Protecting Heme Enzyme Peroxidase Activity from H 2 O 2 Inactivation by Sol–Gel Encapsulation †

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Notes

Protecting Heme Enzyme Peroxidase Activity from H_2O_2 Inactivation by Sol−Gel Encapsulation†

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Introduction

The heme enzymes horseradish peroxidase (HRP) and metmyoglobin (metMb) both exhibit peroxidase activity[1−3] (catalytic oxidation of substrates by peroxides). The reaction of hydrogen peroxide with both proteins oxidizes the heme iron to the ferryl [Fe(IV)═O] state and produces either a porphyrin cation radical[4−6] (compound I) or transient protein radicals[3,7,8] (intermediate I) or both in the reactive intermediate. Each of these ferryl intermediates produced by reaction of the enzyme with H_2O_2 is capable of performing one-electron oxidations on two substrate molecules, regenerating the ferric heme resting state of the enzyme. The peroxidase reactions of these enzymes are shown below in Schemes 1 and 2 with S representing the substrate molecule:

Scheme 1 (HRP)

$$\text{HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + S \rightarrow \text{Compound II} + S^{+\bullet}$$

$$\text{Compound II} + S \rightarrow \text{HRP} + S^{+\bullet}$$

Scheme 2 (Myoglobin)

$$\text{metMb} + H_2O_2 \rightarrow \text{Mb Intermediate I} + H_2O$$

$$\text{Mb Intermediate I} + S \rightarrow \text{Mb Intermediate II} + S^{+\bullet}$$

$$\text{Mb Intermediate II} + S \rightarrow \text{metMb} + S^{+\bullet}$$

Oxidation of either HRP or metMb by peroxide in the absence of substrates, or in the presence of large H_2O_2/substrate concentration ratios, leads to an inactivation of these enzymes.[1,3,9−13] Inactivation of HRP is thought to occur by reaction of the oxidized ferryl intermediate with an additional molecule of H_2O_2;[11] this reaction yields the nonfunctional verdohemoprotein P-670.[14] Inactivation mechanisms of metMb by excess H_2O_2 are poorly understood, with both intramolecular and intermolecular radical transfer reactions proposed.[15] Hydrogen peroxide-treated metMb solutions have been shown to oxidize and damage other proteins.[15−17] Additionally, when pure metMb solutions are treated with excess peroxide, loss of the heme chromophore is concurrent with inactive protein aggregate formation, presumably via intermolecular radical cross-linking reactions.[18]

Intermolecular protein−protein interactions can be minimized by sol−gel immobilization in a silicate matrix. Encapsulation of enzymes through the solution−sol−gel process yields an optically clear matrix with pores large enough to allow diffusion of reactants to the enzyme but small enough to prevent the enzyme from either escaping the matrix or interacting with other enzyme molecules within the matrix.[19] The peroxidase activity of sol−gel-encapsulated HRP has been demonstrated previously,[20] but that of metMb has not been reported.

Inactivation of HRP and metMb by excess peroxide during their reactions with substrates results in a lower observed substrate turnover number, N_s.[11,12] (N_s is defined as the number of molecules of substrate oxidized per number of enzyme molecules present in the presence of excess substrate and peroxide; this is potentially twice the number of times that the enzyme cycles through its oxidized intermediates.) To determine the effect of sol−

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gel encapsulation on the peroxidase substrate turnover number of HRP and metMb, the proteins were encapsulated in a sol−gel matrix and reacted with a substrate in the presence of various amounts of hydrogen peroxide. These results are compared with the findings from identical reaction conditions of the proteins in solution.

Experimental Section

HRP 1-C was purchased from Tyobo (Osaka, Japan) and used as supplied (Rz = 3.06). Horse skeletal muscle myoglobin (90% pure) was acquired from Sigma (St. Louis, MO); it was purified by gel filtration chromatography (Sephacryl S-100 HR) before use. The tetramethoxysilane and methyltrimethoxysilane were supplied by Aldrich Chemical Co. (Milwaukee, WI). Hydrogen peroxide (30% solution in water) and sulfuric acid were obtained from Fluka (Milwaukee, WI). The peroxidase substrate (1-Step Turbo TMB) was provided by Pierce Chemical Co. (Rockford, IL). [The 1-Step Turbo TMB contains both the substrate (3,5,3′,5′-tetramethylbenzidine) and buffered hydrogen peroxide.]

The silica sol−gels were prepared using a procedure similar to one described previously.[19] Tetramethoxysilane (13.6 g), methyltrimethoxysilane (2.0 g), deionized and distilled water (3.5 g), and 0.04 M HCl (0.22 g) were combined and placed in an ultrasonic bath at room temperature until one phase was observed. This sonicated mixture was thoroughly combined with equal parts of 0.01 M phosphate buffer (pH 7.0); 40 μL of the silane/buffer mix was combined with 10 μL of a protein solution (dissolved in 0.01 M phosphate buffer, pH 7.0) in the wells of a 96-well microplate. (The use of microplates for both the production of the sol−gels and conducting the reaction allowed many replications to be easily performed.) Thin metMb-containing sol gels were prepared by adding ≥2 mL of the silane/buffer/protein mix to 6-well cell culture plates; after aging and drying they were cut (ca. 8 × 20 × 4 mm) to fit into a standard 1-cm cuvette. All of the sol−gels were aged (2 weeks) and dried (1 week) at 4 °C.

The peroxidase reactions were monitored with a BioRad Benchmark microplate reader (Hercules, CA). Enzyme-catalyzed oxidation of the substrate (TMB) was accomplished by adding 100 μL of either the commercial peroxidase substrate solution (≤0.03% H_2O_2) or the substrate solution fortified with an additional 0.5% H_2O_2 to the microplate wells containing the protein sol−gels or protein solutions. Substrate solutions were incubated with the enzymes containing known amounts of protein at 37 °C until the reaction had stopped. Peroxidase-catalyzed oxidation of TMB results in several products that coexist in equilibrium;[21] addition of 100 μL of 2 M H_2SO_4 to the reaction solution shifts the equilibrium to a single colored product that absorbs light at 450 nm.

The number of substrate molecules oxidized by each solution or sol−gel (containing a known concentration of enzyme) was determined from the absorbance measured by the microplate reader and the molar extinction coefficient[21] of the product. Any contribution of nonenzymatic TMB oxidation was removed by using a blank consisting of the substrate added to either a sol−gel or solution containing no protein. The number of enzyme molecules present during the reaction was calculated from their respective molar extinction coefficients in solution; for purposes of this calculation it was assumed that sol−gel encapsulation did not denature any of the enzymes. N_s was calculated as the number of molecules of substrate oxidized per number of enzyme molecules present per unit volume in the presence of excess substrate and peroxide. The observation that N_s is determined by enzyme inactivation, and not substrate depletion, was confirmed by treating some of the enzyme solutions and sol−gels with additional substrate solution before the addition of acid. The results of 16 replicate experiments were used to calculate N_s.

Inactivation of metMb by H_2O_2 was measured with a Hewlett−Packard model 8452A diode array spectrophotometer. The sol−gel-encapsulated myoglobin was immersed in a 0.01 M sodium phosphate buffer, pH 6.0, containing 0.5% H_2O_2. The metMb was reacted with H_2O_2 in a solution of 0.01 M phosphate buffer, pH 6.0, to which enough 35% H_2O_2 had been added to bring the final peroxide concentration to 0.5%. These reactions were done at 20 °C.

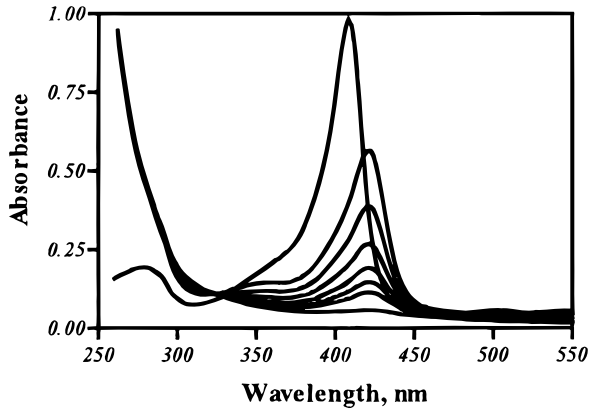

Figure 1. Decay of the Soret chromophore as metMb is reacted with H_2O_2 in solution. (The red-shift of the transition is assigned to a ferryl intermediate.[18]) A precipitate of polymerized myoglobin also forms as the reaction proceeds. The spectra shown were taken 0, 10, 60, 120, 220, 280, and 500 s after the addition of peroxide.

Results and Discussion

The results of the observed substrate turnover number are summarized in Table 1. Under relatively low H_2O_2/substrate ratio conditions (≤5), sol−gel immobilization made no measurable difference to N_s relative to the enzyme reactions in solution. However, when H_2O_2/substrate ratios were large (≥50), encapsulation in the silicate matrix preserved the activity of the enzymes under conditions that lead to enzyme inactivation when in solution.

Figure 1 shows the spectra of metMb in solution at different times after the addition of excess H_2O_2 (0.5%). The characteristic red-shift and decrease in the intensity of the Soret band are observed for formation of ferryl intermediates[18,22] 10 s after addition of the peroxide. Within 5 min the Soret transition disappeared and the solution became cloudy because of protein aggregate formation.

Figure 2 shows the spectra of sol−gel-encapsulated metMb at different times after immersion in a 0.5% H_2O_2-buffered solution. Interestingly, the rate at which the protein's Soret transition disappears is much slower than it is in solution. Also, the red-shift of the Soret, characteristic of the ferryl intermediate,[18,22] is not observed in the encapsulated system. The sol−gel remained clear (i.e., no aggregate formation) even after 24 h in the peroxide solution.

The observed substrate turnover number data for HRP and metMb show that encapsulation in a silicate matrix protects the enzyme at relatively high H_2O_2 concentrations. Because the values for N_s from the sol−gel HRP experiments are similar at the two peroxide concentrations, it is possible that encapsulation greatly inhibits the reactions that inactivate this enzyme in solution. For metMb, a protein more susceptible to peroxide inactivation, encapsulation actually increases N_s. The stabilization of the sol−gel-immobilized metMb with respect to H_2O_2 in the sol−gel matrix may be due to a change in the structure of the reactive intermediate as evidenced by the difference in the spectra of sol−gel and solution peroxide-treated protein.

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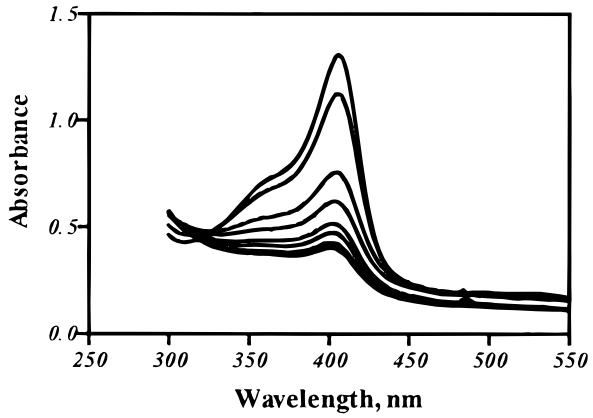

Figure 2. Decay of the Soret chromophore as metMb is reacted with H_2O_2 in a sol−gel. The red-shift associated with a ferryl intermediate is absent and no protein precipitate forms. The spectra are taken 0, 15, 30, 60, 90, 120, and 180 min after the addition of the peroxide. The sol−gel matrix absorbs the light at $\lambda < 300$ nm.

Table 1. Peroxidase Substrate Turnover Numbers of Heme Enzymes in Different Matrices and Peroxide Concentrations

enzyme	matrix	H_2O_2/substrate < 5	H_2O_2/substrate > 50
HRP	solution	307 ± 11	18 ± 5
HRP	sol−gel	290 ± 16	316 ± 19
myoglobin	solution	41 ± 6	4 ± 2
myoglobin	sol−gel	34 ± 9	52 ± 4

Conclusion

Generally, when enzymes are encapsulated in a sol−gel they lose some of their activity. We have observed up to a 15 and 25% loss in the heme chromophore intensity of HRP and metMb respectively, upon drying 2 weeks in unaged sol−gels.[23] This chromophore intensity loss may be due to denaturation of some of the protein during the sol−gel production process. The inactivation of some portion of enzymes when encapsulated in a sol−gel results in a reduction of the number of substrate molecules that can be turned over. This may explain why the turnover numbers are lower for sol−gel-encapsulated enzymes when H_2O_2/substrate <5 in Table 1. As the substrate turnover numbers calculated in this paper were done with the assumption of no enzyme loss, the protective effects of encapsulation on peroxide inactivation protection are actually greater than stated.

The effect of sol−gel immobilization on the peroxidase activity of these heme enzymes demonstrates the usefulness of protein encapsulation in the study of biological systems where protein−protein interactions are important. Additionally, because sol−gel encapsulation of peroxidase enzymes retards H_2O_2 inactivation, this technique may find uses in systems where peroxidases are used to degrade wastes.[24]

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