

BIOC 463A

2011-2012

Alkaline Phosphatase Manuscript Preparation

Over-all Manuscript Preparation

The general format of the manuscript is as done previously: Introduction, Materials and Methods, Results, Discussion, Tables, Figure Legends, Figures, References.

The information/topics that need to be covered in the final manuscript are:

1. Purification procedures: what fundamental principle was each procedure based upon, what was each step's intended purpose (purification vs. concentration). This will be related to considerations addressed in (2).

2. Characterization: appropriate data from your purification table (especially Yield and % Purification). Did you lose enzyme at different steps and why? What do the SDS-PAGE results tell you about purity and molecular weight of the purified protein? How does the mass determined from SDS-PAGE compare with those obtained from ESI mass spectrometry (discussed below), and compared to the theoretical mass determined from ExPASy? Why do you think you see two peaks in the ESI mass spectrum? How is this related to protein translocation across the cytoplasmic membrane?

3. Steady-state kinetics: K_m and k_{cat} as well as the value for k_{cat}/K_m .

4. Competitive inhibition by inorganic phosphate: determination of K_i along with a discussion of the method by which you determined K_i .

5. Inclusion of your Special Research Project data, including references obtained through your literature search. This information will be tied into a consideration of how the three dimensional structure of AP is related to the data presented above (this is called the Structure-Function relationship). This will be based on the knowledge you gained from the Stec et. al. (2000) paper.

6. Information from the Molecular Graphics Assignment (see below). Knowledge gained from this exercise should be demonstrated in primarily the Introduction and Discussion of your final report.

7. Information from the Mass Spec Assignment (see below). Again, this should be included in the Introduction and Discussion.

Unlike your previous write-ups, there are several literature references that you either have been given as hand outs or determined from your PubMed searches during the Special Research Project. Specific information (i.e., topics we discuss in class) from these references should be included in the Introduction, Results, and Discussion sections of your manuscript and cited accordingly.

KEEP READING!

Below are "helpful" hints when writing up your manuscript on the specific sections.

Initial Purification

See 1 and 2 above.

Steady State Kinetic Data

We have determined four important enzymatic properties of alkaline phosphatase: K_m , k_{cat} , and k_{cat}/K_m for PNPP. We have also determined K_i for inorganic phosphate (see below). In composing your final AP manuscript, you will want to add all of the steady-state material to the manuscript you already have written covering purification and characterization of the enzyme. This information will be included primarily in the form of figures and explanation of how you determined the appropriate parameters from these figures in the Results section. You should demonstrate some understanding of what K_m and k_{cat} actually mean with respect to the two things and enzyme must do in order to work in a biochemical reaction. You should also be able to demonstrate how these parameters are related to [Substrate]. We then want you to go to the literature (i.e. the three papers handed out in class) and compare your results with those given in the papers. This comparison should be done in the Discussion section of the report, making the appropriate literature citation. We would also like you to compare the value for K_i/K_m , and how does that compare to the literature value. What do you think the physical interpretation of this ratio means with reference to substrate or inhibitor binding to the active site of the enzyme, based on the high resolution crystal structure?

Competitive Inhibition of AP by Inorganic Phosphate

In class, we looked at the Lineweaver-Burke plot for determining the inhibition constant, K_i , for in competitive inhibition of AP by phosphate. This graphical (or the mathematical equation used to generate the plot) method determines how the experiment is performed. Therefore, in addition to discussing the competing equilibria involved in the experiment, you should also show some knowledge of how the mathematics of the Lineweaver-Burke plot dictates how the experiment is actually done. Finally, you should also discuss why only K_m is apparently altered in the presence of a competitive inhibitor.

Molecular Graphics Assignment

In this exercise you will be required to use ExPASy and download two structures from the Protein Data Bank for alkaline phosphatase (AP). Hopefully, at the end of this exercise you will have a much more intimate understanding of the structure AP which is directly related to its function. *The results will be included in an overall description of the enzyme, in the Introduction as well as the Discussion portion of your final manuscript. Do not address them as questions per se in your final lab report; rather use them as a guide to your thinking as you compose your manuscript.*

1. If you have somehow forgotten to do so, determine from ExPASy the calculated molecular weight for the mature (i.e. after the signal peptide has been cleaved) protein and its pI. Again, make note of how the calculated mass compares with that you determined by SDS-PAGE electrophoresis as well as the mass of the protein determined by ESI mass spectrometry. Remember, the masses determined by these experimental techniques are all for the monomeric form, but remember in solution AP exists as a dimer. Garen and Levinthal determined the molecular weight for the dimer by what technique? How well does the calculated pI (from ExPASy) for the mature protein compare with that determined by Garen and Levinthal? How is the pI related to the type of ion exchange chromatography used to purify the protein?

2. While still in ExPASy, you might want to make note of the amino acid sequence of mature AP, specifically what is the first amino acid at the N-terminus. This might prove useful for the Mass Spec Assignment given below.

3. Now, go to the Protein Data Base where you will find the two 1.75 Å structures (+ and – inorganic phosphate) of alkaline phosphatase published by Stec et. al. in 2000, that will be examined in the remainder of this exercise. For most, using Jmol will suffice to answer the following questions, if you wish to use PyMOL, go for it! To answer the following question, it is best to switch to a cartoon representation of the enzyme with the active site metals displayed as CPK structures. Describe the overall shape of the protein in the homo-dimer (sphere, oblate spheroid, square bi-pyramidal, etc.). The majority of the enzyme consists of alpha helices and beta strands arranged in a very specific folding motif. Describe the motif. The result of this folding motif is the formation of a beta-sheet that runs through the interior of the protein. How many strands are in the beta sheet? Are the beta strands parallel or anti-parallel? How are these orientations of the strands related to the overall folding motif? Based on the location of the large beta sheet, what would be the chemical nature of most of the amino acid side chains comprising this secondary structure?

4. Before beginning this problem, remember the conclusions that we came to regarding Table 1 in Garen and Levinthal with respect to the dependence (or lack thereof) of activity on the R-group of phosphorylated substrates for AP. Now, rotate the molecule so you can locate both active sites, and describe the geometry of the surface(s) of the molecule in the region surrounding the active sites. By far and away, the best way to see surface topology is to display a molecular surface (Surface under Jmol; there are several different types of surfaces that you can explore to see which one best answers displays surface topology). A molecular surface is built on top of the displayed molecule (no matter what the representation, however it is calculated using the van der Waals radii of each atom and for some surfaces the radii of a water molecule rolling on the surface of the CPK structure giving the surface a smooth contour). The color of the surface can be varied (Color>Surface) and it can be displayed either opaque or translucent (which is cute but often confusing). Before generating the surface for the –Pi structure, display the metal atoms at the active site as CPK structures and color code them. It will be easier to find the active site once the molecular surface has been generated (i.e., one or two metals might be peeking through the surface). Another “trick” you can try with the +Pi structure is to display the active site metals and Pi as CPK structures, create your molecular surface, then go back and delete the Pi atoms. This might “open up” the active site. The primary questions you are trying to answer are:

- relative to the entire structure of the enzyme, how big is the active site? (An interesting corollary to this point is why does a protein need so much mass to create a relatively small portion of the protein actually involved with its function?).
- describe the general structure of the protein around the active site and how is that structure related to substrate binding (i.e., does it act as a channel for the substrate to the active site)?
- looking at the +Pi structure, where would you expect the R-group of the substrate to be located once the substrate has bound to the active site? How is this related to the relative promiscuity of this enzyme towards different R-groups (Garen and Levinthal and Coleman papers)? In other words, are there any steric constraints for the R-group of the substrate when it binds to the enzyme?

- switching back to a CPK or stick diagram and taking into consideration the chemical nature of the substrate, are there any charged residues (i.e. the electrostatic component to S binding) that might facilitate its being attracted to the active site? List their residue numbers and what their identity.

5. Display the side chain of Ser102 in both the $-P_i$ and $+P_i$ structures. In the $-P_i$ structure, the side chain is shown in a single conformation, whereas in the $+P_i$ structure, the side chain is shown in two orientations. Postulate an explanation for the differences in the orientation of the Ser102 side chain in these two structures. Locate the Ser102 side chain O atom (listed as the gamma O or OG in PDB nomenclature) in the $-P_i$ structure. How is it oriented with respect to the two Zn and the Mg atoms? Determine the distance from the Ser102 OG and the Mg. How is the relatively large distance between the Ser102 OG and the Mg accounted for in the postulated involvement of the Mg in the catalytic mechanism suggested by Stec. et. al.?

6. The reason why enzymes catalyze reactions faster than the non-catalyzed reaction is because the enzyme can stabilize the transition state as the substrate is converted to product. As Ser 102 attacks the P atom, an important amino acid is critically located to assist in transition state stabilization. What is that amino acid and how does it stabilize the transition state?

7. We have discussed several times, and some have actually examined, the roles played by the two disulfide bonds (i.e., Cys-### - Cys ###) within the enzyme. First display the molecule as a cartoon diagram with the disulfide bonds connecting the two Cys residues in order to get your orientation. Then switch to a CPK structure, making sure the SG atoms are displayed in yellow (the CPK default color for sulfur). How accessible are these atoms? This will determine their susceptibility to attack by reducing agents. Based on its proximity to the active site, which disulfide bond, if reduced, would you speculate to have a greater impact on enzyme activity?

AP Mass Spec Assignment

The following is a description of the analysis of the mass spectrometry data and questions that you should consider in discussing this data in your lab report, especially when comparing with the mass you determined for AP from the SDS-PAGE gel. This will take some time, but hopefully it will be an enriching intellectual process that I know you will all enjoy.

The data obtained from the Mass Spectrometry Facility for analysis of the Stage 4 enzyme can be downloaded as **ap_massspec_data.pdf** from the website. The spectra, as well as a discussion of its significance are to be included in your write-up as part of the biophysical characterization of the enzyme that you have purified.

The technique that was used to obtain these spectra was HPLC-ESI mass spectrometry. In other words, the sample was first run through an HPLC column that separates the proteins according to size (this corresponds to the top left hand box in the figure). The numbers in this chromatograph correspond to the time required for the fraction to come off the column. Each fraction is directly fed into the ESI (electro-spray ionization) mass spectrometer, where the mass of the protein is determined. The figure in the bottom right corresponds to the average mass spectrum of the sample emitting from the HPLC column at 18.17 minutes.

There are two obvious peaks in the average mass spectrum that comes from the individual spectra shown in the bottom left and top right boxes. First, calculate the mass

difference between the two peaks. This mass difference may correspond to two forms of the enzyme, in which there has either been an amino acid substitution, a deletion, or an insertion in one of the proteins, relative to the other. A table of Mutation Mass Shifts has also been included that should be helpful in determining the cause of the difference in masses.

Once you have determined the most likely cause of the mass difference, we want to examine if there is some literature verification for the two forms of protein being present in the Stage 4 enzyme solution. In order to determine this, go to the ExPASy website searching for alkaline phosphatase from *E. coli*. As you have done in the past, recalculate the mass of the mature protein (minus the signal peptide). How well does the calculated mass agree with the actual mass spec results? How well does the ExPASy-calculated mass and the observed mass from the spectrum agree with the mass you determined from the SDS-PAGE gel? How well do each of these data agree with the mass given in Garen and Levinthal?

Now, using ExPASy, attempt to determine if different isomers of alkaline phosphatase are commonly observed that might account for this mass difference seen in the mass spectrum. If you find an isomeric form of the enzyme, determine which amino acid has been mutated, deleted, or inserted. Based on the physical location of this amino acid in the two forms of the protein, would you expect to see a difference in the enzyme activity of the two forms of AP? In other words, would this mutation be expected to have any effect on enzyme activity (i.e., is this residue involved in either coordination of the metals in AP or is involved in the catalytic mechanism)? (This question might be easier to answer once you have read the Stec et al. (2000) manuscript). A recent paper by Kajava et al. (2002, *J. Biological Chemistry*, **277**, 50396-50402) discusses the amino acid sequence specificity for the enzyme that AP binds to in the cell membrane, signal peptidase (SPase), and subsequently carries out two function. This enzyme binds the precursor protein at the cytoplasmic membrane and hydrolyzes the signal peptide, yielding the mature protein that can then be transported across the membrane. Based on the mass spectrometry data, briefly discuss the “fidelity” of cleavage of the signal peptide by SPase (i.e. why do you see two forms of AP in the periplasmic space).

Remember, as this material is included in your write-up, the appropriate literature citations need to be made!!!

Special Research Project Data

Finally, each individual's final AP report will contain the data and discussion of those results from the Special Research Project. Each group will compose this section of the lab report **AS A GROUP**, which will be included in each individual lab report. And, in case you were wondering if anyone will actually look at it, the answer is YES! Dr. H will go through the lab report for each type of experiment and pull out the useful information that can be used for the next semester's class. Please remember to include all the appropriate information (i.e. concentrations, incubation times and temperatures, extinction coefficients, etc.) for this material, it will make it much easier to decipher and work with you results. In some cases, Dr. H. might contact you after the semester is over to get more information, so if you do not want to bothered, include ALL the details.