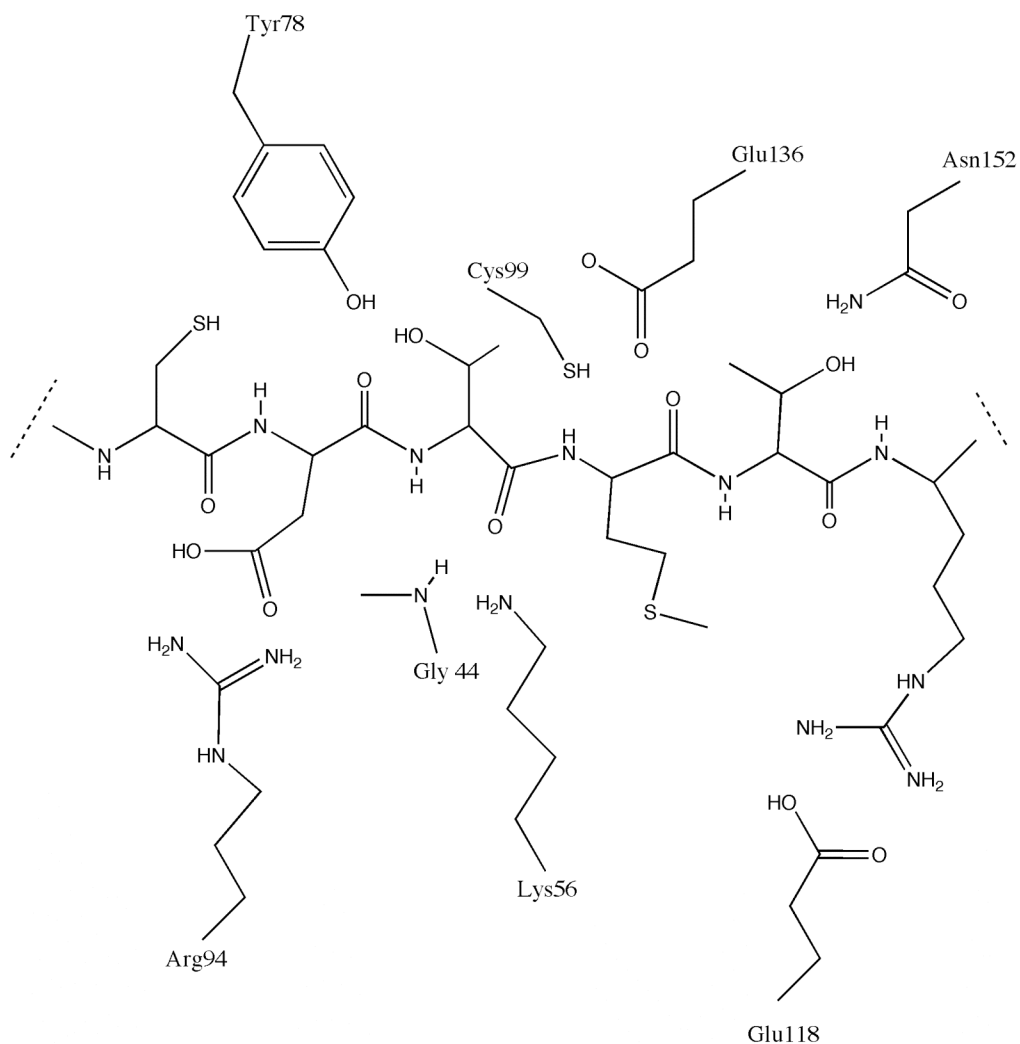
The “activation” reaction is actually a group transfer reaction, but it serves to activate the peptide, so that the subsequent amide formation will be favorable. This makes a convenient way to divide up the overall reaction into two discrete steps.

- e) Binding of enzyme to ATP or BFP-21- P_i are mutually incompatible – it can only bind to one. As the concentration of one increases, the affinity of the enzyme to the other decreases.
- f) You find that you are able to use short peptides corresponding to the N-termini of the A-form enzymes and get transfer of BP-21 to them. This simplifies the biochemical analysis. One of the best artificial substrates is a simple heptapeptide: MTRYAAA. The MTRYAAA peptide will bind with medium affinity to the enzyme ($K_d \approx 70 \mu\text{M}$) in absence of ATP, but with very poor affinity ($K_d \approx 10 \text{mM}$) in presence of ATP.
- g) One of your group members hypothesizes that the enzyme might have an effector site for ATP, even though another site for ATP binding was not seen in the crystal structure. However, careful binding analyses indicate that there is only one site for ATP in the enzyme.
- h) You can perform this simple ligation reaction with BFP ligase:



You find that it is slow, but can be sped up by the presence of ATP, even though ATP is not consumed in the reaction. In fact, you can accelerate this reaction with nonhydrolyzable ATP analogs, such as AMP-PNP. While performing this analysis, you found that much of the enzyme accumulates in the product-bound form if there is no ATP present. If you performed the reaction without ATP and with high amounts of phosphate, you can accumulate almost all of the enzyme in this form, enabling you to crystallize it and determine its structure.

You see immediately that this represents a different conformation of the enzyme. You call this conformation the “L state” (since it seems to represent the “ligation” form of the enzyme), and the former structure the “A state” (which seems to be the form catalyzing the activation step). In the L state several of the residues seen in the A-state active site have moved, especially the ones involved in the binding Mg^{2+} and ATP.



- i) The molecular biology group has cloned the gene for BFP ligase, enabling you to do site-directed mutagenesis to change specific amino acid residues. Based on your crystal structures, you make several mutations. The mutations fall into 3 main classes, based upon their effects upon the activation and ligation steps.

The following mutations primarily affect the activation reaction:

- Asp83→Asn: slower activation reaction and lower affinity for ATP
- Glu127→Gln: slower activation reaction and lower affinity for ATP
- Ser130→Glu: much lower affinity for ATP

The following mutations primarily affect the ligation reaction:

- Cys89→Ser, Ala: slows the ligation reaction ~1000-fold (almost no effect upon affinity for BFP-21-amide or MTRYAAA)

- Glu136→Gln: slows the ligation reaction ~100-fold
(almost no effect upon affinity for BFP-21-amide or MTRYAAA)
- Glu118→Gln: lowers affinity for MTRYAAA (~no effect on k_{cat})
- Asn152→Ala: lowers affinity for MTRYAAA (~no effect on k_{cat})

The following mutations affect both activation and ligation:

- Tyr78→Phe: lowers affinity for BFP-21-amide and BFP-21-P_i
- Arg94→Met: lowers affinity for BFP-21-amide and BFP-21-P_i
- Lys56→Met: slows the rate of both reactions (mainly an effect on k_{cat}), but the effect is more drastic for ligation.

- j) During the stimulation process a specific phosphatase is activated, which removes a phosphate from Ser130 on BFP ligase. You find that Ser130 is normally a phosphoserine before stimulation. The phosphorylated enzyme has much lower activity.

Propose a mechanism for this enzyme that would explain all of these observations. Be complete and show how your model would explain these data. (And be imaginative!)