Overview

We will study the kinetic properties of Alcohol Dehydrogenase (ADH) using ethanol as the main substrate. This is the reaction used by the liver to metabolize ethyl alcohol (ethanol). The kinetics of this reaction will be followed using a spectrophotometer.

Does the name of this enzyme sound familiar? You are right, it does.

This is the same enzyme we used in the “yeast mediated REDUCTION of ethyl acetoacetate” earlier in the semester. The product of this reduction was as alcohol. In this experiment, we will analyze the kinetics of the OXIDATION of an alcohol to the corresponding aldehyde. This illustrates a very important characteristic of enzymes; they accelerate a reaction in both directions, depending on the available substrates and cofactors.

During the first week we will analyze the effects of enzyme/substrate concentrations on the reaction kinetics. The work will be mostly qualitative with the purpose of familiarizing ourselves with the experiment and improving our consistency and accuracy. We will also choose suitable conditions for the quantitative determination of Michaelis-Menten parameters (Week 2).

During the second week of this project, we will determine the Michaelis-Menten parameters, $K_M$, $V_{max}$, and $k_{cat}$.

IMPORTANT: Each group needs to have a laptop so we can analyze your data as you go. You will need to make adjustments to your experiments based on this data analysis!!!

Introduction:

The enzyme Alcohol Dehydrogenase (ADH) catalyzes the oxidation of ethyl alcohol (ethanol) to acetaldehyde. This enzyme uses nicotinamide adenine dinucleotide (NAD$^+$), a cofactor, as the ultimate electron acceptor in this reaction.

$$\text{H}_3\text{C}-\text{CH}_2 + \text{NAD}^+ \xrightarrow{\text{Alcohol Dehydrogenase}} \text{H}_3\text{C}-\text{CH} + \text{NADH} + \text{H}^+$$

While the oxidation reaction is thermodynamically favorable (spontaneous), it has a high activation energy, which makes the reaction very slow in the absence of a catalyst. Enzymes are very powerful catalysts that speed up reactions millions of times as compared to uncatalyzed reactions. They do that by bringing reactants (which are called substrates when they bind to the enzyme) close together into
ideal orientation for the reaction to occur in the enzyme’s active site. Enzymes are able to stabilize the transition state species which results in a lower activation energy for the reaction, and an increased reaction rate.

Enzymes are most often proteins, so their structure is maintained by weak forces such as hydrophobic interactions, ion-ion interactions, hydrogen bonding, and Van der Waals forces. As these intermolecular forces are weak and vary greatly with conditions such as pH and temperature, the structure of proteins is very sensitive to the changes of the environment. Sudden changes in the temperature or pH, or presence of certain salts or solvents might “denature” proteins, that is, destroy their catalytic activity by modifying the enzyme’s active site in some way. Typical enzymes exhibit a definite preference for pH/Temperature conditions that are specific for the type of enzyme and the biological context of the enzyme.

The ADH reaction using an alcohol as a substrate, produces NADH which allows us to follow the kinetics of this reaction. NADH is NAD\(^+\) which has been reduced by addition of electrons and H\(^+\). NADH has a distinct absorption in the UV region of the spectrum, (our eyes can’t detect it), and changes in the concentration of NADH can be followed by monitoring its absorbance using a UV-spectrometer.

\[
\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{Alcohol Dehydrogenase}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \\
\]

UV absorption

In this experiment, we will couple the ADH reaction to the reduction of a colored compound (DCIP), which will allow us to detect the reaction by visible spectrophotometry, as well as allow students to qualitatively assess the progress visually. To this end, we will use two additional chemicals, phenazine methosulfate (PMS) and 2,6-dichloroindophenol (DCIP) to help us with the task of monitoring the reaction. NADH reduces PMS\(_{\text{ox}}\) to PMS\(_{\text{red}}\), then the PMS\(_{\text{red}}\) reduces DCIP\(_{\text{ox}}\) (which is blue) to DCIP\(_{\text{red}}\), which is colorless:

\[
\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \\
\]

Colorimetric detection

The disappearance of the blue color can be followed visually or by use of a spectrophotometer (also called a spectrometer, photometer, or colorimeter). This instrument measures the amount of light that goes through the solution in a cuvette, and reports it in either % transmittance (%T, the light that goes through the sample) or absorbance (A, the amount of light absorbed by the sample). The absorbance (A)
at a specific wavelength is directly related to the concentration of the colored species by the following mathematical equation, known as Beer’s Law,

\[ A = \varepsilon b C \]

Where “\(\varepsilon\)” is the **molar absorptivity or extinction coefficient** (specific for the colored species), “\(b\)” is the pathlength through the sample (1cm in most spectrophotometers), and \(C\) is the molar concentration of the colored species.

Since the reactions 2 and 3 are significantly faster than reaction 1, the rate of disappearance of the blue dye is directly proportional to the rate of consumption of ethanol. Thus, the kinetics of the enzymatic reaction can be followed in the visible region of spectra by measuring rates as the change of absorbance per minute in the reaction mixtures with different ethanol concentrations.

**Michaelis-Menten kinetics…..**

Please STUDY the enzyme kinetics handout posted on moodle!!!

**NOTE**

When we study the progress of the reaction in this experiment by monitoring the change in \(A\) with time, we may observe plots like the one on the right. The initial part “a” indicates an initially very low rate, then the rate increases in segment “b”. This is where the PMS and DCIP reactions are “catching up” with the ethanol oxidation. The main segment “c” appears to be a straight line through most of the run, *even when the concentration of ethanol is too small to saturate the enzyme*. Under these conditions, the reaction should be first order in substrate. The reason that it doesn’t is that the change in ethanol concentration over the time of our experiment is very small. In order to see the curvature in the first order plot, the reaction has to proceed over several half lives (where the initial concentration is cut in half several times). This does not happen in our experiment. DCIP needs to be at only about 70 \(\mu\)M (70 x 10\(^{-6}\) M) to give an absorbance close to 1, so all of the DCIP is consumed stoichiometrically when 70 \(\mu\)M of ethanol is consumed. This is an insignificant change in the initial concentration, so the rate changes insignificantly, and the plot appears to be a straight line. The rates calculated as the slope of any segment of this line are virtually indistinguishable, so *an average slope in section c of the plot can be used to represent a single rate for the reaction at the initial ethanol concentration*. 
WEEK I: BECOMING FAMILIAR WITH THE REACTION

Objective:

The objective of the first week of this project is to complete a control run, a standard experimental run, and several additional runs aimed at analyzing the effects of enzyme concentration and substrate concentration. By the end of the lab period, you should be able to qualitatively describe the effects of both (ADH and Ethanol) concentrations in the A vs time graph, as well as the effect on the values of Vo (umol DCIP/min)

Calibration of spectrophotometer:

Follow the instructions detailed in the “taking a spectrum with the spectrovis Plus Spectrophotometer” posted in the lab, and uploaded to moodle.

Enzyme Assay:

In order to study the activity of this enzyme, you need to know what reagents are required. Enzyme is one of them. That will be supplied to you in a stock solution for this first assay. The assay also requires a buffered solution and some salts. We will use sodium phosphate and a Tris buffer. The assay also requires NAD\(^+\) and ethanol, as well as the DICP and PMS for the coupled colorimetric reaction. Take only enough for what you need that day!!! Do not waste reagents.

You will need the following solutions:

- **Ethanol Stock**: 1.8 M Ethanol
- **DCIP/PMS/NAD\(^+\) mix**: 0.2mM DCIP, 0.08mM PMS, 0.75mM NAD\(^+\) in 10 mM Tris pH=9
- **Buffer**: 0.06M Tris HCl buffer pH 9.0
- **Working ADH solution (1mg/mL)**: dissolve 100 \(\mu\)L of 1mg/mL stock ADH in 50mL 0.1% BSA
  - Each group should make this solution fresh each day and **kept on ice all the time**
  - You can adjust your enzyme concentrations by making a more concentrated/diluted ADH working solution as needed
- **Stock ADH**: 1mg ADH per 1mL of 0.1M NaH\(_2\)PO\(_4\) pH 7.5  (This solution will be available to your group and you will use it to make your working solution)

*Most of these solutions will be ready for you to use the first week. Please take ONLY the volume you will need to your work area (in a 15 ml or 50 ml plastic tube).

*Please estimate how much of each solution you will need to run approximately 15-20 reactions and take/prepare ONLY that amount.*
• Start by calibrating the spectrometer and indicated in the instructions (posted on moodle)

• Run a full spectrum of your reaction mix as described below (BEFORE ADDING THE ENZYME) to detect the λmax. This needs to be done only once.

• Once you are ready to start the reaction, switch to a time based experiment. You will monitor the reaction for 200 seconds at the right wavelength (setup to read A every 1 or 2 seconds). Add your enzyme, MIX (either by quick inversion of by using the pipette) and start monitoring.

• \textit{Preparing your reaction mix:} In general, an assay will be setup in a cuvette and will involve the addition of 1.0 mL of the DCIP, PMS, NAD⁺ solution, 0.5 mL of the 1.8 M Ethanol solution, 0.5 mL of 0.06M Tris-HCl buffer pH 9.0, and 0.8 mL dH₂O. You should use a different pipette tip for each different solution to avoid contamination.

\textbf{Running your reaction:} 0.20 mL of the working ADH solution will be added \textbf{LAST, in the cuvette (in the spec!)}, to start the reaction. Briefly mix this solution using the micropipette, and begin the experimental run by clicking on the collect icon.

\textbf{IMPORTANT NOTES:}

😊 The final volume of the reaction mixture is 3.0 mL. Adjust the volume of dH₂O to reach this final volume when you are varying the volumes of the different components of your reaction mixture.

☼ Always setup your experiment table (table I below) ahead of time and checkmark your reagents as you add them, so you can keep track of what is in each cuvette.

♥♥ Remember, the reaction begins as soon as the enzyme is added, so for best results, little time should be wasted between the addition of the enzyme and the collection of data.

• Now that you were able to run your reaction once, fine tune the reagents volumes to achieve a change in A of about 0.2/min. An acceptable range is 0.15 – 0.3/min.

• Your reaction must be near completion after about 2-3 minutes… if you don’t see it starting to slow down by then, you may stop the reaction, adjust the substrate OR enzyme concentrations accordingly, and run it again. \textbf{NOTE: NEVER change more than one concentration at once!!!}

• Using excel, Plot A vs. time for your reaction and calculate the rate as ΔA/Δmin from the linear portion of the graph.

• The changes in A per minute must be converted to change in umol of product per minute (µmol/min). Assume one molecule of DCIP will be will be reduced for every molecule of
NADH formed. Use an extinction coefficient of 21,000 M$^{-1}$ cm$^{-1}$ for DCIP. **How you will do this conversion (sample calculation)?**

- As you develop proficiency with this assay, you will study the effects of
  a) varying the concentration of ADH on the rate of the reaction:
  b) Varying the amount of ethanol.

**Data analysis**

You will save your data as a csv file so you can easily open it using excel.

- Graph your data of A vs. time for your runs. Make sure you can obtain a good rate that can be comfortably measured and allows you to determine the initial velocity AS WELL AS the end point of your reaction

- While varying the concentration of ADH on the rate of the reaction, determine the $V_0$ ($\Delta A$/min) for each enzyme concentration, convert it to $\Delta$[DCIP]/$\Delta$min using Beers-Lambert law. Plot the initial rate of the reaction (uM DCIP/min) as a function of enzyme concentration (ug./mL) and record your observations. **Start thinking about what enzyme concentration will you use for your Michaelis-Menten experiment?**

- While varying the amount of ethanol. Analyze qualitatively the effect of substrate concentration on the reaction rate by graphing $\Delta$[DCIP]/$\Delta$min as a function of ethanol concentration (M). **What concentration is necessary to reach saturation? How do you determine this value? This value is IMPORTANT for planning your experiments for next week!!!**

- Please upload your graphs of $V_0$ vs [ADH], and $V_0$ vs [Ethanol] on the assignment folder created for that purpose on moodle so we can discuss SOME of them in pre-lab lecture on week II.

The units MUST be uM/min for the initial velocity, M for ethanol, and ug/mL for ADH concentrations!!!

**Table I: Sample Experiment table**

<table>
<thead>
<tr>
<th>Cuvette #</th>
<th>DCIP/PMS/NAD$^+$ solution (mL)</th>
<th>Buffer pH 9 (mL)</th>
<th>1.8 M Ethanol (mL)</th>
<th>ADH Working solution (mL)</th>
<th>dH$_2$O (mL)</th>
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<td>1 (example)</td>
<td>1.0</td>
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**WEEK 2: DETERMINATION OF $K_M$, $V_{MAX}$, AND $K_{CAT}$**
**Objective:**

The objective of the second week of this experiment is to determine the kinetic parameters $K_M$, $V_{max}$, and $k_{cat}$ for ADH using Ethyl alcohol as a substrate.

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**Plan your experiments BEFORE you come to the lab !!**

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From the enzyme assays that you ran last week, you should have an idea of the concentrations to use for the kinetics. Review the enzyme kinetics handout or consult your biochemistry textbook to refresh your memory on how does Michaelis-Menten kinetics works.

**Data analysis:**

- Obtain $V_0$ in values of $\Delta[DCIP]/\Delta\text{min}$ at different ethyl alcohol concentrations.
- Create a Michaelis–Menten plot of $V_0$ vs [Substrate]. Make sure you have enough data points at low [S] as well as under saturation conditions (at high [S]). On this plot ESTIMATE the values for $V_{max}$ and for $K_M$, indicate them on the plot.
- Create the double reciprocal plot (Lineweaver-Burk plot), and calculate the values of $K_M$ and $V_{max}$.
- Using the enzyme concentration in your experiments, calculate the turnover number $k_{cat}$ for ADH.
Prelaboratory Questions:

Week 1:

2. From the description of Michaelis-Menten kinetics, make a rough sketch of how you would expect the plot to look like if you graphed Rate vs. Concentration of Substrate.

3. From the concentration of the stock ethanol solution used to prepare the solution for the Standard Experiment, calculate the concentration of ethanol in the solution used for the sample kinetics run.

4. Why is the enzyme added to the reaction mixture last?

5. If the "regular runs" shared between you and your partner are strongly dissimilar, why should you not precede any further?

6. Why do you run a control in both weeks of the experiment?