Experiment 4 - Kinetics of Lactate Dehydrogenase

Lactate dehydrogenase is a tetrameric enzyme found almost ubiquitously in nature. It catalyzes the final step in glycolysis under anaerobic metabolism, the reduction of pyruvate by NADH to produce L-lactate and NAD$^+$ (Figure 1).

![Figure 1. The lactate dehydrogenase-catalyzed reaction. At pH 7.0, the reaction to the right is distinctly favored, with an equilibrium constant of $10^4$. (R = ADP-ribose)](image)

Lactate dehydrogenase is one of a class of 128 other known enzymes that catalyze the same NAD-dependent class of redox reaction upon alcohols and carbonyls. Dehydrogenases make attractive enzymes to study kinetically due to the strong absorbance of NADH at 340 nm ($a = 6220 \text{ M}^{-1}\text{cm}^{-1}$). The rate of the reaction can easily be followed using a UV/visible spectrophotometer to monitor the loss in absorbance at 340 nm due to decreasing concentration of NADH. The objective of this experiment is to obtain kinetic parameters for the reduction of pyruvate by LDH and to determine the mode of inhibition by oxamate.

**Plotting Kinetic Data**

Good introductions to enzyme kinetics are available in any textbook, and my notes are linked on the Chem 394 web page. By applying the steady state approximation to the following mechanism,

\[
\begin{align*}
E + S & \quad \xrightleftharpoons[k_1]{k_2} ES \\
& \quad \xrightarrow{k_{cat}} E + P
\end{align*}
\]

one obtains the so-called Michaelis Menten equation:

\[
v = \frac{k_{cat}[E]_{tot}[S]}{K_m + [S]}\]

Where $[E]_{tot}$ is the total amount of enzyme present in the assay and $K_m$ is defined as follows:

\[
K_m = \frac{k_{cat} + k_{-1}}{k_1}
\]

While this equation was derived for a single substrate - single product reaction, its formula holds true for LDH so long as one of the substrates (in this experiment, NADH) is held at
constant initial concentration. By also working at constant enzyme concentration it is possible to reduce this equation to a two variable problem, with [S] as the independent variable and v as the dependent. The kinetic constants $K_m$ and $k_{cat}$ (or the related value $V_{max}$, defined as $k_{cat}[E]_o$) are the desired kinetic constants to be determined from this equation.

There are three commonly used methods of plotting enzymatic rate data, each with its own advantages and disadvantages. Part of the goal of this experiment will be to determine where these strengths and weaknesses lie.

- Lineweaver-Burk
The Briggs-Haldane equation can be rewritten to give

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \left( \frac{1}{[S]} \right).$$

A plot of $1/v$ vs. $1/[S]$ gives a line with slope $= \frac{K_m}{V_{max}}$ and a y-intercept of $\frac{1}{V_{max}}$.

- Eadie-Hofstee
Through further manipulation, the B-H equation can be written as

$$v = -K_m \left( \frac{v}{[S]} \right) + V_{max}$$

A plot of $v$ vs. $v/[S]$ will give a line with a slope of $-K_m$ and a y-intercept of $V_{max}$.

- Non-Linear Least Squares Fit
G. N. Wilkinson observed that both of the above methods of plotting enzymatic rate data involve problems in weighting data unevenly [Biochem. J., 80, 324(1961)]. He suggested an algorithm to perform a least squares fit analysis directly on the B-H equation. Rather than submit to the tyranny of linear plots, his method allows one to perform a direct plot of $v$ vs. [S] and to fit a curve satisfying the B-H equation to the data. Since the computing power necessary to routinely perform these calculations was developed relatively recently (the 1980’s), the less complex methods of Lineweaver and Burk and Eadie and Hofstee still predominate the older literature.

Inhibition
In addition to determining the $K_m$ value for pyruvate and the $V_{max}$ for the reaction catalyzed by LDH, inhibition by a pyruvate analog, oxamate:
will be studied. Three types of inhibition behavior are possible: competitive, non-competitive and uncompetitive. Each type of behavior is mechanistically distinct and results in an altered rate law.

**Competitive:**

\[ v = \frac{V_{\text{max}} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \]

**Non-competitive:**

\[ v = \frac{V_{\text{max}} [S]}{(K_m + [S]) \left(1 + \frac{[I]}{K_i}\right)} \]

**Uncompetitive:**

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S] \left(1 + \frac{[I]}{K_i}\right)} \]

[I] is the concentration of the inhibitor and \(K_i\) is the inhibition constant. A fuller explanation of these equations can be found in any discussion of enzyme kinetics. By adding a known concentration of oxamate to the series of reaction mixtures and measuring the impact of that concentration upon the rate vs. [S] data, one can determine a value for \(K_i\) using the appropriate plotting method.

**Procedure**

A reaction mixture will be prepared from buffer (50 mM sodium phosphate, pH 7.3, 100 mM NaCl), NADH and varying concentrations of pyruvate. A quantity of LDH will be added to this mixture and the decrease in absorbance at 340 nm will be observed for each of the different concentrations of pyruvate. If one observes only the early stages of a reaction of this sort, the velocity of the reaction will be essentially constant and will be proportional to the slope of a curve matching absorbance at 340 nm against time. You will use the slopes of \(A_{340}\) vs. time curves to determine velocities for this reaction. An appropriate plot of velocity data for uninhibited reactions will allow the calculation of \(V_{\text{max}}\) and \(K_m\). \(k_{\text{cat}}\) can be calculated knowing the concentration of the enzyme. A plot of velocity data from an inhibited reaction will allow you to determine the competitive or non-competitive nature of the inhibition and to determine \(K_i\).
I. Measurement of $k_{cat}$ and $K_m$

Performing the kinetic runs

• Turn on a spectrophotometer and allow it to warm up for 15-20 min. Connect a temperature regulating water bath to the spectrophotometer and set it to 25°C.

• Make sure that your reaction mixture is equilibrated to 25°C before use and standardize the instrument using blanks prior to the first run, and periodically thereafter, though this is not too important since you will be monitoring change in absorbance rather than absolute absorbance.

• You will need to prepare stock solutions of sodium pyruvate and NADH daily (1 mM solutions of each in phosphate buffer are recommended). The concentration of NADH should be held constant at about 0.25 mM in all reaction mixtures (so that its variation does not complicate the rate vs. [pyruvate] study). Sodium pyruvate can be varied from 0.05 to 0.5 mM in an initial study to get rough estimates of $K_m$ and $k_{cat}$. Typically it is preferable to set three pyruvate concentrations above $K_m$ and three below.

• Add a precise amount of diluted LDH (you should know the number of moles of LDH being added). The amount that needs to be added can be reached by trial and error. It should be enough to provide for a 0.05-0.1 change in $A_{340}$ per minute for the most dilute pyruvate sample, but not so concentrated as to cause significant curvature of the plot at higher concentrations. The source of the enzyme should be noted. A control, with no added enzyme, should provide a background rate that should be subtracted from the other runs. It should be run first. Low quality runs should be repeated. Patience and perseverance are crucial to success in this endeavor. It is strongly recommended that duplicate runs be made at each concentration, and better yet in triplicate, once you are assured that the system is working appropriately.

Plotting the data

From your plots of $A_{340}$ vs. time, calculate initial rates based on the slopes of lines at the initial time of data collection (that is, if curvature appears later, discard that part of the curve). This information will permit you to calculate $\mu$moles of lactate produced per minute.

Be sure to do Lineweaver-Burk, Eadie-Hofstee and Wilkinson plots (using R) of your velocity data and include them in your reports. These captions to these figures should include statistics data regarding the quality of the values determined from each plot. If the data from a single reaction doesn't agree with one of these plots, assess the cause of this deviation and repeat the runs if necessary. (N.B. No individual data point should be collected alone. If you lack sufficient data to get a good plot, start again from scratch. It is not advisable to plot, simultaneously, data collected on separate days.)
II. Velocity Data for an Inhibited Reaction

Performing the kinetic runs
Repeat all of the above, but now run each concentration of pyruvate with 0 mM, 0.05 mM and 0.1 mM oxamate. These runs should all be performed on the same occasion, but duplicate trials are not required in this part of the experiment.

Analyzing the data
One of the difficulties associated with measuring inhibition constants is that there are three unknowns ($k_{cat}$, $K_m$ and $K_i$) and three variables (rate, $[S]$ and $[I]$) in the equations. It is desirable to fit the kinetic data to one of the equations describing inhibition by performing a simultaneous fit. This is possible using the program R. This program will fit the data to each of the three different equations and provide estimates of $k_{cat}$, $K_m$ and $K_i$ along with standard error estimates. Ideally, one of the equations will fit the data best and that will allow a determination of mode of inhibition that is taking place.