

DUE: December 3, 2002

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 noon on Tuesday, December 3.

The problems are each worth **24** points each; there are **6** of them.

If you are a CH461 student, you must answer **5** 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 **6** for full credit.

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

Problems:	1	
	2	
	3	
	4	
	5	
	6	
Total		

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

1. Phosphohexose isomerase is the enzyme responsible for interconverting glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P). You have been studying the mouse enzyme, and discover that there are at least 2 different isoenzymes. A 60-kDa form is expressed predominantly in the fetus, and a 72-kDa form begins to be expressed soon after birth and then becomes the dominant one within a few weeks. You refer to these as the “fetal” and “adult” forms.

You have recently purified these two enzymes and wish to perform a kinetic analysis to determine their properties. You initiate each reaction by adding **1 μL** of a solution of 10 $\mu\text{g}/\text{mL}$ enzyme to each assay tube, which already contains buffer and various amounts of glucose-6- ^{32}P phosphate in a total volume of **100 μL** . After each reaction has proceeded for **30 seconds**, during which time it should still be in the initial phase, you terminate it by adding 20 μL of 0.1 M EDTA. You then assay the reaction for fructose-6-phosphate by HPLC analysis and scintillation counting. The results are tabulated below (expect some error):

[G-6-P] in tube before reaction (mM)	After reaction, the tube contains _ nmol F-6-P	
	fetal enzyme	adult enzyme
0.05	0.36	0.40
0.1	0.61	0.66
0.2	1.07	0.93
0.5	1.58	1.31
1	1.93	1.43
2	2.27	1.52

From these data, calculate v_{max} and K_m of each enzyme for G-6-P. Assuming that all of the enzyme is active, calculate k_{cat} . (Why is this assumption necessary?) Calculate the specificity constants. Discuss the main differences between the fetal and adult forms.

Why is EDTA (ethylene diamine tetraacetic acid) used to terminate the reaction? In the case of each set of reactions, in which one was the conversion of G-6-P to F-6-P (as a percent of starting G-6-P) the highest and what was this extent of conversion (as a percent of starting G-6-P)? In this reaction, what was the $\Delta G'$ of the reaction at the point of termination? Why is this important?

2. You work for a biotech company that is attempting to increase the sugar content of lettuce leaves. The idea is to inhibit the activity of the enzyme sucrose-6-phosphate phosphatase (see Figure 20-17). After removal of the phosphate, the neutral sucrose molecule can be transported across the cellular membrane and down to the roots. If you can inhibit this activity, then perhaps more sucrose would be retained in the leaves, and they would be sweeter. (You might ask why people would want sweeter lettuce, but it is not your job to question marketing analyses...)

The synthetic division has come up with an inhibitor they call TR-54. Your job is to test it and determine what kind of inhibitor it is. Here is the relevant data:

[TR-54] (nM)	initial velocity (nM s ⁻¹) when [sucrose-6-phosphate] = ___ μM						
	1	3	9	27	81	243	729
0	16	45	109	208	297	346	367
10	15	42	102	199	291	343	366
30	13	36	90	183	279	338	363
100	8.4	24	64	143	244	320	356
300	4.2	12	35	88	181	277	337
1000	1.5	4.6	13	38	94	189	283

What are the v_{max} & K_m of the enzyme for sucrose-6-phosphate?

What sort of inhibitor is TR-54: competitive, uncompetitive, or mixed?
What is the K_i and/or K_i' ?

While you are characterizing the synthetic inhibitor, the plant physiology section makes an important discovery – they have found a natural inhibitor. It appears that under certain conditions, sucrose export from lettuce leaves slows down, and this decrease correlates with the presence of a small molecule secreted by cells in the plant vascular tissue when the external sucrose concentration rises. They call the molecule “retainin”, since it causes sucrose to be retained. You obtain a sample of retainin and test it as well, and get the data on the next page.

[retainin] (nM)	initial velocity (nM s ⁻¹) when [sucrose-6-phosphate] = ___ μM						
	1	3	9	27	81	243	729
0	16	51	139	236	280	294	298
1 nM	15	48	134	234	279	294	298
20 nM	5.8	21	80	199	272	293	298
400 nM	0.49	1.91	9.48	53	184	276	296
8 μM	0.078	0.27	1.18	6.6	42	170	274
160 μM	0.057	0.19	0.74	3.7	23	118	251

Careful analysis of this data causes you to re-examine the nature of the enzyme. As you suspected, you find that sucrose-6-phosphate phosphatase is a dimer. Explain why the enzyme must be multimeric and how it likely works, and then explain how retainin works.

3. Although aldolase is commonly thought to catalyze the splitting of fructose-1,6-bisphosphate (FBP) to dihydroxyacetonephosphate (DHAP) and glyceraldehyde 3-phosphate (G3P), it also catalyzes the reverse reaction (like any enzyme):



There are several reasons to assay the enzyme using the reverse reaction: it is more favorable in that direction, the reverse reaction is important in gluconeogenesis, and it allows separate analysis of interaction with DHAP and G3P.

You make 25 tubes containing buffer, ions (to mimic the intracellular environment), and various combinations of DHAP and G3P, with each ranging from 10 μM to 1 mM. You initiate each reaction by adding aldolase (final concentration = 1 nM), and you measure the initial rate of the reaction in each tube, which is tabulated below:

[DHAP] (mM)	initial velocity (nM/s) when [G3P] (mM) =				
	0.01	0.03	0.1	0.3	1
0.01	1.3	3.9	12.1	35.5	80.4
0.03	3.0	8.6	29.0	65.2	137
0.1	6.1	16.4	47.1	101	167
0.3	7.7	21.3	58.2	124	169
1	8.7	24.0	67.3	132	182

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. Discuss the implications of this for the forward reaction. Examine the structure of aldolase from rabbit muscle (PDB code = 1ADO; this can also be downloaded from my website); it is bound to DHAP in this structure. Does the mechanism make sense in light of this structure? Explain your answer.

Determine the following parameters for aldolase:

K_m and/or K_d for DHAP

K_m and/or K_d for G3P

k_{cat} of the enzyme

4. 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:

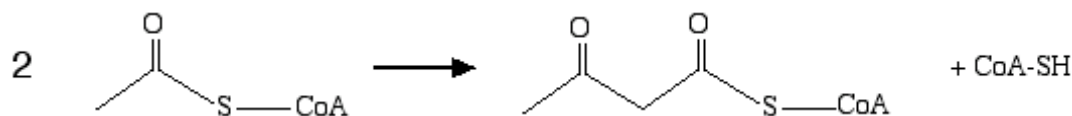
5. Examine the crystal structure of yeast enolase. These are in the PDB files 2ONE and 7ENL, which can be downloaded from the website.

a) Describe the overall tertiary and quaternary structure of enolase. What kind of domains does it have (*e.g.* what class)?

b) Briefly describe the dimerization interface.

c) Examine the active site. Which amino acids are part of the metal-binding site(s)? Which amino acids are important for catalysis? Write out a simplified model of the active site, based on the structure. Fill out the table on the next page for as many residues as you think are important for enolase's function (up to 12).

6. Your biodiversity team discovers a new bacterium, *Acidophilus carbonera*, that lives in very acidic conditions in a former coal mine. It has a very active respiratory chain, requiring it to produce large amounts of heme, quinones, etc. The precursor many of these molecules (and others) is acetoacetyl-CoA. It is formed by condensation of 2 molecules of acetyl-CoA:

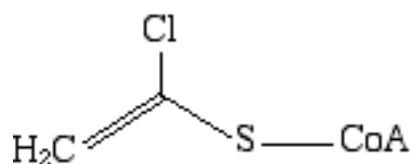


The enzyme thiolase catalyzes this reaction. There are two kinds of thiolase:

- a catabolic thiolase involved in α -oxidation of fatty acids that is used to drive the reverse of the above reaction using a variety of α -keto acyl-CoA substrates
- an anabolic thiolase that is committed to using only acetyl-CoA to make acetoacetyl-CoA

When grown under aerobic conditions with certain carbon sources, *A. carbonera* expresses an anabolic thiolase in relatively high amounts, allowing you to purify large amounts of it. Your team embarks upon an intensive characterization of the enzyme, using kinetic analysis, site-directed mutagenesis, and X-ray crystallography. After almost two years of work, here is what you know:

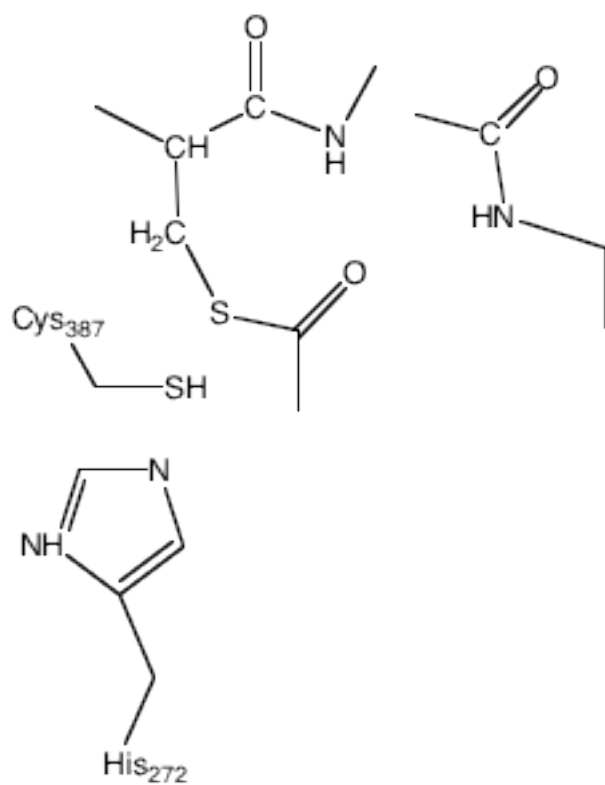
- If the enzyme is denatured by rapid treatment with urea during a reaction with acetyl-CoA, then it is found that Cys89 is acetylated (thioester linkage). Use of [^{14}C]-acetyl-CoA (with the isotope on the methyl group of the acetyl moiety) demonstrates that the acetyl group on Cys89 originates from acetyl-CoA. This serves as the starting point for examining the acetylation reaction: enzyme is incubated with various concentrations of acetyl-CoA, rapidly denatured, and the amount of acetylated enzyme can be quantified.
- The enzyme works well under basic conditions (unless the pH goes over 11-12, at which point it begins to denature), but it becomes inactive at lower pHs. Overall activity is lost with a pK_a near 8, but the formation of acetylated Cys89 requires even lower pH to inhibit (pK_a of inactivation is near 6).
- The enzyme is also inhibited by molecules that chelate divalent cations. It can be restored by addition of Zn^{2+} . (Addition of Co^{2+} can also restore some activity.)
- You have a chlorinated version of acetyl-CoA that inhibits the enzyme with a low K_i . Interestingly, it does not have such a large effect on the acetylation reaction. In fact, the inhibitor binds much more tightly to the acetylated enzyme than to non-acetylated thiolase.



- e) You crystallize the acetylated enzyme with and without bound inhibitor and determine the structures by X-ray diffraction. This helps you to identify the active site. (See last page). Panel A is without inhibitor, and panel B is with the inhibitor bound. The two sketches emphasize different aspects of the structure, (Since these are 2-dimensional sketches, it is hard to see how all the residues fit in 3-dimensional space, so I have shown different parts in the 2 sketches.) In panel A, it is obvious that the acetylated Cys89 is very close to Cys387, which in turn is close to His272. The carbonyl oxygen of the thioester is placed in a little pocket, in which the amide nitrogens of Gly90 and Gly92 seem to be pointing toward the oxygen. In panel B, you can see the bound Zn atom is coordinated by two His residues and a Glu residue and is near the inhibitor. Also nearby and on the other side is the Lys113 sidechain. The Coenzyme A (CoA) moiety makes extensive interactions with several residues lining a pocket (the “CoA pocket”). Note that there is only one such pocket. The active site is deep within the enzyme. Only one acetyl-CoA can fit at any one time. In fact, modeling studies indicate that, in order for the CoA pocket to interact maximally with the CoA portion, the acetyl group will be somewhat strained when sitting in the acetylated enzyme.
- f) You make several mutants to test the role of certain amino acid residues identified in the crystal structure:
- 1) **Cys89:** Mutation to Ser lowers activity 1000-fold. Mutation to Ala knocks out activity.
 - 2) **Gly90:** Mutation to Pro destabilizes the enzyme and causes it to be degraded, so you cannot analyze this mutant.
 - 3) **Gly92:** Mutation to Pro lowers activity ~600-fold. Acetylation is lowered ~50-fold.
 - 4) **Cys387:** Mutation to Ser lowers activity 200-fold. Mutation to Ala lowers activity 2,500-fold. Acetylation is similarly effected.
 - 5) **His272:** Mutation to Gln lowers activity 20-fold. Acetylation is similarly effected.
 - 6) **Lys113:** Mutation to Arg knocks out activity. Mutation to His lowers activity 10,000-fold. Neither of these mutations has much effect on the acetylation of Cys89.

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

