Abstract

The rate constant for the formation of Compound II of horseradish peroxidase (HRP-II) was found using second-order kinetic conditions. Ascorbic acid was used as the reducing agent for HRP-II formation and reduction of HRP-II to the ferric HRP state. The rate constant for HRP-I formation was unable to be obtained due to the speed of the reaction making it impossible to monitor with the equipment available. The rate constant for the formation of HRP-II from HRP-I was also unable to obtained due to the complicated second-order conditions used making the curve of an absorbance against time plot difficult to fit as HRP-II continued on to form ferric HRP before all of the HRP-I was reacted to completion. The rate constant of the formation of ferric HRP from HRP-II was found to be $2.298 M^{-1}s^{-1}$ using isoenzyme C at room temperature and a pH of 7. This value is lower than published values using better peroxidase reducing agents as was expected since ascorbic acid is not as good of a reducing agent for peroxidases.

Introduction

Motivation

Chemical kinetics is a field of chemistry concerned with measuring and determining the rates of chemical reactions.[1] It is the study of what happens when molecules collide and form a new product, and how quickly this reaction happens. Understanding how and how fast a reaction occurs is very important. Our own lives depend on a balance of many reactions that go on in our bodies and determine the growth of animals and the plants that we eat. Without knowledge of chemical kinetics we wouldn't be able to create chemicals, such as pharmaceuticals, at an efficient rate, or design drugs that are appropriate for treating a particular ailment.

Kinetics can tell us much more about a reaction than just how fast it occurs. It can also give us an idea of the mechanisms of complex reactions.[2] Overall reactions take place in a series of elementary reactions, the simplest type of reactions. The study of kinetics allows the determination of these elementary reactions so that the complete mechanism of the whole reaction can be determined.

There are thousands of reactions happening within the cells of an organism at any time. These reactions do not occur very quickly on their own, so they need the help of enzymes to mediate them and lower their activation energy, allowing them to occur more rapidly and readily to meet the needs of the organism. In 1913, Lenor Michaelis and Maud L. Menten proposed the theory of enzyme kinetics that observes the Michaelis-Menten Equation, the fundamental equation used in the study of enzyme kinetics.[3]

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

The enzyme (E) does not suddenly turn the substrate (S) into the product (P), but must first form an enzyme-substrate complex as shown below.

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} E + P \qquad (2)$$

K_m is Michaelis-Menten constant and is defined as

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad (3)$$

In most enzyme-catalyzed reactions, the formation of the product is usually the rate-limiting step, which is the slowest step, so the Michaelis-Menten equation can be simplified to

$$K_m = \frac{[E][S]}{[ES]} \qquad (4)$$

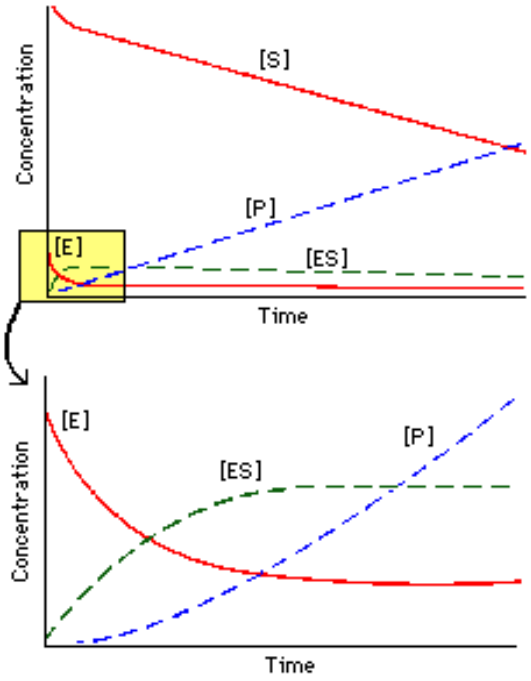
The Michaelis-Menten equation assumes that the concentration of the enzyme-substrate complex reaches a constant value very quickly in the system. In other words, the complex goes on to form the product of the reaction as soon as the enzyme and substrate form the complex. This is known as the steady-state assumption and is essential when studying Michaelis-Menten kinetics of enzymes. Mathematically, since the complex (ES) concentration will not change much under these conditions, we have:

$$\frac{d[ES]}{dt} = 0 \qquad (5)$$

The kinetics of the enzyme and the substrate before they reach this steady state of Michaelis-Menten kinetics are often complicated and can be of interest. These kinetics

are known as pre-steady state kinetics because they occur before the concentration of the enzyme-substrate complex reaches a constant concentration, that is before the relationship in equation (5) is observed. This period of time before the ES concentration is zero can be visualized as the highlighted region in Figure 1.

Figure 1- Pre-steady state enzyme kinetics[4]



Pre-steady state kinetics can allow for the postulation of possible intermediate reactions that occur during the process of forming the final products from the reactants. Since the study of steady-state kinetics only deals with observing how fast the reactants go to form the final product, it does allow for study of the intermediate reactions. Once the steady-state is reached, all of these intermediate steps are in equilibrium and their rates do not change.

Dr. Ann Kleinschmidt, a professor of biology at Allegheny College, works with many types of plant peroxidases that are isolated from various plants. Peroxidases are a

type of enzyme that reduce hydrogen peroxide (H_2O_2) to water (H_2O). These enzymes are found everywhere in nature, including in plants, animals, bacteria, and fungi.[5] They are heavily studied due to their commercial uses in diagnostic assays and their potential to be used as bioremedies.[6] Horseradish peroxidase is used to reduce phenols in pollutants from petroleum refineries, resin manufacturing plants, and coal processing factories.[7] Peroxidases are used for the preparation of enzyme conjugated antibodies, and can also replace many harsh chemicals, such as the carcinogenic formaldehyde in glues. Companies are looking into using peroxidases in the manufacturing of computer chips, car parts, and linings for cans.[7] They have also been implicated in a number of important biological processes, including cell wall synthesis and degredation, and stress response during oxidative stress.[7]

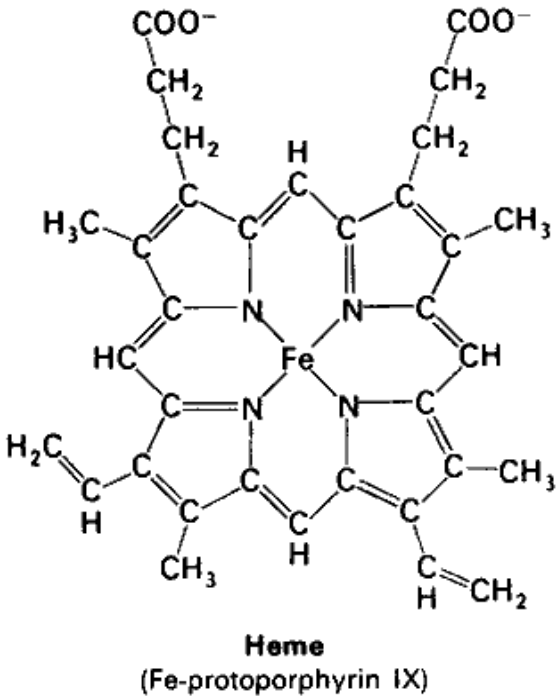
Kinetic studies of some of these ascorbate peroxidases have not been performed yet and would be of strong interest to Dr. Kleinschmidt in her further studies of the enzymes. Before the pre-steady state kinetics of the ascorbate peroxidases can be examined, a model for their study is needed so that these difficult to extract peroxidases are not wasted. Horseradish peroxidase (HRP) is an enzyme that has been highly studied and is very cheap and easy to access.. Though it is not an ascorbate peroxidase, it will still provide a useful model for understanding the procedures that can be used to study the costly ascorbate peroxidases. The knowledge of the kinetics of horseradish peroxidase has been used in the proposal of active site mechanisms that the enzyme uses.[5,20] If ascorbate peroxidase produced kinetic data similar to that of HRP, then it can be proposed that this enzyme follows a mechanism similar to those that have been proposed are used by HRP.

Theory

Peroxidases are classified according to the kingdom of organism in which they are found (animal and plant superfamilies) and their primary, secondary, tertiary, and quarternary structures. On the basis of amino acid sequence and presence of specific helices, there are three families of peroxidases within the plant superfamily.[8] Ascorbate peroxidases are Class I, which are peroxidases of prokaryotic origin. Class I peroxidases have no calcium ions, no cystine bridges, and no signal peptide for excretion. Their C-terminal end has a heme attached, but the N-terminal end does not. This is in strong contrast to horseradish peroxidase which is a Class III classical secretory plant peroxidase. Class III peroxidases contain two calcium ions, a signal peptide for excretion, and cystine bridges. These differences are what set ascorbate peroxidase (APX) apart from horseradish peroxidase. The structural differences between the two enzymes results in different specificities for substrates and differences in kinetic properties. The structure of HRP allows for a low specificity for ascorbate, while APXs have a low specificity for phenolic substrates.[9] Also, the first intermediate form of the enzyme in the accepted mechanism is much more stable for HRP than for APX.

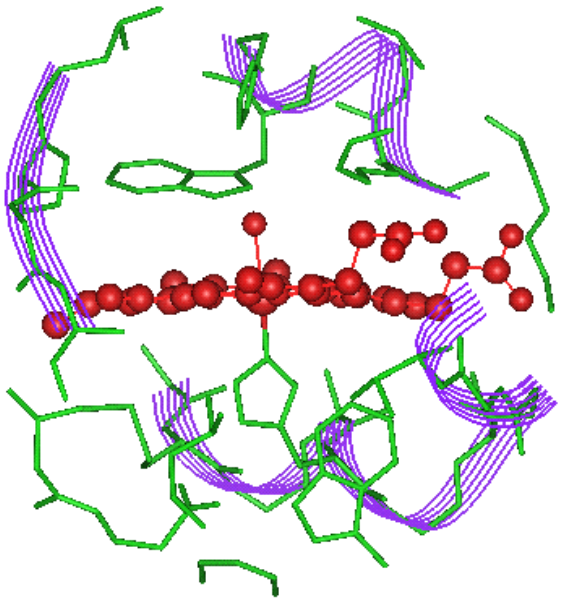
The heme group of nearly all plant peroixdases is ferriprotoporphyrin IX.[10] The structure of this heme is shown in Figure 2 below.

Figure 2- Structure of ferriprotoporphyrin IX[11]



Ferriprotoporphyrin IX has four pyrrole molecules joined by methene centered around a central Fe(III) molecule. Though in its inorganic compounds iron is typically six-coordinate, in its native resting ferric state, the Fe(III) is more likely five-coordinate. The pyrroles are located on positions 1-4 on the Fe(III). Position 5 is the proximal side of the heme and is occupied by a side chain of a histidine residue. This linkage between the histidine of the protein and the Fe(III) is what connects the heme to the peroxidase, along with hydrogen bonds between the propionate side chains of the heme and neighboring amino acids of the protein. Position 6 is the distal side and is where reactions with the peroxidases usually occur. The linkage of histidine to Position 5 and the availability of Position 6 for reactions can be seen in Figure 3.

Figure 3- Orientation of the heme in the peroxidase[12]



Work on ascorbate peroxidases didn't begin until 1989, so it is a newer member of the peroxidase family of enzymes.[13] It has been studied from chloroplasts, plant cystol, and root nodule sources thus far. It has become clear since its initial studies that ascorbate peroxidases have the purpose of catalyzing the reduction of hydrogen peroxide by ascorbate. This is shown below in Scheme 1.

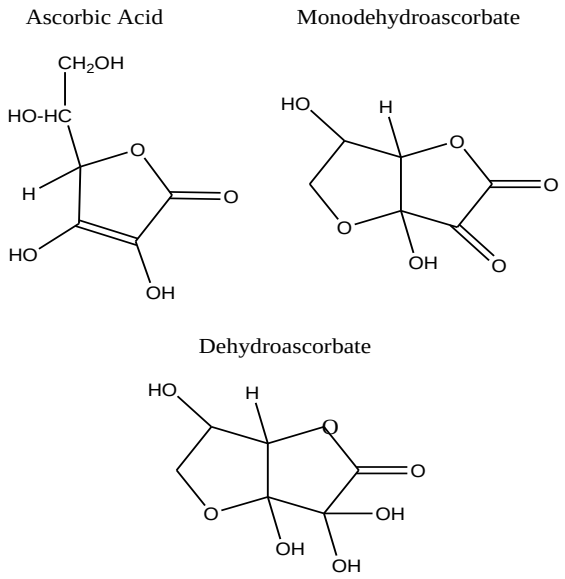
Scheme 1- Reaction of ascorbate with hydrogen peroxide

$$H_2O_2 + 2\ C_6H_7O_6 \rightarrow H_2O + 2\ C_6H_4O_6\cdot$$

Free radicals are highly reactive and unstable molecules that react with components of cells and cause damage to DNA, cell membranes, and mitochondria,

eventually lead to cell death.[14] This is known as oxidative stress since free radicals are usually good oxidizing agents. Hydrogen peroxide is produced when molecular oxygen oxidizes reduced enzymes and metabolites inside of a cell.[15] Mitochondria in cells can produce it as a byproduct of aerobic respiration. It can also be produced by peroxisomal β-oxidation of fatty acids.[16] Certain foods, such as chemically-altered fats from vegetable oils and vegetable shortening, contain free radicals that can enter the body when consumed.[17] When ascorbic acid is oxidized by these free radicals, it forms monodehydroascorbate. Monodehydroascborate is a fairly unreactive free radical, making it good for preventing oxidative damage in plants. The monodehydroascborate may be oxidized further by the free radicals to form dehydroascorbic acid. The structures of all three of these ascorbic acid derivatives are shown in Figure 4[18] below.

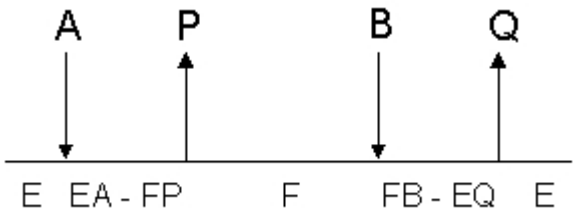
Figure 4- Different forms of ascorbic acid



Enzymes that react with two different substrates generally follow a double-displacement (ping-pong) mechanism. HRP and APX are such enzymes, as they react

first with hydrogen peroxide and then with a reducing substrate. This mechanism works by first binding the enzyme (E) to the first substrate (A) and releasing the product of the reaction (P) and a modified form of the enzyme (F). The modified enzyme then reacts with the second substrate (B) to form a second product (Q) and regenerate the original form of the enzyme (E).[19] The ping-pong mechanism can be represented as in Figure 5 seen below.[20]

Figure 5- Ping-Pong Mechanism



Peroxidase kinetics are similar to double-displacement kinetics.[21] The most commonly accepted general mechanism is represented in scheme 2.[4]

Scheme 2- General Peroxidase Mechanism

$$\text{Native Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{CompoundI} + H_2O$$
$$\text{CompoundI} + AH \xrightarrow{k_2} \text{CompoundII} + \cdot A$$
$$\text{CompoundII} + AH \xrightarrow{k_3} \text{Native Peroxidase} + \cdot A + H_2O$$

In this representation, AH is a reducing substrate and ·A is the corresponding free radical. Compound I and Compound II are the modified enzymes that form as intermediates during the reactions. The cycle of catalytic activity for peroxidases begins with a two electron transfer from hydrogen peroxide to the ferric native enzyme.[22] In the following

two reactions, a single electron is transferred from the reducing substrate to the intermediate forms of the enzyme in each of these steps. This returns the enzyme to its native ferric form.

The catalytic cycle of HRP has been heavily studied and is known to follow scheme 3.[5,7,8]

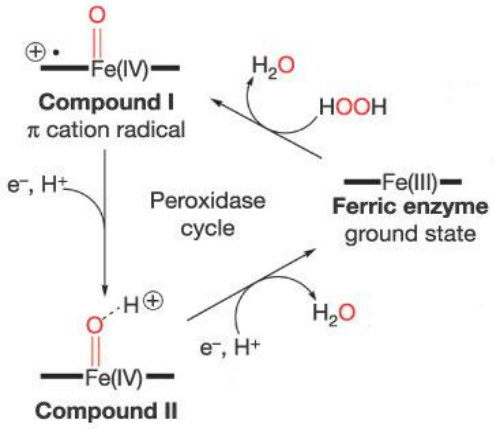
Scheme 3- Mechanism for HRP oxidation

$$HRP + H_2O_2 \xrightarrow{k_a} HRP - I + H_2O$$
$$HRP - I + RH_2 \xrightarrow{k_b} HRP - II + RH\cdot$$
$$HRP - II + RH_2 \xrightarrow{k_c} HRP + RH\cdot + H_2O$$

The cycle starts with a two electron transfer from the ferric Fe(III) state of HRP to form the HRP-I intermediate with its heme iron oxidized to the [Fe(IV)=0] ferryl state and a π-cation radical on the porphyrin ring.[23] The rate constant for the formation of HRP-I from HRP and hydrogen peroxide has been measured to be $1.7 \times 10^7 M^{-1}s^{-1}$ at room temperature and pH 7.0 for isoenzyme C .[4] The HRP-I then accepts an electron from a reducing substrate to neutralize the cation radical on the porphyrin ring, but the ferryl heme remains unchanged. This results in the formation of HRP-II. Kinetic studies have not been done on the k_b for the formation of HRP-II from HRP-I and ascorbate since ascorbate is not a good reducing substrate for HRP. When *o*-diphenols were used as the reducing substrate, k_b was determined to be $2.17 \times 10^3 M^{-1}s^{-1}$at room temperature and pH 7.0 for isoenzyme C.[4] Since phenols are much better reducing substrates for HRP than ascorbate, then one predicts that k_2 for the reaction of HRP with ascorbate will be much lower than $2.17 \times 10^3 M^{-1}s^{-1}$. HRP-II then accepts another electron from a reducing

substrate to return the enzyme back to its ferric Fe(III) state. When p-aminobenzoic acid (PABA) was used as a reducing agent, k_c was found to be $590 M^{-1}s^{-1}$ at room temperature and pH 7 for isoenzyme C.[24] As was the case for the k_b value, the value when using ascorbic acid should be lower than for the better peroxidase reducing agent p-aminobenzoic acid. The schematics of the structures of these modified hemes can be seen in Figure 6 below.

Figure 6- Peroxidase Cycle[17]

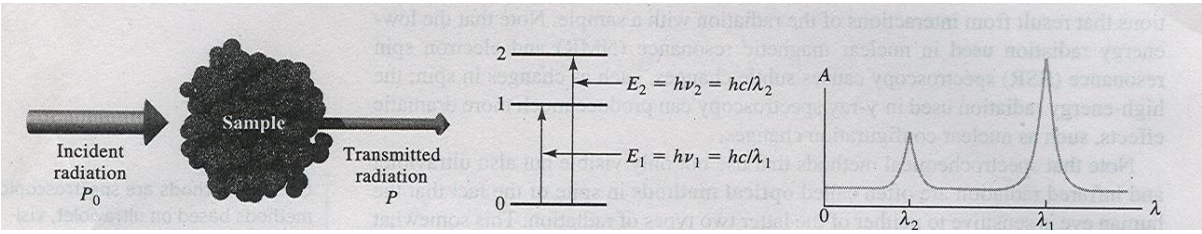


Each of these states of the enzyme has their own distinct absorption spectra. This allows them to be monitored with a stopped-flow spectrometer instrument which can detect changes in absorbance at a certain wavelengths over a period of time.

The absorption spectra of each form of the enzyme intermediates can be found using UV-visible absorption spectroscopy. The sample being studied is excited by light of ultraviolet and visible wavelengths, resulting in an excited state. The amount of

electromagnetic radiation that is absorbed due to the excitation of electrons is measured.[25] This is illustrated in Figure 7 below.

Figure 7- Absorption Spectroscopy[18]

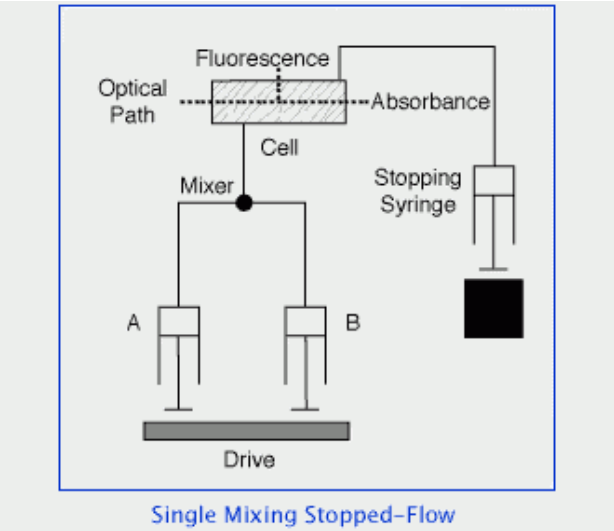


Beer's Law was valuable in this experiment as a tool for finding the exact concentrations of peroxidases, hydrogen peroxide, and reducing substrate that will used in the experiment. This was done by finding the molar absorptivity constants published by literature and using them to solve for concentration with Beer's Law. The absorption spectra of the different forms of the enzyme will also be useful in determining wavelengths where changes in absorption can be observed when the enzymes are being reacted. Heme peroxidases usually have a maximum absorbance around 407nm know as the Soret region.[26] Absorbance of light at this wavelength is due to the π-π^* transition of the porphyrin ring.[27] Violet light is absorbed, resulting in the orangish coloration of the HRP solution.

Any time two substances are reacted for kinetic study there is a dead-time between the time they are mixed and the time that reliable kinetic data can be measured. Reducing this dead time is essential when studying pre-steady state kinetics because the

two substances need very little time to reach their steady-state. The best way to reduce this dead-time is by using flow methods.[28] The most often used of the flow methods is the stopped-flow spectrometer. This method mixes two samples very rapidly and stops the flow of the mixture promptly. The reaction is then monitored slightly after the point where it is mixed to determine the progress of the reaction.[29] The set-up of this instrument is shown in Figure 8.[30]

Figure 8- Stopped-Flow Set-up



The samples to be mixed are placed in syringes A and B. The drive mechanism then pushes the plungers up rapidly to the mixer. Here the samples are mixed and moved instantly to the observation cell. The solutions are pressed into the system until the stopping syringe is filled, causing the flow to end promptly. In the observation cell, a light beam of desired wavelength is transmitted through the recently mixed samples to a photo-detector so that absorption measurements can me made. The quick movement of the mixture to the observation cell minimizes the dead-time to the order of 2-4 ms.[8] This makes the stopped-flow technique a good one to use when studying rapid enzyme

reactions where reactions occur in milliseconds. The faster the reaction, the shorter the dead time must be.

Rates of reaction can be described as the rate of decrease of a reactant concentration with time.[31] The general reaction shown below will be used as an example.

$$aA+bB \rightarrow P \qquad (6)$$

In this equation, a and b are the coefficients of the reactants in the balanced elementary reaction.

The rate of reaction for the decrease in reactant A would be described as the derivate

$$\frac{-d[A]}{dt} \propto [A]^m[B]^n \qquad (7)$$

where the square brackets indicate the concentration of the reactants, t is time, and m and n are the coefficients of the balanced elementary reaction. This is true only when the reaction is an elementary reaction. This proportionality of the rate of reaction to the concentrations of the reactants can be described by the constant of proportionality (k). k is also often known as the rate constant. Using this rate constant simplifies to:

$$\frac{-d[A]}{dt} = k[A]^m[B]^n \qquad (8)$$

The order of reaction is the sum of m and n.[32] If the order of reaction is two, such as it is for each reaction in scheme 2, and it is most likely that the reaction is an elementary one, then the reaction is bimolecular. If m=1 and n=1 then it is likely to be an bimolecular elementary step. However, m=2 and n=0, or m=0 and n=2, in which case it is definitely not an elementary step. It is difficult to determine the rate constant for a second order reaction because it requires the monitoring of the changes in concentration of both A and B simultaneously. One way to simplify this is to use an excess of B so that

its concentration stays relatively constant during the reaction and only [A] is changed.[33] This is a method known as flooding.[34] It works by changing the second order conditions into pseudo-first order conditions which are much easier to monitor. Since the concentration of B will not change, equation (8) can be simplified to

$$\frac{-d[A]}{dt} = k_{obs}[A] \qquad (9)$$

where k_{obs} is the observed rate constant which is related to the true rate constant, k, by

$$k_{obs}=k[B] \qquad (10)$$

Integration of equation (11) with the time limits being taken as 0 to the time between concentration limits $[A]_0$ and $[A]_t$ results in the equation

$$[A]=[A]_0e^{-kobs(t)} \text{ or } \ln([A]/[A]_0)=-k_{obs}t \qquad (11)$$

The changes in concentration of A can be found by using the stopped-flow spectrometer to monitor the changes in absorbance of the reaction mixture against time. Equation (11) can be fitted to the graph of the absorbance against time so that k_{obs} can be determined at various excess concentration of B when using pseudo-first order conditions. For our system, A=HRP and B=H_2O_2 and m and n are both expected to one based on previous work.

Once the k_{obs} are determined for several excess concentrations of B, then graphing the k_{obs} against the corresponding concentration of B will provide a linear relationship as shown in equation (10) with a slope of the true rate constant k. This can be seen by looking at equation (12) which relates k_{obs} to [B] by true rate constant k.

Both the horseradish peroxidase and the ascorbate peroxidases will have very large rate constants for all 3 intermediate steps because both are very rapid reactions. Also, the formation of the second peroxidase intermediate is expected to be the rate-

limiting step.[23] The relationship of absorbance to time has been seen to be double-exponential by some due to the binding equilibrium between the enzymes and the substrates.[23,36] Before the 1st step in scheme (3) is completed, the HRP and H_2O_2 first form a complex as shown in equation (12) below.

$$HRP+H_2O_2 \leftrightarrow HRP\text{-}H_2O_2 \qquad (12)$$

Since this reaction is reversible, HRP- H_2O_2 can revert back to their individual components as they do in equation (14) or this complex can go on to form HRP-I, both of these reactions will have an exponential dependence on time under pseudo-first order conditions which ultimately results in a double-exponential curve.

Experimental Plan

Horseradish peroxidase (HRP) isoenzyme C was purchased from Sigma-Aldrich and used without further purification. The concentration of the HRP was determined with an Ocean Optics spectrophotometer and the extinction coefficient for the Soret region of $102 mM^{-1}cm^{-1}$.[35] Hydrogen peroxide (35% v/v) was obtained from Sigma-Aldrich and its concentration was calculated using the published extinction coefficient of $\varepsilon_{240}=39.4 M^{-1}cm^{-1}$.[36] Ascorbic acid was obtained from Fluka (UK) and its concentration was determined using volumetric techniques to make the aqueous solutions used. E-pure water with conductivity better than 16MΩ-cm was used throughout the research.

Maximum absorbances and isobestic points for HRP, HRP-I, and HRP-II were determined with a Perkin-Elmer UV-visible spectrophotometer. An HRP solution was prepared by dissolving the enzyme in a 10μM pH 7 phosphate buffer. Mixing 1 molar equivalent of HRP with a 1 molar equivalent of hydrogen peroxide produced HRP-I. Mixing a 1 molar equivalent of HRP-I with a 0.9 molar equivalent of ascorbic acid produced HRP-II.

Pre-steady-state kinetics were monitored in a KinTek SF-E100 Stopped-Flow spectrometer. The data was recorded and analyzed on a PC running software provided by the manufacturer. The temperature was controlled at 25ºC with a Neslab Endocal bath with a heater-cooler.[35] A 4.24μM HRP solution was mixed with varying excess concentrations of hydrogen peroxide (10-100 fold) in the stopped-flow and the absorbance was measured against time at 421 nm where there is the greatest difference in absorbance between HRP and HRP-I as determined using the Perkin-Elmer UV-visible spectrophotometer.

Second order rate constants for the formation of HRP-II were determined by preparing an HRP-I solution by mixing a molar equivalent of HRP with a 1 molar equivalent of hydrogen peroxide and allowing a suitable time period of a few seconds to allow the HRP-I formation to fully occur. This HRP-I solution was then mixed with a molar equivalent concentration of ascorbic acid in the stopped-flow and absorbance was measured against time at the HRP-I absorbance peak of 419nm. Varying equal concentrations of HRP-I and ascorbic acid of 10, 8, 6, and 4μM were used to obtain multiple k_{obs} values.

Second-order rate constants for the formation ferric HRP from HRP-II were measured at the HRP peak absorbance around 402nm. Mixing a molar equivalent of HRP with a molar equivalent of hydrogen peroxide and 0.9 molar equivalent of ascorbic acid produced HRP-II. Equivalent concentrations of HRP-II and ascorbic acid were run through the stopped flow to obtain k_{obs} values. Multiple equivalent concentrations of HRP-II and ascorbic acid were used to obtain several k_{obs} values using the same concentrations as were used for the mixing of HRP-I and ascorbic acid.

The curve of absorbance against time was fitted to the first order integrated rate equation to obtain pseudo-first-order-rate constants (k_{obs}).[1]

$$A=Ce^{-kobs*t}+b \qquad (12)$$

In the equation, A is the absorbance change over time, C is a constant related to the initial absorbance, t is the time in seconds, b is the absorbance at $t=\infty$.

When second-order kinetics were used, equivalent concentrations of enzyme and substrate were used so that the rate of reaction would be equal to the product of the true

rate constant and the concentrations of the enzyme and substrate as shown in equations (13) and (14).

$$r=k[S][E] \tag{13}$$

Since equal concentrations of substrate and enzyme are being used then, the rate is equal to rate constant times the square of that equivalent concentration, which can be represented as c. The rate of reaction can then be represented by

$$r=\frac{k}{c^2}=\frac{dp}{dt}=\frac{-dc}{dt} \tag{14}$$

Integrating equation (14) from time 0 to time t gives the integrated second-order rate equation

$$A \propto c=\frac{c_0}{1+c_0 kt}+c_\infty=\frac{A_0}{1+k_{obs}t}+A_\infty \tag{15}$$

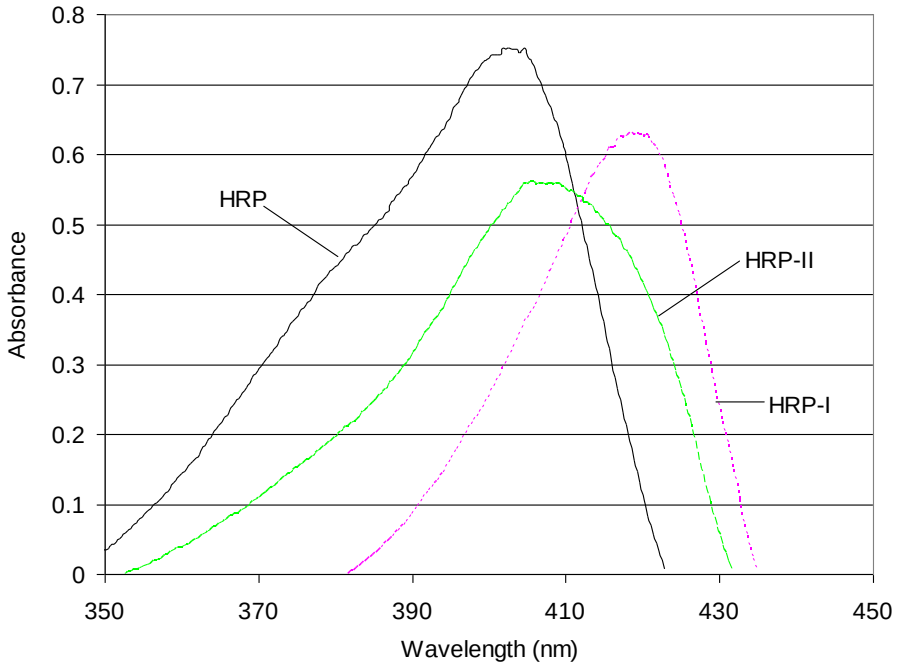
where c_0 is the initial concentration of substrate, $k_{obs}=c_o k$, A_0 is the initial absorbance, and A_∞ is the absorbance the sample approaches at time goes to infinity.

When using second-order conditions, fitting equation (15) to plot of absorbance against time provides k_{obs} values that are graphed against the varying concentrations of enzyme and substrate to find the true second-order rate constant.

Results and Discussion

The spectra of native horseradish, Compound I, and Compound II obtained using UV-Vis photospectroscopy are presented in Figure 7.

Figure 7- UV-visible spectra in the Soret region of HRP, HRP-I, and HRP-II

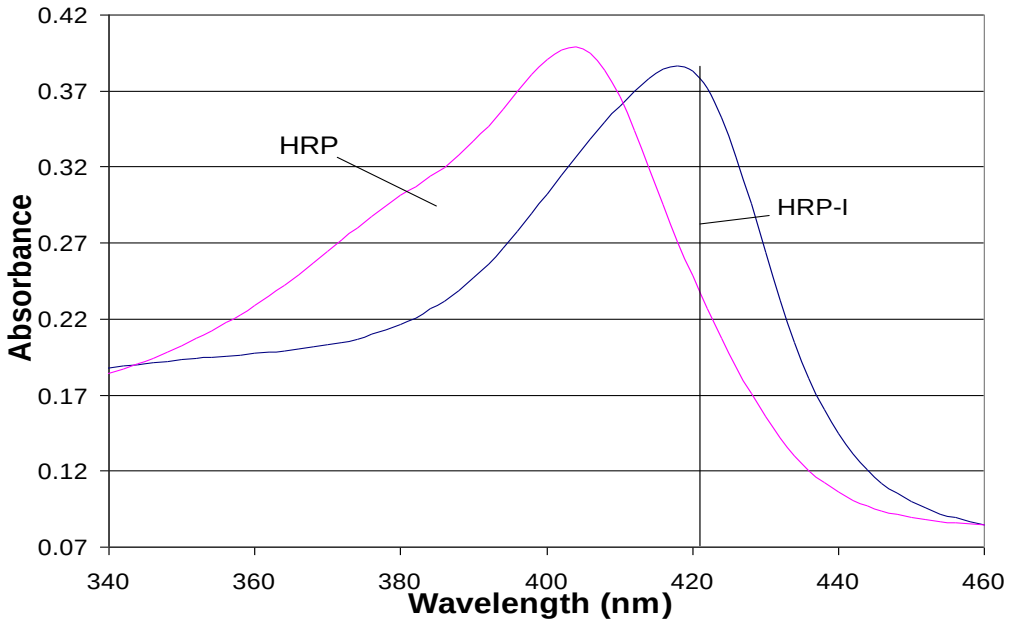


The maximum absorbance of native HRP is at 402.89nm, of HRP-I is at 418.87nm, and of HRP-II is at 407.99nm. The isobestic point of native HRP and HRP-I occurs at 411.89nm. The isobestic point of HRP-I and HRP-II occurs at 412.00nm, and that of HRP and HRP-II occurs at 410.17nm.

Observed pseudo-first-order rate constants ($k_{a,obs}$) for Compound I formation found by using a single exponential fit showed a linear dependence on the concentration of hydrogen peroxide. Significant change in absorbance was not found at the wavelength

of maximum absorbance of HRP (402.89nm). A separate set of spectra for native HRP and HRP-I were obtained from a Perkin-Elmer photospectrometer. These spectra are presented in Figure 8.

Figure 8- Spectra of HRP and HRP-I



These spectra indicate that the wavelength where there is greatest difference between absorbances of HRP and HRP-I is around 421nm. In practice, 418nm showed the greatest changes in absorbances when stopped-flow tests were run.

The plots of the absorbances against time for the varying concentrations of hydrogen peroxide at 418nm are shown in Figure 9.

Figure 9

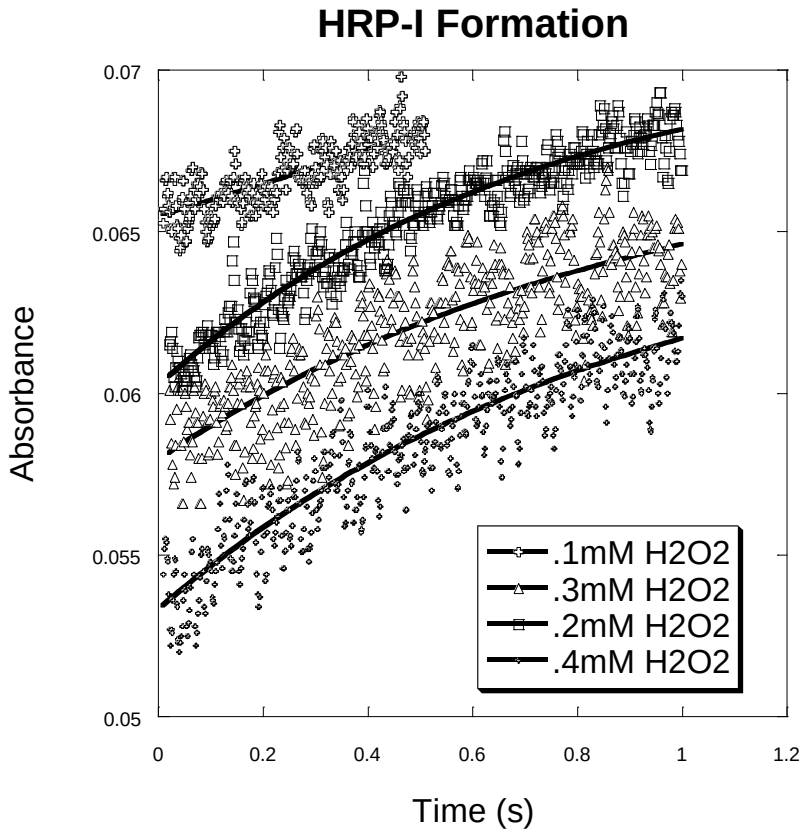


Figure 9: Stopped-flow time courses at 418nm for HRP-I formation. Pseudo-first order conditions were created by testing a 25-100-fold excess concentration of H_2O_2 against 4µM HRP. No significant change in absorbance was observed.

The change in absorbance for each of the concentration was less than 0.005 which is the limit of what can be measured. This indicated that no significant change in absorbance was observed using pseudo-first conditions. The experiment was repeated using second-order conditions. This method also did not display any significant change in absorbance over a 10 second course of time. A possible reason that no absorbance was observed could be because the reaction was too fast to be monitored by the stopped-flow apparatus.

The reaction might be occurring in the dead-time before the reaction is able to be monitored. This is possible as the published rate constant for HRP-I formation is very high (k_a=1.7x$10^7$$M^{-1}s^{-1}$).[4]

Reduction of HRP-II to ferric HRP by ascorbic acid was monitored using second-order kinetics instead of pseudo-first-order kinetics. The HRP-II solution and ascorbic acid solution were run through the stopped-flow at equal concentrations in order for the second-order kinetics to be used. Absorbance-time plots for ferric HRP formation are shown in Figure 11.

Figure 11

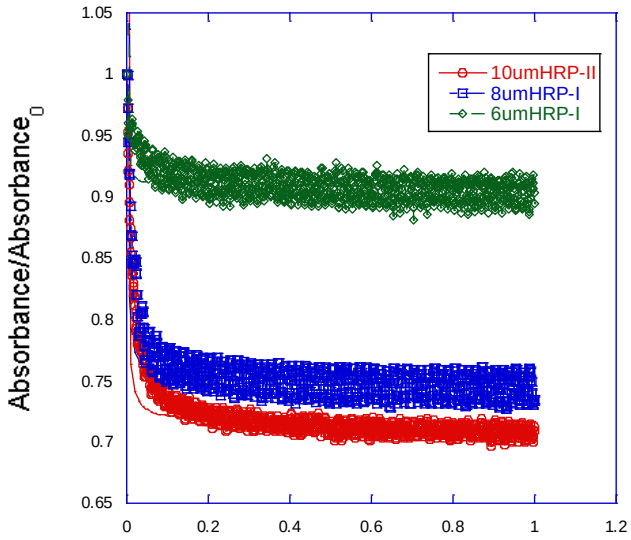


Figure 11: Stopped-flow time courses at 402nm for the reaction of HRP-II in 10μm phosphate buffer, pH 7.0, at room temperature. The curves were fitted to the second-order rate equation $\frac{A_0}{1+k_{obs}t} + A_\infty$ **in order to obtain the k_{obs} values. In each experiment, equal concentrations of HRP-II and ascorbic acid were used to create second-order conditions.**

A linear dependence of $k_{c,\,obs}$ on ascorbic acid concentration was observed. The graph $k_{c.obs}$ against concentration of ascorbic acid is presented in Figure 12.

Figure 12

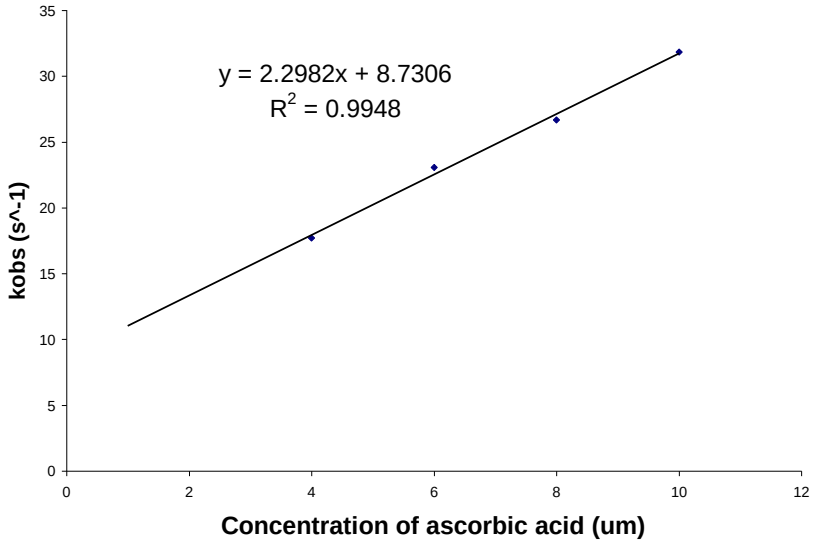


Figure 12: True rate constant for the formation of ferric HRP from HRP-II. The k_{obs} values obtained using the second-order conditions for 4, 6, 8, and 10µM ascorbic acid were graphed against their respective ascorbic acid concentrations.

The slope of the plot revealed a true rate constant of HRP formation from HRP-II of k_c=2.298$M^{-1}s^{-1}$ using isoenzyme C at room temperature in a 10µM ascorbic acid pH 7.0 buffer. This value is much smaller than that obtained from HRP-II reduction by p-aminobenzoic acid, which was k_c=590$M^{-1}s^{-1}$.[24] This was expected because p-aminobenzoic acid is a much better reducing agent for peroxidases than ascorbic acid.

The formation of HRP-II from HRP-I and ascorbic acid was also monitored using second-order conditions. Equal concentrations of HRP-I and ascorbic acid were run through the stopped-flow. The absorbance-time plots are displayed in Figure 13.

Figure 13

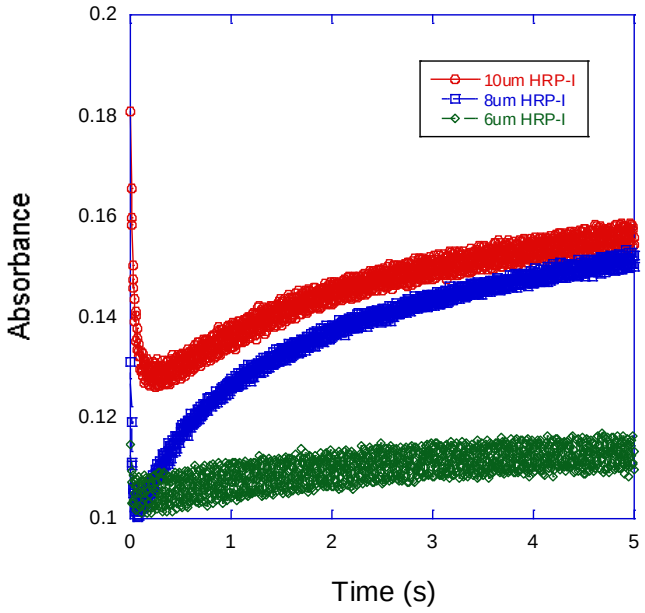


Figure 13: Stopped-flow time courses of HRP-II formation from HRP-I and equal concentrations of ascorbic acid at 419nm in a 10µM phosphate buffer, pH 7, at room temperature. The sharp decrease in absorbance between 0 and about .2s is due to the formation of HRP-II. The upturn in the absorbance is due to the formation of ferric HRP from the HRP-II before all the HRP-I was reacted completely. Because of this complexity, the data cannot be fitted to a second-order curve.

The resulting absorbance-time plot showed a sharp decrease in absorbance initially from HRP-II formation, but the absorbance then quickly began to increase following the formation of ferric HRP from HRP-II before all of the HRP-I was reacted completely. This created complications that make the data difficult to fit to a curve that would include k_b and k_c.

In order to demonstrate the complications of creating an equation to fit this curve, let the absorbance of ascorbic acid be represented by $A=a_0-x-y$, where a_0 is the initial absorbance, let HRP-I be represented by a_0-x-y also, as ascorbic acid and HRP-I are in

equal concentrations originally, HRP-II=x-y, and HRP=y. The two reactions observed in Figure (13) can be represented as

$$\begin{aligned} &HRP-I(a_0-x-y)+A(a_0-x-y)\xrightarrow{k_b}HRP-II(x-y)\\ &HRP-II(x-y)+A(a_0-x-y)\xrightarrow{k_c}HRP(y) \end{aligned} \qquad (16)$$

The concentration of ferric HRP is [HRP]=x-y, and the concentration of HRP-II is [HRP-II]=a_0-(x+y). The change in HRP concentration with respect to time could then be represented as

$$\frac{d(x-y)}{dt}=k_c(x-y)(a_0-(x+y)) \qquad (17)$$

and the change in (x+y) component of the HRP-II concentration with respect to time as

$$-\frac{d(x+y)}{dt}=k_b(a_0-(x+y))^2-k_c w(a_0-z) \qquad (18)$$

Mathematica 5.2 was unable to solve the integral for equation (18), so an equation to fit the absorbance-time plots in figure (13) was not possible.

Though it was not possible to obtain quantitative data for the formation of HRP-II from HRP-I, qualitatively figure (13) indicated from the sharp decrease in absorbance due to the HRP-II formation, and the slow increase in absorbance from HRP formation from the HRP-II, that the latter reactions is much slower, indicating that it is the rate-limiting step. This was expected, as in previous studies of horseradish peroxidase, the formation of HRP from HRP-II has had the lowest rate constant, making it the slowest, and therefore rate-limiting step.[24]

This study of horseradish peroxidase kinetics was unable to yield a k_a value because of the reaction occurring in the dead-time of the stopped-flow spectrometer. Although no quantitative data was found for the k_b value due to difficulties fitting a

proper equation to the absorbance-time plot, qualitatively it was determined that HRP formation from HRP-II and ascorbic acid was the rate-limiting step. Using second-order conditions, a k_c value of $2.298 M^{-1}s^{-1}$ was determined at room temperature using isoenzyme C of HRP in a 10μM ascorbic acid buffer, pH 7.0.

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