MDH Assay Determining Km & Vmax:

Using Initial Rate Kinetics to Determine Km & Vmax

Overview: Determination of the Basic Kinetic Parameters, K_m and V_{max}

Does the Enzyme Obey Simple Michaelis-Menten Kinetics?

In experiments concerning the measurement of initial rates, a number of critical features involved with accurately measuring the initial rate of an enzyme catalyzed reaction and some of the uses of such measurements must be considered. In this experiment, we start to answer some of the questions about what sort of information do we want to know about enzymes: specifically, how well do they bind their substrates [as assessed by their Michaelis Constant, K_m], what is the maximum rate of the catalyzed reaction [as assessed by the Maximum Rate of the catalyzed reaction with saturating substrate concentrations, V_{max}] and how efficiently does the enzyme operate, as estimated by V_{max}/K_m.

Why do we want to know these things about an enzyme? Knowing each of the above parameters can help us understand how the enzyme may operate in vivo, how changes in the enzyme as a result of mutation may contribute to a clinical condition, or how an enzyme might be engineered to be more useful in a biotechnological setting. Knowing the K_m for Glutamate of the enzyme N-AcetylGlutamate Synthase and realizing that the in vivo concentrations of Glutamate are much lower helps us understand how the Urea Cycle is regulated [small changes in Glutamate concentrations have a direct and proportional effect on the concentration of N-AcetylGlutamate, an absolutely required allosteric effector of the Enzyme Carbamoyl Synthase- the key first step in Urea Synthesis]. Understanding how fast an enzyme can go at saturating substrate concentrations can help elucidate whether or not the enzyme plays an overall rate limiting role in a pathway, and hence may be subject to regulation. Isocitrate Dehydrogenase in the Krebs Cycle has the lowest V_{max} of any enzyme in the cycle and hence is the rate limiting enzyme of the cycle. As a result it is the major point of activation of the cycle [you must speed up the overall rate limiting step if you want to speed up the cycle]. The catalytic efficiency of an enzyme can give not only information about its evolutionary status [for example the enzyme Triose Phosphate Isomerase, a very primitive enzyme whose catalytic efficiency has evolved to near perfection], but also the potential to improve the enzyme for biotechnological purposes.

Introduction to the Design of the Experiment.

The Michaelis-Menten Equation:

$$v_o = V_{max}[S]/\{K_m + [S]\}$$

Together with its linear transformation, the LineWeaver Burk Equation:

$$1/v_o = 1/V_{max} + \{K_m/V_{max}\} \times 1/[S]$$

are the basic equations of enzyme kinetics which allows us to not only calculate values for K_m and V_{max} from the appropriate experiments, but helps us to understand the design of such experiments. These equations are used to analyze simple one substrate kinetics and calculate Km and Vmax. The basic equations also apply to multi-substrate enzyme kinetics except that the values for Km and Vmax obtained from the simple experiment used earlier do not take into account the possibility that the second substrate may have some influence on the values for each parameter. For a given two substrate experiment where values for Km and Vmax are obtained from a single experiment with one of the substrate concentrations varied, they are the values determined at a fixed concentration of the other substrate or substrates and are usually referred to as "apparent" values. Furthermore, they are values obtained assuming that the enzyme obeys Michaelis-Menten kinetics. This, too, is not always the case.

Using the Predictions of the Equation to Design the Experiment:
Both equations predict a particular response of the initial rate, v_o, to changes in the concentration of a substrate. The parameters Km and Vmax are obtained from experiments where the initial velocity is measured as a function of the substrate concentration. In these experiments, it is important to keep in mind how you plan to display and analyze the resultant data. If you are using the Michaelis Menten equation, which gives a plot of v_o vs [S], the experiment should be designed to have regular spacing along the [S] axis. If you plan to use the LineWeaver Burk plot to display the data, which utilizes a plot of 1/vo vs 1/[S], then 1/[S] should be regularly spaced. Are there any other considerations in the design of the experiment? Two. First, the most accurate data is likely to be obtained from $0.5K_m$ to $5K_m$ and hence a preliminary experiment to give a rough value of K_m can be useful. Second, you are testing the hypothesis that the enzyme obeys the fit to the appropriate equation, and to test this assumption as thoroughly as possible you need as wide a range of substrate concentrations as possible.

Why the Data May Not Fit the Predictions of the Equations
The activity of many enzymes is "regulated" by the concentration of their own substrates. In such cases, simple Michaelis Menten kinetics are not observed. Three scenarios are possible: First, the enzyme could have a regulatory binding site for the substrate in addition to the active site. At concentrations of substrate where there was significant binding to the "regulatory" site, the activity could be increased or decreased. Think what this would do to the simple assumption made in the Michaelis Menten or LineWeaver Burk equations. If we assume that the "regulatory" site has lower affinity than the active site, the apparent activity of the enzyme would disproportionally increase or decrease depending upon whether the binding to the regulatory site increased or decreased the activity of the enzyme.

Second, if the enzyme has multiple subunits with active sites, there could be some type of allosteric interaction between the sites such that substrate affinity or catalytic activity increased or decreased as a higher degree of saturation was achieved. Such "homotropic" allosteric interactions could give rise to sigmoidal saturation curves. Like those obtained with oxygen saturation of hemoglobin in the case where the substrate binding was enhanced as saturation increased-positive cooperativity. Or, saturation curves which decreased substrate binding-so called negative cooperativity.

While both of these mechanisms require either a second binding site for the substrate or allosteric interactions between subunits, there is an explanation for both the above types of "deviation" from Michaelis-Menten behaviour that requires no such causes. Consider a two substrate enzyme with subsites for each substrate. In many cases, the products of a reaction closely resemble the substrates- Malate Dehydrogenase for example where the substrate NAD^+ is very similar to the product NADH and similarly the substrate Malate is very similar to the product Oxaloacetate. If in a two substrate-two product system such as malate dehydrogenase, malate were the first product to leave, but the rate limiting step in the overall reaction was the release of NAD^+ from the resultant Enzyme-NAD^+ complex, it is conceivable that at high concentrations of oxaloacetate, oxaloacetate would bind to the E-NAD^+ complex before NAD^+ was released to form an E-NAD^+-Oxaloacetate Complex. If this complex released NAD^+ faster than the E-NAD^+ complex, the result would be that oxaloacetate would speed up the overall rate limiting step and cause "substrate activation." If the E-NAD^+-oxaloacetate complex released NAD^+ more slowly that the E-NAD^+ complex, oxaloacetate would slow the overall reaction, and a "substrate inhibition" effect would be observed. Such complexes, containing one product and one substrate, often called "abortive" complexes, play key roles in many enzyme catalyzed reactions and result in significant deviations at high substrate concentrations from expected Michaelis-Menten behavior. As a result, there is a necessity to decide whether or not the collected data has in fact obeyed the predictions of the Michaelis Menten or LineWeaver Burk equations.

As discussed in the "Quantitative Analysis of Data" section this involves an analysis of the residuals of the fit to the appropriate equation to determine whether in fact the data obeys the predicted equation.

An Equation for Substrate Inhibition

$$v_o = Vmax*X/(Km + X*(1+X/Ksi))$$

Where v_o, Vmax and Km have their usual meanings, X is the concentration of the substrate causing the inhibition, and Ksi is the inhibition constant for the substrate (X) causing the inhibition either by non-productive binding or by abortive complex formation