Oxidation Reactions Catalyzed by Horseradish Peroxidase

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1. Introduction

The objective of this investigation was to characterize the number of peroxidase units per mg of solid in a commercially available sample of horseradish peroxidase, and initiate a systematic investigation involving four distinct HRP-catalyzed oxidation reactions that are of potential interest for use in antibody-directed enzyme prodrug therapy (ADEPT).[1] In this study, indol-3-acetic acid[2], acetylacetone[3], acetaminophen[4], and diethylstilbestrol[5] were investigated as potential drug candidates for ADEPT. These oxidation reactions were investigated at 37^{o}C in 0.1 M phosphate buffer, pH 7.3, and as a function of substrate, enzyme, and hydrogen peroxide concentration.

2. Materials and Methods

4-Acetamidophenol (Acetaminophen), potassium phosphate monobasic (anhydrous), pyrogallol, peroxidase (Type VI from Horseradish), diethylstilbestrol, acetaminophen, and indole-3-acetic acid from sigma, hydrogen peroxide from Fisher, ethanol from AAPER alcohol and Chemical Co., and potassium hydroxide from Aldrich were used as received. Water was distilled, filtered and deionized prior to use (Milli-Q system from Millipore). Unless otherwise stated, the experiments were carried out at 37°C in potassium phosphate buffer 7.3.

Spectrophotometric studies were performed with a Shimadzu spectrophotometer model UV-2101 PC. Disposable plastic cuvettes were used for the characterization of HRP units, and double-chamber quartz cuvettes were used in experiments dealing with the characterization of peroxidase reactions with ADEPT prodrug candidates. In this last case, before starting the reaction by mixing the components present in the two distinct cuvette chambers, an initial spectrum of the sample was recorded. After that, the components of both chambers were mixed and data acquisition immediately initiated. Typically, the first chamber was filled with 1 ml of HRP solution of concentration twice as high as the concentration required for the reaction under investigation, and the second chamber with 1 ml of a solution containing the substrate, buffer, and H_2O_2 (when present) in concentrations also twice as high as the respective concentrations required for the reaction. Therefore, upon mixing the final concentrations were half of the initial concentrations while the optical path for each constituent of the system was made twice as long. Under these conditions, any spectroscopic change observed upon mixing of the components initially present in the distinct cuvette chambers represents evidence of occurrence of a chemical reaction. Buffer solutions were prepared with the help of a pHmeter model 520A from

Orion and a combination pH electrode model PerpHec ROSS from Thermo. Data treatment was done with the IGOR Pro Version 4.0 software from WaveMetrics, using a Dell computer Model Dimension 5150.

3. Results and Discussion.

3.1 Characterization of the number enzyme units per mg of solid found in a commercially available sample of peroxidase.

We have initially characterized the number of enzyme units per mg of solid present in a commercially available sample of horseradish peroxidase using the standard pyrogallol method.[6] Figure 1 shows the changes in absorption at 420 nm that take place when HRP catalyzes the transformation of pyrogallol into purpurogallin in the presence of H_2O_2. When using this standard method, one HRP unit is defined as the amount of enzyme required for the formation of 1.0 milligram of purpurogallin from pyrogallol in 20 seconds of reaction at pH 6.0 and 20 ^{o}C. Changes in absorption are measured using the maximum linear reaction rate, and the number of enzyme units calculated as previously described. For this method to work well, the reactions must be carried out under condition in which the rate of formation of purpurogallin depends only upon the HRP concentration (that is, under conditions of great excess of pyrogallol and H_2O_2).

The results presented in Figure 1 indicate that our experimental conditions were adequate. While the reaction rate was found to be highly dependent upon HRP concentration, a two-fold decrease in pyrogallol and H_2O_2 concentration had no effect on reaction rate. Using this method we have found that our HRP sample can be described as having 182.7 ± 1.8 units (standard pyrogallol units) per mg of solid. This information will facilitate the comparison of the results obtained in the present study with those obtained in future studies in which distinct samples of HRP will inevitably be used.

Because our studies involving oxidation reactions of substrates considered as candidates for use as prodrugs in ADEPT are carried out at pH 7.3, and not at the standard pH of the pyrogallol method (pH 6.0), we have compared the rate of reaction of pyrogallol with HRP under identical conditions but with the reactions run either at pH 6.0 or pH 7.3 (Figure 2). At pH 7.3 the pyrogallol reaction actually is faster then at the standard pH (6.0).

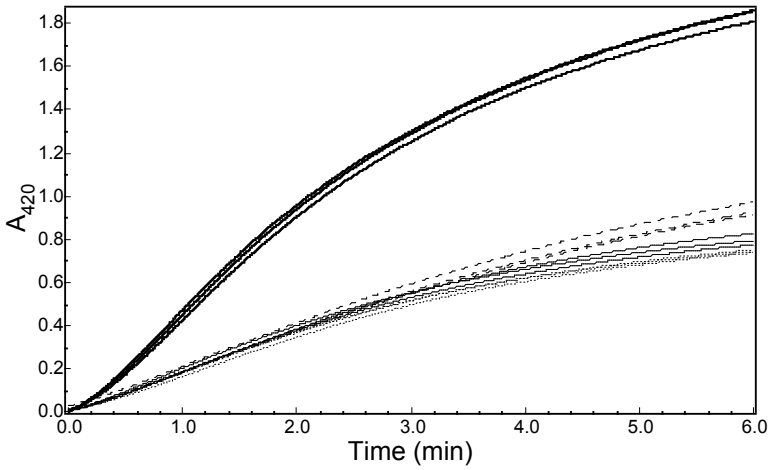

Figure 1. Changes in 420 nm absorbance as a function of reaction time for the standard reaction of oxidation of pyrogallol catalyzed by HRP. Measurements carried out in triplicates. Upper (solid) curves: [Pyrogallol] = 42 mM, [HRP] = 1.6 nM, [H_2O_2] = 8 mM. From the top of the lower set of (9) curves, and at 6 minutes of reaction: dashed lines, [Pyrogallol] = 42 mM, [HRP] = 0.8 nM, [H_2O_2] = 4 mM; solid lines, [Pyrogallol] = 42 mM, [HRP] = 0.8 nM, [H_2O_2] = 8 mM; dotted lines, [Pyrogallol] = 21 mM, [HRP] = 0.8 nM, [H_2O_2] = 8 mM. All experiments carried out at 20 ^oC in Pi buffer 11 mM pH = 6.0.

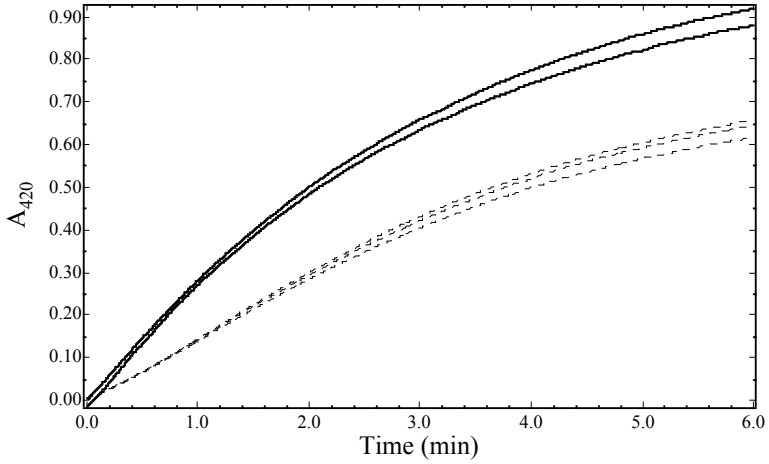

Figure 2. Changes in 420 nm absorbance as a function of reaction time for the standard reaction of oxidation of pyrogallol catalyzed by HRP. Reaction carried at pH 7.0 (upper, solid lines) and pH 6.0 (lower, dashed lines). Phosphate buffer 11 mM, [Pyrogallol] = 42 mM, [HRP] = 1.6 nM, [H_2O_2] = 8 mM, T = 20^oC.

3.2 Oxidation reactions catalyzed by HRP of potential interest for ADEPT.

Four oxidation reactions catalyzed by HRP that are of potential interest for use in ADEPT have been investigated. Figure 3 shows the spectroscopic changes that take place, as a function of reaction time, when the oxidation of diethylstilbestrol is catalyzed by HRP. Under the experimental conditions described in Figure 3 the rate of reaction depends upon the concentration of enzyme, substrate and hydrogen peroxide (data shown). The reaction does not occur when in the absence of H_2O_2 (Figure 3, inset). In this reaction, and under the experimental conditions described in Figure 3, no well defined band is observed before the mixing of all reaction components (dashed line), but the

band characteristic on native HRP with maximum at 403 nm. Immediately after the mixing of all reaction components, new absorption bands are noticed, with maxima around 310 nm and 350 nm, respectively. The spectroscopy changes shown in Figure 3 actually better represent the subsequent consumption of this (these) initially and rapidly formed reaction product(s) by the HRP/H_2O_2 system.

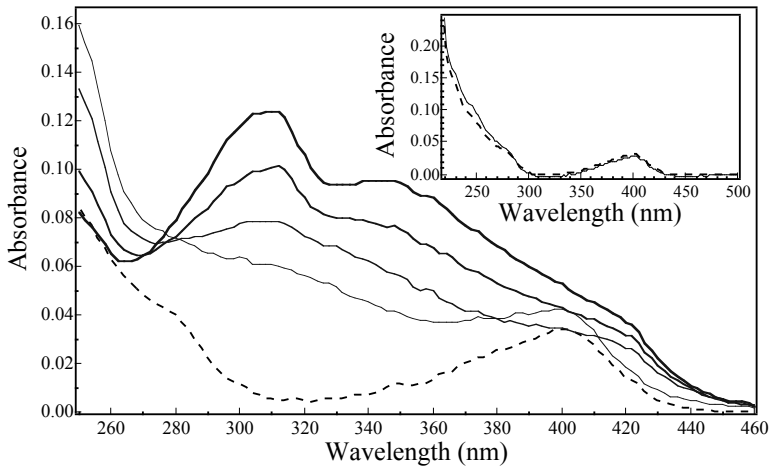

Figure 3. Spectroscopic changes associated with the oxidation of diethylstilbestrol catalyzed by HRP. Dashed line: before mixing the components present in the distinct chambers of the tandem uv-vis cuvette). Solid lines, from the top at 310 nm, the reaction times were (min): 1, 3, 10 and 30. T=37 ^oC, 0.1 M Pi buffer. [HRP] = 0.5 mM, [H_2O_2] = 250 mM, [diethylstilbestrol] = 100 mM. Inset. Changes in absorbance when the reaction is run in absence of H_2O_2. Dashed line: before mixing; solid line, 30 minutes after mixing.

Figure 4 shows the spectroscopic changes that take place, as a function of reaction time, when the oxidation of acetaminophen is catalyzed by HRP. Under the experimental conditions described in Figure 4, the absorption spectrum of native HRP is easily observed (band with maximum at 403 nm) before the mixing of all reaction components takes place (dashed line). After the mixing, the most direct evidence of occurrence of a chemical reaction is the appearance of a new absorption band with maximum in the 320 nm region. No reaction is observed in the absence of H_2O_2. Under the conditions described in Figure 4 the reaction rate depends upon the concentration of substrate and enzyme (data not shown).

Figure 5 shows the spectroscopic changes that take place as a function of reaction time when the oxidation of indole-3-acetic acid is catalyzed by HRP. Under the experimental conditions described in Figure 5, the absorption spectrum of HRP can be observed before the mixing of all components as a small band with maximum at 403 nm (dashed line). After the mixing, a shift to the red is observed in this HRP band, and the substrate is consumed (decrease in absorption in the 280 nm region) concomitantly with the appearance of a reaction product that absorbs in the 240 nm – 250 nm region. Under the conditions described in Figure five (e.g. [H_2O_2] = 500 mM) the reaction actually is basically

independent on H_2O_2 (Figure 5, inset). Virtually the same spectroscopic changes are observed both in the presence and in the absence of H_2O_2. Upon increasing H_2O_2 concentration from 500 mM to yyy mM in the reaction system, the reaction date appears to actually decrease (data not shown).

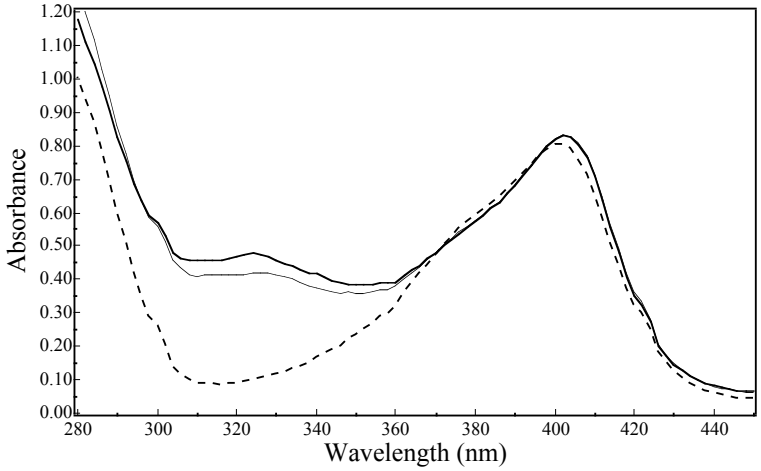

Figure 4. Spectroscopic changes associated with the oxidation of acetaminophen catalyzed by HRP. Dashed line: before mixing the components present in the distinct chambers of the tandem uv-vis cuvette. Solid lines, from the bottom at 320 nm, the reaction times were (min): 0 (data recorded immediately after mixing), 30. T=37 ^{o}C, 0.1 M Pi buffer. [HRP] = 0.5 mM, [H_2O_2] = 250 mM, [acetaminophen] = 100 mM. No spectroscopic changes have been observed in the absence of H_2O_2.

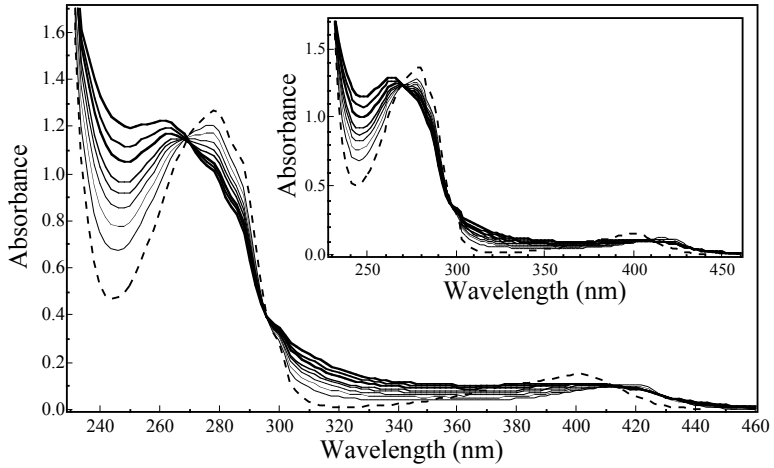

Figure 5. Spectroscopic changes associated with the oxidation of indol-3-acetic acid catalyzed by HRP. Dashed line: before mixing the components present in the distinct chambers of the tandem uv-vis cuvette). Solid lines, from the bottom at 245 nm, the reaction times were (min): 2, 4, 6, 8, 10, 15, 20, 37. T=37 ^{o}C, 0.1 M Pi buffer. [HRP] = 2 mM, [H_2O_2] = 500 mM, [indole-3-acetic acid] = 300 mM. Inset. Same reaction (and reaction times) carried out in the absence of H_2O_2.

Figure 6 shows the spectroscopic changes that take place as a function of reaction time when the oxidation of acetylacetone is catalyzed by HRP. Under the experimental conditions described in Figure 6, the absorption spectrum of HRP can be observed before the mixing of all components as a small band with maximum at 403 nm (dashed line). After the mixing, a shift to the red is observed in this HRP band, as the substrate is consumed as indicated by a decrease in absorbance in the 280 nm region. Small increases in absorption in the 240 nm region are also observed over the course of the reaction. Also in this case, and at least under the conditions investigated in this study, the reaction of oxidation of acetylacetone catalyzed by HRP does not appear to depend upon the presence of H_2O_2 in the reaction mixture (inset of Figure 6).

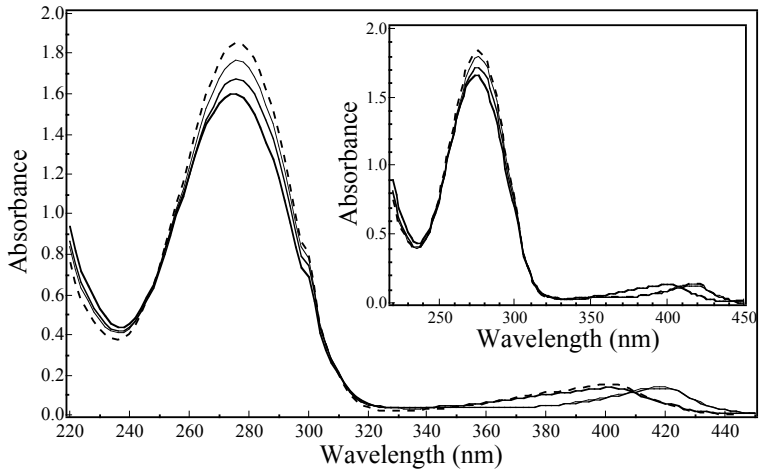

Figure 6. Spectroscopic changes associated with the oxidation of acetylacetone catalyzed by HRP. Dashed line: before mixing the components present in the distinct chambers of the tandem uv-vis cuvette). Solid lines, from the top at 275 nm, the reaction times were (min): 3, 20, 30. T=37 ^{o}C, 0.1 M Pi buffer. [HRP] = 2 mM, [H_2O_2] = 500 mM, [acetylacetone] = 1000 mM. Inset. Same reaction (and reaction times) carried out in the absence of H_2O_2.

4. Conclusions.

We have searched for and found experimental conditions that can be seen as appropriate for the study of four HRP-catalyzed reactions involving prodrug candidates for use in ADEPT. The oxidations of two of these candidates, diethylstilbestrol and acetaminophen, are clearly H_2O_2 dependent processes, while the respective reactions of two other candidates, acetylacetone and indole-3-acetic acid apparently do not require H_2O_2 to occur. Although the presence of peroxide contaminants is always a possibility when HRP-catalyzed reactions are found to occur in the absence of H_2O_2, compelling evidences have previously described in the literature pointing to the somehow surprising fact that fact the reactions of oxidation of both acetylacetone[3] and indole-3-acetic acid[2,7,8] catalyzed by peroxidase are indeed independent of H_2O_2.

5. References.

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