Reading Guide to "Two Site-Directed Mutations . . ."[1]

Do not worry about the technical details of how the x-ray crystal structure was solved (ignore Table1, and related material in the *Materials and Methods* section).

The last pages of this packet contain diagrams about bacterial cell walls that may be helpful.

Study figures and tables carefully, they are information-rich. Be sure to look up all terms you don't know.

Come to class ready to discuss the paper and answer the questions herein. What questions do you have that are not covered here? Write them down and bring them to class.

Bring your copy of the paper with your notes to class!

Monday Questions

1. Why is this study important or worthwhile? Think big picture.

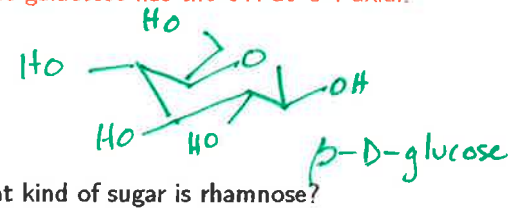
   This study is mainly about how enzymes evolve, how one enzyme becomes a different enzyme via small changes at the active site.

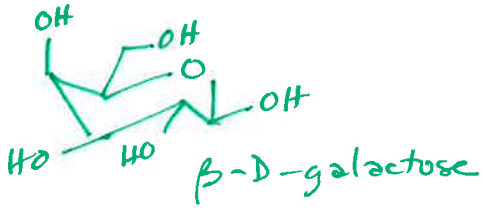
2. Why is the *context* of this study important?

   The context is the biosynthesis of components of LPS. If one can understand how LPS is put together, one can potentially design inhibitors that stop the biosynthesis and presumably, infection by the particular bacterium.

3. What term describes the relationship between galactose and glucose? Draw both in a chair conformation.

   Galactose is the C-4 epimer of glucose. They are also diastereomers. β-D-glucose has all attachments equatorial; hence galactose has the OH at C-4 axial.

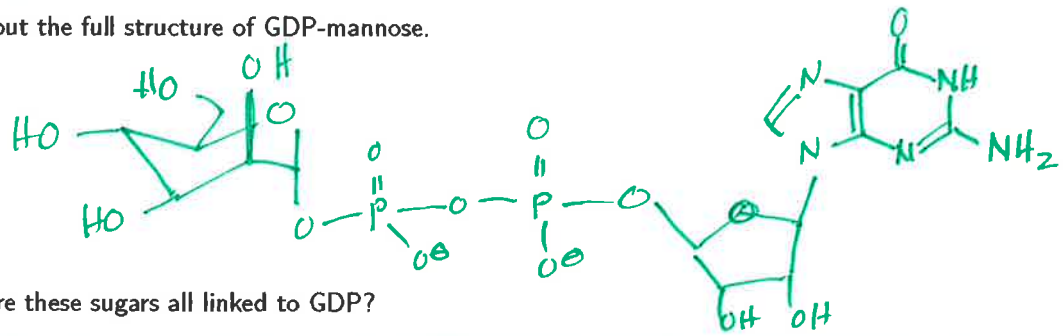




4. What kind of sugar is rhamnose?

   Rhamnose is a 6-deoxysugar and also in the L-series (this is very rare, most naturally occuring sugars in the D-series).

5. Draw out the full structure of GDP-mannose.



6. Why are these sugars all linked to GDP?

   When these sugars are put into a growing LPS structure, it is the glycosidic bond that is made. GDP, as a diphosphate, is a good leaving group.

7. What is an aldimine? An internal aldimine? An external aldimine? A ketimine?

   An aldimine is an imine made from an aldehyde, and a ketimine is an imine made from a ketone. Internal means the imine is made from a the amine group of a lysine side chain from the enzyme; external means the imine is made from an amine bearing molecule from the outside, meaning not from the enzyme.

[1] Cook *et. al. Biochemistry* vol. 48, pgs 5246-5253 (2009)

8. What is meant by a "ping-pong" mechanism generally? In this paper, what is the ping? The pong?

   Ping-pong means there are two completely separate steps in which the first must complete before the second can begin. In this paper the ping is the formation of PMP from glutamine (product is α-ketoglutarate). The pong is the second set of reactions shown in Scheme 2.

9. These are PLP-dependent enzymes. Why then is PMP the reactant in Scheme 2? Write a balanced reaction showing what happened prior to the start of Scheme 2. What kind of transformation is this? Optional: Draw the mechanism of this transformation.

   The answer is above: there first must be a transamination between glutamate and PLP prior to the reactions given in Scheme 2.

10. What is site-directed mutagenesis in general? Why is it a useful tool when studying enzyme mechanisms?

    It is a collection of techniques that allows one to change individual amino acids in a protein. By changing side chains in the active site, one can deduce the importance and role of each side chain methodically.

11. Regarding the reactions that were run (described under *Functional Assays*):

    (a) When the reaction was over, the crude reaction mixtures were "...filtered through an Amicon membrane to remove the enzymes." What's going on here? What is another related technique?

    This is essentially the same process as dialysis: proteins cannot pass a semi-permiable membrane with small pores and thus the protein can be removed from the low MW reactants and products. Using a filter keeps the solution relatively concentrated and one doesn't have to concentrate as much as when one conducts dialysis.

    (b) The crude reaction mixtures were purified using anion-exchange. Why did the authors choose this method/

    All the substrates and products have the GDP group at the anomeric carbon and the phosphates make them negative. Anion exchange would therefore allow one to quickly remove any neutral or positive side products/impurities.

    (c) What is the purpose of the NH_4HCO_3 gradient?

    The anions stick to the column via electrostatic attractions. To get them to move, you have to give the column an excess of ions (in this case HCO_3^- so that the anions will move off the column.

    (d) In Figures 3a and 4a the results of HPLC of the reaction mixtures are shown. What is HPLC? How are the peaks detected? Why do they use 253 nm?

    HPLC = high performance (also sometimes pressure) liquid chromatography. Peaks are detected via UV-Vis spectroscopy. The choice of 253 nm is because this is at or near λ_{max} for the guanine base (remember 260 nm is a compromise wavelength for quantifying DNA generally, since all bases absorb near this value).

    (e) What is ESI? Why does GDP-4-keto-3,6-dideoxymannose give two peaks in Figure 3b?

    ESI is electrospray ionization, a soft ionization technique used for proteins and any molecule when you don't want the parent ion to decompose and give fragments. ESI gives peaks according to $\frac{m}{z}$ where z is 1 to dozens.

12. Figure 3 gives data about S187N. From this data, what do the authors conclude?

    They conclude there is change in reaction mode, i.e. the mutant is still a dehydratase.

13. What is K_m? k_{cat}? $\frac{k_{cat}}{K_m}$? Besides a simple definition, how does one interpret these quantities?

    You can look up the definitions. K_m for our purposes is the binding or affinity of the substrate to the enzyme; smaller numbers mean stronger binding. k_{cat} is the turnover number, how fast the enzyme can go when saturated with substrate. $\frac{k_{cat}}{K_m}$ is catalytic perfection or efficiency

14. Regarding Table 2:

    (a) Why are there two values for K_m for each enzyme?

    There are two substrates for the enzyme

    (b) Which substrate binds most tightly to WT enzyme?

    GDP-4-keto-6-deoxymannose

(c) Both WT enzyme and the double-mutant catalyze the same reaction. Which one is more efficient?

WT, by about 1000x

Wednesday Questions

1. The authors state that "ColD is an unusual PLP-dependent enzyme in that it does not utilize an active site lysine to covalently attach the PLP cofactor...".

   (a) Give an example from the book of a "usual" (typical) PLP-dependent enzyme that does have an active site lysine.

   Any of the enzymes conducting an aldol reaction, technically Class I aldolases, e.g. Fig. 4.5 or Fig. 4.20

   (b) Give an example of an enzyme from one of the metabolic pathways that uses an internal aldimine not involving PLP.

   Skip this question is it not worded well.

2. This paper is about a double mutant, but the authors discuss two single mutations they prepared in earlier work.

   (a) What were these two mutations, and how did they perform as catalysts?

   They are ColD H188K and GDP-PS K___H. Both were completely inactive.

   (b) Regarding your prior answer, the authors prepared those mutants with a reason (theory) in mind. What was it? Explain the theory with reference to Scheme 2.

   Their initial theory was that the H vs K issue determined the reaction mode of the enzyme, so it was either the pK_a of the side chain or the size of the side chain or both that made the difference.

3. Figure 2 compares the active sites of GDP-PS and ColD in the "...external aldimine forms with their respective sugar products." In this figure:

   (a) Which portions of the figure are part of the protein?

   See the caption. Also, the AA side chains are numbered.

   (b) Which portions are part of the product?

   See above

   (c) Draw the full chemical structure (you may ignore stereochemistry) of this product, or alternatively, find the structure elsewhere in the paper.

   See the last full structure, right hand branch, in Scheme 2.

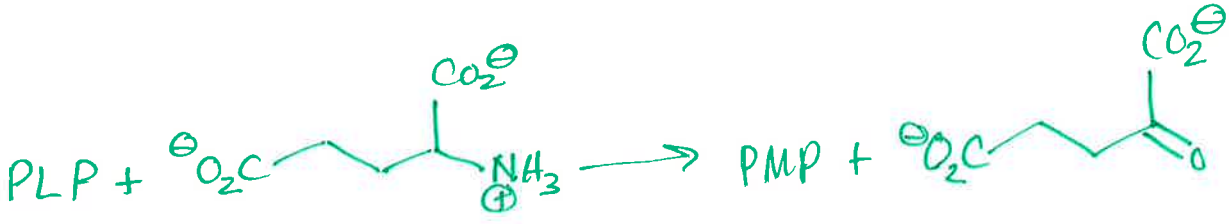
   (d) How did the authors know the product was in the aldimine form, as opposed to the ketimine form? Be as specific as possible.

   By looking at the lengths of the C to N bonds. A single bond will be longer than a double bond.

4. Figure 5b shows the active site in the double mutant, the subject of this paper.

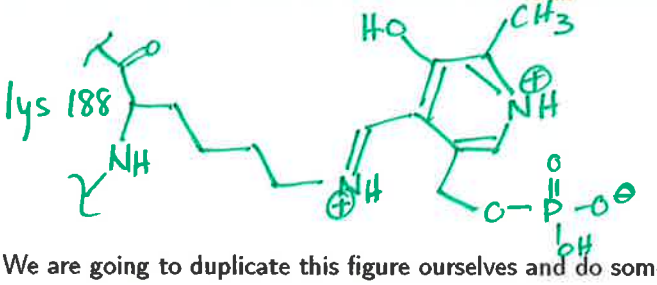
   (a) Why is α-ketoglutarate in this structure? Account for its origin chemically.

   It is the product of the ping. See the discussion above.



(b) In Figure 2 we were looking at the *internal* aldimine. In this figure, we are looking at the *external* aldimine. Draw the full structure for this external aldimine (you may ignore stereochemistry).

This question is worded wrong. Fig. 2 is the external aldimine, not the internal. Fig. 5 is the internal, since the K188 is an imine with the substrate. Here is the structure from 5b:



(c) We are going to duplicate this figure ourselves and do some additional investigations.

i. Open Chimera and fetch PDB entry 3GR9. This is the double mutant reported in the paper.

ii. There are several copies of the structure in the unit cell. Delete all chains except chains A and B.

iii. Select all atoms of the aldimine.

iv. Go to Select → Zone... and set the zone to 5.0 angstroms and check the "Select all atoms/bonds of any residue...". Then click OK. This creates a larger selection centered on the active site.

v. Go to Action → atoms/bonds → side chain/base → show. This ensures that all side chains are visible.

vi. Invert the selection.

vii. Go to Action → atoms/bonds → delete. Now you are looking at just the active site.

viii. Do File → Save session as... and give a file name.

(d) Using this view of the active site, prove that we are looking at the external aldimine.

Look at the C to N bond lengths.

(e) More questions coming later!