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Oxidation of Dibenzothiophene Catalyzed by Heme-Containing Enzymes Encapsulated in Sol-Gel Glass

A New Form of Biocatalysts

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ABSTRACT

We have encapsulated several hemoproteins in the sol-gel glass to catalyze the oxidation reaction of dibenzothiophene (model for organic sulfur compounds in coal) with hydrogen peroxide. In addition to cytochrome c and myoglobin, which have previously been encapsulated in sol-gel glasses, two other hemoproteins, horseradish peroxidase and bovine blood hemoglobin, have now been successfully immobilized in sol-gel media with the retention of their spectroscopic properties. All four hemoproteins studied also demonstrate similar catalytic activities toward the oxidation of dibenzothiophene as compared with the results obtained with the proteins in solution. In the case of encapsulated cytochrome c, the more water-soluble S-oxide was obtained with much higher selectivity over the less water-soluble sulfone (S-oxide/sulfone = 7.1) as compared to what was obtained in the aqueous/organic medium (S-oxide/sulfone = 2.3). Because of the advantage of easy separation of the encapsulated proteins from the liquid reaction mixture, it is clear from these studies that the immobilization of active hemoproteins in the solid glass media could serve as more practical biocatalysts.

Index Entries: Dibenzothiophene; biooxidation; biocatalyst; encapsulation; sol-gel glass, hemoglobin; cytochrome c; horseradish peroxidase; myoglobin.

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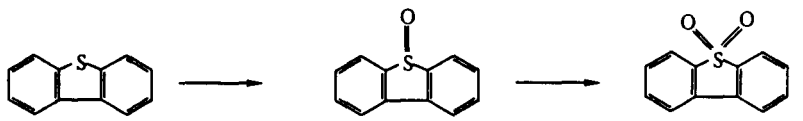
INTRODUCTION

The hemoproteins catalyze the oxidation of the organic sulfides at the expense of hydrogen peroxide (*1–6*). Organic sulfur-containing compounds from the combustion of coal represent an important class of environmental chemicals, whose presence often causes poisoning of catalysts, corrosion of surfaces, and air pollution. Biocatalytic sulfoxidation often produces products with increased water solubility and enhanced reactivity, which can then be readily removed from the substrate, e.g., coal.

Among the proteins studied, it has been shown that chloroperoxidase, lactoperoxidase, and horseradish peroxidase catalyze the oxygenation of benzyl methyl sulfide, thioanisole, and thiobenzamide to their respective sulfoxides (*2–6*). A very recent study has demonstrated that dibenzothiophene can be oxidized to form its S-oxide and sulfone (S-dioxide) by hydrogen peroxide in the presence of horseradish peroxidase, cytochrome c, and bovine blood hemoglobin, and so on (*1*).



DIBENZOTHIOPHENE DIBENZOTHIOPHENE--S-OXIDE DIBENZOTHIOPHENE-S-DIOXIDE

Dibenzothiophene is a widely accepted model compound for organic sulfur in coal because of its poor water solubility ($< 10\ \mu M$) and the difficulties associated with its transformation into other products by chemical methods (*1*).

Recently, successful encapsulation of a series of proteins in sol-gel glass has been reported (*7–9*). It was shown that the encapsulated biomolecules retained their characteristic enzymatic activities inside the glass matrix. These studies have provided excellent prospects for the use of these solid state composite materials containing biochemically active macromolecules as potential bio-devices, such as biosensors or biocatalysts.

In this paper, we report the oxidation of dibenzothiophene catalyzed by cytochrome c, horseradish peroxidase, hemoglobin, and myoglobin encapsulated in a solid sol-gel glass matrix. It is found that all four encapsulated hemoproteins possess similar catalytic activities as compared with the proteins present in solution. The apparent advantages of using the encapsulated proteins are easy separation of the catalyst from the liquid reaction mixture as well as stabilization of the catalysts in the glass matrix. This work should extend the use of hemoproteins as catalysts for sulfoxidation to more practical applications. It also offers the exciting possibility of encapsulated biomacromolecules as the potential biocatalysts.

EXPERIMENTAL

Materials

Horseradish peroxidase (type II), bovine blood hemoglobin, bovine heart cytochrome c, and bovine heart myoglobin were all purchased from Sigma Chemical Co. (St. Louis, MO). Dibenzothiophene, dibenzothiophene S-oxide, and dibenzothiophene sulfone, tetramethoxysilane (TMOS), acetonitrile (HPLC grade), ethanol (spectrophotometric grade) were supplied by Aldrich Chemical Co. (Milwaukee, WI). Hydrogen peroxide (30% solution in water) was obtained from Fisher Scientific (Pittsburgh, PA).

Apparatus

Dibenzothiophene and its oxidation products were analyzed by a Shimadzu LC-6A high performance liquid chromatograph (HPLC) equipped with a SPD-6AV UV-Vis absorbance detector at 230 nm. A Vydac C_{18} column (10 × 200 mm) was used for separation. An acetonitrile-water mixture was used as the mobile phase and the flowrate was set at 2 mL/min. A linear gradient of acetonitrile-water (40:60–60:40 for 8 min; 60:40–80:20 for 4 min; 80:20–100:0 for 2 min, and held for 10 min to obtain the peak of dibenzothiophene) was used for separation. In a typical chromatogram, the retention times for dibenzothiophene-S-oxide, dibenzothiophene sulfone, and dibenzothiophene was found to be 10.55, 12.32, and 20.27 min, respectively. Products were identified with authentic samples purchased from Aldrich.

Absorption spectra of proteins were recorded on a HP-8452A UV-Vis diode array spectrophotometer.

Procedure

Encapsulation of the Hemoproteins in Sol-Gel Matrix

The silica sol was prepared following the procedure described previously (7) by sonication of tetramethoxysilane (TMOS, Aldrich, 15.25 g), deionized water (3.38 g), and 0.04*M* HCl (0.22 g) in an ice-cooled ultrasonic bath for 30–40 min. The silica sol (0.4 mL) was mixed with different concentrations of proteins (0.6 mL) in 10 m*M* sodium phosphate buffer, pH 7.0 for the encapsulations. The silica sol was also mixed with phosphate buffer only to provide the baseline control for the optical spectroscopic measurements. The hemoproteins sol was quickly mixed in polystyrene microcuvets and then stored at 4°C for aging and drying. The aging usually takes approx 2 wk. After aging, the samples were exposed to air for drying at 4°C. Usually 95% of the water was removed from the samples. Typically, an additional 2–3 wk were needed for the completion of the drying process. Thus, the age of the samples used in our studies approached 4–5 wk.

Oxidation of Dibenzothiophene

The following reaction conditions were chosen (*1*). The solvent system consisted of 25% (v/v) ethanol/water buffered at pH 5.2 (10 m*M* sodium acetate and 10 m*M* sodium phosphate). The pH of 5.2 has been shown to be optimal for the oxidation reaction under consideration in this work. In a typical experiment, 25 μM protein, 250 μM dibenzothiophene, and 10 m*M* hydrogen peroxide were employed. For the encapsulated proteins, the reaction conditions were the same except that the encapsulated proteins, which were prepared at the same concentration, were premoistened (to prevent cracking of the samples) and then immersed in the reaction mixture under constant stirring. The reactions were mostly carried out at 30°C. For the time course measurements the reactions were performed at room temperature (22°C) in order to prolong the reaction time. A 50-μL aliquot of the reaction mixture was taken at different times. The reaction was first quenched at liquid N_2 temperature before the reactants and products were separated by HPLC. Usually it took less than 2 min for the reaction mixture to warm up before the HPLC separation.

RESULTS AND DISCUSSION

Immobilization of Hemoproteins in Sol-Gel Glasses

The optical absorption spectra of cytochrome c and myoglobin encapsulated in sol-gel glasses in either aqueous buffer solution (a) and in encapsulated sol-gel glass (b) are compared in Fig. 1. No significant changes in the spectroscopic properties of the proteins were observed as a result of the encapsulation, in agreement with the results reported previously (*7*). As an indication of the chemical activity of these two proteins in the glass matrix, encapsulated ferricytochrome c, and myoglobin were found to form ferrocytochrome c and deoxymyoglobin, respectively, when these proteins are soaked with the sol-gel glass in a solution of the reductant sodium dithionite (data not shown).

The absorption spectra of the horseradish peroxidase and bovine blood hemoglobin in aqueous buffer solution (a) and in sol-gel glass (b) are also compared in Fig. 1. Once again, it can be seen that the spectroscopic properties of the proteins are preserved during the glass formation. This is the first report of encapsulation of these two proteins in silica glass. To check the chemical activity of the peroxidase encapsulated in sol-gel glass, we carried out an experiment to produce the Complex II by a simple addition of hydrogen peroxide and ascorbate to the peroxidase gel sample (*10*). As reported previously (*10–12*), during the steady state of the peroxidative oxidation of donors, peroxidase is retained in the form of Complex II. Figure 2 shows the difference spectrum between Complex II and peroxidase in immobilized sol-gel glass, and it is in agreement with

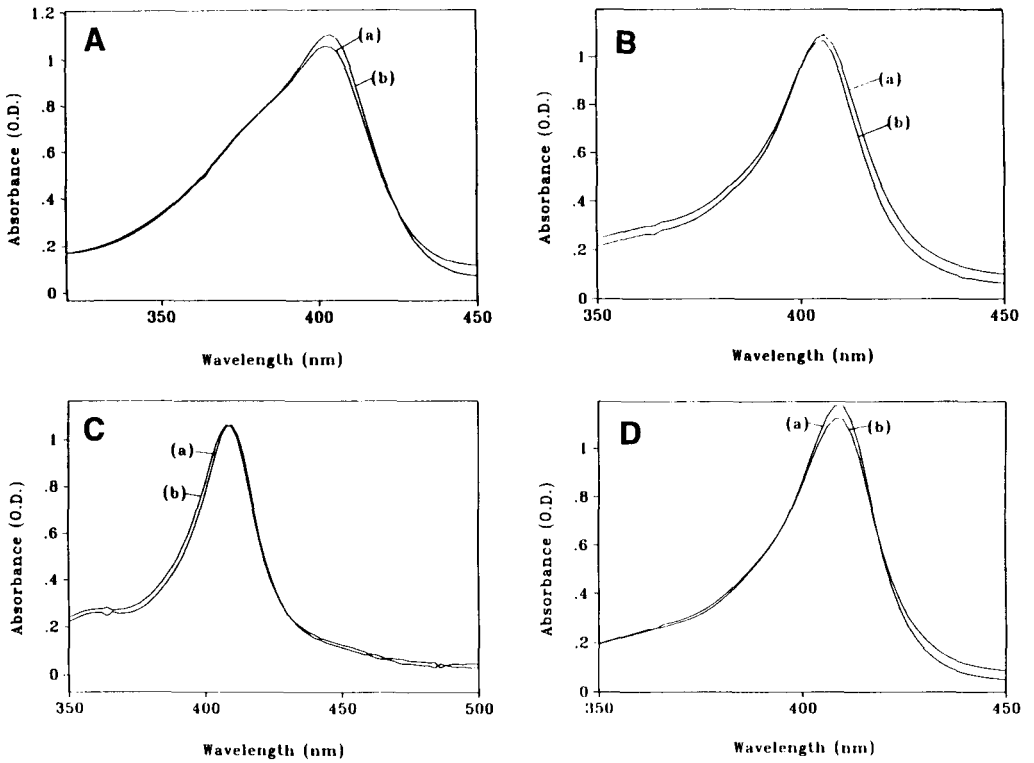


Fig. 1. Soret bands of hemoproteins in aqueous buffered suspensions (a) and encapsulated in sol-gel glass (b). (**A**) Horseradish peroxidase; (**B**) Bovine blood hemoglobin; (**C**) Bovine heart cytochrome c; and (**D**) Bovine heart myoglobin. Protein concentrations are 60–90 μM.

the spectrum obtained before (*10*) for the peroxidase in the aqueous solution. This result indicates that the chemical activity of peroxidase is retained during the process of the encapsulation.

The above results demonstrate that the biochemical properties of the selected encapsulated hemoproteins are well retained inside the glass matrix. Therefore, it should be of interest to ascertain whether the encapsulated hemoproteins can catalyze the same chemical reactions as in aqueous solution.

Oxidation of Dibenzothiophene Catalyzed by Encapsulated Hemoproteins

The oxidation of dibenzothiophene to its S-oxide and sulfone by various hemoproteins in a series of mixed water and organic solvents was recently studied in great detail (*1*). Accordingly, in our work, no effort was made to characterize this reaction further in the aqueous/organic medium. Instead, we have carried out the oxidation reaction by using the encapsulated hemoproteins as catalysts and have compared their catalytic activities with the proteins in aqueous solution.

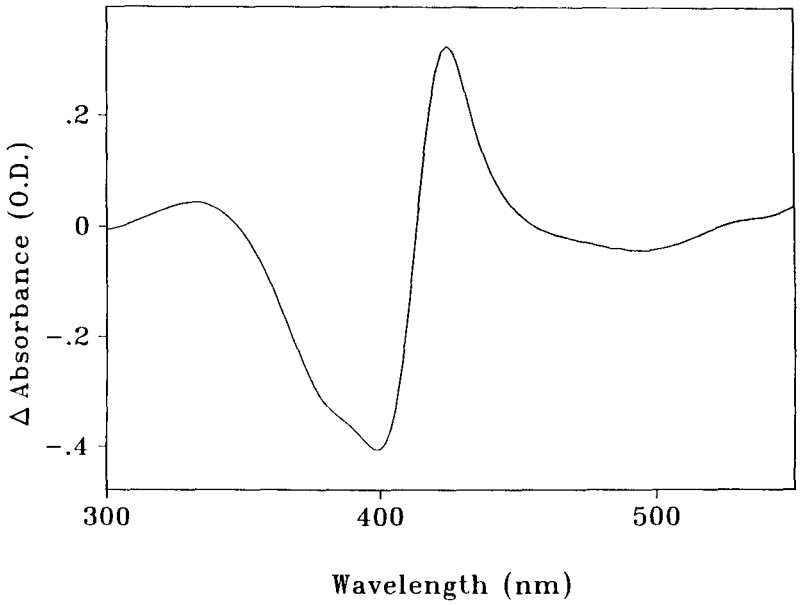


Fig. 2. Difference absorption spectrum between Complex II and horseradish peroxidase encapsulated in sol-gel glass. The spectrum of the Complex II was recorded immediately after the addition of 0.66 m*M* ascorbate and 0.29 m*M* hydrogen peroxide to 6.6 μM encapsulated peroxidase (pH 6.0 phosphate buffer) under constant stirring.

The glass pores in dried gels usually have an average diameter around 50–100 Å (*7,9*). Thus, dibenzothiophene should be able to readily diffuse into the porous glass medium to reach the encapsulated protein where the reaction can take place. Similarly, it should be feasible for the oxidation products (either S-oxide or sulfone) to diffuse through the porous glass medium back into the solution phase.

Table 1 summarizes the results of oxidation of dibenzothiophene catalyzed by different hemoproteins encapsulated in sol-gel glass in 25% (v/v) ethanol/water solvent. The results of control experiments with the proteins in the solution are presented in Table 1, which are found to agree well with those previously reported (*1*). First, all four hemoproteins studied here exhibit similar catalytic activity in the sol-gel glass matrix as in solution. The maximal extent of conversion of the substrate was also found to be similar to the corresponding controls for all four hemoproteins. No protein moieties were detected in the solution phase at the end of reaction indicating effective encapsulation of the proteins in the solid state.

In order to study the effect of diffusion of the substrate from the reaction mixture to the glass medium, the oxidation reaction was investigated at different times following the initiation of the reaction. Figure 3 shows the typical time course of the cytochrome c catalyzed oxidation of dibenzothiophene by hydrogen peroxide at 22°C for proteins both in encapsulated

Table 1
Oxidation of Dibenzothiophene Catalyzed by Different Hemoproteins[a]

Hemoprotein	Oxidation of dibenzothiophene, %	S-oxide, %	Sulfone, %
Horseradish peroxidase			
in solution	18.4	18.4	0
in glass	18.1	18.1	0
Hemoglobin			
in solution	94.8	87.6	7.2
in glass	90.6	82.4	8.2
Cytochrome c			
in solution	90.8	63.3	27.5
in glass	93.4	80.9	12.4
Myoglobin			
in solution	91.2	86.8	4.4
in glass	90.1	89.0	1.1

[a]Experimental condition: 25 μM protein, 230 μM dibenzothiophene, 10 mM hydrogen peroxide, 20 mM acetate-phosphate buffer (pH 5.2) containing 25% (v/v) of ethanol solution. Reaction time: 1 h for proteins in solution; 2 h for proteins encapsulated in sol-gel medium. Reaction temperature: 30°C.

form and in the solution, respectively. The total yield of conversion as well as the percentage yield for both S-oxide and sulfone are plotted against the time at which the reaction mixtures were quenched at liquid N_2 temperature for the HPLC analysis. For the proteins in the solution, it can be seen that the reaction is fast during the first 10 min and that the reaction is complete within 60 min. On the other hand, with the cytochrome c encapsulated in glass, the reaction took about twice as long to reach its maximal transformation ($\sim$110 min) indicating that the diffusion of the reactant (and/or products) indeed partly controls the apparent reaction kinetics. The time-course measurements were also performed for other hemoproteins and similar behaviors were observed as for cytochrome c (data not shown).

Interestingly, with cytochrome c in solution, both S-oxide and sulfone formation yields increased during the time course examined. However, with encapsulated cytochrome c, the yield for sulfone formation remained constant, whereas the yield for S-oxide increased during the time course of the reaction. In other words, with encapsulated cytochrome c, the oxidation reaction favors the formation of the S-oxide over the sulfone. To highlight this unexpected effect, the ratio of S-oxide to sulfone is plotted against the time course for both encapsulated cytochrome c and cytochrome c in solution in Fig. 4. A ratio of 2.3 is observed in the case of

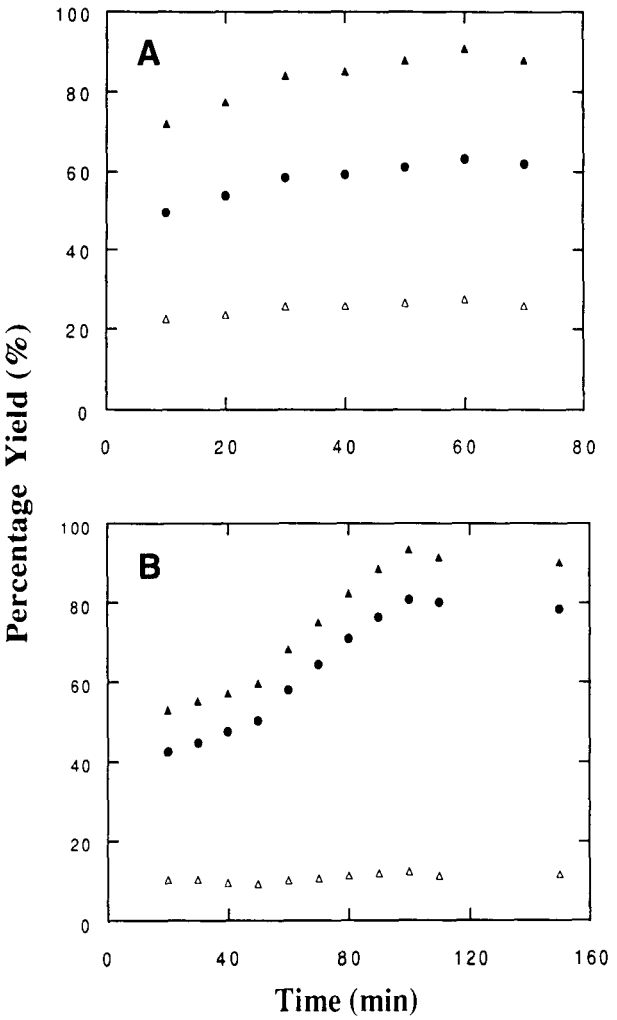


Fig. 3. The time course of oxidation of dibenzothiophene catalyzed by bovine heart cytochrome c in solution (**A**) and in sol-gel glass (**B**). (●) percentage yield for S-oxide; (△) percentage yield for sulfone; (▲) total percentage yield. Experimental conditions: 25 μM peroxidase, 230 μM dibenzothiophene, 10 mM hydrogen peroxide, and 20 mM acetate-phosphate buffered at pH 5.2 in 25% (v/v) ethanol/water solvent. Reaction temperature: 22°C.

the protein in solution. With the encapsulated cytochrome c, a much higher ratio of ~7 is observed at the end of the reaction. This result is of practical significance, as the S-oxide is more soluble in water than sulfone. In attempts to remove organic sulfur compounds such as dibenzothiophene, an improved selectivity toward the more water soluble S-oxide should facilitate the aqueous extraction of the sulfur compound from the substrate. The mechanism for this improved selectivity to S-oxide is not

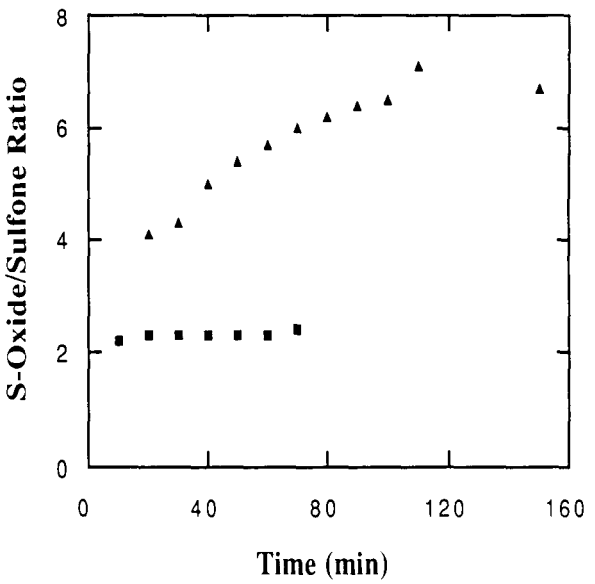


Fig. 4. Plot of the ratio of the reaction products S-oxide to sulfone versus reaction time observed for dibenzothiophene oxidation catalyzed by bovine heart cytochrome c in encapsulated form (▲) and in the solution (■). Data taken from Fig. 3.

understood at the present time although some structural perturbation by the formation of glass matrix around the protein or decreased accessibility of the S-oxide substrate to its binding domain on the protein may account for this observation. Further investigation is needed to understand this phenomenon. Enhanced selectivity toward the S-oxide was also observed for the reaction catalyzed by horseradish peroxidase, hemoglobin and myoglobin; in fact, the selectivity toward the S-oxide is more pronounced for these proteins than in the case of cytochrome c. Thus, differences in the affinity or accessibility of the S-oxide to a hydrophobic binding domain on the surface or within the protein might be important factors.

Finally, it should be pointed out that the inactivation of hemoglobin and cytochrome c by the hydrogen peroxide has also been observed for the encapsulated systems as has been reported for proteins in aqueous organic media (*1*). It was proposed that the deactivation of the proteins may be caused by the oxidation of methylene bridges of the heme by hydrogen peroxide leading to an irreversible destruction of the heme structure (*1*). This inactivation of the proteins by hydrogen peroxide, of course, would limit the lifetime of the encapsulated enzymes. However, the encapsulation of the heme proteins should protect the protein from digestion by proteases. Although chemical coupling of the proteins to porous silica or glass would immobilize the proteins, this immobilization does not necessarily alter the access of the proteins to proteases. Thus,

encapsulation of the proteins in sol-gel can enhance the lifetime of these enzymes. In our work the extent of inactivation of the proteins was always found to be less severe than that observed for the proteins in solution. Further research regarding this aspect is in progress.

SUMMARY

A new form of biocatalysts based on the encapsulation of hemoprotein in sol-gel glass has been shown to catalyze the oxidation of dibenzothiophene in the presence of hydrogen peroxide. Similar catalytic efficiencies have been found for these encapsulated systems as compared with the proteins in solution except that encapsulated cytochrome c shows greater selectivity toward formation of the more water soluble S-oxide. Easy separation of the encapsulated catalysts from the homogeneous reaction mixture should offer a distinct advantage in the exploitation of this system for potential environmental and industrial applications. In future work, it would also be important to study methods for improving the stability of the hemoproteins encapsulated in the glass matrix by retarding the inactivation of the proteins brought about the exposure to the hydrogen peroxide.

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