MDH Kinetic Introduction:

Deep background on enzyme assays and kinetics

Deep Understanding: 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:

$$v = -d[A]/dt = d[P]/dt$$

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.

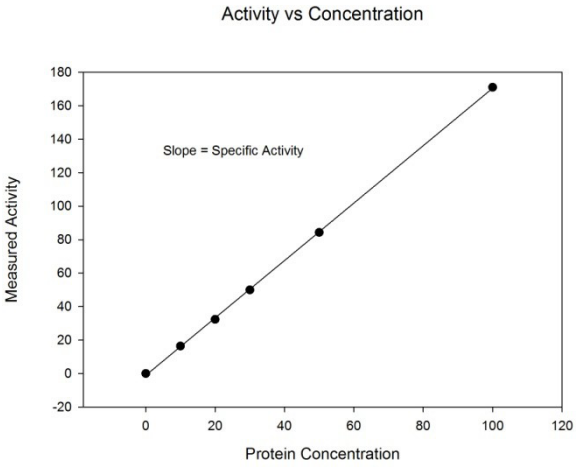

Figure 2 Activity is directly proportional to concentration

The use of saturating substrate concentrations in reaction mixtures 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.

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.

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 added much too much and probably need to dilute the enzyme 50-100 fold (curve a). If you added too littleof 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^+

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NADH has an absorption band centered at 340 nm with an extinction coefficient of 6.22 x 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.

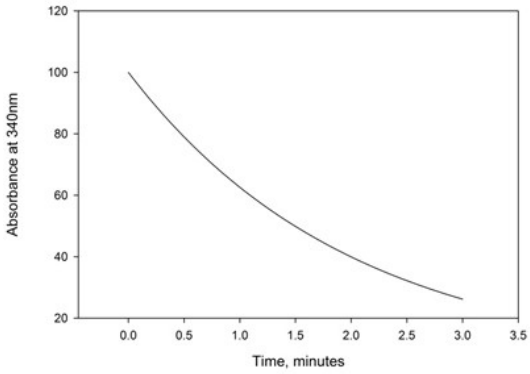

Figure 4 Time course of the reduction of OAA catalyzed by MDH using NADH

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. 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)

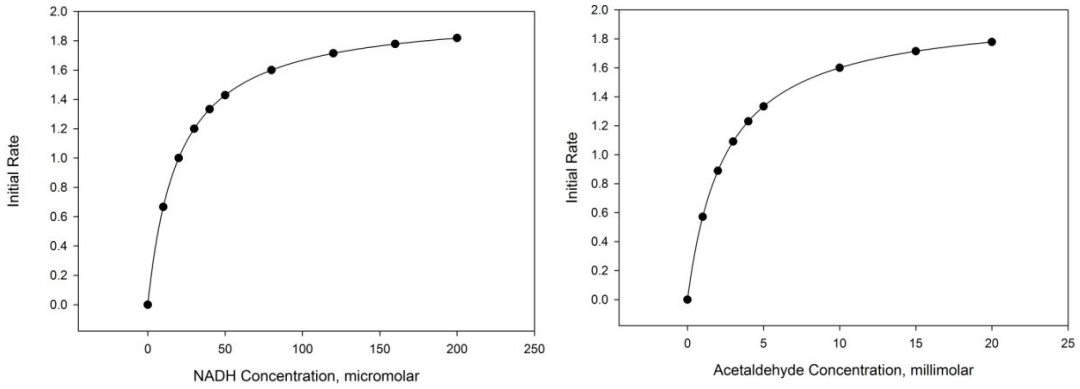

Figure 5. Dependence of the rate of the reaction catalyzed by alcohol dehydrogenase on theconcentrations of NADH and acetaldehyde.

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

to dilute the enzyme 50-100 fold (curve a). If you added too littleof 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 no it 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).

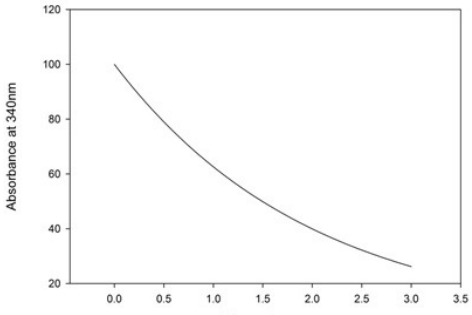

Figure 6 Time course of the reduction of oxaloacetate catalyzed by malate dehydrogenase using NADH as coenzyme

You then calculate the initial rate (dA/dt) from the linear regionthat extrapolates back to the correct absorbance at t = 0. Usually in 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.

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