

Name _____

DUE: November 27, 2006

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 Monday, November 27.

There are **5** problems worth **24** points each.

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. The Gram-positive bacterium, *Clostridium botulinum*, is the causative agent of botulism, one of the most dangerous forms of food poisoning. The bacterium secretes Botulinum toxin (Botox), a neurotoxin that is the most acutely poisonous substance on earth. (The lethal dose is ~250 pg/kg, meaning that one hundred grams could kill every human on earth.) Although it has found some therapeutic use, Botox can still kill. It does so by getting into neurons and cleaving the SNAP-25 protein, which is required for the release of neurotransmitters from the axon endings. By inhibiting neurotransmitter release, the toxin interferes with nerve impulses and causes paralysis of muscles. You are working for a pharmaceutical company that is looking for a new drug that will inhibit Botox, to provide a cure in cases of acute botulism.

- a) You wish to start by understanding the properties of Botox type A, the most common form of the enzyme (Botox-A). The toxin has 2 subunits with a 100-kDa heavy chain joined by a disulfide bond to a 50-kDa light chain; the light chain has the protease active site. In order to make the assay faster and easier, you have made a peptide that corresponds to the part of SNAP-25 where Botox-A cleaves it. To each end of the peptide is attached a different fluorophore. The system is set up such that the C-terminal fluorophore quenches the fluorescence emitted by the N-terminal fluorophore due to its proximity. (This phenomenon is called FRET, for fluorescence resonance energy transfer.) After cleavage in the middle of the modified peptide (called SFP, for SNAP-25 FRET peptide), the fluorescence from the N-terminal fluorophore will increase. Use of fluorescence allows you to detect activity with small amounts of substrate. You initiate each reaction by adding 10 μL of a solution of 50 ng/mL enzyme to a cuvet containing buffer and peptide in a total volume of 1 mL, mix and put it in a fluorometer. You monitor the reaction over the course of 30-100 seconds and the computer calculates the initial velocity for you based on the increase in fluorescence.

[SFP] in tube before reaction starts (μM)	Initial velocity (pM/s)
1	180
3	483
10	1146
30	1906
100	2479

From these data, calculate k_{cat} and K_m of Botox-A for SFP.

What is the specificity constant?

Comment on the efficiency of the Botox-A – is it a “catalytically perfect” enzyme?

- b) Your team has done a high-throughput screen for molecules that seem to inhibit Botox-A. Preliminary assays indicated that one of them (code-named BTAI-27) is a promising candidate. You repeat the assays exactly as in part (a), except that the tubes also contain BTAI-27 in various amounts. Here is the relevant data:

[SFP] in tube before reaction starts (μM)	Initial velocity (pM/s) when [BTAI-27] = (nM)				
	1	4	16	64	256
1	180	179	169	160	139
3	465	475	445	393	265
10	1104	1124	993	773	383
30	1866	1841	1551	1026	453
100	2446	2281	1902	1227	471

What sort of inhibitor is BTAI-27: competitive, uncompetitive, or mixed?

What is the K_i and/or K_i' of BTAI-27?

Based on these, do you think BTAI-27 will be a good therapeutic agent?
(Briefly explain why or why not.)

2. Thioredoxin (Trx) is a protein with two reactive surface Cys residues that can reversibly form an intra-polypeptide disulfide bond. **Thioredoxin reductase** uses the reducing power of NADPH to convert oxidized thioredoxin (disulfide form) to the reduced form:



There are many different forms of this enzyme, and they vary in their polypeptide composition, substrate specificity, and perhaps even in their mechanism. You wish to characterize the thioredoxin reductase from a newly discovered multi-drug-resistant strain of enterobacterium that is causing problems in hospitals with post-surgery patients. In order to do this, you set up 25 tubes containing buffer (pH 7.0), ions (to mimic the intracellular environment), and various combinations of Trx(ox) and NADPH. You initiate each reaction by adding thioredoxin reductase (final concentration = 0.5 nM), and you monitor the absorbance of NADPH at 340 nm (NADP⁺ does not absorb at this wavelength) over time. From these traces, you estimate the initial rate of the reaction in each tube, which is tabulated below:

[NADPH] (mM)	initial velocity (nM/s) when [Trx(ox)] (μM) =				
	1	3	10	30	100
0.1	6.6	13.3	18.4	22.0	24.2
0.3	8.1	18.7	37.8	47.1	54.0
1	9.0	23.1	51.9	83.0	107
3	8.6	25.6	63.3	109	157
10	8.9	24.4	65.4	123	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.

Determine the following parameters for phosphorylase kinase:

K_m and/or K_d for Trx(ox)

K_m and/or K_d for NADPH

k_{cat} of the enzyme

It is known that this enzyme uses a covalently bound flavin to transfer electrons from NADPH to Trx, but has no other cofactors. Based on your analysis, answer the following questions:

- Do you think that the sites where NADPH and Trx bind are distinct or overlapping?
- If you added NADPH alone to thioredoxin reductase, what would happen to the flavin?

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/>. Hit the “Search” tab on the left. Take the “**Enzyme Classification**” link, which will take you to a new page.
2. There will be a list of the 6 enzyme types. You can use this to browse the structures of the various enzymes. Pick one and click the “caret” symbol to the left to open the folder. (Don’t click the blue link until you are down to the lowest level, or it will take you *all* the structures that fit that description.) Using this, you can browse through each class of enzyme; at each step, you are given new choices until you have a 4-digit EC number that defines a unique enzyme. If the link is blue, there is at least one representative in the PDB of that enzyme type. Clicking the link will take you to a page with all those structures.
3. 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. Once you are satisfied, download (at least 2 of the files).

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*.]

Enzyme name _____

E.C. number _____

What reaction does this enzyme catalyze?

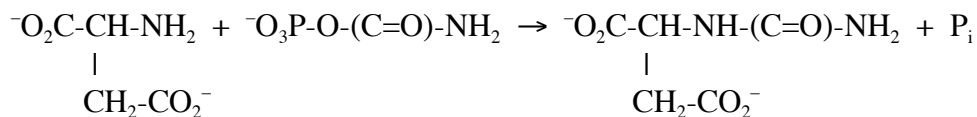
The two examples you chose to examine:

	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.)

4. The enzyme aspartate transcarbamylase (ATCase) has been used as a prime example of an allosteric enzyme. It catalyzes this reaction:



Its activity shows cooperative behavior with respect to either of the substrates (Asp or carbamoyl phosphate). In this problem you will explore the structural basis of this behavior. The enzyme is made of 6 catalytic (C) subunits and 6 regulatory (R) subunits. Two catalytic trimers (C_3) are held together by 3 regulatory dimers (R_2). You should use the following PDB files to answer the questions below (you can get them from the PDB or from my website):

PDB ID#	Description
1EKX	Catalytic trimer (C_3) in the absence of regulatory subunits, bound to PALA
1Q95	R-state bound to PALA, shows the entire $(C_3)_2(R_2)_3$ structure
5AT1	T-state bound to CTP
4AT1	R-state(?) bound to ATP

Note that the last 3 files only show one R_2 dimer, with each R subunit associated with one C subunit. However, the PDB has made a file in which it has created a model of the whole $(C_3)_2(R_2)_3$ structure. Download the “Biological Unit Coordinates” to see this. (I recommend that you use the “structure” command in RasMol for these; otherwise they will look strange.) PAM is short for phosphonoacetamide (${}^{-}\text{O}_3\text{P-CH}_2\text{-(C=O)-NH}_2$), which mimics carbamoyl phosphate, while PALA stands for *N*-(phosphonoacetyl)-L-aspartate, which is thought to mimic an intermediate state during catalysis.

- a) Briefly describe the symmetry relations in ATCase (C_2 , C_3 , D_2 , etc) – how would you classify each of these:
 - a. The C_3 catalytic trimer (e.g. 1EKX)
 - b. The R_2 regulatory dimers (e.g. 3AT1)
 - c. The entire $(C_3)_2(R_2)_3$ dodecamer (e.g. 1Q95)

- b) Briefly describe the tertiary structure of each subunit (R & C). What kind of domains does it have (e.g. what class)?

- e) Which amino acids are important for binding the effector (CTP)? Identify 5 amino acids involved in binding CTP. For each, indicate how it interacts with CTP. Then examine the same residue in the ATP-bound structure and indicate if it is playing the same role with ATP (if different, describe how it interacts with ATP, *if* it does).

Residue (amino acid & #)	Likely role in binding CTP	Similar or different role with ATP?

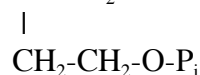
- f) Based on your analysis above, suggest a mutation that would make the enzyme more resistant to CTP (i.e. decrease affinity for CTP), but that would have less effect upon binding ATP. Just tell me the residue (amino acid name and number) and what you would change it to. Sketch 2 sets of curves below for how you would expect your mutant to behave compared to the wild-type version of ATCase. In each, plot activity vs. [Asp] with (1) no additions, (2) + 0.5 mM CTP, (3) + 0.5 mM CTP + 1 mM ATP.

5. You are part of a team working on a new class of bacteria found growing near a toxic waste dump that has high quantities of methanethiol ($\text{CH}_3\text{-SH}$). You discover that this organism has come up with an interesting way to use $\text{CH}_3\text{-SH}$ to make methionine. It possesses a novel enzyme, that you provisionally name Methionine Synthase II (MS2, to distinguish it from the more conventional Methionine Synthases). It catalyzes this reaction:



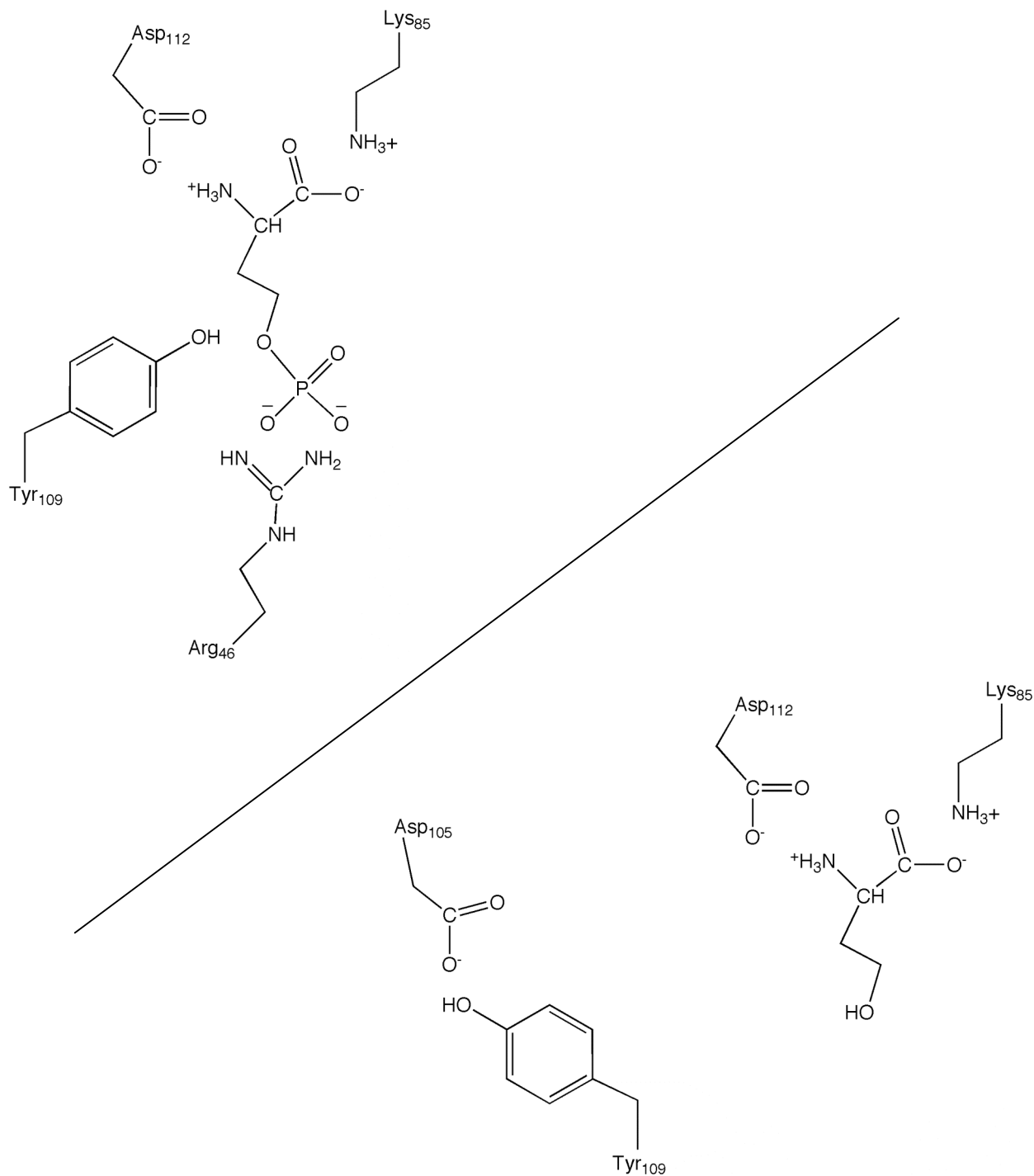
Your job is to figure out how MS2 carries out this reaction. After several years of work, you know the following:

- a) MS2 is a homodimer, with 2 copies of a 57-kDa subunit.
- b) If you add homoserine and ATP to MS2 in the absence of methanethiol, incubate for a few minutes, extract the enzyme with solvent and run the extract on an HPLC column, you get a new product, which you identify as *O*-phosphohomoserine: $\text{H}_2\text{N-CH-CO}_2\text{H}$

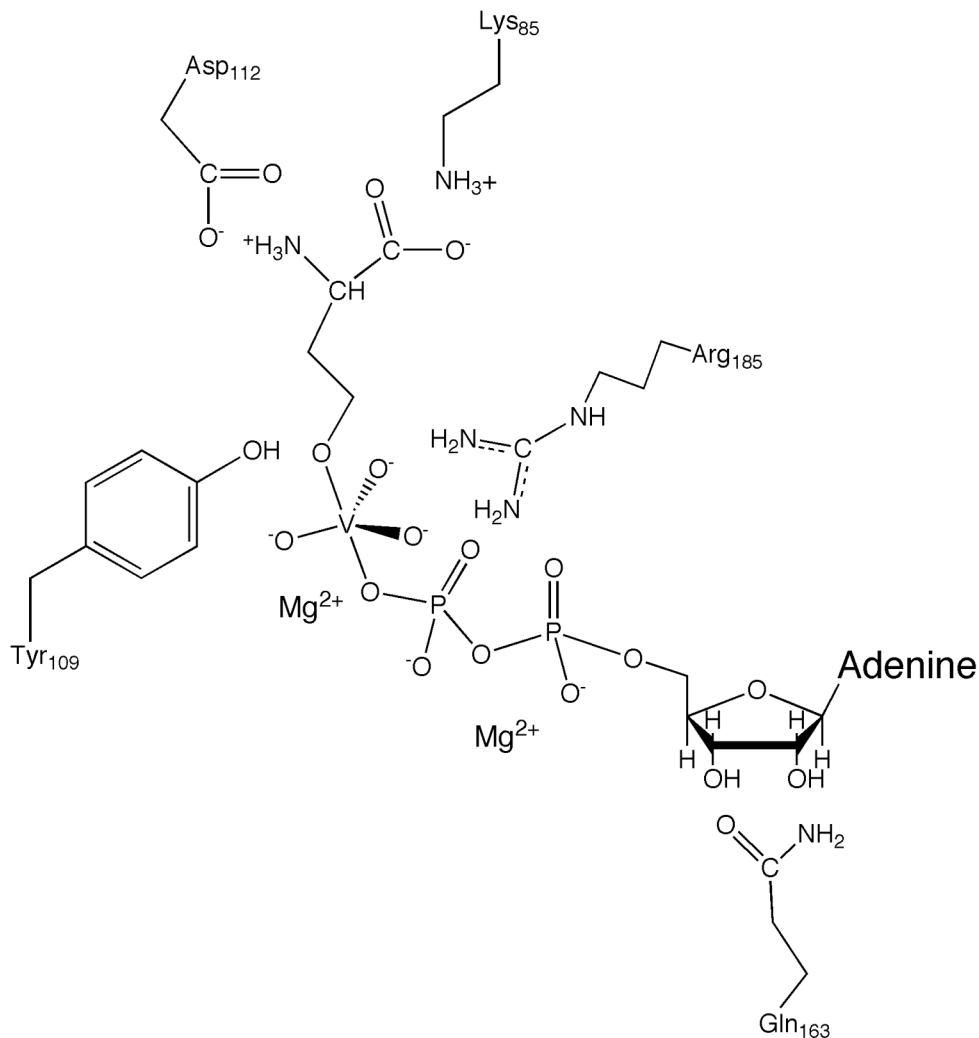


- c) Careful quantification of this reaction reveals that 1 mole of phosphohomoserine is formed per mole of enzyme. If you run the reaction with the γ -phosphate of ATP labeled with ^{32}P , and then run the reaction on a gel filtration column (with a separation range of 0.2 – 10 kDa), the ^{32}P -labeled homoserine comes off in the void with the enzyme, while the excess [^{32}P]-ATP and the ADP come off much later. You call this reaction *sub-reaction #1*:
 $\text{homoserine} + \text{ATP} \rightarrow \text{phosphohomoserine} + \text{ADP}$
- d) Subsequent work with a mutant (see below), in which the phosphohomoserine is released as a product, allows you to perform a kinetic analysis of sub-reaction #1:
- 1) The mutant enzyme behaves as if it forms a ternary complex with ATP and homoserine. Furthermore, the data is most compatible with an ordered pathway in which homoserine binds first.
 - 2) The mutant enzyme works well under neutral or somewhat basic conditions, but it becomes inactive at lower pHs. Activity starts to be lost with a pK_a near 6; this is mainly due to a (relatively weak) effect upon k_{cat} . At even lower pH, a further decrease in activity is seen, but this is due mainly to an increase in the K_M for homoserine. With increasing pH, activity is lost with a pK_a around 11; this is mainly an effect upon K_M for homoserine.
- 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 normal enzyme with phosphohomoserine and determine the structure by X-ray diffraction. This helps you to identify the active site. (See figure on next page.) The interaction between the phosphate and Arg46 seemed to be especially important, which is what inspired you to make the Arg46→Glu mutation. This mutant slightly impairs sub-reaction #1, but it also causes release of the phosphohomoserine product. (This is the mutant that you used in the kinetic analysis described above.)
- g) The Tyr109 residue also seems to be important. Based on this, you decide to do an NMR analysis, which requires assignment of couplings to every residue. The interesting result

from this is that the phenol group of Tyr109 is transiently deprotonated during the catalytic mechanism. When bound to homoserine alone, it is strongly hydrogen-bonded to Asp105, which is nowhere close to the Tyr109 sidechain in the structure. Intrigued by this, you obtain a crystal structure of the enzyme bound to homoserine (shown below), demonstrating that Tyr109 must undergo a conformational change upon binding to ATP.

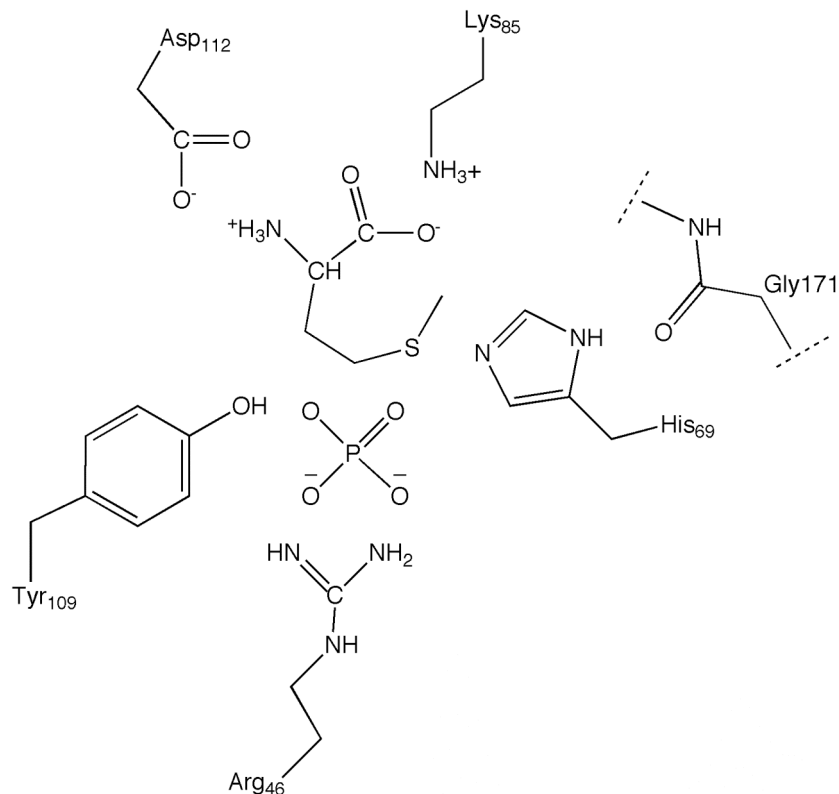


- h) Binding to ATP also triggers the transient deprotonation of Tyr109. Concomitantly, Asp105 becomes protonated.
- i) You use a trick to mimic a pentavalent phosphorous, which is a common intermediate in phosphotransfer and phosphoester hydrolysis reactions, and substitute a vanadate moiety for the γ -P_i of ATP. You reason that this molecule, called VIMI (“vanadyl intermediate mimic”), should resemble an intermediate in the reaction. As it turns out, this molecule binds very tightly to the enzyme, and is a strong competitive inhibitor (*i.e.* it has a very low K_i) for both sub-reaction #1 (in the R46E mutant) and for the full reaction in the normal enzyme. You obtain a structure of the enzyme bound to this inhibitor (see below).



- j) If you add phosphohomoserine and methanethiol to MS2, it readily produces methionine and phosphate. This is *sub-reaction #2*: phosphohomoserine + CH₃-SH → Met + P_i. This reaction is also inhibited by chelators, but not as much as sub-reaction #1. By NMR analysis, you find that Tyr109 is deprotonated again during the reaction. A pH analysis of sub-reaction #2 reveals that the k_{cat} decreases as pH lowers, with a pK_a ≈ 6.5.

- k) If you soak crystals made from empty enzyme with a solution containing high amounts of phosphate and methionine, you can force the two products into the active site. The resulting structure is shown below. (If you bind methionine to MS2 in the absence of phosphate, the structure looks similar, except that the overall conformation resembles the structure with homoserine bound, and Tyr109 is next to Asp105.)



- l) You make several mutants to test the role of certain amino acid residues identified in the crystal structure:
- 1) **Asp112:** Mutation to Asn increases K_M for homoserine ~ 50 -fold, but has little effect on k_{cat} .
 - 2) **Lys85:** Mutation to Glu increases K_M for homoserine ~ 100 -fold, but has little effect on k_{cat} .
 - 3) **Arg46:** Mutation to Glu increases K_M for ATP ~ 10 -fold, decreases the k_{cat} of sub-reaction #1 by ~ 5 -fold, and allows release of phosphohomoserine; it also increases the K_M for phosphohomoserine in sub-reaction #2 by ~ 100 -fold.
 - 4) **Asp105:** Mutation to Asn has no effect upon K_M of any of the substrates, but reduces k_{cat} of sub-reaction #1 by about 80-fold. Mutation to Leu reduces k_{cat} ~ 200 -fold. These changes to k_{cat} are the same whether it is the whole reaction or sub-reaction #1 that is assayed. (In order to check sub-reaction #1, you have to make the double mutant – R46E/D105N.)

- 5) **Tyr109:** Mutation to Phe has little effect upon K_M of any of the substrates, but reduces k_{cat} of sub-reaction #1 by ~ 900 -fold. It has a weaker effect upon sub-reaction #2: k_{ca} decreases ~ 75 -fold.
- 6) **Arg185:** Mutation to Gln increases K_M for ATP by ~ 20 -fold; k_{cat} of sub-reaction #1 decreases almost 200-fold, but no effect upon sub-reaction #2.
- 7) **His69:** Mutation to Phe has no effect on sub-reaction #1, but reduces k_{cat} of sub-reaction #2 by ~ 200 -fold. However, if you do a pH study with the H69F mutant, you find that carrying out the reaction out at higher pHs reduces the effect upon k_{cat} .
- 8) **Gln163:** Mutation to Leu increases K_M for ATP by ~ 50 -fold.

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!)