BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 5
Enzyme Kinetics

The purpose of this laboratory session is to study the kinetics of beef heart L-lactate dehydrogenase. You will use the same partially purified sample of LDH you used in Laboratory 4 when you first measured the activity of the enzyme. As part of this lab, you will vary the NAD^+ concentration and determine the V_{max} for the reaction and the K_m for this substrate.

I. PreLab Preparation

Before the lab, please read Chapter 8 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor along with the sections of your Biochemistry textbook that deal with enzyme kinetics. Also read the **Additional Supplement** on **Enzyme Kinetics**. You will carry out **Experiment 8a** in the lab manual as described on page 231, but use the partially purified LDH enzyme rather than your own sample of LDH. Because this experiment involves many of the same calculations you did in Laboratory 4, please review the handout for that lab and the sample calculations shown in it.

II. Laboratory Procedures

A. Measurement of LDH Activity in a Stock LDH Enzyme Solution

The purpose of this part of the experiment is to find a volume of the stock enzyme to use for the kinetic experiments. This volume may be different from the volume you used in Laboratory 4 because it will be a new enzyme solution. Also, you will want to add a sufficient amount of enzyme to the cuvette so that the initial rate of the reaction is **0.1 to 0.2 ΔA_{340}/min**. The rate of the reaction will decrease as the one of the substrates (NAD^+) becomes limiting and so the absorbance at 340 will increase more slowly. You want to start, therefore, with a relatively rapid rate of reaction.

1. Turn on the Genesys 20 spectrophotometer and allow it to warm up for 15 minutes. Set the wavelength to 340 nm.

2. You will be provided with the following stock solutions for the LDH assay:

   Assay Buffer Solution = 0.15 M CAPS, pH 10.0
   NAD^+ = 6 mM
   L-lactate = 150 mM, pH 7.0
   stock bovine LDH = stock ammonium sulfate preparation diluted **1/200** in 0.1 M KPO_4 buffer, pH 7.5

3. To set up a standard assay, add 1.9 ml of the Assay Buffer to a 4.5 ml methacrylate cuvette. Then add 0.5 ml (500 µl) of the NAD^+ solution and 0.5 ml (500 µl) of the L-lactate solution. Note that the total volume at this point is **2.9 ml**. It will be easiest if you use a 5 or 10 ml pipet to add the buffer and a P-1000 micropipetter to add the NAD^+ and L-lactate solutions. Be sure to use a clean tip for each solution.

4. Since you cannot assume that the activity in the stock solution of bovine LDH is exactly the same as it was last week, you may need to try several different volumes in order to find a volume that gives a reasonable rate of reaction. **The final total volume in the cuvette should always be 3.0 ml**, so you can add up to **100 µl (0.1 ml)** of the enzyme solution and water.

5. Start by assaying the **same volume** of the stock enzyme solution you used last week for the pH analysis. Add an appropriate volume of the water to the cuvette containing the CAPS buffer, NAD^+, and L-lactate first. Place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Insert the cuvette into the spectrophotometer and set the instrument to zero absorbance. Then remove the cuvette and add the appropriate volume of the stock LDH enzyme solution. Rapidly place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Place the cuvette back in the instrument and read the absorbance at 340 nm at 15 second intervals for a total of 3 minutes. Record the absorbance values in your lab notebook.

6. If the reaction occurs at a reasonable rate (for example, an increase in A_{340} of 0.1-0.2 per minute), repeat the assay two more times so that you have three replicate assays.

7. If the rate of the reaction is too slow, try adding a larger volume of the stock enzyme solution to the reaction. **Be sure to reduce the amount of water, so that the final total volume 3.0 ml in each case**.

8. If the rate of the reaction is too fast, try adding a smaller volume of the stock enzyme solution to the reaction. Change the amount of water as necessary to the total volume is **3.0 ml**.

9. Once a suitable volume or dilution of the stock enzyme solution has been identified, repeat the assay as necessary so that you have three replicates.

10. Plot the results for each reaction on a piece of graph paper or in a graph made with Excel. Identify the linear portion of the rate curve and use it to determine the initial velocity (V_o) of the reaction in ΔA_{340}/min. Then calculate the average initial velocity in ΔA_{340}/min and in µmole/min as you did last week. You can use the conversion factor of 0.48 since all of the other conditions are still the same.

11. Finally, express the rate of the reaction in µmole/min ml or units/ml as you did last week. This calculation takes into account the volume or dilution of the stock enzyme solution you actually used in the assays.

B. Effects of Variations in NAD^+ Concentration on Enzyme Activity

The purpose of this part of the experiment is to vary the NAD^+ concentration and to determine the effect of this change on the enzyme activity. The volume of the enzyme, the concentration of the CAPS buffer, and the concentration of the other substrate (L-lactate) will be kept constant.

1. Set up a new reaction in which the volume of NAD^+ added to the reaction mixture is reduced. **Because the standard assay is designed to be run under saturating conditions, reduce the NAD^+ concentration by at least ½.** Add enough water so that the total volume of NAD^+ and any extra water is always **500 µl**. Run the reaction and measure the change in absorbance at 340 nm. If the rate of the reaction does not decrease very much, set up a new reaction with less NAD^+.

2. If you get down to 50 µl of 6 mM NAD^+ and there is still not much decrease in activity, make a 1/10 dilution of the stock NAD^+ solution by adding 100 µl to 900 µl of water in a microcentrifuge tube.

3. Once you find a NAD^+ concentration that reduces the rate of the reaction significantly (<75% of the control reaction), begin to do a series of careful replicate assays. **You should run the reaction at each substrate concentration at least twice**.

4. Gradually reduce the NAD^+ concentration until there is almost no detectable activity. **You want to have at least six (6) sets of assays with a wide range of sub-maximal rates of reaction**.

5. Make graphs of your assays and determine the initial velocity in each case. Calculate the average initial velocity of the replicates in ΔA_{340}/min. Then, using the standard conversion factor of 0.48, express the initial velocity ($\mathbf{V_o}$) in µmoles/min.

6. Calculate the NAD^+ concentration (the variable substrate) in μmoles/liter (μM) in each of these reactions. For example, the standard reaction contains 500 μl of 6 mM NAD^+, in a total volume of 3.0 ml. One way to determine the substrate concentration is to calculate the number of moles of NAD^+ in the reaction mixture.

$$0.5 \text{ ml} \times \frac{6 \text{ mmole}}{\text{liter}} \times \frac{\text{liter}}{1000 \text{ ml}} = 0.003 \text{ mmole}$$

$$\frac{0.003 \text{ mmole}}{3 \text{ ml}} \times \frac{1000 \text{ ml}}{\text{liter}} = \frac{1 \text{ mmole}}{\text{liter}}$$

$$= 1 \text{ mM}$$
$$= 1000 \text{ μM}$$

Another way to determine the substrate concentration is to use a dilution factor. Since 0.5 ml is diluted to a total volume of 3.0 ml, the dilution factor is

$$\frac{0.5 \text{ ml}}{3.0 \text{ ml}} = \frac{1}{6}$$

The final concentration is thus $\frac{1}{6} \times 6 \text{ mM} = 1 \text{ mM or } 1000 \text{ μM}$

Do a similar calculation for each of substrate concentration in which the NAD^+ concentration has been reduced and duplicate assays have been run.

C. Analysis of the Results

The purpose of this part of the experiment is to determine the V_{max} for the reaction and the K_m for NAD^+ as a substrate. You can make the graphs either on graph paper or by using Excel. While it is relatively easy to make the standard hyperbolic plot and the Eadie-Hofstee plot in Excel, it is more difficult to make the Lineweaver-Burke plot. It can be done, however, if you extend (forecast) the line toward the negative values on the X axis. You will probably not be able to make the Eisenthal-Cornish-Bowden direct plot using Excel.

1. Prepare a standard hyperbolic $\mathbf{V_o}$ vs. **[S]** plot of the data as shown in Figure 8.3. Estimate the $\mathbf{V_{max}}$ for the reaction and the $\mathbf{K_m}$ for NAD^+ from the graph.

2. Prepare a double-reciprocal (Lineweaver-Burke) plot of the data as shown in Figure 8.4. Calculate the values of $1/V_o$ and 1/[S] from your data, and then plot $\mathbf{1/V_o}$ as a function of **1/[S]**. **Note that in this graph, the Y axis is placed in the middle of the paper so there is room to extrapolate and to determine $-1/K_m$.** Estimate the $\mathbf{V_{max}}$ for the reaction and the $\mathbf{K_m}$ for NAD^+ from the graph.

3. Prepare an Eadie-Hofstee plot of the data as shown in Figure 8.6. Plot $\mathbf{V_o}$ vs. $\mathbf{V_o[S]}$. Estimate the $\mathbf{V_{max}}$ for the reaction and the $\mathbf{K_m}$ for NAD^+ from the graph.

4. Prepare an Eisenthal-Cornish-Bowden direct plot of the data as shown in Figure 8.7. Estimate the $\mathbf{V_{max}}$ for the reaction and the $\mathbf{K_m}$ for NAD^+ from the graph. **Note that in this graph, the Y axis is placed in the middle of the paper.** The substrate concentrations are placed to the left of the Y axis and the lines extended to the right of the Y axis. Draw lines to connect the related [S] and the V_o values and then extend them to the right of the central axis. In a good data set, they will tend to collect together in one spot, although they may not intersect perfectly. Use your best estimate of the intersection point to determine V_{max} and K_m.

5. Compile a table in which you compare the $\mathbf{V_{max}}$ and $\mathbf{K_m}$ values from the various plots. What was the average value for the V_{max} of the reaction? What was the average value of the K_m for NAD^+?