Biochemistry Lab

ENZYME ASSAY BACKGROUND

***Enzyme assays**: Just a few simple notes and helpful hints to guide your way along the fun world of enzyme kinetics. This can be a time where you generate a ton of interesting fun data or where you generate more than your fair share of frustration. Most of the problems with assays are due to simple mistakes that are often* due to a lack of attention to detail. Enzyme assays need lots of concentration and attention to detail.

The purification, characterization and quantitation of proteins depends upon the accurate determination of both the enzyme activity and the protein concentration. These are related in the term specific activity. Protein activityranges from enzymatic activity to binding to structural roles in cells and can be assayed in a variety of ways including continuous or discontinuous (stop time) enzyme assays.

Each type of assay presents particular problems that must be considered and taken into account to obtain accurate activity measurements. Similarly, there are a number of ways to determine the concentration of the protein in a given solution, and again each type of assay has its own particular limitations and assumptions. Thevarious types of assays and some examples of using such data to characterize a pure protein and to quantitate active protein are discussed below.

Chromophores: The concept of enzyme assays relies on measuring the loss of a substrate or the increase of a product. If either is readily identifiable by UV/VIS spec then your world just got easier. You can simply create the conditions necessary for the analysis of your chromophore, and you are ready to go. If not then there are a number of other means to measure your substrate or product and it is beyond the scope of this page. For MDH assays, NADH and NAD^+ absorb at two different wavelengths. You can look for changes of NADH at 340 nm. ***REMEMBER that an increase in absorbance corresponds to an increase in the concentration of NADH in the cuvette***. Look for the conversion from absorbance per min to units per ml on the MDH assay.

***Detection Method**: The study of an enzymatic reaction or assay is to follow either the loss of the substrate (a reactant) or the formation of one or more of the products. There are two main ways to measure an enzyme's reaction, coupled or direct. If the substrate or product has a characteristic absorbance or spectral "fingerprint," the changes in concentration can be directly measured. This is the case for many of the dehydrogenase enzymes. Both NAD^+ and NADH strongly absorb have strong UV absorbances, but at 340 nm, NADH has a much higher absorbance than NAD^+. Therefore, the enzymes activity can be directly measured.*

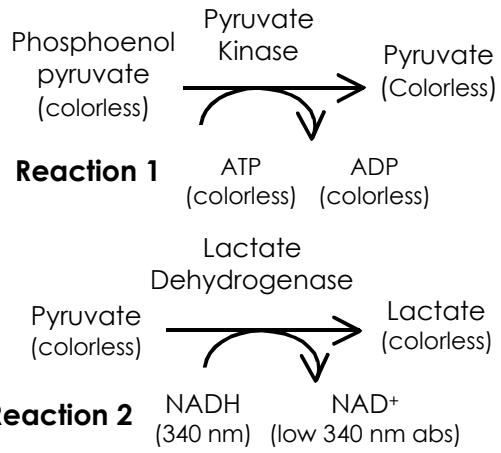

Figure 1. Example of a coupled assay. *The pyruvate kinase reaction is measured indirectly by the loss of absorbance at 340nm.*

A coupled reaction (Fig 1) uses one of the products as a reactant for an additional enzyme. That enzyme is typically easy to measure. There are lots of considerations with this type of assay. There must be enough of the second enzyme present, so that it isn't limiting the rate. The reactants for the second reaction also must be in excess, so the rate is limited only by the production of the reactant for the second enzyme.

Assay method: There are two common methods of determining the activity of an enzyme: stop time assay and a real-time assay, also called a continous assay. A stop time assay is just that; start the reaction and stop or read the results at a given time. This is the easiest way to do many assays at one time, **BUT** there are two things that need to be considered before doing this form of the assay. First, is the assay linear? In other words, in the time that I am running the assay,, is the product being produced (or substrate converted) at a linear rate? If the conditions of the assay tube are such that the reactants (substrate) are depleted or the products are inhibiting the enzyme, then you **CANNOT** use this assay. Second, is the compound you are measuring stable enough to wait to read and are the conditions used to stop the enzyme, i.e. acid or base too harsh to maintain the structure of the readout? We will be doing both stop time and real time/continous assays. Meaning the change in absorbance (also known as optical density – OD) vs. time. From this graph (done on the spectrophotometer), you will select a region that is reasonably linear and determine the dOD/min and then convert it to Units of enzyme activity per ml.

Absorbance: Most specs can only read between 0.01 and 3.0 abs units. At either end of this range there will be too much noise.

Always run a control assay – This is an assay that does not contain enzyme. It will tell you any drift in the baseline absorbance. If you get an appreciable amount of drift, you will have to subtract this dOD/min from your enzyme assay tube. If it is about zero, then baseline corrections are not needed. The control assay will also tell you what the starting absorbance is. Remember, if after an assay your results show the opposite absorbance but no change in absorbance per min. If your enzyme is too concentrated, that is if E>>S. it is likely that the time it took you to add the components together, mix and close the lid of the spec, the assay was already completed.

Proper Rates: This depends on each enzyme. For MDH, a rate of 0.05 to 0.4 dOD/min is good enough. If the rate is over too fast (*see above*,) then dilute the enzyme. If you are not certain how much to dilute the enzyme, do a 1:2 or 1:5. I have included notes in the MDH assay for our favorite expressed enzyme.

Run a positive and negative control: Always include a sample that has every component except NADH. The absorbance from these samples represent the absorbance when no/little NADH is left and the reaction is exhausted substrate. ALSO include a sample with NADH but NO enzyme. This is the starting concentration/absorbance. Samples that have the same absorbance did not have an active enzyme or enough enzyme to accurately be measured (below threshold of detection). ALWAYS keep the total volume the same; thus replace your NADH or MDH with an equivalent volume of enzyme assay buffer.

Run a positive enzyme assay control: Use a sample that you know has the enzyme. Often this can be from an extract or some purified protein already prepared.

Temperature: Bring all solutions to room temp before starting assays. The easiest way to do this is mix the next set of tubes while assaying one set. Enzyme should always be on ice before adding to the enzyme cocktail or it will denature. 10°C can bring about a 2 fold change in kinetics. Be consistent.

Measuring and Pipetting: This is another problem area. Day to day variations or even batch to batch changes in how you make up your enzyme or substrate solutions will cause a lot of error. For the MDH assay, always prepare at lest 25% more reagents than you need.

Mixing: Pipetting the enzyme up and down a few times with your micropipet DOES NOT EFFECTIVELY MIXTHE REACTION. You will get irreproducible data. With the cuvette in the spectrophotometer, hold the Pasteurpipette, air expelled in one hand and the micropipettor with the enzyme in the other. As soon as you pipet the enzyme into the reaction mix, gently suck up the reaction mix into the Pasteur pipet and expel back into the cuvette without introducing bubbles. Start recording A340nm versus time. This effectively mixes the enzyme and reaction mix. You should get reproducible data with this method. Because of the "deadtime" phenomenon discussed early, you should develop a consistent pattern of these steps before starting the recording at A340nm.

Calculating enzyme units: 1 Unit of enzyme catalyzes the conversion of 1 µmole of substrate to product per minute. To calculate the units in any spectrophotometric based assay, Beer's law is used: $A = \varepsilon l C$

Where A = absorbance (M^{-1} cm^{-1}), b = pathlength of the cell (1 cm), C = concentration of the absorbing species (M) and ε = the molar extinction coefficient.

When assaying enzyme activity we use Δ A/min (change in absorbance per time).
So $\Delta A = \varepsilon l (\Delta C)$ - as the concentration of chromaphore changes so will the absorbance.

FOR PLATE READERS, ADJUST THE CALCULATIONS FOR THE PROPER PATHLENGTH.

$\Delta A/min = \varepsilon l (\Delta C/min)$ *adds in the time factor*

$\Delta C / min = (\Delta A/min)/ \varepsilon l$ *rearrange factors*

$\Delta C / min = (\Delta A/min)/ (6.2 \times 10^3 \times 1)$

Example of enzymes that use *NADH* in a standard 1 mm pathlength cell.

- NADH extinction coefficient = 6.2×10^3 $m^{-1}cm^{-1}$

$\Delta C/min = (\Delta A/min \times 0.161 \times 10^{-3})$ M/min *inverse of the denominator*

$\Delta C/min = (\Delta A/min \times 0.161 \times 10^{-3})$ (mole/liter)/min *convert M to mole/liter*

$\Delta C/min = (\Delta A/min \times 0.161 \times 10^{3})$ (µmole/liter)/min *convert to µmole*

$\Delta C/min = (\Delta A/min \times 0.161)$ (µmole/ml)/min *convert to ml*

* µmole/ml is the same as mM thus mM/ml

This is the mM/ml of enzyme in the assay itself. Units are expresssed in µmole/min not µmola**r** But you only measure a few µl of actual enzyme from a test tube that are placed into an enzyme assay cuvette, the concentrations must be accounted for… use the ratio of total assay volume X to enzyme added vol Y

- *Δ A/min x 0.*061 ((µmole/ml)/min) x total assay vol/enzyme added volume

Then: Units of enzyme / ml of the enzyme in your test tube. Using a 1.0 ml total assay volume with 0.01 ml of enzyme sample: Δ A/min x 0.161 x (Y/X) Δ A/min x 0.161 x (1.0/0.01)

Δ A/min x 16.1 is the U/ml in your assay cuvet with a pathway of 1.0 ml and a 10 µl enzyme addition.

What is Specific Activity?

In conjunction with the determination of the activity of an enzyme solution, you can use the protein concentration and activity measurement to determine a parameter known as the "specific activity" of an enzymecontaining solution:

Specific Activity = Enzymatic Activity/Protein Concentration

As discussed above, the specific activity of a pure protein is a characteristic of that protein just as is its molecularweight or amino acid sequence and can be utilized to follow the purification of the protein.

What are the units of specific activity? The answer is complicated. If enzyme activity is measured in change of absorbance of the substrate as it is converted to product (often the case with Malate Dehydrogenase), the units of enzyme activity could be D Absorbance/minute. If the protein concentration in the cuvette is in mg/mL, then the units of specific activity would be:

(Δ Absorbance/minute)/(mg/mL)

What is the Turnover Number?

The turnover number of an enzyme (also known as Kcat or catalytic rate constant) is the maximal number of molecules of substrate converted to product per active site per unit of time. This is calculated as:

k_{cat} = Vmax / [E_T] where E_T is the enzyme concentration used to determine Vmax.

k_{cat} is also \ expressed as molecules converted per enzyme molecule active site (equivalent to moles per mole) per sec, whereas the specific activity of a pure enzyme is in units (μmoles per min) per mg enzyme

Making Measurements of the Initial Rate of an Enzyme Catalyzed Reaction

Overview: The measurement of the rate of a reaction depends upon being able to estimate either the amount of substrate [A]present or the amount of product [P] present as a function of time. The rate [often referred to as the velocity, v] of the reaction is simply:

$$\mathbf{v = -d[A]/dt = d[P]/dt}$$

The rate of the reaction being proportional to the concentration of the reactant, A, where v = k[A], where k is therate constant of the reaction. Measurement of the velocity as a function of the concentration of A allows the rateconstant, k, to be determined.

Enzyme catalyzed reactions are a little more complex and derivation of the Michaelis-Menten equation is based on the ability to measure the initial velocity, vo, defined as the velocity of the reaction immediately after the enzyme steady state has been achieved. Since the determination of the properties of an enzyme depend upon various applications of the Michaelis-Menten equation, it is critically important that the initial velocity of an enzyme catalyzed reaction is accurately measured. The following experiment illustrates this point and will familiarize you with the types of calculations that are involved in measuring the rates of enzyme catalyzed reactions.

General Considerations: The use of saturating substrate concentrations in reactionmixtures to minimize experimental errors has been emphasized. It is also important that reaction rates be measured under conditions where a sufficiently small amount of substrate is utilized so that the rate does not change during the assay as a result of substrate depletion. Similarly, aproduct buildup, which may lead to product inhibition, is to be avoided. In general, a convenient wayto test that these factors do not become a problem is to measure activity at a series of protein concentrations: The rate should be directly proportional to the protein concentration, as in Figure 2. Deviations below the line indicate that substrate depletion or product accumulationmay be occurring. Deviations from linearity can also result from protein aggregation or subunit dissociation affecting the rate of the catalyzed reaction.

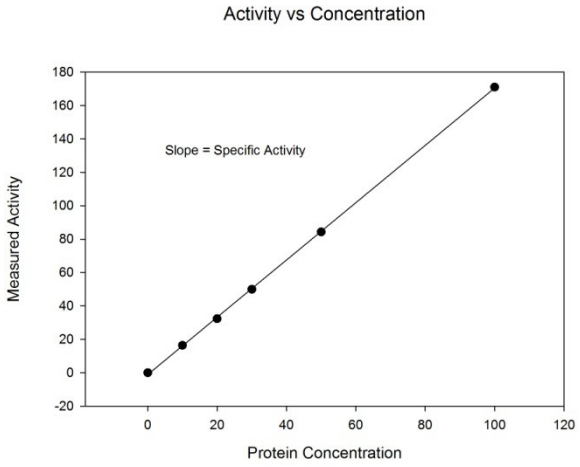

Figure 2 Activity is directly proportional to concentration

For the study of enzyme kinetics, it is important that the rate that is measured is the "Initial" rate of the reaction.In addition to being linearly dependent of the amount of enzyme added, an important criterion of the initial rate is that whatever change is being measured to follow the activity extrapolate to zero change at the start of the reaction- this ensures that the measured rate is indeed the initial rate of the reaction and that some change in the rate of the reaction did not occur in whatever "deadtime" the physical measurement of the rate involves- for example in the direct assays described below for dehydrogenases the deadtime is the time interval between introducing the enzyme, mixing and starting the actual absorbance measurements. This problem is illustrated in Figure 3. With an enzyme, such as Malate Dehydrogenase, which catalyzes a reaction that proceeds quickly to equilibrium, a small "deadtime" can lead to a large error in the estimated "initial" rate- however, such a situation is easily detected by the "must extrapolate to zero change at the start of the reaction" rule.

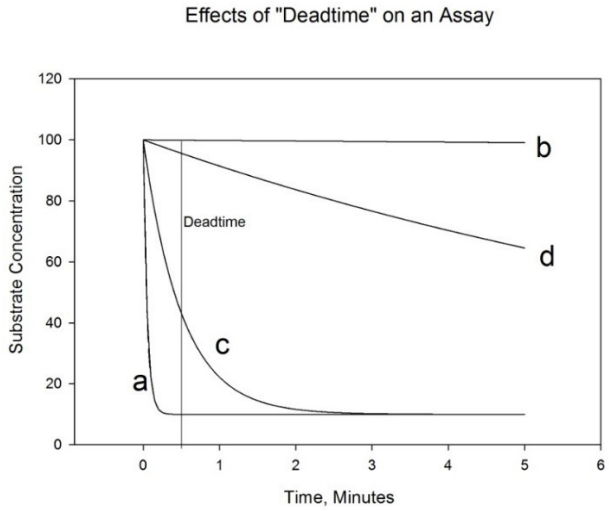

Figure 3: Effects of "Deadtime" on Accurate Rate Determination

How do you decide how much enzyme to use? When dealing with an enzyme where you do not know the specific activity, it is important to establish the correct amount of enzyme to use in assays. The trial and error approach is the only option you have. Try some amount (say 10µL of the solution you have) and measure the "rate"- there are three possible outcomes of this experiment- too much was added, too little was added, or approximately the right amount was added, as shown in figure 3- curve d. If too much was added you can make a best guess as to how much too much from the shape of theresultant curve- if by the time you initiated the measurement the reaction was already at, or close to equilibrium you much too much and

probably need to dilute the enzyme 50-100 fold (curve a). If you added too little of the enzyme to get a reasonably measurable rate (curve b) you need to concentrate the enzyme or simply add more volume of the enzyme until you get a reasonably measurable rate. If you added approximately the right amount the issue is whether or notit extrapolates back to the starting absorbance (usually about 0.6 in an MDH assay) at t = zero, in which case it is fine to continue with your experiment (curve d), or whether the enzyme needs some dilution- curve c- (by either adding a smaller volume- this depends upon how small a volume you are comfortable being able to add accurately- or by diluting maybe 5-10 fold).

You then calculate the initial rate (dA/dt) from the linear regionthat extrapolates back to the correct absorbance at t = 0. Usuallyin an MDH assay you can establish conditions where the plot is linear for about 30 seconds or more. Do not include any data from the curved region of the plot as this will distort the initial rate estimate.

Once you have established how much enzyme you need to add to give an accurately measurable initial rate it isprobably a good idea to test the highest and lowest combinations of substrates that you will use to ensure that you can make good measurements throughout the range you will use during a given experiment.

Continuous Assays: The activity of an enzyme can often be conveniently measured by following either the production of a product or the removal of a substrate. With certain classes of enzymes (e.g., dehydrogenases) the natural substrates are chromophoric and exhibit spectral changes that can be followed directly. For example,malate dehydrogenase catalyzes the reduction of oxaloacetate by the coenzyme NADH:

Oxaloacetate + NADH ⇔ Malate + NAD^+

NADH has an absorption band centered at 340 nm with an extinction coefficient of 6.22×10^3 $cm^{-1}M^{-1}$, while NAD^+ has no absorbance at this wavelength. When malate dehydrogenase is added to a mixture of oxaloacetateand NADH, there is a time-dependent loss of absorbance at 340 nm.

If the reaction is allowed to proceed to equilibrium, the "rate" progressively slows until equilibrium is reached. Clearly, the reaction "rate" changes during the time course of the reaction as a consequence of both utilization of substrate and approach to equilibrium.

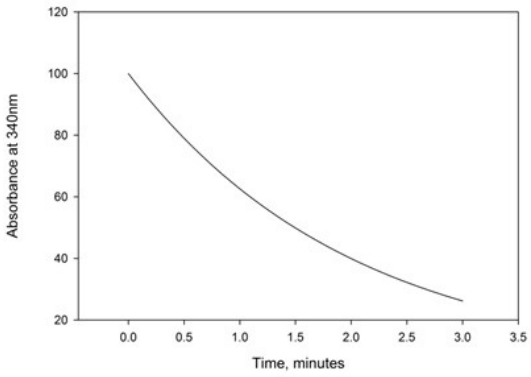

Figure 4 Time course of the reduction of OAA catalyzed by MDH using NADH

To enable reproducible rate determinations, two aspects of the reaction are determined: (1) the initial rate, as shown in Figure 4, and (2) the rate at saturating substrate concentrations. This rate (the "maximum rate") is calculated using concentrations of, in the case of alcohol

dehydrogenase, for example, acetaldehyde and NADH that give an experimentally determined maximum rate (Figure 5a&b).

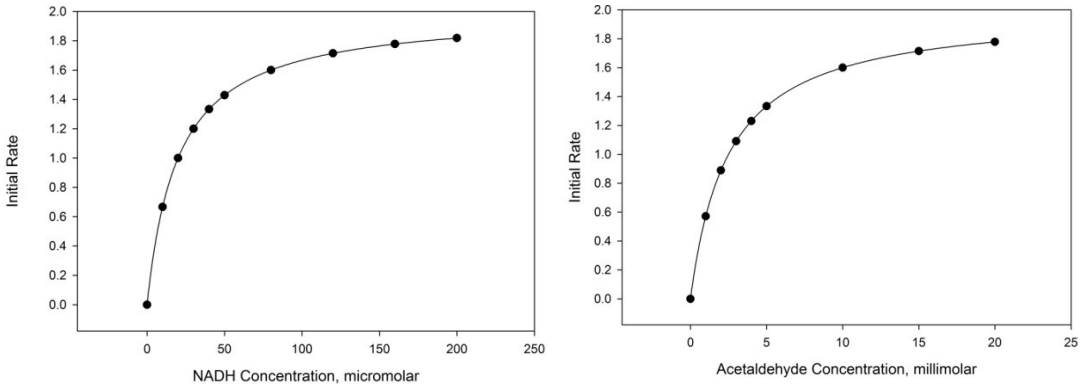

Figure 5 Dependence of the rate of the reaction catalyzed by alcohol dehydrogenase on theconcentrations of NADH and acetaldehyde.

These substrate concentrations are used for two reasons, one pragmatic and the other theoretical. If substrate concentrations sufficient to give this maximum rate are used, any experimental error in making up the assay is minimized. From the theoretical standpoint, under these conditions the measured rate of the reaction is dependent only on the concentration of the enzyme, a situation necessary if the enzyme assay is used to determine the activity of the enzyme. The case of malate dehydrogenase is complicated by the fact that the enzyme is known to exhibit substrate inhibition at high concentrations of Oxaloacetate and hence the appropriate Vmax concentrations of oxaloacetate cannot be used. Similarly it is not practical to use "saturating"concentrations of NADH because the Km of the wild type for NADH is in the range of 150-250 µM. Since 200mM NADH would have a concentration of 1.24 (usually spectrophotometers have their most accurate measurements between 0.05 and 1 absorbance units) it is unwise to use any NADH concentration above about 200µM. Established "Standard" Assay concentrations for NADH are usually 100µM, in the most accurate rangeof the spectrophotometer. With Malate Dehydrogenase it is essential to record the concentrations of both NADH and Oxaloacetate that are used in a "Standard" assay