

DUE: December 1, 2003

Name \_\_\_\_\_

ID number \_\_\_\_\_

## 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 9:05 AM on Monday, December 1.**

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.

(I will drop the one with the lowest points, if you answer all.)

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

The maximum amount of points is thus **120** (CH461) or **144** (CH561).

Problems:	1	
	2	
	3	
	4	
	5	
Total		

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

\_\_\_\_\_

1. You have recently isolated a new soil bacterium, called *Terreococcus novelum*. This soil bacterium competes with a certain fungus, *Streptomyces competicus*, for the same ecological niche. *S. competicus* secretes an enzyme that hydrolyzes the peptidoglycan cell wall of the bacterium. This enzyme, called lysozyme Sc1 (since it is the first lysozyme enzyme isolated from *S. competicus*), hydrolyzes the linkage between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (Mur2Ac; see Fig. 9-19 of your book). It behaves as a monomer with a molecular mass of 25 kDa.

- a) You wish to perform a kinetic analysis of lysozyme Sc1. Using peptidoglycan is obviously difficult, so you use an artificial substrate: [<sup>14</sup>C]-GlcNAc(β1→4)Mur2Ac. You initiate each reaction by adding **5 μL** of a solution of 1 μg/mL enzyme to each assay tube, which already contains buffer and various amounts of GlcNAc(β1→4)Mur2Ac in a total volume of **95 μL**. After each reaction has proceeded for **2 minutes**, during which time it should still be in the initial phase, you terminate it by adding 20 μL of 3 M NaOH. You then assay the reaction for [<sup>14</sup>C]-GlcNAc by HPLC analysis and scintillation counting for detection and quantification of the radioactive product. The results are tabulated below (expect some error). Note that the specific activity of the radioactive isotope is such that 1 nanomole = 245,000 cpm (counts per minute).

[GlcNAc(β1→4)Mur2Ac] in tube before reaction starts (mM)	amount of GlcNAc after reaction (cpm)
0.01	248
0.03	682
0.1	1737
0.3	3150
1	4351
3	4918

From these data, calculate  $k_{cat}$  and  $K_m$  of lysozyme Sc1 for GlcNAc(β1→4)Mur2Ac. What is the specificity constant?

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

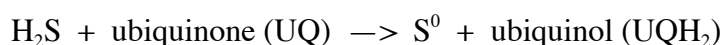
- b) As it turns out, *T. novelum* does not just take this kind of treatment lying down. It secretes an inhibitor of lysozyme Sc1. You have isolated a strain that makes lots of this inhibitor, called LSCI (for lysozyme Sc1 inhibitor), and purified it. Using your assay as before, you run the assay at different concentrations of LSCI. Here is the relevant data:

[GlcNAc( $\beta$ 1 $\rightarrow$ 4) Mur2Ac] in tube before reaction starts (mM)	Amount of GlcNAc after reaction (cpm) when [LSCI] = ( $\mu$ M)					
	0.1	0.3	1	3	10	30
0.01	225	180	113	54	19	6.8
0.03	594	515	321	160	56	20
0.1	1559	1359	914	503	186	67
0.3	2912	2641	2087	1229	524	192
1	4282	4002	3629	2634	1401	583
3	4701	4718	4483	3950	2681	1480

What sort of inhibitor is LSCI: competitive, uncompetitive, or mixed?

What is the  $K_i$  and/or  $K_i'$  of LSCI?

2. There are some unusual lithotrophic bacteria that use sulfide as an electron source and perform aerobic respiration. You have isolated the enzyme sulfide:quinone oxidoreductase (SQR) from a newly-discovered bacteria, *Sulfolobus malodori*. It catalyzes the following reaction:



(Actually, it can use  $\text{Na}_2\text{S}$  as a substrate as well, in which case protons from the medium are consumed. The other complication is that UQ is a lipid-soluble substrate. In this problem, we will treat it as if it is like a soluble substrate.)

In order to obtain insight into the mechanism of this enzyme, you perform a kinetic analysis with varying amounts of sulfide ( $\text{S}^{2-}$ ) and UQ. Each reaction contains 1 nM enzyme.

[ $\text{S}^{2-}$ ] ( $\mu\text{M}$ )	initial velocity (nM/s) when [UQ] ( $\mu\text{M}$ ) =				
	10	30	100	300	1000
10	18.4	45.1	105	157	178
30	52.0	124	270	399	489
100	130	336	738	1095	1388
300	262	664	1385	2275	2690
1000	366	964	2143	3106	4098

Which mechanism are these data more consistent with?

- "Ping-Pong" mechanism
- ternary complex with ordered pathway
- ternary complex with random pathway

Give the reasons for your conclusion.

Determine the following parameters for your enzyme:

- $K_m$  for sulfide
- $K_m$  for ubiquinone
- $k_{\text{cat}}$  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 8-3). 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. 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 discussion, use the PDB codes to refer to the different structures. Your grade will be based on how thoroughly you demonstrate your knowledge.

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:

4. This problem is about the relationship between the structure and function of hemoglobin.

- a) Examine the crystal structures of the T & R forms of hemoglobin. These are in the PDB files 1HGA (T state) and 1BBB (R state, bound to CO), which can be downloaded from the website. List all of the interactions *between subunits* in the T state that *do not exist* in the R state. Use the table on the next page as a guide. (You do not need to list more than the table provides room for. If you have more interactions than 11, list the most important ones.)
- b) Examine the structure of the T state of hemoglobin bound to 2,3-bisphosphoglycerate (BPG) shown in PDB file 1B86. List the top 6 interactions you can see between the protein and the effector.

Subunit ( $\alpha$ , $\beta$ , etc)	Residue (amino acid type and residue number)	Type of interaction (charge-charge, H-bond, etc)

Examine those same residues in the R state – would they be able to bind BPG in the R state?

- c) In the mutant,  $\alpha$ -D126N, the interaction between subunits in the T state is weakened. Examine the structure of the T state. Explain why mutation of the Asp126 residue in the  $\alpha$ -subunit to Asn would weaken the T state. Sketch an  $O_2$ -binding curve of the normal and mutant hemoglobins. Note: this does not have to use real values of  $[O_2]$ ; it is more for comparison purposes. Explain your curve – why did you draw it that way?
- d) Using the values below and the symmetry model for cooperativity in oxygen-binding to hemoglobin, calculate the equilibrium constant for the conversion of  $T(O_2)_4 \rightarrow R(O_2)_4$ :

$$K_{eq}^{TR} = [R]/[T] = 3 \times 10^{-4}$$

$$K_D^T = 8 \times 10^{-4} \text{ M}$$

$$K_D^R = 2 \times 10^{-6} \text{ M}$$



5. In many organisms, phospholipids are synthesized using an intermediate in which diacylglycerol (DAG) is activated by linkage to CDP (cytidine diphosphate). In bacteria, the enzyme phosphatidylserine (PS) synthase catalyzes this reaction:



In mammalian cells, PS can also be synthesized by an alternate pathway, in which serine displaces ethanolamine from PE (see pages 792-795 of your book). Most bacteria lack this alternate pathway, so PS synthase might be a good target for antibiotic design. You reason that an inhibitor that binds this enzyme specifically might inhibit growth of pathogenic bacteria without harming the host cells. But before you can make such an inhibitor, you need to know how this enzyme works. You start by cloning it from a bacterium that is affected by few antibiotics, *Pneumococcus megaresistans*.

Your research team embarks upon an intensive characterization of the enzyme, using kinetic analysis, site-directed mutagenesis, and X-ray crystallography. After several years of work, this is what you know:

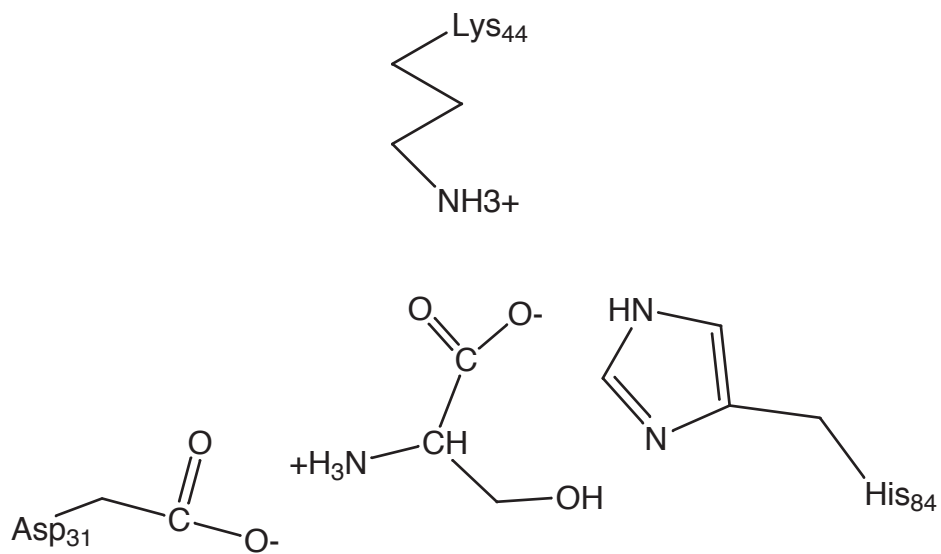
- a) The enzyme can bind Ser in the absence of CDP-DAG. It will not bind Thr or any larger amino acid. While it can bind Ala or Gly, it cannot (of course) use them as substrates. It will only use L-Ser; D-Ser is not a substrate. If you acetylate the amino terminus of Ser or amidate the carboxyl terminus, affinity is reduced about 100-fold.
- b) During the course of this analysis, you find that the carboxylic acid of the Ser is critically important for catalysis. While acetylation of the amine group increases the  $K_M$  about 100-fold (in line with the change in affinity), amidation of the carboxyl group raises  $K_M$  about 100-fold *and* decreases  $k_{cat}$  ~200-fold. A similar effect is seen if the carboxyl group is converted to a methyl ester. The enzyme will work only very weakly with ethanolamine (that is, it can make PE too, but it is a very poor PE synthase). The  $K_M$  for ethanolamine is ~200-fold higher than the  $K_M$  for serine, and the  $k_{cat}$  is ~200-fold lower.
- c) PS synthase is not so specific for the other substrate (CDP-DAG), and it seems to be more particular about the nucleotide portion than it is about the lipid moiety. While a glycerol backbone is required, it can be esterified to fatty acids as short as butyrate. Neither CMP-DAG nor CTP-DAG will work. There is also specificity towards the base of the nucleotide. Substitution of cytosine for purines (*i.e.* ADP-DAG or GDP-DAG) results in no activity, but UDP-DAG can function as a substrate, albeit with a  $K_M$  about 10-fold higher. The 2-deoxy version, dCDP-DAG, reacts with ~5-fold higher  $K_M$ .
- d) The enzyme works well under neutral or somewhat basic conditions, but it becomes inactive at lower pHs. Activity is lost with a  $pK_a$  near 5.5; this is mainly an effect upon  $k_{cat}$ . With increasing pH, activity is lost with a  $pK_a$  around 10; this is mainly an effect upon both  $K_M$  for Ser.
- e) The enzyme is also inhibited by molecules that chelate divalent cations. It can be restored by addition of  $Mg^{2+}$ .
- f) You crystallize the acetylated enzyme with Ser and determine the structure by X-ray diffraction. This helps you to identify the active site. (See top panel on last page.)

- g) You use a common trick to mimic a pentavalent phosphorous, which is a common intermediate in phosphotransfer and phosphoester hydrolysis reactions, and substitute a vanadate moiety for the  $\beta$ -P<sub>i</sub>. You reason that this molecule, called VMI-1 (“vanadyl mimic inhibitor # 1”) should resemble an intermediate in the reaction. As it turns out, this molecule binds very tightly to the enzyme, and is a very strong competitive inhibitor (*i.e.* it has a very low K<sub>p</sub>). You obtain a structure of the enzyme bound to this inhibitor (see bottom panel on last page).
- h) Several interactions can be seen in these structures, not all of which are obvious in these pictures.
1. The bound Mg<sup>2+</sup> ion shows up prominently in the vanadyl derivative co-crystal, but it is located behind the vanadyl moiety, where it can interact with the two free oxygens. It is somewhat closer to the oxygen bridging the phosphate and vanadate than it is to the other two oxygens (of Ser and DAG).
  2. Trp77 is located behind the cytosine residue; the faces of the indole group and the cytosine are within van der Waals contact.
- i) If you bind CDP alone to the enzyme and crystallize it, the CDP binds as shown in the VMI-1 co-crystal, although the  $\beta$ -P<sub>i</sub> is in a somewhat different place than the vanadyl group, which has a different geometry. If you bind CDP-DAG to the enzyme, the cytosine moiety is not able to fit into the same binding niche in the same way; because DAG is restricted from entering the active site, it pulls the CDP moiety away from its optimal binding arrangement.
- j) You make several mutants to test the role of certain amino acid residues identified in the crystal structure:
- 1) **Asp31:** Mutation to Asn reduces affinity for Ser ~100-fold and K<sub>M</sub> for Ser increases ~100-fold; almost no effect on k<sub>cat</sub>.
  - 2) **Lys44:** Mutation to Gln reduces affinity for Ser ~100-fold and K<sub>M</sub> for Ser increases ~100-fold; almost no effect on k<sub>cat</sub>.
  - 3) **Trp77:** Mutation to Leu reduces affinity for CDP ~10-fold and K<sub>M</sub> for CDP-DAG increases by about the same amount; k<sub>cat</sub> decreases ~3-fold.
  - 4) **His84:** Mutation to Gln reduces affinity for Ser ~15-fold and K<sub>M</sub> for Ser increases ~20-fold; k<sub>cat</sub> decreases almost 500-fold.
  - 5) **Thr99:** Mutation to Ala reduces affinity for CDP ~10-fold and K<sub>M</sub> for CDP-DAG increases by about the same amount; k<sub>cat</sub> decreases ~5-fold.
  - 6) **Asn107:** Mutation to Leu reduces affinity for CDP ~10-fold and K<sub>M</sub> for CDP-DAG increases by about the same amount; k<sub>cat</sub> decreases ~5-fold.
  - 7) **Gln134:** Mutation to Leu reduces affinity for CDP ~10-fold and K<sub>M</sub> for CDP-DAG increases by about the same amount; k<sub>cat</sub> decreases ~3-fold.

- 8) **Glu172:** Mutation to Gln has almost no effect upon  $K_M$  for either Ser or CDP-DAG, or upon  $k_{cat}$ . However, the mutation has an interesting effect: it allows a side-reaction (CDP-DAG + Ser  $\rightarrow$  CDP-Ser + DAG), which normally does not occur to any appreciable amount to occur  $\sim 20\%$  of the time. In effect, the mutation *increases* the  $k_{cat}$  of this unproductive reaction.
- 9) **Arg185:** Mutation to Leu reduces affinity for CDP  $\sim 10$ -fold and  $K_M$  for CDP-DAG increases by about the same amount;  $k_{cat}$  decreases  $\sim 80$ -fold. This mutation also allows the side-reaction described above (to make CDP-Ser) to occur  $\sim 5\%$  of the time.

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!)

**A**



**B**

