Project 6B: Alcohol Dehydrogenase Kinetics—

Influence of pH, Inhibitors, Denaturation, etc. on Michaelis-Menten Parameters

LABORATORY REPORT: Informal Report

PRE-LAB ASSIGNMENT

• Read the entire laboratory project described in the following pages, as well as the source paper by Bendinskas *et al*.[1]

• Prepare, on a typed sheet of paper, the Project Objectives of this lab; on the same sheet, complete the assignment below:

1) The UV chromophore that is assayed in the ADH-catalyzed oxidation of alcohol is _____. The alcohol:chromophore stoichiometry in this oxidation reaction is: _____.

2) ADH maximal activity (at infinite substrate) is estimated to be $\Delta A_{340}/\Delta t$ = 0.652 min^{-1}. Calculate V_{max} in units of μM/s.

3) If the solution above was made by adding 5.0 μL of ADH stock solution (0.50 mg/mL) to a cuvet with a final volume of 1.00 mL, and MW(ADH) = 141 kDa, calculate k_{cat}.

4) If 0.150 M substrate gives an activity of $\Delta A_{340}/\Delta t$ = 0.427 min^{-1}, use the information in question (3) above to calculate K_m.

5) Using the solution composition details for the initial uncatalyzed reaction cuvet, as specified in "Experimental: Characterization of ADH at pH 9," calculate the final concentrations of NAD^+, EtOH, and Tris buffer in this control cuvet.

6) The website below presents views of yeast alcohol dehydrogenase, YADH: http://www.biochem.arizona.edu/classes/bioc463a/molecular_graphics_gallery/jmol/adh_juhyung/adh.html
   a. How many Zn^{2+} cations are bound to a single ADH monomer? _____.
   b. The catalytic Zn^{2+} is tetrahedral, bound by _____ cys-S:$^-$, and _____his≡N residues. The fourth site is used to bind the ____ atom of the _____ substrate.
   c. In addition to binding this substrate, Zn^{2+} aids catalysis by stabilizing _____.
   d. NAD^+ oxidizes alcohols (R_2CHOH) by accepting a(n) _____ that originated on the alcohol's _O *or* C_ atom.

INTRODUCTION

[1] Bendinskas, K. et al "Kinetics of Alcohol Dehydrogenase-Catalyzed Oxidation of Ethanol Followed by Visible Spectroscopy" *J. Chem. Educ.* **2005,** *82,* 1068-1070.

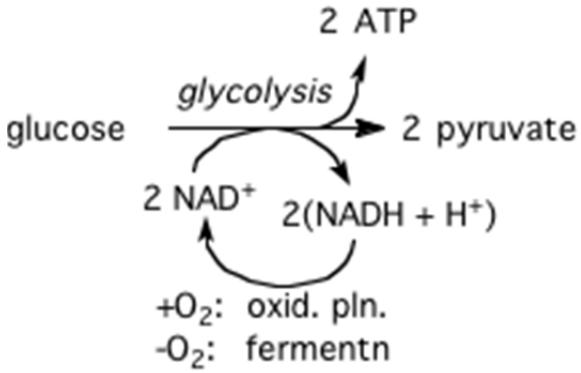
Enzymes, like other proteins, must be folded properly in order to function; therefore, denaturants (e.g., heat, detergent, non-polar solvent) cause catalytic activity to decline as the enzyme unfolds. Many enzymes utilize bound cofactors (e.g., metal cations, organic ligands) at key sites to affect substrate binding or catalysis. Reagents that interfere with these cofactors, or with amino acid side chains at these key sites, will inhibit activity; such reagents include acids, bases, and salts. Finally, many enzymes also have their activity reversibly controlled by endogenous ligands that can either inhibit or activate enzyme activity. In this project, you will study the effects of many of these reagents on the activity of the enzyme alcohol dehydrogenase (ADH).

Alcohol Dehydrogenase and Glucose Metabolism

ADH, a zinc-containing metalloenzyme, catalyzes the reversible oxidation of alcohols to ketones or aldehydes:

$$\underset{\textit{alcohol}}{R_2CHOH} + NAD^+ \rightleftharpoons \underset{\textit{aldehyde/ketone}}{R_2C{=}O} + NADH + H^+ \qquad (1)$$

As you have learned, in the ten-step glycolysis pathway, glucose is oxidized to 2 pyruvate, 2 NAD^+ are reduced to 2 NADH, and a net of 2 ATP are produced (Scheme 1). NADH must subsequently be reoxidized to NAD^+ in order for glycolysis to continue. As depicted in Scheme 1, under *aerobic* conditions, NADH is reoxidized to NAD^+ by O_2 (ultimately), via mitochondrial electron transfer chains that carry out oxidative phosphorylation. Under *anaerobic* conditions, NADH is reoxidized by fermentation, either lactate (in muscle) or ethanol (in yeast).

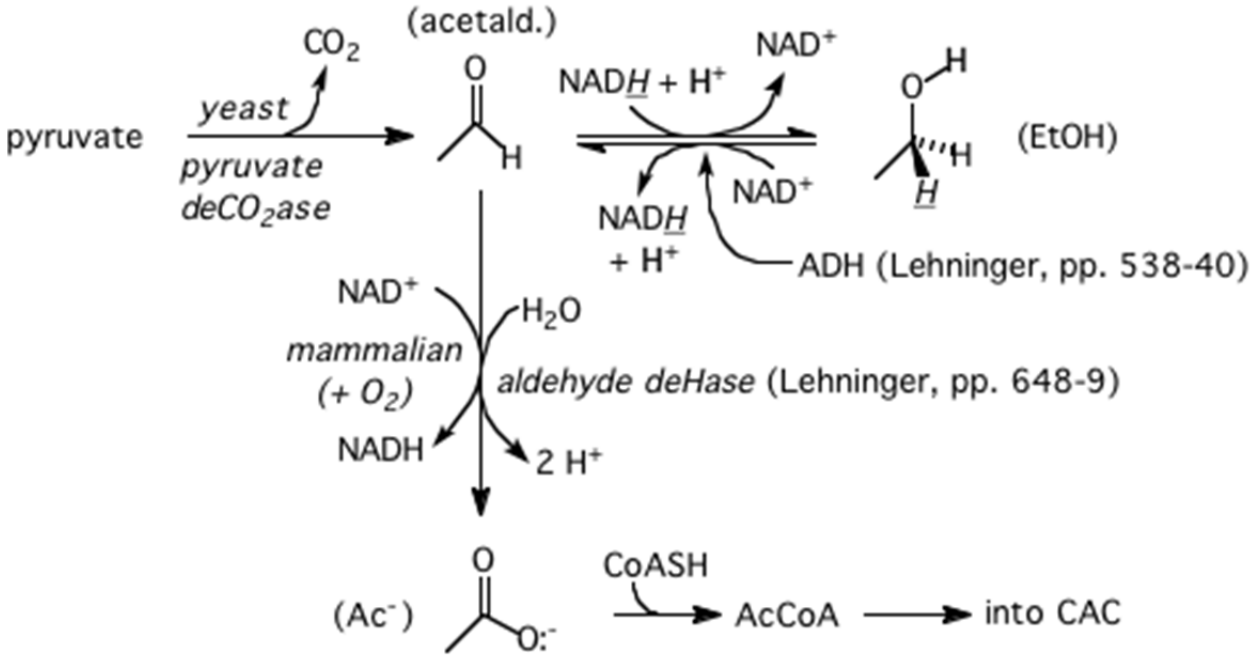


Scheme 1: A general overview of glycolysis and the regeneration of NAD^+ by oxidative phosphorylation under aerobic conditions, or by fermentation under anaerobic conditions.

Ethanol Fermentation:

ADH is a key enzyme in the ethanol fermentation process. The enzyme is named for the forward reaction depicted in equation (1), where it catalyzes the reversible oxidation of ethanol to acetaldehyde, using NAD+ as oxidizing agent along with a critical Zn^{2+} ion at the active site. In anaerobic yeast, the reaction runs in the *reverse* direction from that depicted in equation (1): excess pyruvate from glycolysis is decarboxylated to acetaldehyde, which is then *reduced* to ethanol, the final waste product of ethanol fermentation (top line of Scheme 2). The NAD^+ produced in this reaction is then used to run glycolysis.

In aerobic catabolism of ingested ethanol, the reaction goes in the *forward* direction: excess ethanol is *oxidized* to acetaldehyde, which is further oxidized (by aldehyde dehydrogenase) to acetate; acetate is then converted to acetyl CoA, which is catabolized in the citric acid cycle (Scheme 2).

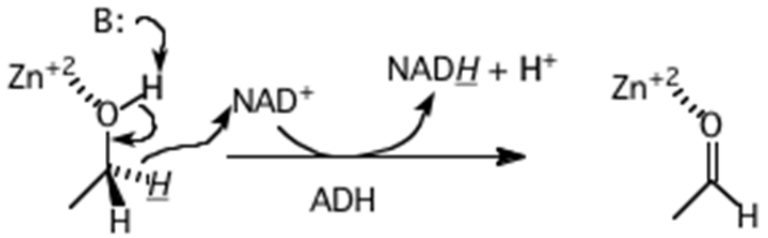


Scheme 2: An overview of ethanol metabolism. The top line, from pyruvate to ethanol, shows anaerobic ethanol fermentation. Starting with ethanol, and running to acetaldehyde and then down to acetate and acetylCoA, shows the aerobic catabolism of ethanol. See Lehninger[2] for background reading on ADH and aldehyde dehydrogenase.

Catalytic Mechanism of the Zn^{2+}-metalloenzyme, ADH

[2] Nelson, D.L. and Cox, M.M. *Lehninger Principles of Biochemistry* 5th edn., W.H. Freeman & Co., NY, 2008; pp. 538-540 (ADH), 648-649 (aldehyde dehydrogenase), and 547-550 (pyruvate decarboxylase).

Scheme 3 depicts the bioorganic mechanism of ethanol oxidation as catalyzed by ADH. Note the importance of the initial base catalyst, as well as the functions of the active site Zn^{2+}, in both binding the ethanol substrate and stabilizing the deprotonated alkoxide intermediate.



Scheme 3: Mechanism of ethanol oxidation catalyzed by ADH.

Assaying ADH Kinetics:

When ADH runs in the forward direction, oxidizing alcohol, the chromophore NADH is produced in a 1:1 stoichiometric ratio to ethanol. NADH has a λ_{max} in the near UVA region, at 340 nm, and $\varepsilon_{340}(\text{NADH}) = 6220\ M^{-1}cm^{-1}$. We can easily follow the reaction by the <u>increase</u> in A_{340} as NAD^+ is reduced to NADH. The initial slope of the absorbance vs. time curve, $\Delta A_{340}/\Delta t$, will then be linearly proportional to the initial velocity of the reaction, v_0.

$$v_0 \equiv \Delta[\text{products}]/\Delta t = \Delta[\text{NADH}]/\Delta t \propto \Delta A_{340}/\Delta t \quad (2)$$

From Beer's Law: $\Delta[\text{NADH}] = \Delta A_{340}/\varepsilon_{340} \bullet \ell (= 1.00\ \text{cm}) = \Delta A_{340}/6220\ M^{-1}$ (3)

Inserting Equation (2) into Equation (1), we get

$$v_0 = \Delta[\text{NADH}]/\Delta t = (\Delta A_{340}/\varepsilon_{340})/\Delta t = (\Delta A_{340}/\Delta t)/\varepsilon_{340} = \text{slope}_{340}/\varepsilon_{340} \quad (4)$$

Hence the initial slope of the A_{340} vs. time curve can easily be converted to the initial reaction velocity, v_0.

Before coming to the pre-lab lecture, familiarize yourself with Michaelis-Menten kinetics, and

the significance of the parameters K_m, V_{max}, and k_{cat} in the signature equation:

$$v_0 = \frac{V_{max}}{1 + K_m/[S]_0} = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_m} \quad (5)$$

EXPERIMENTAL: DAILY PLAN

Prior to beginning laboratory work, students should have in their notebooks a daily plan based on pre-lab discussions. Have the instructor check your plan every day before you begin work. Note that our procedure will differ from that outlined by Bendinskas et al.[1] on p. 1068: Instead of using the visible redox dyes PMS and DCIP, we will simply assay [NADH] by measuring changes in A_{340}. On day 1 you will do a Michaelis-Menten assay of ADH at pH 9. After that you will build a pH profile of ADH, and then do a Michaelis-Menten assay at a suboptimal pH. Next, you will test the effect of an inhibitor, then do a Michaelis-Menten assay of partially inhibited ADH. Finally, you will test the effect of a denaturant, as well as several other parameters (e.g. temperature, ionic strength, different concentrations of enzyme or NAD^+, or different types of alcohol substrates).

EXPERIMENTAL: STOCK SOLUTIONS AND BUFFERS

The basic reaction mixture for this initial set of experiments will contain ADH, buffer, NAD^+, and ethanol. EtOH concentrations will be varied **from 2 mM until near saturation is achieved** (i.e., very little increase in slope with increase in [EtOH]; depending on the batch of ADH, saturation could occur anywhere **from 100-400 mM EtOH**).

The prepared stock solutions available to you will be:

0.50 mg/mL ADH (Sigma catalog # A-7011); each pair takes ≈ 1 mL to use per day; store on ice.
1.00 mM NAD^+
0.10 and 1.0 M ethanol (aqueous)
0.20 M Tris buffer, pH 9.0
0.10, 1.0, and 3.0 or 6.0 M NaOH and HCl (for buffers)

Buffer salts available to you are: Na_3PO_4, Na_2HPO_4, NaH_2PO_4, glycine, Tris, and NaOAc. pK_as for the acid/conjugate base pairs are as follows:

H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	HOAc	Tris-NH_3^+	Glycine-NH_3^+
pK_{a1} =2.15	pK_{a2} =7.20	pK_{a3} =12.375	4.756	8.30	9.90

Student teams are responsible for making 250 mLs of 0.200 M buffer stock solutions at the following pHs: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 10.8, 11.5, and 12.5. You may have to calibrate the pH meter in order to make these solutions: Check with instructor and stockroom attendant.

You will be diluting these buffer stock solutions 10-fold in your kinetic runs; this dilution may change the pH slightly. In order to determine this, make three 10-fold dilutions for each buffer that you make, e.g., 1 mL stock + 9 mL water, mix and measure final pH. Report the average final pH ± standard deviation: (a) on the label of the buffer stock solution; (b) in your formal report; and (c) to the class as a whole.

EXPERIMENTAL: MICHAELIS-MENTEN CHARACTERIZATION OF ADH AT pH 9

Start each day of the enzyme kinetics lab by pouring out for yourself sub-portions of these four stock solutions: 1 mL of the ADH stock, 4 mL of the NAD^+ stock, 2 mL of EtOH stocks, and 2 mL of buffer stock. At the end of the day you may discard leftover portions, or leave for other students. Set up the Genesys UV-Vis spectrophotometer to measure ADH kinetics: absorbance at 340 nm vs. time; data collection interval of 1 s; and total run time of 1 min. You must first test the rate of the uncatalyzed reaction, in the absence of enzyme. Obtain a several clean semi-micro plastic cuvets that can be used in the near UV. The reference cuvet (in slot 'B'lank) will contain water. Add both substrates, buffer, and water to a cuvet: 250 µL of 1.00 mM NAD^+; 150 µL of 1.0 M EtOH; 100 µL of 0.20 M Tris buffer (pH 9.0); and 500 µL of deionized water: total volume = 1000 µL. Cover the cuvet with Parafilm, mix by inverting 2-3 times, place in slot 1, and run A_{340} vs. time. The absorbance change should be minimal. If it is not, then you must subtract this "background" slope ($\Delta A/\Delta t$) from experimental mixtures of catalyzed reactions.

Enzymes generally saturate hyperbolically with substrate. Because response to added substrate is not linear, enzyme kinetics measurements generally employ a logarithmic series of substrate concentrations. For an enzyme that saturates at ≈ 300 mM substrate, a typical experiment would

employ [substrate]= 1, 2, 5, 10, 20, 50, 100, 150, 300 mM. In a typical ADH run, you will use final concentrations of 0.250 mM NAD^+, and 20 mM buffer, in a final volume of 1.00 mL.

Make a table with six columns, for volumes of: (a) NAD^+ stock (1.00 mM); (b) buffer stock (0.20 M); (c) EtOH stock (0.10 or 1.0 M)[3]; (d) ADH enzyme stock (0.50 mg/mL); (e) water; and (f) total volume. Make nine rows, one for each of the logarithmically spaced EtOH concentrations stipulated above. Fill in each cell in the table with the appropriate volume; for now, assume that 5 µL of ADH stock is added. Before you proceed with the experiment, check your calculations with the instructor.

To run the first enzyme-catalyzed reaction, add NAD^+, EtOH (150 µL of 1.0 M), buffer, and water (only 495 µL), as in the uncatalyzed reaction above. Start the enzyme-catalyzed reaction by adding 5 µL of ADH stock last; as **quickly as possible**, mix the solution, insert the cuvet in the spectrophotometer, and measure the increase in A_{340} for at least 40 s. The slope should be at least $\Delta A_{340}/\Delta t \geq 0.002$/s (0.12/min); if it is not, then add more enzyme (e.g., 10, 20, 30 µL). **Every day that you work with ADH, you must start the day by checking enzyme activity in this manner**; adjust the volume of enzyme aliquot to be used that day accordingly.

Now perform the enzyme-catalyzed experiment at the other eight concentrations of EtOH, adjusting the volume of added water to give final volumes of 1000 µL. For each [EtOH], determine the initial slope of the *A vs. t* curve; as discussed in pre-lab, you will use the "Tabulated" data to calculate the initial $\Delta A_{340}/\Delta t$, using only the most linear part of the *A vs. t* curve. Ascertain whether the slopes vary with concentration as expected. If not, find your instructor and do some trouble-shooting.

EXPERIMENTAL: INFLUENCE OF pH ON ADH ACTIVITY

Enzymes are influenced by inhibitors, activators, and denaturants, in addition to solution parameters like temperature, ionic strength, and pH. In characterizing such influences, one generally starts with a "profile" of how enzyme activity changes as the parameter of interest

[3] Keep in mind that although 20 µL of a 0.10 M stock and 2 µL of a 1.0 M stock give you the same number of moles, the former is more accurately dispensed with a 20 µL pipettor.

changes (e.g., pH, ionic strength, inhibitor concentration, etc.). In this first set of measurements, one must keep all other kinetics parameters the same, especially the concentrations of enzyme, substrates, and buffer. Select a high EtOH concentration that gave good activity (e.g., 0.150 M), and replace the pH 9 buffer with pH 4-12.5 buffers, and determine $\Delta A_{340}/\Delta t$ slopes at each pH. The enzyme pH profile tells you where the enzyme is optimally active, minimally active (at high and low pHs), and partially active (i.e., partially inhibited). It is important to determine whether partial inhibition is due to problems at the active site causing k_{cat} to fall, at the substrate binding site causing K_m to rise, or both. This question is answered by doing a Michaelis-Menten plot for the partially inhibited enzyme: Select an appropriate pH, and determine $\Delta A_{340}/\Delta t$ slopes at the logarithmically spaced ethanol concentrations stipulated above.

EXPERIMENTAL: ENZYME INHIBITORS

You have available to you a number of ADH inhibitors:
Cd^{2+}, Al^{3+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, EDTA (Zn^{2+} chelator, incubate overnight);
dithithreitol; 2,4-dinitrophenol or 4-nitrophenol; thiourea; acetamide;
4-methyl pyrazole, imidazole, N-ethyl maleimide

Select a high EtOH concentration and optimal pH that gave good activity (e.g., 0.150 M, pH 9), and do a run with no inhibitor to determine the control v_0. Then determine v_0 at progressively higher inhibitor concentrations, until the enzyme is completely inhibited. This maximum [inhibitor] varies, but for the organic inhibitors it is roughly 10,000 times the final concentration of enzyme in the cuvet; for the metals, it is roughly 1 μM for Cd^{2+} (use gloves when handling the toxic heavy metal Cd). In these runs, incubate the enzyme first with buffer, water, and inhibitor for about 5-10 minutes, then start the reaction by adding NAD^+ and ethanol.

The enzyme inhibition profile reveals a range of inhibitor concentrations in which the enzyme is partially inhibited. Prepare a Michaelis-Menten plot of the partially inhibited enzyme at pH 9 to determine whether inhibition is due to problems at the active site causing k_{cat} to fall, at the substrate binding site causing K_m to rise, or both. Select an appropriate intermediate [inhibitor], and determine $\Delta A_{340}/\Delta t$ slopes at the logarithmically spaced ethanol concentrations stipulated above.

EXPERIMENTAL: ENZYME DENATURATION

You have available to you a number of protein denaturants:
SDS detergent, urea, guanidineHCl, acetone (and other nonpolar solvents), and heat[4]. Study one of these by collecting slopes at different denaturant concentrations to make an ADH denaturation profile. You will reach complete denaturation at approximately 10% SDS, 1 M urea, and 0.5 M guanidine. As with the inhibitors, incubate the enzyme first with buffer, water, and denaturant for about 20-30 minutes, then start the reaction by adding NAD^+ and ethanol. You are not *required* to prepare a Michaelis-Menten plot of the partially denatured enzyme to characterize the effects on substrate binding vs. catalysis, but you may *choose* to do so as one of your final further studies, outlined below.

EXPERIMENTAL: FURTHER STUDIES OF ADH ACTIVITY

You have studied the effects of pH, inhibition, and denaturation on ADH catalysis of ethanol oxidation by NAD^+. There are many other parameters that influence ADH activity, including temperature[4] (Arrhenius equation), ionic strength (add NaCl, KCl, KNO_3, etc.), [NAD^+], [enzyme], and different types of alcohol substrates. Devise and carry out experiments to study the influence of two of these parameters on ADH activity.

DATA ANALYSIS: CALCULATING v_0 AND MICHAELIS-MENTEN FITS

Convert all $\Delta A_{340}/\Delta t$ slopes to v_0 in units of μM/s, using the molar absorptivity of NADH.

For your Michaelis-Menten experiments (pH 9, partial inhibition at suboptimal pH, partial inhibition with inhibitor), use Kaleidagraph to make Michaelis-Menten plots: v_0 vs. [EtOH]. Fit the data to the equation for hyperbolic saturation, equation (5).

1. *For all three Michaelis-Menten plots, report, in addition to R^2, K_m and V_{max}, all with associated uncertainties and correct significant figures.*

[4] Using temperatures other than room T is a bit tricky, as the Genesys spectrophotometers lack temperature control.

2. *Calculate the final concentration, of enzyme in the cuvet (mg/mL). Using a MW of 141 kDa for Saccharomyces cerevisiae tetrameric YADH, calculate and report k_{cat} for all three fits.*

3. *Is your inhibitor competitive, mixed, uncompetitive, or none of these? Support your conclusion. Does the inhibitor affect substrate binding, catalysis, or both?*

DATA ANALYSIS: pH PROFILE

Using Kaleidagraph, plot the pH profile of ADH, v_0 vs. pH. Fit the data to the following equation:

$$v_0 = \frac{v_0(@opt.pH)}{1+10^{(pK_{a,lo}-pH)}+10^{(pH-pK_{a,hi})}} \qquad (6)$$

where $pK_{a,lo}$ = the pK_a of the side chain that is deprotonated at lower pH to activate the enzyme, and $pK_{a,hi}$ = the pK_a of the side chain that is deprotonated at higher pH to deactivate the enzyme. For the derivation of equation (6), see Appendix 1 at the end of this project.

4. *Report, in addition to R^2, $pK_{a,low}$, $pK_{a,hi}$, and v_0(at optimal pH), with their uncertainties and correct significant figures.*

5. *Speculate as to the identity of a critical side chain that must be deprotonated (base catalyst?), and another side chain that must be protonated (substrate binding?).*

6. *Is partial inhibition at suboptimal pH due to changes at the substrate binding site, the active site, or both? Support your answer; explain, based on your side chain speculations above.*

DATA ANALYSIS: ENZYME INHIBITION

Using Kaleidagraph, plot the inhibitor profile of ADH: v_0 vs. [inhibitor]. Fit the data to the following equation, derived from the reversible inhibitor kinetics equations in your biochemistry textbook (see Appendix 2):

$$v_0 = \frac{V_{\max}}{1 + \frac{K_m}{[S]_0} + x[I]_0} \quad (7a)$$

where x represents the sensitivity of the enzyme to inhibitor (I).

Recall that for the control, uninhibited enzyme, $v_0(\text{control}) = V_{max} \cdot (1 + K_m/[S]_0)^{-1}$, so dividing both the numerator and the denominator of Equation (7a) by $V_{\max}$, and using the above equation for v_0(control), we get

$$v_0 = \frac{1}{\frac{1}{v_0(control)} + \frac{x}{V_{\max}}[I]_0} \quad (7b)$$

In Kaleidagraph, this equation would be:

$$v_0 = (\text{m1}\text{\^{}}\text{-1} + \text{m2} \cdot \text{m0})\text{\^{}}\text{-1} \quad (7c)$$

Note that m1 = v_0(control); m0 = $[\text{I}]_0$; and m2 = $x/V_{\max}$(control); m2 has units of inverse rate·inverse [inhibitor], so if your [inhibitor] is in nM, then the units of m2 are $\text{s} \cdot \mu\text{M}^{-1} \cdot \text{nM}^{-1}$. Since you know $V_{\max}$(control) from your earliest Michaelis-Menten plot of the uninhibited control enzyme at this pH, you can calculate $x = \text{m2} * V_{\max}(\text{control})$; x will have units of nM^{-1}.

7. *Compare the fitted value of v_0(control) from this data set to the value from when you first studied the uninhibited enzyme at this pH and this $[S]_0$. How close are these two values?*

From the reversible inhibitor kinetics equations, given K_m(control) at this pH, we can derive (see Appendix 2) that if the inhibition is reversible and

a. **competitive**, then $x = \frac{K_m}{K_i[S]_0}$ (8a)

b. **uncompetitive**, then $x = 1/K_i'$ (8b)

c. **mixed**, then $$x = \frac{K_m}{K_i[S]_0} + 1/K_i' \quad (8c)$$

In the **noncompetitive** special case, when $K_i = K_i'$, equation (8c) reduces to

$$x = \frac{(1 + K_m/[S]_0)}{K_i} \quad (8c')$$

8. *Using K_m(control) from your early Michaelis-Menten plot at this pH, $[S]_0$ used in this inhibitor experiment, and the nature of your inhibitor from question #3 above, determine K_i (or K_i') for your inhibitor.*

DATA ANALYSIS: ENZYME DENATURATION

As denaturant is added, the enzyme denaturation process reaches a new equilibrium, featuring higher concentrations of denatured enzyme. This causes enzyme activity to fall, according to the equation:

$$v_0 = \frac{v_{0,H_2O}}{1 + \exp[\frac{\Delta G^o_{U,H_2O}}{RT}(\frac{[denaturant]}{C_{50}} - 1)]} \quad (10a)$$

where $v_{0,H2O}$ = initial reaction velocity in the absence of denaturant

$\Delta G°_{U,H2O}$ is the free energy of unfolding in the absence of denaturant,

and C_{50} = the [denaturant] that gives 50% denaturation, where $K_U = 1$, and $\Delta G°_U = 0$.

For the derivation of this equation from thermodynamic principles, see Appendix 3.

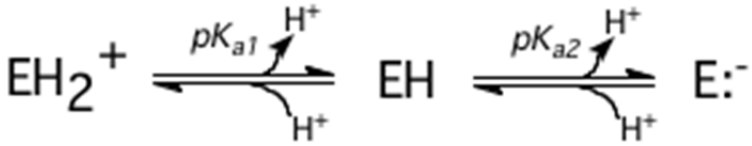
Use Kaleidagraph to fit your v_0 vs. [denaturant] data to this version of equation (10):

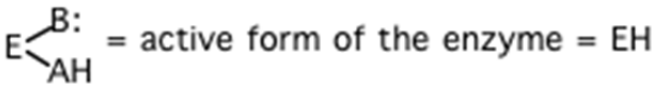
$$v_0 = \frac{m1}{1 + \exp[\frac{m2}{RT}(\frac{m0}{m3} - 1)]} \quad (10b)$$

9. *Report your best-fit values for $v_{0,H2O}$, $\Delta G°_{U,H2O}$, and C_{50}.*

APPENDIX 1: THE ENZYME pH PROFILE EQUILIBRIUM

Enzymes typically have bell-shaped pH profiles featuring a pH optimum centered between rising activity as pH is first increased, followed by falling activity upon further pH increase. This behavior can be modeled simply by assuming that a single acidic residue with a low pK_a must be *de*protonated to activate the enzyme, while a second acidic residue with a high pK_a must be *protonated* for the enzyme to maintain high activity. The enzyme can then be modeled as an inactive diprotic acid, EH_2^+; $pK_{a1} = pK_{a,lo}$ describes the first deprotonation to EH, which is the active form of the enzyme; and $pK_{a2} = pK_{a,hi}$ describes the second deprotonation to $E:^-$, which is an inactive form of the enzyme:





(A1.1)

From these two successive equilibria, we can write

$$K_{a1} = [EH]_{eq}[H^+]_{eq}/[EH_2^+]_{eq} \rightarrow [EH_2^+]_{eq} = [EH]_{eq}[H^+]_{eq}/K_{a1} \quad \text{(A1.2a)}$$

and $$K_{a2} = [E:^-]_{eq}[H^+]_{eq}/[EH]_{eq} \rightarrow [E:^-]_{eq} = K_{a2}\cdot[EH]_{eq}/[H^+]_{eq} \quad \text{(A1.2b)}$$

The mass balance equation, $$[E]_{total} = [EH] + [E:^-] + [EH_2^+] \quad \text{(A1.3)}$$

can be rewritten as $$[EH] = [E]_{total} - K_{a2}\cdot[EH]_{eq}/[H^+]_{eq} - [EH]_{eq}[H^+]_{eq}/K_{a1} \quad \text{(A1.4)}$$

The fraction of enzyme in the active EH form, $f_{EH} = [EH]/[E]_{total}$ is then

$$f_{EH} = 1 - (K_{a2}/[H^+])f_{EH} - ([H^+]/K_{a1})f_{EH} = (1 + K_{a2}/[H^+] + [H^+]/K_{a1})^{-1} \quad \text{(A1.5)}$$

Since v_0 decreases from a maximum of its value at the optimum pH, $v_0(opt.\ pH)$, to lower values that are proportional to f_{EH} at each pH, we can write

$$v_0 = \frac{v_0(opt.pH)}{1+\frac{K_{a2}}{[H^+]}+\frac{[H^+]}{K_{a1}}} = \frac{v_0(opt.pH)}{1+10^{(pH-pK_{a2})}+10^{(pK_{a1}-pH)}} \qquad \text{(A1.6)}$$

APPENDIX 2: REVERSIBLE ENZYME INHIBITION

Most biochemistry textbooks give kinetic equations for the influence of reversible inhibition on enzyme activity (for example, see Lehninger[5]). ***Competitive*** inhibitors bind only to free enzyme (E), and not to the enzyme-substrate complex (E·S). Competitive inhibitors thus compete with substrate for enzyme binding, often at the same binding site. The Michaelis-Menten equation in the presence of competitive inhibitor is:

$$v_0(w/inh.) = \frac{V_{max}}{1+\frac{\alpha K_m}{[S]_0}} \qquad \text{(A2.1)}$$

where $\alpha \equiv 1 + [I]/K_i$, and $K_i \equiv K_d(E{\cdot}I) = [E][I]/[E{\cdot}I]$. In all Appendix 2 equations, both V_{max} and K_m refer to the values obtained previously for the control enzyme at this pH, in the absence of inhibitor. Note that V_{max} remains the same, but K_m in the presence of inhibitor is higher, because $\alpha > 1$. Furthermore, because $\alpha > 1$, in the presence of inhibitor v_0 always declines. Given the definition of α, equation (A2.1) can be rewritten as

$$v_0(w/inh.) = \frac{V_{max}}{1+\frac{K_m(1+[I]/K_i)}{[S]_0}} = \frac{V_{max}}{1+\frac{K_m}{[S]_0}+[I]\frac{K_m}{K_i[S]_0}} \qquad \text{(A2.2)}$$

Uncompetitive inhibitors bind only to the enzyme-substrate complex (E·S), not to the free enzyme (E). Uncompetitive inhibitors thus do not compete with substrate for enzyme binding; in

[5] Nelson, D.L. and Cox, M.M. *Lehninger Principles of Biochemistry* 5th edn., W.H. Freeman & Co., NY, 2008; pp. 201-203.

fact, they *must* bind at an inhibitory site that is distinct from the substrate binding site. The Michaelis-Menten equation in the presence of an uncompetitive inhibitor is:

$$v_0(w/inh.) = \frac{V_{\max}}{\alpha' + \dfrac{K_m}{[S]_0}} = \frac{V_{\max}/\alpha'}{1 + \dfrac{K_m/\alpha'}{[S]_0}} \qquad \text{(A2.3)}$$

where $\alpha' \equiv 1 + [I]/K_i'$, and $K_i' \equiv K_d(S \cdot E \cdot I) = [E \cdot S][I]/[S \cdot E \cdot I]$. Note that both V_{max} and K_m decline by a factor of $1/\alpha'$ (again, $\alpha' > 1$); but because $\alpha' > 1$, the denominator $(\alpha' + K_m/[S]_0)$ is larger in the presence of inhibitor, and v_0 always declines. Given the definition of α', equation (A2.3) can be rewritten as

$$v_0(w/inh.) = \frac{V_{\max}}{\alpha' + \dfrac{K_m}{[S]_0}} = \frac{V_{\max}}{1 + \dfrac{K_m}{[S]_0} + \dfrac{[I]}{K_i'}} \qquad \text{(A2.4)}$$

Mixed inhibitors can bind to the enzyme-substrate complex (E·S), and also to the free enzyme (E). Mixed inhibitors also do not compete with substrate for enzyme binding; they too *must* bind at an inhibitory site that is distinct from the substrate binding site. The Michaelis-Menten equation in the presence of an uncompetitive inhibitor is:

$$v_0(w/inh.) = \frac{V_{\max}}{\alpha' + \dfrac{\alpha K_m}{[S]_0}} = \frac{V_{\max}/\alpha'}{1 + \dfrac{K_m(\alpha/\alpha')}{[S]_0}} \qquad \text{(A2.5)}$$

Note that V_{max} declines by a factor of $1/\alpha'$ (again, $\alpha' > 1$), whereas, because α and α' are usually within an order of magnitude of each other, K_m is approximately unchanged. Again, because both α and α' exceed 1, the denominator $(\alpha' + \alpha K_m/[S]_0)$ is larger in the presence of inhibitor, and v_0 always declines. Given the definitions of α and α', equation (A2.5) can be rewritten as

$$v_0(w/inh.) = \frac{V_{max}}{(1+\frac{[I]}{K_i'}) + \frac{K_m(1+[I]/K_i)}{[S]_0}} = \frac{V_{max}}{1+\frac{K_m}{[S]_0} + [I](\frac{1}{K_i'} + \frac{K_m}{K_i[S]_0})} \quad \text{(A2.6)}$$

Noncompetitive inhibition is the special case of mixed inhibition where $K_i = K_i'$, $\alpha = \alpha'$. In this case, equation A2.6 reduces to

$$v_0(w/inh.) = \frac{V_{max}/\alpha}{1+\frac{K_m}{[S]_0}} = \frac{V_{max}}{1+\frac{K_m}{[S]_0} + [I](\frac{1+K_m/[S]_0}{K_i})} \quad \text{(A2.6')}$$

APPENDIX 3: THE ENZYME DENATURATION EQUILIBRIUM

As denaturant is added, the enzyme denaturation process reaches a new equilibrium, featuring higher concentrations of denatured enzyme, E_D: $E_N \rightleftharpoons E_D$. The equilibrium constant for denaturation/unfolding is given by $K_U = [E_D]_{eq}/[E_N]_{eq}$. Furthermore, since the denatured enzyme is inactive, enzyme activity is proportional only to $[E_N]$. As denaturant is added, enzyme activity falls from the control value in the absence of denaturant, $v_{0,H2O}$, to a value of zero when the enzyme is fully denatured at high [denaturant]. At any particular [denaturant], $[E_N] \propto v_0$, while $[E_D] \propto (v_{0,H2O} - v_0)$. Combining these equations, we get

$$K_U = \frac{(v_{0,H_2O} - v_0)}{v_0} = \frac{v_{0,H_2O}}{v_0} - 1 \quad \text{(A3.1)}$$

which can be rearranged to $v_0 = v_{0,H2O}/(1 + K_U)$ (A3.1')

Denaturation clearly becomes more spontaneous as denaturant concentration increases. In fact, $\Delta G°_U$ falls linearly with [denaturant]:

$$\Delta G°_U = -RT\ln K_U = \Delta G°_{U,H2O} - m[\text{denaturant}] \quad \text{(A3.2)}$$

which can be rearranged to $K_U = \exp\{(-1/RT)(\Delta G°_{U,H2O} - m[\text{denaturant}]\}$ (A3.2')

where $\Delta G°_{U,H2O}$ is the free energy of unfolding in the absence of denaturant, and m is an index of the sensitivity of the protein to added denaturant; m has units of kcal/mol/M. At C_{50}, the [denaturant] that gives 50% denaturation, $K_U = 1$, and $\Delta G°_U = 0$; from equation (A3.2) then:

$$m = \Delta G°_{U,H2O}/C_{50} \tag{A3.3}$$

Equations A3.1', 3.2', and 3.3 can be combined to give

$$v_0 = \frac{v_{0,H_2O}}{1+\exp[\frac{\Delta G^o_{U,H_2O}}{RT}(\frac{[denaturant]}{C_{50}}-1)]} \tag{A3.4}$$