Enzyme Kinetics of Horseradish Peroxidase

CH454 Physical Chemistry Lab #1

Introduction

Enzymes are widely used in the chemical, biochemical and biotechnological applications. The range of chemical synthesis that is possible using enzymes is quite broad and new methods are under development that will greatly expand the field of enzyme use. Enzymatic catalysis is also one of the best understood types of catalysis. Many catalysts are difficult to characterize because they function at low concentration in complex mixtures and the intermediates are nearly impossible to isolate. In many chemical applications the addition of catalysts is understood as kind of "pixie dust" that just works, but no one knows why. It is difficult to detect transition state intermediates, which are altered by the catalyst to accelerate the rate. Although proteins are larger and more complex than inorganic or heterogeneous catalysts, the known structures provide insight into enzymatic catalysis. There are many very well characterized reactions, for which we know the structure of the enzyme, the details of the active site, and often we know how the substrate binds and precisely what aspects of protein structure are responsible for lowering the transition state energy. For these reasons enzymatic catalysis can be considered a model for how we would like to understand chemical catalysis in general.

The importance of enzymatic catalysis has been appreciated for more than 100 years. An early approach to characterization of the kinetics of enzymes is attributable to Menten and Michaelis. While there are many variations of Michaelis-Menten catalysis the simplest version treats and enzyme, E, and substrate, S, that combine to form a complex known as ES, the enzyme-substrate complex and then to form product, P, and reform the original enzyme. We can write this mechanism as follows:

$$E + S \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} ES \xrightarrow{k_{cat}} P + E$$

Often the Michaelis-Menten equation is plotted as the initial rate, V_0, which is equal to d[P]/dt. In this form we have,

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}$$

Where the Michaelis constant is:

$$K_M = \frac{k_{off} + k_{cat}}{k_{on}}$$

There are several special regimes that can be useful to understand the Michaelis-Menten equation:

Maximal rate: If there is excess substrate present the rate is limited by the rate at which the ES complex falls apart. The rate of formation of products is a maximum and $V_{max} = k_{cat}[E]_0$ is called the maximum velocity.

Half-maximal rate: If $V = V_{max}/2$, then $[S] = K_M$.

Second order regime: If $[S] << K_M$ then the rate of formation of products is $d[P]/dt = k_{cat}/K_M [E]_0[S]$. The rate depends on [S] as well as $[E]_0$.

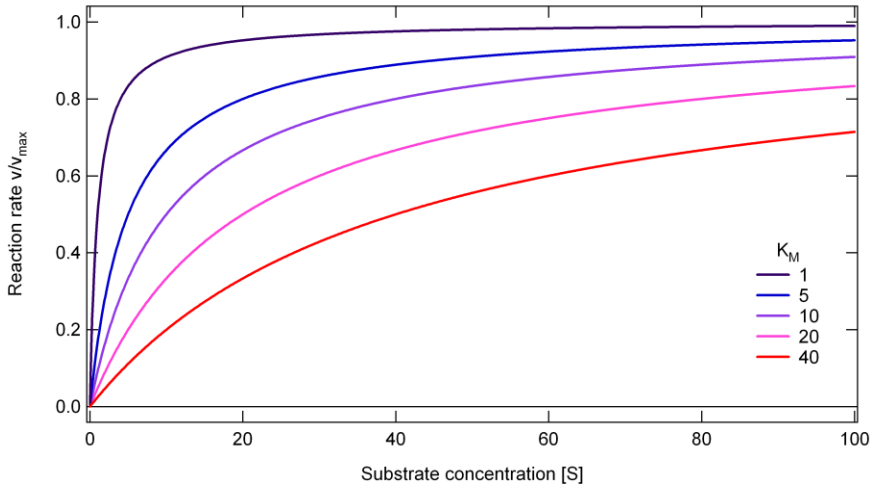

Figure 1.1. Michaelis-Menten curves showing the saturation of the kinetics at high [S]

Our experiment will determine the Michaelis-Menten parameters for the heme peroxidase enzyme known as horseradish peroxidase (HRP). HRP is the standard peroxidase enzyme that has been studied for many years. It is sold as a lyophilized powder. Published work suggests that we should be able to use approximately a 30-60 nanomolar solution of HRP as the enzyme in the turnover experiment described in this laboratory. Using 30 nanomolar concentration of HRP slows down the reaction relative to 60 nanomolar since V_{max} is proportional to the initial enzyme concentration. It is also interesting to see the saturation region, which is easier using 30 nanomolar. Make sure to consistently use the same HRP concentration in all experiments. HRP is a highly versatile and interesting enzyme. HRP was first discovered as the secretory peroxidase of horseradish root. It can oxidize a number of substrates because the only requirements are that a molecule must bind to the surface of the protein near the heme (not very strongly) and have a redox potential in the correct range to be oxidized by the ferryl intermediate formed when HRP reacts with H_2O_2. There are many studies in the literature of horseradish peroxidase oxidation of 2,4,6-trichlorophenol (TCP), which is a known naturally occurring pollutant in shallow coastal waters. In this study we will investigate the kinetics of HRP. Studies can be conducted as a function of pH from 5.0 to 7.5 or as a function of temperature.

The mechanism of the peroxidase reaction involves activation of HRP by H_2O_2. We can think of the reaction with H_2O_2 as a preliminary step that creates active form of the enzyme that we call compound I. It is compound I that binds to the substrate oxidizes substrate in two steps. We will ask the student to read the publications and to make the discussion of mechanism part of the laboratory report.

Experimental

You can measure the time-resolved kinetics of enzymes using a photo-diode array spectrophotometer. This type of spectrophotometer reads all of the wavelengths from 200-1000 nm simultaneous in less than 1 second. The advantage of this technology is that the instrument can be set up to run in kinetics mode, in which successive spectra are obtained each 3 seconds. If the kinetic change during catalysis by an enzyme has an optical signal in the range of the instrument and a time course that is longer than a few seconds, but shorter than a few hours it is appropriate for measurement using a photodiode array. One of the nice features of enzyme kinetic experiments is that the overall rate can be tuned by changing the enzyme concentration. Since we measure rates relative to the maximal rate, V_{max}, and

$$V_{max} = k_{cat}[E]_0$$

we see that we can get V_{max} to have a range of values by changing the enzyme concentration. Of course, there are limitations since the enzyme concentration cannot be higher than the solubility of the enzyme and there are practical limitations to how dilute the enzyme can be to function in a reliable way.

In order to obtain data appropriate for a Michaelis-Menten analysis, you will need to make 6 dilutions of the substrate with constant concentrations of enzyme and co-substrate hydrogen peroxide. The hydrogen peroxidase concentration should be high enough that complete conversion to product can be achieved at the higher substrate concentration. The volume of solution will need to be sufficient that the optical path of the light in the photodiode array passes only through solution and there is no air space on the top that could give rise to spurious absorption. In a small volume cuvette (with a 0.4 cm pathlength) this volume is 1.2 mL. We can summarize the requirements for this experiment as follows:

$[E]_0$ is constant (usually $[E]_0 = 3\text{-}6 \times 10^{-8}$ M)

$[H_2O_2]$ is constant at 1.2 mM

Ideally, $[H_2O_2] > [S]$ for all measurements, but this is not crucial since the reaction does not proceed to 100% completion. The substrate is not completely consumed. We do not want to have too high a concentration of H_2O_2 to avoid oxidative side reactions.

[S] ranges from zero to a maximal value of approximately 1.5 mM.

Note finally that [S] will need to have a higher coverage at low concentrations since the Michaelis-Menten curve has a greater rate of change at lower concentrations. For an enzyme that has an unknown catalytic rate, we will need to make a run to estimate the kinetics. Then

once we have an idea what the value of k_{cat} and K_m are we can determine the values. Typically, we will want 3 values of substrate concentration below K_m and two values above Km. An approximate distribution of substrate concentrations is:

[S] = 0.1, 0.2, 0.5, 0.7, 1.0, and 1.5 M

You will need to make stock solutions of the H_2O_2 and substrate. These solutions should be made fresh for each experiment since H_2O_2 tends to react at room temperature and phenols also degrade by slow light and oxygen-dependent reactions.

Setting up the data acquisition

Using the Cary spectrophotometer software in Kinetics mode. You will want to monitor at 272 and 314 nm. The 272 nm wavelength is used to monitor the appearance of the quinone product. The 314 nm wavelength is to monitor the disappearance (consumption) of substrate TCP. Kinetics mode can also scan a region of the spectrum every 2-3 seconds, depending on the bandwidth selected. Scanning from 240-320 will give a sufficient time resolution, but also show the spectra region of both substrate and product as a function of time. We will use the data at 272 nm for fitting the initial slope to obtain V_0 as needed for the Michaelis-Menten protocol discussed in the introduction.

Stock solutions

The concentration of HRP protein stock solution is determined by using UV-Vis in the Standard mode. The absorbance at 400 nm which is the λ_{max} of the Soret band is recorded and used to calculate the concentration of HRP stock solution according to Beer's law: $c=A/(\varepsilon_{400}\, d)$. The path length d of the quartz cuvette is 0.4 cm, and ε_{400} for HRP is 103,000 M^{-1} cm^{-1}. The TCP stock solution in 100 mM KPi buffer (~ 30 mM TCP) can be made by dissolving in methanol. The concentration can be determined spectroscopically using the molar absorptivity of $\varepsilon_{312\ nm}$ = 3752 M^{-1} cm^{-1}. The hydrogen peroxide stock solution is prepared freshly before the kinetic experiments. For a typical HRP kinetic reaction with the final hydrogen peroxide concentration at 1200 μM. You can prepare the hydrogen peroxide stock solution by having 10 mL KPi buffer mixing with 7.4 μL of 30% concentrated hydrogen peroxide solution (from Sigma-Aldrich). To test the concentration of H2O2 in the commercial 30% solution you may use the molar absorptivity of $\varepsilon_{240\ nm}$ = 43.6 M^{-1} cm^{-1}.

Mixing protocol

Calculate the volume of each component (protein, substrate, buffer) you need for each kinetic assay, add the buffer to the cuvette first, then substrate solution and finally mixed with protein solution in the cuvette. The volume for hydrogen peroxide solution is fixed at 200 μL and will be added in the end to initiate the reaction. Place the solutions in the cuvette, wait 3 min for it to reach the desired temperature. Make a new file for each kinetic assay and set the experimental parameters. When you are ready to start the reaction, press F7 to start the experiment while at the same time add the H_2O_2 solution, mixing the solution once or twice quickly with the syringe tip.

Analysis

Data transfer

The data can be extracted from the software by copying and pasting into an Excel spreadsheet. The spreadsheet can be transferred to your UNITY account using Secure Shell software. Secure Shell is a windows based program that is based on the LINUX sftp (secure file transfer protocol) command. You will find the Secure Shell 3.2.9 Icon on the desktop. You can set up the path for transfer using the software. The procedure is shown on the website using the screen shots of the SSH software.

Uploading the data into IgorPro

To upload data from an Excel spreadsheet into IgorPro the easiest method is the cut and paste method. You may open the Excel spreadsheet and select the rows and columns you would like to analyze. Then these values can be copied (<ctrl c>) and pasted (<ctrl v) into the table in IgorPro. When IgorPro is opened there is always a table presented as a default. When you paste the data into this table the data columns will have the labels wave0, wave1, wave2 etc. As long as you keep careful records there is no need to rename all of these columns. For example, if you have a typical spectroscopic data set with wavelengths from 400 – 600 nm, there will be 200 columns. It would be a bit painful to change the names of 200 data waves. If you do need to change a name of a wave you can do it on the command line of IgorPro.

- Rename wave0 time
- Rename wave1 lamda400

Note that IgorPro waves must have names that begin with a letter and not a number.

Plot all the time courses of the absorbance at the given wavelength. Select all the corresponding absorbance waves as the y axis and the only wave "time" as the x axis at Windows -> New Graph and go ahead to plot them.

Fitting the kinetic data using the method of initial rates

Igor has a number of standard fitting functions. The fit to a straight line is a standard fitting function. As you have learned, fits to a straight line are known as linear least squares fitting and there is a unique solution for the slope and intercept. In this problem the intercept is not important for the kinetic analysis, but the slope tells you how ΔA changes with time. Once you know this you will need to convert ΔA to $\Delta[P]$, the change in concentration. For this step you will use Beer's law.

To fit to a straight line you will need to plot the kinetic data and then use the cursors to select the first few points (6 to 10 points). The data are only linear over a very small range of time. Make sure that you have selected a short enough range that it is linear. However, you will need a minimum number of points to make the fitting meaningful. Experience suggests that 6 points is the minimum.

The procedure for plotting, selecting data with the cursors and defining the fitting function are given on the website. Use the website to guide you in this step. Record the values of your fit for each of your kinetic runs at each of your concentrations. Make a table with the following entries

	$\Delta A_1/\Delta t$	$\Delta A_2/\Delta t$	$\Delta A_3/\Delta t$	$<\Delta A/\Delta t>$	$\sigma(\Delta A/\Delta t)$
$[S]_1$	0.00478	0.00512	0.00499		
$[S]_2$	0.00839	0.00812	0.00806		
…					

Constructing and fitting the Michaelis-Menten plot

Once you have obtained the average values of the initial rate, $V_0 = <\Delta A/\Delta t>$, for each substrate concentration you will need to construct a plot of the initial rates vs. substrate concentration, V_0 vs. [S]. This is the Michaelis-Menten plot. The data in this plot will be fit using non-linear least squares fitting. In IgorPro this can be done by adding a macro to the software. IgorPro has a number of standard fitting functions for non-linear least squares fitting, but the Michaelis-Menten model is not one of them. You may use the macro below, which is available for download on the website. You will need to add these lines of text to the Procedure Window of IgorPro. When you close the Procedure Window it will automatically compile the macro and make it available in the Analysis menu. You may select this fitting function in that menu.

Non-linear least squares fitting differs from least squares in that an initial guess for the parameters is required. In the case of M-M fitting you will need to input the V_{max} and K_m values. How can you "guess" these values? It seems a bit tautological since the whole point of fitting is to obtain the values. In the case of M-M you can estimate V_{max} since that is the limiting value of the initial rate at large [S] concentration. You can inspect the plot and either simply use the largest value or perhaps a somewhat larger value based on your intuition of how much the curve is increasing over the observable range. Km can be estimating finding the value of [S] the corresponds to $V_{max}/2$, since $K_m = [S]$ gives $V_{max}/2$ in the M-M formula. You will still need to do the fitting in order to obtain accurate values of these parameters. The fitting menu allows you to input an estimated value of the standard deviation (also called the weight). When this is entered the fitting function will return a value of chi-squared, χ^2. In non-linear least squares fitting this is the figure of merit that is most frequently used to indicate the goodness of it. If the errors are properly estimated a good fit should have $\chi^2 = 1$.

SVD analysis of the spectral components of the data

Singular value decomposition (SVD) is a powerful method for global analysis of spectral data sets. The SVD method is a mathematical approach to decomposition of a data set in two dimensions (e.g. wavelength and time or wavelength and pH etc.) into orthogonal basis spectra.

Lab Report

The laboratory report for this protocol should include the usual sections, Abstract, Introduction, Materials and Methods, Results, Discussion, and Conclusion. The references should include some general considerations of Michaelis-Menten enzyme kinetics and other fundamental aspects. The relationship to the peroxidase reaction scheme should also be included. The field of peroxidase chemistry is quite large, and it is important to see the context of the study. Thus, references on kinetics studies of peroxidases as a comparison to the study here would be

considered appropriate. It would be appropriate to discuss how the peroxidase mechanism fits within the Michaelis-Menten kinetic scheme (and the assumptions needed for that adaptation). A well-known peroxidase enzymologist, Dr. Dunford has written that it is an egregious error to treat peroxidases using the Michaelis-Menten (M-M) scheme and yet he shows readers how to do it. What is meant by this error? Why is it that we still use the M-M scheme? Try to write a thoughtful report based on the literature that you can. Keep in mind that one recent piece of information comes from the book on "Heme Peroxidases" edited by Drs. Dunford and Raven.

Be sure to use the three replicates of the M-M curve to discuss the error analysis associated with fits of the linear date (method of initial rates) and the non-linear least squares fit of the M-M curve. We can recommend the use IgorPro to create nice figures with error bars. Please inquire if you are interested in this option. Excel is always acceptable for use in data analysis and for figure preparation. cm^{-1}

Discuss the method of initial rate and its use. Some kinetics applications use the integrated rate law. The M-M rate expression is not an integrated rate law. Why not use the integrated rate law? The answer is not necessarily a simple reason, but rather is based on both experimental expediency (i.e. what the data look like) and mathematical limitations (i.e. the mathematical form of the integrated rate law). Give some thought to these aspects and also see if you can find any information on this question in the literature.

References

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