

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

Name \_\_\_\_\_

DUE: December 1, 2004

## 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 Wednesday, 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.

(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.

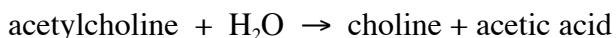
The maximum amount of points is thus **120** (CH461) or **144** (CH561).

Problems:	1	
	2	
	3	
	4	
	5	
<b>Total</b>		

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

\_\_\_\_\_

1. You have a job with the Department of Homeland Security. Lake Oopaloochi is the main source of drinking water for Fort Sylvester. There are rumors that the lake has been tampered with. Your HPLC analysis of the lake water reveals a novel small peptide, and mass spectrometry reveals that it is a toxin produced by a fresh-water cyanobacterium, *Buggi verdeblu*. It is known that this peptide inhibits acetylcholinesterase, which is essential for clearing the neurotransmitter in the synapse by catalyzing this reaction:



This kind of toxin can cause seizures, because the muscles continue to contract after the signal is over. Although there is data on the interaction of this toxin (called CAI-2, for cyanobacterial acetylcholinesterase inhibitor #2) with the enzyme from rats, there is no data on the human enzyme. It is important to establish this system with human acetylcholinesterase, so that the danger to the human population can be assessed.

- a) You have isolated the acetylcholinesterase enzyme from recent human cadavers. It behaves as 125-kDa monomer, and your solution is 0.5  $\mu\text{g}/\text{mL}$ . You also have in hand some purified CAI-2. You initiate a series of reactions by addition 1  $\mu\text{L}$  of enzyme to a tube containing acetylcholine in a total volume of 100  $\mu\text{L}$ . The concentration of the acetylcholine and CAI-2 during the reaction is reported in the left-hand column and top-most row, respectively. After 30 seconds have elapsed, you add some EDTA to stop the reaction (the enzyme requires  $\text{Mg}^{2+}$ ). You analyze each reaction by HPLC and quantify the amount of choline produced, which is shown in the table below (in picomoles of choline).

[Acetylcholine] ( $\mu\text{M}$ )	Amount of choline after reaction (pmol) when [CAI-2] = (nM)					
	0	10	30	100	300	1000
10	171	145	111	63	28	9.6
30	466	400	319	180	84	28
100	1240	1090	915	562	256	93
300	2350	2110	1880	1300	681	260
1000	3300	3190	3020	2580	1710	785
3000	3950	3840	3630	3410	2790	1690

From these data, calculate  $k_{\text{cat}}$  and  $K_m$  of human acetylcholinesterase for its substrate.

What is the specificity constant?

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

What sort of inhibitor is CAI-2: competitive, uncompetitive, or mixed?

What is the  $K_i$  and/or  $K_i'$  of CAI-2 for human acetylcholinesterase?

b) With this data in hand, you decide to figure out how much CAI-2 is in the lake. You take a sample of lake water and set up reactions this way:

- 1) For each reaction tube, you have 50  $\mu\text{L}$  of lake water.
- 2) You add acetylcholine to have the desired amount in the tube, and water to bring the volume to 99  $\mu\text{L}$ .
- 3) You initiate the reaction by addition of 1  $\mu\text{L}$  of stock enzyme.
- 4) After 30 seconds have elapsed, you terminate the reaction and analyze product as usual.

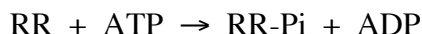
(*i.e.* The reactions are done exactly the same as before, except now half the volume of each reaction is water from the lake.)

The data is shown below:

[Acetylcholine] ( $\mu\text{M}$ )	Amount of choline after reaction (pmol)
10	126
30	355
100	982
300	1990
1000	3090
3000	3680

From this, estimate the concentration of CAI-2 in the lake, assuming that all observed inhibition is due to CAI-2. With the amount of cyanobacteria found in Lake Oopaloochi, one would expect the concentration of CAI-2 to be around 5 - 30 nM. Based on this, do you have any evidence that the lake has been tampered with – that somebody has added CAI-2 to the lake?

2. There is a receptor kinase involved in signaling in bacteria. When it is turned on by an extracellular signal, it catalyzes the following reaction:



“RR” stands for *response regulator*, a small protein that becomes phosphorylated on a specific Asp residue, forming a mixed acid anhydride (RR-P<sub>i</sub>).

In order to obtain insight into the mechanism of this kinase, you perform a kinetic analysis with varying amounts of ATP and RR. Each reaction contains 1 nM of the kinase. You do the assay in the presence of a saturating concentration of the signaling molecule, which keeps the enzyme maximally active.

[ATP] (mM)	initial velocity (nM/s) when [RR] ( $\mu\text{M}$ ) =			
	3	10	30	100
0.1	5.9	6.9	7.0	7.6
0.3	11.4	17.3	19.6	20.0
1	18.3	32.9	44.5	47.9
3	20.0	45.0	67.6	85
10	20.9	51.6	89	122

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:

- $K_m$  (and/or  $K_d$ ) for ATP
- $K_m$  (and/or  $K_d$ ) for RR
- $k_{\text{cat}}$  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 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?

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 Fructose-6-P<sub>i</sub> (Frc-1,6-bisP<sub>i</sub>)

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

## (2) Residues important for binding ATP (ADP) as substrate (product)

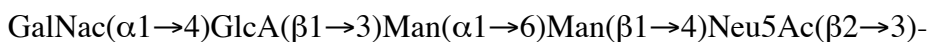
Residue (amino acid & #)	Type of secondary structure element	Likely role	Distance to nearest atom of substrate



- d) You have created a mutant in which Glu241 has been converted to Gln. Sketch below a graph of reaction velocity vs. [Frc-6-P<sub>i</sub>], comparing mutant to normal PFK in these 2 conditions:
- a. in the presence of ADP
  - b. in the presence of high ATP (1mM, and no ADP)

It might be best to make 2 sketches – one in the first condition, and one in the second condition. These do not need to be quantitative. I just want to know in what way you would expect the mutant enzyme to be different from the normal enzyme.

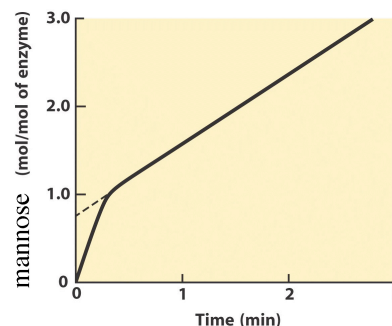
5. Before a sperm can fuse with an egg and create a zygote, it must penetrate the thick extracellular matrix surrounding the plasma membrane of the oocyte. This is one area in which species compatibility can be checked. Consider simple marine animals. Often both gametes are free in the marine environment, allowing sperm from one organism to encounter eggs from another species. One of the many ways used by organisms to ensure compatibility is the type of extracellular matrix surrounding the egg. In a certain species of jellyfish, *Yellus ouchi*, the egg's plasma membrane has a major glycoprotein, to which are attached chains of an unusual anionic polysaccharide, consisting of many repeats of this basic unit:



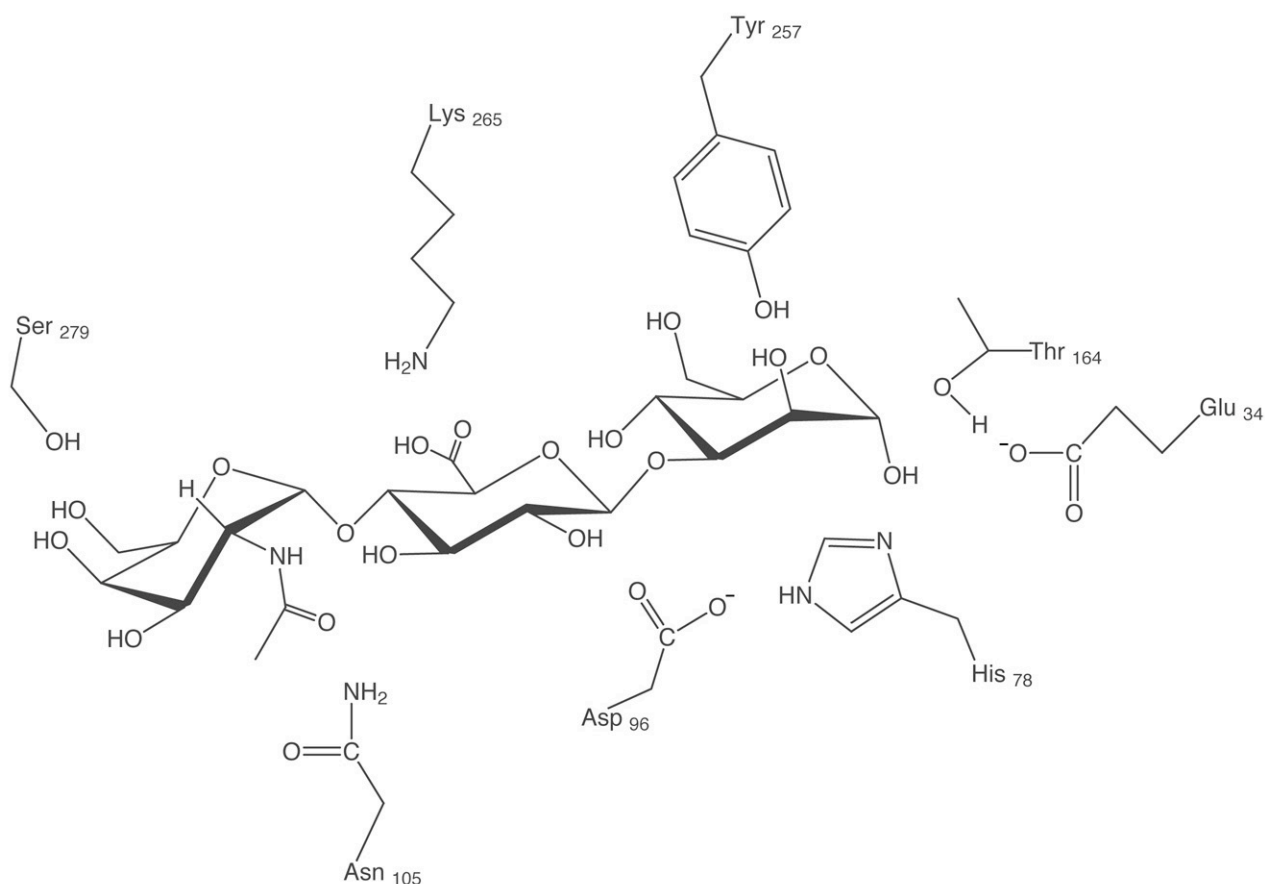
(refer to Table 7-1 in your book for abbreviations)

The sperm of *Y. ouchi* possess a specific glycosidase that hydrolyzes the  $\text{Man}(\alpha 1 \rightarrow 6)\text{Man}$  linkage in this polysaccharide, allowing the sperm to break through the dense extracellular matrix of the egg. This combination of polysaccharide and glycosidase is almost unique to this species and ensures that no other sperm can fuse with a *Y. ouchi* egg. In order to understand the basis for this interaction and to understand how the enzyme 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) It is possible to get good activity with a small substrate, consisting of a single repeat of the polymer. In fact, one can use a tetrasaccharide, consisting of GalNac-GlcA-Man-Man, and the enzyme will cleave it efficiently. This becomes your standard substrate for all subsequent kinetic analysis of the enzyme. The glycosidase exhibits kinetic parameters of  $K_m = 120 \mu\text{M}$  and  $k_{\text{cat}} = 10 \text{ s}^{-1}$  for this substrate.
- b) You have tried to see how specific the glycosidase is for its substrate:
  - It requires an acetylated amine at position 2 in the first carbohydrate residue. This can be replaced with GlcNac, but replacement with Gal increases  $K_m \sim 20$ -fold.
  - It requires a carboxylate at position 6 in the second carbohydrate residue (position #2). This can be replaced with galacturonic acid, but replacement with Glc increases  $K_m \sim 50$ -fold.
  - Replacement of either of the mannoses with any other sugar results in a large decrease in activity of the enzyme towards the substrate.
  - Also important is the stereochemistry of the substrate. Changing the linkages to  $\text{GalNac}(\beta 1 \rightarrow 4)\text{GlcA}$  or  $\text{GlcA}(\alpha 1 \rightarrow 3)\text{Man}$  lowers activity greatly, due to a big increase in  $K_m$ . Change of the scissile bond to  $\text{Man}(\beta 1 \rightarrow 6)\text{Man}$  abolishes activity.
- c) The glycosidase has a pH optimum near 6-6.2. It loses activity as the pH rises with a  $\text{pK}_a$  around 7, and as pH drops with a  $\text{pK}_a$  around 4.5, both primarily an effect on  $k_{\text{cat}}$ .
- d) The enzyme displays “burst kinetics”. If you assay for free mannose, this appears almost immediately (with an amount equivalent to the amount of glycosidase), followed by a slower linear rate (in the initial stage).



- e) The reason for the burst kinetics appears to be due to slow release of the trisaccharide product (GalNac-GlcA-Man). In fact, the enzyme exhibits a phenomenon called “product inhibition”. Significant amounts of GalNac( $\alpha$ 1 $\rightarrow$ 4)GlcA( $\beta$ 1 $\rightarrow$ 3)Man can inhibit the enzyme, and analysis of this effect confirms that the trisaccharide is a competitive inhibitor. You use this fact to try and identify the active site by co-crystallizing the glycosidase with the trisaccharide. You obtain X-ray diffraction data from the crystals and eventually (after much effort) obtain a model of the structure. Below is a model of how the trisaccharide is bound to the protein. The trisaccharide is bound in a cleft between 2 domains, and it seems that there might be some flexibility between the two domains (*i.e.* perhaps they could move slightly relative to each other). Residues 1-128 are in the N-terminal domain, and residues 135-312 are in the C-terminal domain. Interestingly, although you supplied the trisaccharide as a racemic mixture, only the anomer with the mannose in the  $\alpha$  configuration is found bound to the enzyme. Keep in mind that hydrogens do not show up in most crystal structures, due to the low resolution. In some cases, hydrogens have been indicated in the structure below, *but keep in mind that the actual protonation states of some functional groups may be different than shown below.*



- f) The results with the specific binding of the  $\alpha$  anomer prompt you to test an idea: which anomer is produced by the glycosidase (or is it a mixture)? You let the enzyme cleave some tetrasaccharide product and then quickly measure the rotation of polarized light produced by the products and find that the trisaccharide product is almost purely the  $\alpha$  anomer.

- g) Meanwhile, your molecular biology group has cloned the gene for the *Y. ouchi* sperm glycosidase, enabling you to do site-directed mutagenesis to change specific amino acid residues. Based on your crystal structure, you make several mutations.
- h) Mutation of any of the following residues primarily affects  $K_m$ :
- Asn105→Leu: increases  $K_m$  ~20-fold
  - Tyr257→Phe: increases  $K_m$  ~15-fold
  - Lys265→Met: increases  $K_m$  ~40-fold (Change to Arg has a very small effect.)
  - Ser279→Ala: increases  $K_m$  ~10-fold

Each of these mutations also has a modest effect upon  $k_{cat}$  (~2-fold *increase*).

However, some of them have another effect – they make the enzyme less specific:

- Asn105→Leu: poor discrimination between GalNac and Gal in position #1
  - Lys265→Met: poor discrimination between GlcA and Glc in position #2
  - Tyr257→Phe: poor discrimination between Man and Glc in position #3
- i) Mutation of these residues affects  $k_{cat}$ :
- Glu34→Gln: lowers  $k_{cat}$  ~300-fold
  - His76→Phe: lowers  $k_{cat}$  ~300-fold
  - Asp96→Asn: lowers  $k_{cat}$  ~20-fold
  - Thr164→Val: Practically abolishes activity, lowering  $k_{cat}$  >1000-fold. (Change to Ser has a much smaller effect, lowering it only ~3-fold.)
- j) The Asp96→Asn mutation does not seem to have a profound effect upon the rate of the reaction, but it has an interesting side-effect: you see that a fraction of the glycosidase exists as a form in which the trisaccharide is covalently linked to the enzyme. It is only transiently linked to the enzyme, but if you rapidly denature the glycosidase (with urea or SDS) while it is cleaving the substrate and then analyze by SDS-PAGE, you see that a small fraction has a slightly higher molecular weight. Cleavage of the glycosidase with trypsin followed by tandem-MS of the peptides allows you to map the point of linkage to Thr164. Similar analysis with the normal enzyme does not provide evidence for such a covalent linkage.
- k) In the crystal structure, the 2-OH of the GlcA residue seems to be just out of range for strong H-bonding to Asp96. However, you realize that such a H-bond might exist during a certain stage of the reaction. The result with the Asp96→Asn mutant prompts you to test this idea by synthesizing a substrate where the GlcA is replaced by 2-deoxy-GlcA (GalNac-deoxyGlcA-Man-Man). Interestingly, hydrolysis of this substrate in the normal enzyme looks like the results with Asp96→Asn mutant on the normal trisaccharide: it is slower, but more importantly, a form of the enzyme appears where Thr164 is linked to the trisaccharide GalNac-deoxyGlcA-Man.

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

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!)