Chem 460 Laboratory – Fall 2008
Experiment 3: Investigating Fumarase: pH Profile, Stereospecificity and Thermodynamics of Reaction

Before Lab

Week 1 -- pH Profile for Fumarase

• Read Box 11-1 (page 323) and Section 16-3G (page 531) and look at Table 16-2 of VV&P
• Review hydration and dehydration reactions of alkenes in your organic chemistry text.
• In your notebook, write the reaction you will be studying.

Week 2 -- Stereospecificity of Fumarase & Thermodynamics of Fumarate to Malate

• Review stereochemistry and proton NMR spectroscopy in your organic chemistry text
• Review the following web documents from Chem 250/251:
  o Overview of Chemical Shifts in H-NMR
  o Introduction to Spin-Spin Coupling in H-NMR
• In your notebook:
  o Draw a Newman projection (down the C2-C3) of malate in its most stable conformation
  o Determine if C2 of L-malate has R or S stereochemistry
  o Describe how you will make the pH 7.00 phosphate buffer specifically for the NMR experiments in this lab (Note: you can’t use NaOH and HCl…why?)

Week 3 -- Report Workshop

• Bring 2 copies of a draft of your Fumarase report. The report should have a cover sheet with your name on it, but your name should not appear in the rest of the report. The instructor will remove the cover sheet before distributing the report for peer review.

Introduction

In this two week lab you will be exploring the reaction catalyzed by the enzyme fumarase, which converts fumarate to L-malate (Figure 1) as part of the citric acid cycle. From your organic chemistry course you should recognize this as the hydration of an alkene. Hydration (or the reverse, dehydration) reactions in the organic lab often involve strong acid and heating, but the enzyme fumarase allows this reaction to take place rapidly at room temperature and neutral pH.
You will investigate three different aspects of this reaction over the next two weeks:

1. Most enzymes show optimal activity at a particular pH. You will determine the pH dependence of the rate of hydration of fumarate by fumarase.
2. Most enzymes catalyze reactions in a stereospecific manner. For example, fumarase converts fumarate only to L-Malate, not its enantiomer D-Malate. You will use isotopically labelled water (D₂O) to determine whether the hydrogen that is added to fumarate is attached in a stereospecific manner.
3. Enzymes, like all catalysts, lower the activation barrier for a reaction, permitting both the forward and reverse reactions to occur at faster rates, thus permitting reactions to come to equilibrium fairly quickly. By measuring the amounts of fumarate and malate present at equilibrium you will be able to determine the equilibrium constant and ΔG° for this reaction.

**Week 1: Fumarase Activity as a Function of pH**

You and your partner will create an ‘activity vs. pH profile’ for fumarase. This requires that you determine the rate at which fumarase converts fumarate to malate as a function of pH. How will you measure fumarase’s activity (i.e. rate of reaction)? Since fumarate contains a conjugated double bond, it absorbs in the UV at a higher wavelength than malate, and can thus be distinguished from malate. By placing fumarate into a cuvet (what type?) and adding fumarase, you can observe spectrophotometrically the disappearance of fumarate as a function of time—and thus get fumarase’s rate of reaction. To generate your ‘activity vs. pH profile’ you will carry out a series of such reaction assays at various pHs. Each assay will consist of the substrate fumarate, buffer at a particular pH and the enzyme fumarase at a concentration that causes 10% of the fumarate to be consumed over at least 1 minute (why?). There are four tasks you need to accomplish prior to acquiring the data:

1) Make 0.5 M buffer solutions at pHs 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9—you need 5 mL of each solution (we will carry out this task as a group). In this case, we want the components in each assay to be the same, so we need to use just a single buffering compound. Because the pKₐ of KH₂PO₄ is close to the middle of this series, it is the preferred buffer for this experiment. Keep the solutions in the 25° water bath (why?).
2) Make ~1 mL of a 100 mM stock solution of fumarate (available as the disodium salt, FW=160.0 g/mol).

3) Acquire a UV-vis spectrum of 10 mM fumarate to determine the wavelength to be monitored. Note that we will not be using the $\lambda_{\text{max}}$. Instead we will use the wavelength at which 10 mM fumarate absorbs 1 AU. Note that you want the spectrophotometer in “Standard” mode for this step. Rinse your cuvet with dH$_2$O followed by EtOH.

4) Test 20 $\mu$L of a 1:20 dilution of the enzyme (in phosphate buffer pH 7) and see if this amount causes ~10% of the fumarate to be consumed over a 1 min period. Note that you want the spectrophotometer in “Kinetics” mode for this step.

Monitor the rate of the reaction at each pH. To do this, add enzyme (the amount determined via step 4 above) to a cuvet containing 10 mM fumarate and 0.1 M buffer, such that the final volume is 1 mL. Using parafilm, invert the cuvet three times, look carefully to see that there are no bubbles in the cuvet, and measure the rate of the reaction over a 1 minute period. Maintain consistency by doing each reaction one at a time—i.e. don’t set up all ten cuvets at once.

Similar to what you did for 4-nitrophenol last week, fit the data you obtained today to a theoretical curve that is created by assuming that the pH dependence of the enzyme is due to the presence of two acidic groups, from amino acid residues, in the active site of the enzyme—one that needs to be protonated and one that needs to be deprotonated. Note how the activity vs. pH curve is like two Henderson-Hasselbalch curves: the left side showing the more acidic residue being deprotonated and the right side showing the more basic residue being deprotonated. Again, use least squares to precisely determine the $pK_a$s of these two groups. Also, calculate $R^2$ (using the equation from last week’s lab) to determine how well your theoretical curve fits your data.

Based on the two $pK_a$s you obtained, can you speculate as to which amino acids might be the catalytic acid and base residues in fumarase’s active site?

**Week 2: Stereospecificity and Thermodynamics of the Fumarase Reaction**

Biochemists often employ isotopes in experiments because isotopes allow us to distinguish between two atoms that otherwise appear identical. In terms of the fumarase reaction, to investigate whether the proton from the water goes to the pro$R$ or pro$S$ position of malate, we will be using the isotope deuterium (D)—which is just a proton ($^1$H) with a neutron ($^2$H). The extra neutron in deuterium causes this atom to behave differently, compared to a proton, in a nuclear magnetic resonance (NMR) spectrometer. The deuterium will be incorporated into malate by carrying out the fumarase reaction in the presence of D$_2$O—which will cause a deuterium, instead of a proton, to be added to the 3-carbon of fumarate (see Figure 1)—and you will use NMR chemical shifts and coupling constants to deduce which position the deuterium occupies in malate.

1) Based on your pre-lab calculations, prepare 5 mL of 0.1 M phosphate buffer in D$_2$O using the pH optimum from last week’s lab. (This solution will actually have a pD
instead of pH since we are working with D_2O not H_2O. You have at your disposal H_3PO_4, KH_2PO_4, K_2HPO_4, K_3PO_4 and D_2O. Check your calculations with your instructor before making up your solutions, and please be careful with the D_2O since it is a bit expensive.

2) Prepare two ~1 mL solutions of 0.1 M fumarate and 0.1 M L-malate in the 0.1 M phosphate/D_2O buffer. You have available fumaric acid disodium salt (FW 160.0 g/mol) and L-malic acid monosodium salt (FW 156.1 g/mol). Again, check your calculations with your instructor before making up your solutions.

3) Acquire and print NMR spectra of 0.1 M fumarate and 0.1 M malate.

4) Assign all peaks in both the fumarate and malate spectra. Understanding the malate spectrum takes a bit of work, as described below. To determine which resonances are due to the proR and proS protons on malate’s carbon-3, you need to consider the magnitude of the various coupling constants (there should be three distinct J values—a small, medium and large). The magnitude of coupling constants between hydrogens on adjacent atoms is a function of the dihedral angle between them; to estimate the dihedral angle, draw the Newman projection down the C2-C3 bond of malate and look at the orientation of the proton on C2 with those on C3 when the molecule is in its lowest energy conformer. The largest coupling occurs when the dihedral angles are near 0 and 180°, and the coupling is smallest at 90°. A curve showing the relationship between the coupling constant and the dihedral angle is often referred to as a "Karplus curve", after Martin Karplus. The exact size of the coupling constants also depends on a variety of factors in addition to the dihedral angle, such as the electronegativity of groups on the carbons (electronegative groups tend to make the coupling constant smaller). One simple formula for generating a Karplus curve that takes into account electronegative groups was developed by Durette and Horton (Durette, P.L.; Horton, D. Org. Magn. Res. 1971, 3, 417):

\[ J_{HR} = (7.8 - 1.0\cos\phi + 5.6\cos2\phi)(1- 0.1\Sigma\Delta\chi_i) \]

\( \phi \) is the dihedral angle between the vicinal HIs.
\( \Sigma\Delta\chi_i \) is the sum of the electronegativity differences between the attached atoms and hydrogen.
\( \chi \) values for some common atoms: H=2.20, C=2.60, O=3.50, N=3.05.

Use this equation in Excel to generate a Karplus curve for L-malate to help you assign the proR and the proS hydrogens on carbon-3 of malate.

5) To determine the stereoselectivity and thermodynamics of the fumarase reaction, set up two NMR tubes each with 800 µL of 0.1 M phosphate buffer and add 200 µL of 0.1 M fumarate (in 0.1 M phosphate/D_2O buffer) to one tube and 200 µL of 0.1 M malate (in 0.1 M phosphate/D_2O buffer) to the other tube. Then, to begin the reactions, add 2 µL of concentrated fumarase to each tube. Mix thoroughly by inverting the capped tube several times. Take a spectrum of the solutions shortly after the addition of fumarase and again near the end of the lab period. If necessary, take spectra later in the week, after equilibrium has been achieved.
6) Analyze your spectra to determine if the addition of the hydrogen to C3 is stereospecific, and if so, determine which hydrogen is the one added (proR or proS).
7) Integrate the peaks to determine the ratio of malate to fumarate at equilibrium (K\text{eq}) and calculate ΔG°.

**Week 3: Report Workshop**

The instructor will lead a brief discussion about writing a scientific paper. Then you will peer review the draft report written by one of your colleagues. After the peer review the instructor will lead a more focused discussion on important elements for this report.

Bring 2 copies of a draft of your Fumarase report. The report should have a cover sheet with your name on it, but your name should not appear in the rest of the report. The instructor will remove the cover sheet before distributing the report for peer review. While you are reviewing each other's reports, the instructor will check your draft report for completeness. The instructor will NOT be proofreading and checking the report in detail, but rather checking that you have complete draft with all the essential elements that are described below. This draft check will be worth 20 pts.

For this report you will write a Results and Discussion section. Since you are not writing an Introduction, please include in the beginning of the Results & Discussion section a figure showing the reaction catalyzed by fumarase. Please have your report double spaced. You may find it useful to look at some Biochemistry articles online at http://pubs.acs.org. Click on the "search the journals" tab and search in the Titles and Abstracts of Biochemistry for references to "pH profile", for example. In addition to writing a formal Results & Discussion section, turn in your lab notebook pages and email your lab instructor the Excel file used to analyze your pH profile. (You should turn in these items when you turn in your final report.)

Your Results & Discussion section should have three main subsections:

**pH Profile**

You should have a figure with the experimental data shown superimposed on a theoretical fit (similar to your plot for Exp 2). Be sure you follow the guidelines for a Biochemistry paper. Also include a figure legend with pertinent details on the experimental conditions and theoretical fit. In the narrative of the text you should describe your results, including some introductory information on how the experiment was performed. You should also elaborate on the model used to fit the data and the pK\textsubscript{a}'s you extracted, and include the pH optimum for fumarase. Based on the fit of the experimental data to the theoretical data, comment on the accuracy of the pK\textsubscript{a}s you report.
**Stereoselectivity**

In this section you should have a figure of the spectrum of malate and your assignments for the 3 protons. You should also have a figure with the spectrum showing incorporation of deuterium into malate. You will need to make a persuasive argument for the assignments you have made based on looking at the conformation of malate and the Karplus curve. Be sure to discuss carefully what you have learned about the stereoselectivity of the reaction.

**$\Delta G^\circ$ for Fumarate to Malate and Malate to Fumarate**

Present your data on the equilibrium constants obtained for both the reaction of fumarate to malate and malate to fumarate. Be sure to provide enough information so that your instructor can see how you went from the NMR information to the $\Delta G^\circ$ values. Again, be sure to comment on the accuracy of your results.

**Final Report (80 pts)**

Your final report will be due in lab the week after your Report Workshop.