

BL483 BASIC BIOCHEMICAL TECHNIQUES

Lecture 3 - Enzyme Kinetics -- Expt 2 Part B

Read in Text: p. 78 to 81 (ignore part about uncompetitive inhibitors)

Lab Expt #2 - 2nd Part in 3rd Week - Expt #9 p. 107

What you will do in Lab: Carry out K_m and V_{max} determination as describe in Lab Manual as well as doing the kinetic analysis of the inhibitors P_i (inorganic phosphate) and sodium fluoride (NaF) of the Phosphatase.

What you will turn in for the Lab Report #2 (due in Week #4): From the first lab of Expt #2, turn in your data and graphics for p-NitroPhenol Standard curve, ranging finding for proper enzyme concentration to use, and the pH optimum of the wheat germ acid phosphatase. All data collected on the enzyme kinetics of wheat germ phosphatase including V_o vs $[S]$ and double reciprocal plots for the reaction catalyzed by acid phosphatase in the absence of inhibitors and the presence of inhibitors. From the double reciprocal plot you derive the K_m and V_{max} as well as the apparent K_m and V_{max} in presence of the inhibitors. By comparing the results in the presence and absence of the inhibitors, you will obtain the information for determining what type of inhibitor each additive is and by using the quantitative results you will obtain the K_i values for P_i and F^- . Using the K_i values, you will decide which inhibitor is a more effective inhibitor (ie which has the smaller K_i).

Special Note: There is a computer program on the PC in the lab called "EnzPack", which you can use to assist you with doing the calculations for K_m and V_{max} in the absence of inhibitors and similar parameters in the presence of inhibitors. The program has an autotutorial portion and also a section for doing calculations on experimental data. The program uses five different types of methods for determining K_m and V_{max} : Direct Linear Plot, Lineweaver Burk (double reciprocal plot), Wolf plot, Eadie Hofsted and a method for fitting data directly to the Michaelis-Menten equation. If you have done the kinetic analysis well and your data is good quality then, all five analysis methods will give similar results for K_m and V_{max} . **The program can be run from the DOS prompt in the root directory by entering: EP.** This runs a batch file that starts the main program.

1. Introduction

Enzymes are biocatalysts. To serve this role in living systems, enzymes must have a specific site for binding the substrates of the reaction they catalyze and the enzyme must form a complex with its substrates. In honor of the first biochemists to describe enzyme kinetic phenomena, the complex of enzyme and substrate is called the Michaelis complex:



Figure 1. Formation of ES complex.

This complex can breakdown to form the free enzyme and substrate or go on to form product, which we will treat as an irreversible reaction:

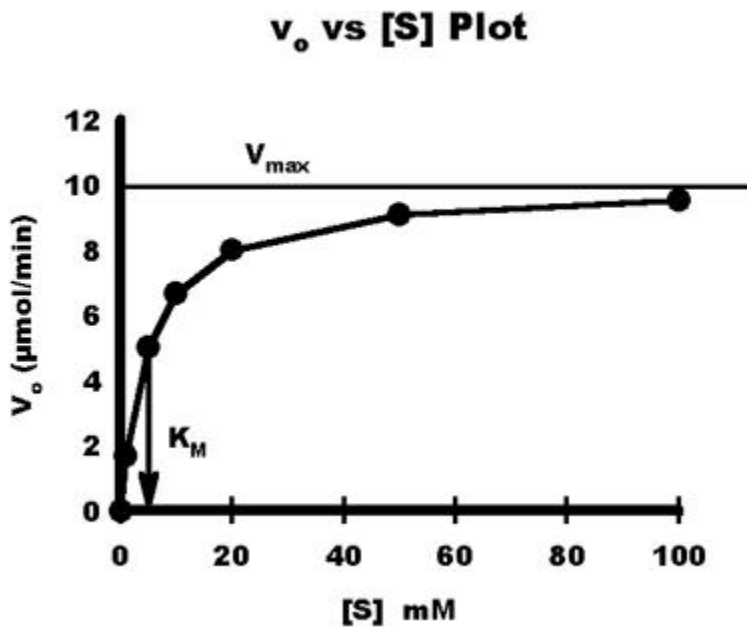


Figure 2. Production of product and regeneration of Enzyme from ES complex.

We assume also that the formation of the ES complex is very rapid and that [ES] reaches a "steady state" or constant level in a few millisecond. Hence, this type of enzyme kinetic analysis is known as steady state kinetic analysis.

2. Substrate Saturation of the Enzyme

The definition of a catalyst is something which accelerates the rate of a reaction without being changed itself. For a biocatalyst or enzyme to be most effective, it should be able to do its job with a much lower concentration of itself as compared to the substrate(s) it processes. This leads to the concept that there are a limited number of catalytic sites in a solution containing enzyme and substrate(s): [E] much less than [S]. Consequently, the enzyme will become "saturated" with substrate and the initial velocity (v) of the enzyme catalyzed reaction will reach a limiting value called the maximum velocity (V_{max}). This is most easily observed by plotting initial velocity versus substrate concentration.



Graph 1. v_o vs. [S] plot.

This response of the enzyme catalyzed reaction can be described by the Michaelis-Menten equation:

$$v_o = \frac{V_{\max} [S]}{K_M + [S]}$$

Figure 3. Michaelis-Menton Equation.

This equation defines the shape of a square hyperbola, which is the shape shown for the plot of V_o vs. $[S]$. The constants -- V_{\max} and K_M --- can be obtained from a set of experimental data where V_o is measured at different $[S]$. You are doing this experiment this week in lab for the acid phosphatase. And while you need to make a plot of V_o vs. $[S]$ and turn it in as part of your lab report to establish that the phosphatase is obeying the Michaelis-Menton equation, it is best to use other methods to obtain values for V_{\max} and K_M .

But before we get to that stuff, let us make a couple of definitions a little clearer. V_{\max} is the asymptote of the graph which V_o approaches at very high $[S]$. The units of V_{\max} are the same as those of V_o which should be in terms of the quantity of product formed per unit time (i.e. for the acid phosphatase V_o and V_{\max} should be in μmol or nmol of pNitrophenolate formed/min or hr). Operationally, the K_M is defined as the substrate concentration which gives 0.5 V_{\max} (verify this for yourself by substituting $[S] = K_M$ in the Michaelis-Menton equation shown above). So K_M values are in the same units as substrate concentration (i.e. for the acid phosphatase if $[\text{pNPP}]$ is in μM then K_M must also be in μM). However, more importantly, the K_M is a measure of the strength of the Michaelis complex between enzyme and substrate. Let us make a clear distinction between the binding constant of substrate to the enzyme (K_s) and the Michaelis Constant (K_M) -- it is often stated that the K_M measures the "affinity" of the enzyme for its substrate. But this is only true in special cases because the K_M also includes a measure of how well the enzyme catalyzes the formation of product after the enzyme substrate complex has formed.

As it turns out the ratio of V_{\max}/K_M is the best way to compare enzymes. The "V/K" constant, as it is sometimes called, is a measure of the "efficiency of catalysis" -- if the V/K is large then the enzyme is very efficient. However, there is an upper limit to efficiency which is determined by how fast the substrate can diffuse to the enzyme in solution. Some enzymes have attained this limit over evolutionary time, but most have not and are limited by chemistry that takes place during catalysis.

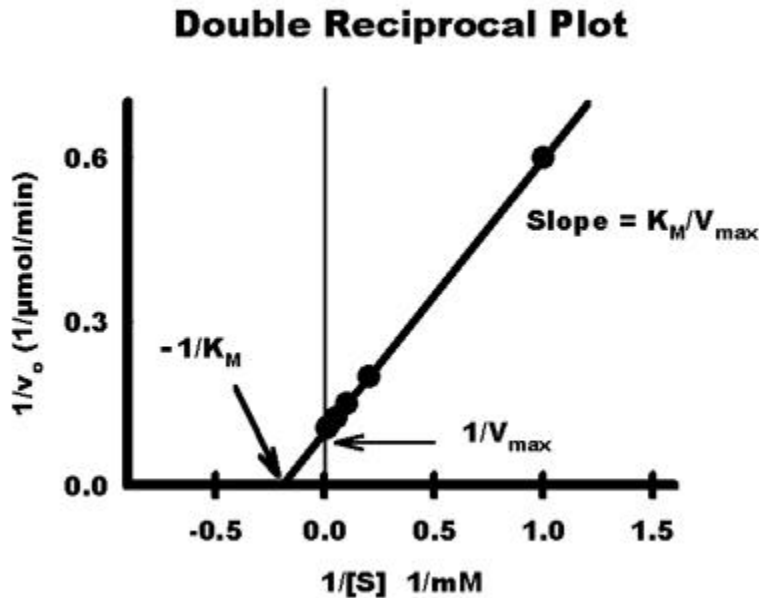
3. Calculating K_M and V_{\max} from Experimental Data

The best solutions to the problem of calculating K_M and V_{\max} are to use a computer program. I have a computer program available in my lab that runs on a PC in DOS (called EnzPlot) which you can use to obtain these kinetic constants for your data (It also has a tutorial on enzyme kinetics). But even if you use the computer, you must also make a graphical representation of these results using the "Lineweaver-Burk" plot, which is a linear transformation of the Michaelis-Menton equation generated by taking the reciprocal of both sides of the equation:

$$1/v_0 = 1/V_{max} + ((K_m/V_{max}) * (1/[S]))$$

Figure 4. Equation for Lineweaver-Burk Plot (Linear Transformation of M&M Equation).

To use this equation and make the graph required for your lab report, you take the reciprocal of each of your datum points or in other words for each v you calculate $1/v$ and for each $[S]$ you calculate $1/[S]$, then you plot $1/v$ versus $1/[S]$:



Graph 2. Double Reciprocal Plot of $1/v$ versus $1/[S]$.

You get the K_m and V_{max} from the graph as shown on it. Since the above equation is the one for a line (i.e. $y = a + bx$), you can make a linear regression or "least-squares fit" of your data for this graph and calculate K_m and V_{max} from the equation of the line.

4. Simple Inhibitors of Enzymes and their Kinetic Analysis

Inhibitors of enzymes have a long history of usefulness in gaining understanding of how enzymes work and unraveling metabolic pathways. You will use two simple inhibitors in the lab this week: 1) the first is inorganic phosphate, P_i , which is the product of the phosphatase reaction and acts as a classical competitive inhibitor of the enzyme; and 2) fluoride, F^- , which is an anion often found to inhibit phosphate metabolizing enzymes and acts as a classical noncompetitive inhibitor of the acid phosphatase. Well, what are competitive and non-competitive inhibitors of an enzyme. A competitive inhibitor is a substance which chemically resembles the substrate and its inhibition can be overcome by high concentrations of the substrate. A noncompetitive inhibitor is a substance with no chemical similarity to substrate and its inhibition can not be overcome by high concentration of substrate. Let us go back to our model of the kinetics of the

phosphatase and see how these inhibitors act:

Competitive Inhibition:

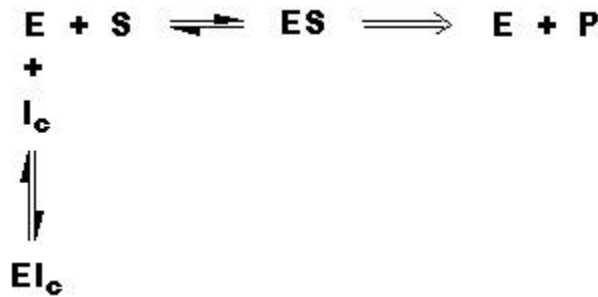


Figure 5. Model for Competitive Inhibitor.

The competitive inhibitor, I_c , forms a complex with the enzyme at the substrate binding site and prevents the substrate from getting in. But the EI_c complex is reversible and so as more substrate is added, the substrate manages to get in and overcomes the effects of the inhibitor. Consequently, the overall effect on the kinetics is to increase the K_m and we call this new kinetic constant, the apparent K_m or K_m' (called K_m prime or K_m apparent). The maximum velocity which is attained is the same in the presence and absence of the competitive inhibitor, so V_{max} is the same for both the uninhibited and competitively inhibited reaction. This is illustrated below using graphes for the response of an enzyme to competitive and noncompetitive inhibitors.

NonCompetitive Inhibition:

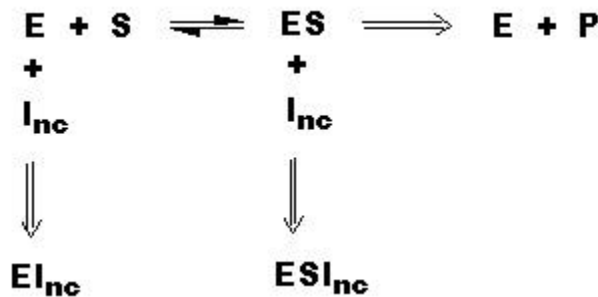
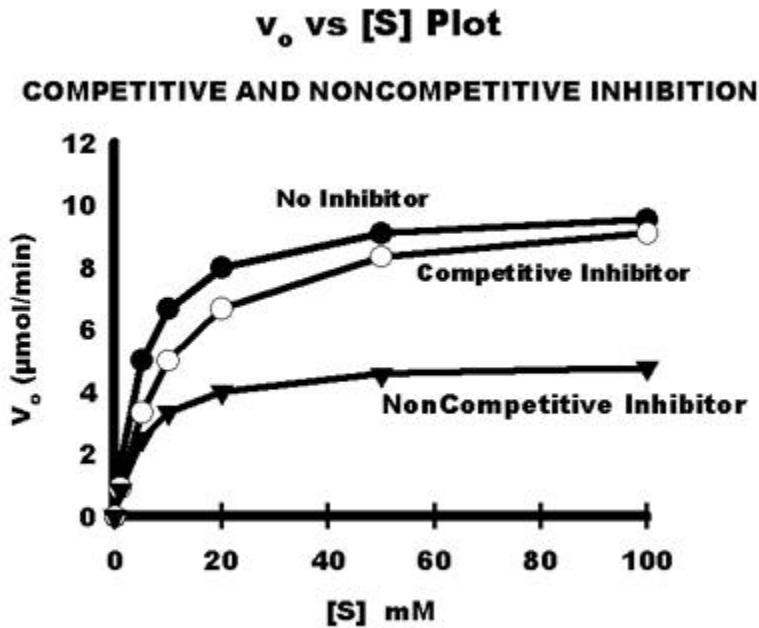


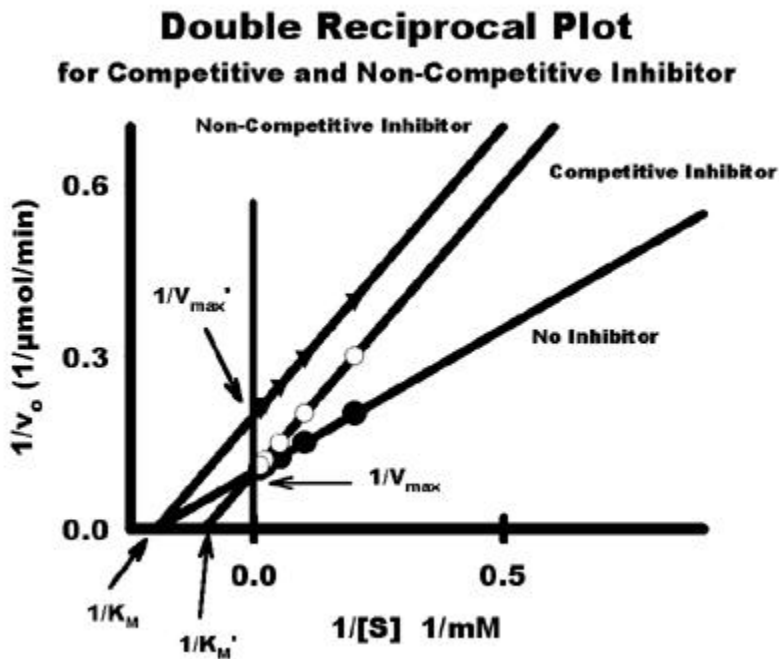
Figure 6. Model for Noncompetitive Inhibitor.

The noncompetitive inhibitor, I_{nc} , forms a complex with enzyme, which is unaffected by the substrate concentration, such that it does not matter if substrate is already bound to the enzyme when the noncompetitive inhibitor binds or not. In this classical type of noncompetitive inhibition, the K_m is not altered and only the V_{max} is decreased. Thus, the real V_{max} remains the same and you obtain an apparent V_{max} or V_{max}' (called V_{max} prime or V_{max} apparent).

Graph 3 and Graph 4 illustrate the expected results for a competitive and noncompetitive inhibitor.



Graph 3: v vs. $[S]$ plot for competitive and noncompetitive inhibitors.



Graph 4: Double Reciprocal plot for competitive and noncompetitive inhibitors.

5. Calculation of the K_i for Competitive and NonCompetitive Inhibitor

The strength of the complex formed between the enzyme and the inhibitor can be calculated from the kinetics constants obtained in the analysis of steady state kinetics:

Table of Equations for Calculating Inhibitor Binding Constants (K_i values).

Type of Inhibition	Michaelis Constant	Maximum Velocity
No Inhibitor	K_m	V_{max}
Competitive Inhibitor	$K_m' = (K_m * (1 + [I]/K_i))$	V_{max}
NonCompetitive Inhibitor	K_m	$V_{max}' = V_{max}/(1 + [I]/K_i)$

The symbols K_m and V_{max} have the same meaning here as in the standard kinetic analysis, while in the inhibited reactions, K_m' represents the " K_m " obtained in the graphical solution of the data collected in the presence of a competitive inhibitor and K_m' will always be greater than K_m . V_{max}' is obtained from the graphical solution of the data collected in the presence of the noncompetitive inhibitor and will always be less than V_{max} . The inhibitors of both types are used at known concentrations ($[I]$) and these must be used to find the strength of the binding of the inhibitor to the enzyme (K_i) and the K_i has the same units of concentration as does the inhibitor. So if $[I] = 1$ mM, then the units of K_i will be mM. So the degree to which the inhibitor effects the kinetics of the enzyme depends on both the strength of its complex with the enzyme, with a low K_i indicating strong binding of the inhibitor to the enzyme, and the concentration of the inhibitor added to the kinetic analysis. When you make your lab report, calculate the K_i constants for P_i and F^- and compare them. The one with the lower K_i is the stronger inhibitor.

Which is the stronger inhibitor of acid phosphatase, P_i or F^- , according to your kinetic data?

You can in another sense compare the K_i constants to the K_m for substrate, which is also expressed as a concentration, and make a general judgment as to whether the strength of the Michaelis complex (sort of the affinity of the enzyme for the substrate) is greater than the strength of the complex of inhibitor and enzyme. Since the competitive inhibitor binds at the same site as the substrate (especially in the case of the phosphatase you are studying since the competitive inhibitor being used is the product of the reaction), how does the K_i for P_i compare to the K_m for pNPP? In another sense, the noncompetitive inhibitor, which apparently binds to the enzyme at a place other than the substrate binding site, will generally have a K_i with less meaningful relationship to the K_m of the enzyme.