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Oxidation of Dibenzothiophene Catalyzed by Hemoglobin and Other Hemoproteins in Various Aqueous-Organic Media

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ABSTRACT

Biocatalytic oxidation of dibenzothiophene (a model of organic sulfur in coal) with hydrogen peroxide was investigated. It was found that various hemoproteins, both enzymic (e.g., horseradish peroxidase) and nonenzymic (e.g., bovine blood hemoglobin), readily oxidized dibensothiophene to its S-oxide and, to a minor extent, further to its S-dioxide (sulfone). This process catalyzed by hemoglobin (a slaughterhouse waste protein) was studied in a number of monophasic aqueous-organic mixtures. Although hemoglobin was competent as an oxidation catalyst even in nearly dry organic solvents (with protic, acidic solvents being optimal), the highest conversions were observed in predominantly aqueous media. The hemoglobin-catalyzed oxidation of dibenzothiophene at low concentrations of the protein stopped long before all the substrate was oxidized. This phenomenon was caused by inactivation of hemoglobin by hydrogen peroxide that destroyed the heme moiety. The maximal degree of the hemoglobin-catalyzed dibenzothiophene oxidation was predicted, and found, to be strongly dependent on the reaction medium composition.

Index Entries: Hemoglobin; peroxidase; coal desulfurization; dibenzothiophene; aqueous-organic media for biocatalysis; biooxidations.

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INTRODUCTION

Sulfur is a natural constitutent of hydrocarbon fuels, such as coal and crude oil (*1,2*). Its presence in these fuels causes poisoning of catalysts, corrosion of surfaces, and air pollution. For instance, sulfurous emissions from the combustion of coal constitute a major source of the acid rain problem (*1,2*). Consequently, sulfur-containing compounds must be removed from coal in order to attain high energy values and satisfactory environmental standards.

Dibenzothiophene is a widely accepted model compound for organic sulfur in coal (*1–3*). This substance possesses low reactivity since the electron density is delocalized by benzene rings; hence, it is difficult to transform by chemical methods. In addition, the poor solubility of dibenzothiophene in water (less than 10 μM) is responsible for the fact that organic sulfur cannot be washed out of coal like most inorganic sulfur compounds (*1,2*). A number of strategies for coal and, in particular, dibenzothiophene, desulfurization have been examined, including microbial approaches (*3,4*). An attractive route for the removal of organic sulfur from coal appears to be the oxidation of dibenzothiphene to its S-oxide and then to its S-dioxide (sulfone) (Fig. 1) (*3*). In the present work, we have explored the biocatalytic oxidation of dibenzothiophene.

Recent studies have demonstrated that peroxidases can participate in the oxidative metabolism of organosulfur compounds. The oxygenation of benzyl methyl sulfide, thioanisole, and thiobenzamide to the respective sulfoxides has been found to be catalyzed by chloroperoxidase, lactoperoxidase, and horseradish peroxidase (*5–9*). Our strategy has involved the use of inexpensive, abundant proteins to oxidize dibenzothiophene to the corresponding S-oxide and/or sulfone. The rationale behind this transformation is as follows: oxidation of dibenzothiophene should improve its solubility in water, thereby facilitating the subsequent aqueous extraction from coal; and oxidized species of dibenzothiophene are more amenable to the subsequent biological or chemical degradation than the parent compound.

MATERIALS AND METHODS

Horseradish peroxidase (EC 1.11.1.7) (type II) with specific activity of 200 U/mg solid, bovine milk lactoperoxidase (EC 1.11.1.7) with specific activity of 76 U/mg solid, horse heart myoglobin and cytochrome c (both type III), bovine blood hemoglobin and hemin, hen egg-white lysozyme, bovine serum albumin, and cumene hydroperoxide were all purchased from Sigma Chemical Co., St. Louis, MO. Dibenzothiophene, its S-oxide

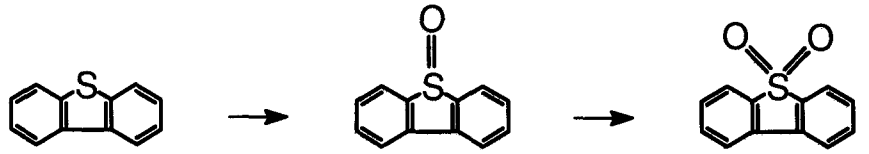

Fig. 1. Initial steps of the oxidation of dibenzothiophene potentially leading to sulfur removal.

and sulfone, tetranitromethane, acetonitrile (HPLC grade), 1-propanol (HPLC grade), methanol (HPLC grade), and ethanol (spectrophotometric grade) were obtained from Aldrich Chemical Co., Milwaukee, WI. All other organic solvents were 99+ pure and were dried prior to use by incubation over 3 Å molecular sieves. Hydrogen peroxide (30% solution in water) was from Mallinckrodt, St. Louis, MO.

In a typical experiment, to 0.25 mL of an organic solvent containing dibenzothiophene, 0.75 mL of a 20 m*M* acetate-phosphate (10 m*M* sodium acetate and 10 m*M* sodium phosphate) aqueous buffer, pH 5.2, was added to bring the final concentration of dibenzothiophene in the system to 230 μM. Then hydrogen peroxide (final concentration in the system of 0.1–10 m*M*) and an aqueous buffered solution of a protein (final concentration in the system of 0.25–25 μM) were added. When high concentrations of organic solvents were used, a protein was first lyophilized from an aqueous solution with pH 4.1, and the resultant dry powder was added to the reaction mixture. Periodically, aliquots of the reaction mixture were withdrawn and analyzed by reverse-phase HPLC, as described below.

Dibenzothiophene and its oxidation products were analyzed using a Waters 712 high performance liquid chromatograph equipped with a Waters μBondapak C_{18} column (3.9×300 mm). The mobile phase consisted of an acetonitrile-water mixture; the flow rate was 2 mL/min. Absorbance peaks were monitored in a Waters 486 tunable absorbance detector at 230 nm. Dibenzothiophene-S-oxide and -sulfone were separated in the following regime: a linear gradient of acetonitrile-water (40:60–60:40 over 7 min), after which the acetonitrile content was increased to 80% (over 3 min) and held for 1 min to obtain the peak of dibenzothiophene. The identities of the substrate and its oxidation products were confirmed by comparision with the authentic compounds. In a typical chromatogram, the retention times of dibenzothiophene-S-oxide, dibenzothiophene sulfone, and dibenzothiophene were 3.76, 5.63, and 10.97 min, respectively. Absorption spectra of proteins were recorded using a Perkin-Elmer (Norwalk, CT) model 552 UV-visible spectrophotometer.

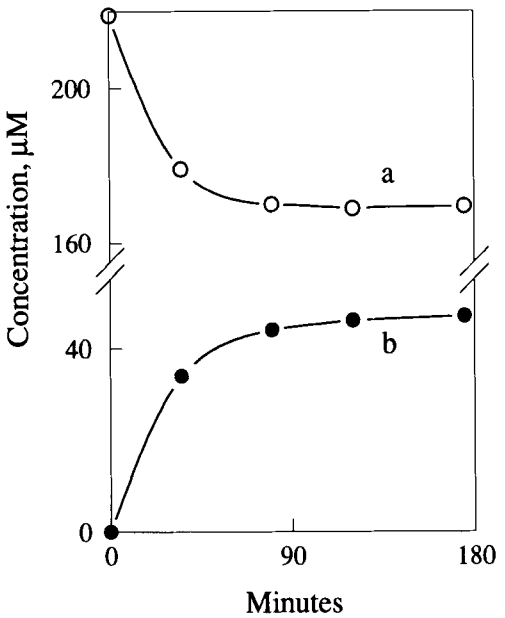

Fig. 2. The time course of horseradish peroxidase-catalyzed oxidation of dibenzothiophene (a) to dibenzothiophene-S-oxide (b). Experimental conditions: 230 μM dibenzothiophene, 25 μM horseradish peroxidase, 10 mM hydrogen peroxide, 20 mM acetate-phosphate buffer (pH 5.2) containing 25% (v/v) of 1-propanol, 30°C.

RESULTS AND DISCUSSION

Oxidation of Dibenzothiophene Catalyzed by Various Hemoproteins in a Mixture of 25% of 1-Propanol and 75% of Water

The oxidation of dibenzothiophene to its S-oxide (Fig. 1) by hydrogen peroxide catalyzed by horseradish peroxidase in a mixture of 25% of dimethylformamide and 75% of an aqueous buffer was recently described by Dordick et al. (*10*). The authors of this study reported a 40% transformation of dibenzothiophene (300 μM) at which point the reaction stopped. In the present work, we investigated this phenomenon mechanistically and in detail and then extended it to other, more economically attractive proteins (such as hemoglobin) and organic cosolvents (such as 1-propanol).

Figure 2 depicts the time course of horseradish peroxidase-catalyzed oxidation of dibenzothiophene to the S-oxide with hydrogen peroxide in a mixture of 25% of 1-propanol and 75% of aqueous acetate-phosphate buffer, pH 5.2 (this pH was found to be optimal for the reaction rate in this system). One can see from the figure that the oxidation is fast, quantitative, and results in dibenzothiophene-S-oxide as the sole product. We

Table 1
Oxidation of Dibenzothiophene
Catalyzed by Different Hemoproteins*

Hemoprotein	Oxidation of dibenzothiophene, %
Horseradish peroxidase	18
Lactoperoxidase	7
Hemoglobin	99
Cytochrome c	98
Myoglobin	95
Hemin	9

*Experimental conditions: 25 μM protein; 230 μM dibenzothiophene, 10 mM hydrogen peroxide, 1 h reaction time, 20 mM acetate-phosphate buffer (pH 5.2) containing 25% (v/v) of 1-propanol, 30°C.

demonstrated that the oxidation did not occur in the absence of any of the components of the reaction mixture, that is, without enzyme or hydrogen peroxide.

The enzymatic oxidation of dibenzothiophene was further studied as a function of hydrogen peroxide and peroxidase concentrations (other conditions were the same as in Fig. 2) in order to optimize the reaction conditions. At 2 mM hydrogen peroxide, when the enzyme concentration was increased from 1 to 25 μM the yield of dibenzothiophene-S-oxide after a 4-h reaction increased from 1.6 to 30 μM (0.7 to 13% conversion). At 25 μM horseradish peroxidase, an increase in hydrogen peroxide concentration from 0.8 to 5 mM enhanced the oxide yield after 4 h from 9.6 to 41 μM (4.2 to 18% conversion); further doubling of the hydrogen peroxide concentration had no appreciable affect on the product concentration.

In order to test the generality of the phenomenon reflected in Fig. 2, we explored the feasibility of the oxidation of dibenzothiophene in the presence of other hemoproteins (both enzymatic and nonenzymic)—lactoperoxidase, myoglobin, cytochrome c, and hemoglobin. The results obtained are presented in Table 1. It is seen that lactoperoxidase also can catalyze the oxidation of the substrate, albeit with a lower efficiency than horseradish peroxidase and close to that of hemin, a nonproteinaceous cofactor present in all hemoproteins (*11,12*). More importantly, we were gratified to discover (Table 1) that even *nonenzymic hemoproteins*, such as myoglobin, cytochrome c, and hemoglobin were able to catalyze the oxidation of dibenzothiophene by hydrogen peroxide, and, in fact, much more effectively than peroxidases. For example, the specific catalytic activity (i.e., the initial reaction rate divided by the protein concentration) for bovine blood hemoglobin is two orders of magnitude greater than that for horseradish peroxidase (1.7 and $1.5 \cdot 10^{-2}$ min^{-1}, respectively). As one can see from Table 1, the maximal degree of conversion of the substrate is

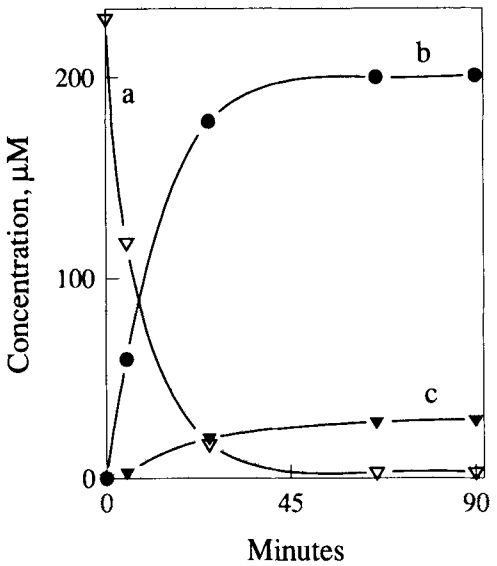

Fig. 3. The time course of hemoglobin-catalyzed oxidation of dibenzothiophene (a) to S-oxide (b) and sulfone (c). Experimental conditions are the same as those in Fig. 2.

close to 100% in the case of the nonenzymic hemoproteins. Importantly, we established that proteins not containing a heme cofactor—bovine serum albumin and lysozyme (25 μM)—were unable to oxidize dibenzothiophene with 10 mM hydrogen peroxide. Therefore, oxidations depicted in Table 1 require a heme moiety (preferably bound to a protein matrix).

The data in Table 1 reveal that the oxidation of dibenzothiophene is truly catalytic (as opposed to stoichiometric): the turnover number of the reaction (i.e., the ratio of the concentration of the product to that of the protein) exceeds 10. It will be demonstrated later that the turnover number can be readily increased further to more than 100 simply by raising the concentration of dibenzothiophene.

Figure 3 shows a typical time course of the hemoglobin-catalyzed oxidation of dibenzothiophene by hydrogen peroxide. It can be seen that the reaction is fast and that an almost complete transformation of the substrate was observed after 45 min. In contrast to the situation with horseradish peroxidase, approx 10% of the S-oxide was further converted to the S-dioxide (sulfone). Note that no appreciable oxidation of dibenzothiophene was observed in the presence of hemoglobin without hydrogen peroxide. We also tested the possibility that the observed oxidation of dibenzothiophene involved molecular oxygen. To this end, in a separate experiment, air was excluded from the system. No significant difference either in kinetics or in the maximal level of substrate transformation was detected when the oxidation of dibenzothiophene by hydrogen peroxide,

catalyzed by hemoglobin, was carried out under anaerobic (under argon) instead of aerobic (under air) conditions. We also established that there was no oxidation of dibenzothiophene when pure oxygen was used instead of hydrogen peroxide as an oxidant. Hence, all the components of reaction mixture are required for the oxidation of dibenzothiophene.

Interestingly, we found that hydrogen peroxide could be replaced with an organic peroxide as an oxidant. When 10 m*M* cumene hydroperoxide was employed instead of hydrogen peroxide (other conditions were identical to those in Fig. 3), the oxidation of dibenzothiophene was complete in 10 min; the product composition was the same as for hemoglobin (i.e., approximately 90% of the oxide and 10% of the sulfone).

Hemoglobin-Catalyzed Oxidation of Dibenzothiophene in Predominantly Organic Media

As mentioned above, the solubility of dibenzothiophene in water depends on the nature and concentration of the organic cosolvent and is greater at high concentrations of organic solvents. Consequently, we raised the concentration of 1-propanol and found that the oxidation occurred even in the 99% solvent (entry number 10 in Table 2). Unfortunately, in this medium the reaction stopped long before the full depletion of dibenzothiophene; in fact, the maximal degree of conversion was less than in water containing 25% of 1-propanol. In an effort to find a more optimal organic solvent, we examined the ability of hemoglobin to catalyze the oxidation of dibenzothiophene by hydrogen peroxide in numerous 99% organic solvents belonging to different classes (e.g., primary, secondary, and tertiary alcohols, as well as diols, amides, ethers, and esters). The data obtained are depicted in Table 2. One can see that in terms of their propensity to allow the oxidation process, the solvents may be divided into two different groups. The first group (entries from 1–20 in the table) are those conducive to a substantial transformation of dibenzothiophene; note that almost all of them are protic solvents. The second group (comprised of the last ten solvents in the table) are those not supporting the oxidation process; nearly all of them are aprotic.

The exact molecular mechanism of the discovered oxidation of dibenzothiophene by hydrogen peroxide, catalyzed by hemoglobin, remains unclear. In fact, only a few examples of S-oxygenations catalyzed by hemoproteins have been reported in the literature (*5–9*). These include thioanisole and thiobenzamide oxidation catalyzed by horseradish, lacto-, and chloro- peroxidases. Although the mechanism of the enzymatic oxygen incorporation into such sulfur-containing compounds is also somewhat obscure (*5–9*), it is thought to occur via a free-radical pathway involving a hydrogen transfer (*5*). The data in Table 2, namely that only protic solvents support the reaction, suggest that the solvent molecules

Table 2
Oxidation of Dibenzothiophene Catalyzed by Hemoglobin in Different Organic Solvents Containing 1% of Water*

Entry	Solvent	Oxidation of dibenzothiophene, %
1	1,2-Propanediol	36
2	Ethylene glycol	30
3	Ethanol	28
4	Methanol	26
5	1,3-Propanediol	25
6	1,2-Butanediol	20
7	2-Methoxyethanol	18
8	Formamide	17
9	2,3-Butanediol	12
10	1-Propanol	11
11	N-Methylformamide	6
12	2-Propanol	5
13	1,4-Butanediol	4
14	1,3-Butanediol	3
15	1-Butanol	2
16	N,N-Dimethylformamide	2
17	1-Pentanol	1.3
18	1-Hexanol	1.2
19	Acetonitrile	1
20	2-Butanol	0.9
21	*tert*-Butanol	0.1
22	Cyclohexanol	0
23	Acetone	0
24	Ethyl formate	0
25	Chloroform	0
26	Ethyl acetate	0
27	Tetrahydrofuran	0
28	*tert*-Butyl methyl ether	0
29	Toluene	0
30	Tetrachloromethane	0

*Experimental conditions: 25 μM hemoglobin (lyophilized from an aqueous solution of pH 4.1), 230 μM dibenzothiophene, 10 mM hydrogen peroxide, 30°C.

could participate in this reaction by means of hydrogen bonding, and hence the acidity of the reaction medium may be important. Indeed, a correlation between the maximal degree of dibenzothiophene conversion and solvent acidity was revealed. For example, we found that the extent of the substrate oxidation (the number in parentheses below) in the series of homologous primary alcohols, isomeric butanols and propanols, and

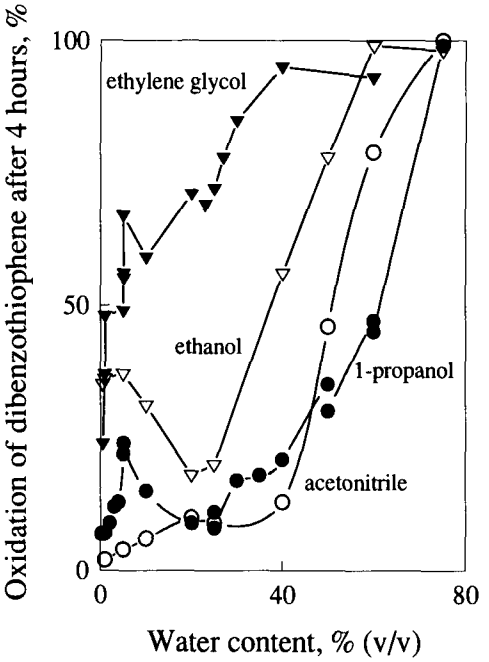

Fig. 4. Hemoglobin-catalyzed oxidation of dibenzothiophene in organic solvents as a function of water content (20 m*M* acetate-phosphate buffer, pH 4.1). Experimental conditions: 25 μM hemoglobin (lyophilized from an aqueous solution of pH 4.1), 10 m*M* hydrogen peroxide, 230 μM dibenzothiophene, 30°C.

substituted formamides and the acidity of the solvents (*13,14*) changed in the same order:

Methanol (26) ≈ Ethanol (28) > 1-Propanol (11) > 1-Butanol (2)
1-Butanol (2) > 2-Butanol (0.9) > *tert*-Butanol (0.1)
1-Propanol (11) > 2-Propanol (5)
Formamide (17) > N-Methylformamide (6) > N,N-Dimethylformamide (1).

Among all the solvents used, water is the strongest acid (*13,14*). Comparing the results in Fig. 3 and Table 2, one can see that the efficiency of the transformation of dibenzothiophene is almost three times higher in water containing 25% of 1-propanol than in the best organic solvent containing 1% of water (1,2-propanediol). In order to ascertain whether this phenomenon is general, we studied the dependence of the degree of oxidation of dibenzothiophene on the water content for four different organic solvents: 1-propanol, acetonitrile, ethanol, and ethylene glycol. The data obtained are shown in Fig. 4. As one can see, although the dependence is quite distinct for varous solvents, the overall trend for all of them is that the higher the water content, the greater the transformation of dibenzothiophene. It is also seen in Fig. 4 that the oxidation of the substrate

Table 3
Oxidation of Dibenzothiophene Catalyzed by Hemoglobin in Water Containing 25% (v/v) of an Organic Solvent*

Concentration of hemoglobin, μM	Solvent	Oxidation of dibenzothiophene, %
25	1-propanol	99
5	1-propanol	96
2.5	1-propanol	56
0.25	1-propanol	5
25	methanol	98
25	ethanol	99
2.5	ethanol	53
25	2-propanol	93
25	*tert*-butanol	94
25	acetonitrile	100
25	ethylene glycol	91

*Experimental conditions: 230 μM dibenzothiophene, 10 mM hydrogen peroxide, 20 mM acetate-phosphate buffer (pH 5.2), 30°C.

reaches the maximum and levels off in the mixture of 25% of an organic solvent and 75% of the aqueous buffer regardless of the solvent. (Note that in some cases, e.g., for ethanol or ethylene glycol, even in a 50% organic solvent the complete transformation of dibenzothiophene was observed.) We widened the range of solvents (25% in aqueous buffer) and established that in all of them nearly complete transformation of dibenzothiophene occurs (Table 3). As seen in Table 3, the maximal degree of oxidation of dibenzothiophene with hydrogen peroxide catalyzed by hemoglobin depends only on the concentration of the latter. Compared to horseradish peroxidase (where at 25 μM enzyme only 18% of the substrate was oxidized [Fig. 2]), in the case of hemoglobin the conversion requires much lower concentrations of the protein. Even at only 5 μM hemoglobin, nearly a 100% transformation of the substrate was attained (Table 3). In this case, a simple calculation indicates that one molecule of hemoglobin turns over more than 40 molecules of dibenzothiophene.

The data in Table 3 show, however, that at low concentrations of hemoglobin the oxidation of dibenzothiophene is incomplete. Specifically, the dibenzothiophene's maximal conversion depends on the concentration of hemoglobin, being 5, 56, and 99% at 0.25, 2.5, and 25 μM protein in 1-propanol, respectively. In order to elucidate why the oxidation ceased prior to the depletion of dibenzothiophene, we carried out the reaction until it stopped. Then the reaction mixture was divided into three portions; 10 mM hydrogen peroxide was added to one, 2.5 μM protein to another, and nothing to the third one, which served as a control. It was found that addition of hydrogen peroxide (or nothing) had no effect. On

the other hand, when the fresh protein was added to the reaction mixture, the oxidation of dibenzothiophene started again to result in a nearly complete consumption of the substrate. These observations suggest that the protein inactivates during the reaction.

The Mechanism of Hemoglobin Inactivation

We established in an independent experiment that there was no inactivation of the protein when present without the substrates in the reaction medium during the entire period of time allowed for the oxidation to proceed. We also made certain that the time course of the dibenzothiophene oxidation did not change if hemoglobin had been preincubated with dibenzothiophene for 1 h prior to the addition of hydrogen peroxide to the reaction mixture. On the other hand, when hydrogen peroxide was added first to the protein solution, then there was no subsequent transformation of dibenzothiophene at all, even if the latter was added after only a 5 min incubation of hemoglobin and hydrogen peroxide (Fig. 5A). Hence the protein is inactivated by hydrogen peroxide.

In order to shed light on the mechanism of hemoglobin inactivation during its incubation with hydrogen peroxide, we examined the visible absorption spectrum of the protein. This spectrum is a characteristic feature of hemoproteins reflecting the heme structure (*11,12*); it is very sensitive even to minor changes in the microenvironment and the structure of the heme. As seen in Fig. 5A, the absorbance at the Soret band dropped more than 10-fold in the first 5 min of the incubation of hemoglobin with hydrogen peroxide in an aqueous acetate-phosphate buffer containing 25% of 1-propanol. Furthermore, the entire spectrum of hemoglobin changed drastically in 5 min after hydrogen peroxide addition (Fig. 5B). These spectral changes suggest that the oxidative degradation of the heme in hemoglobin occurred, probably through a Fe(III)-bile pigment-protein complex as an intermediate (*15–17*). Thus, a likely reason for the observed inactivation of the protein is the oxidation of methylene bridges of the heme by hydrogen peroxide leading to an irreversible destruction of the heme structure. As Fig. 5A illustrates, the rate of the hemoglobin inactivation depends on the concentration of hydrogen peroxide: the process markedly slows down when the H_2O_2 concentration is lowered 20-fold. The fact that there is a clear correlation between the decay in absorbance of hemoglobin (Fig. 5A) and that in its catalytic activity (Fig. 5C) suggests a causal relationship between the two.

In order to establish whether the observed destruction of the heme moiety is general, we studied the spectral change of horseradish peroxidase during the oxidation of dibenzothiophene by hydrogen peroxide. It was found that the decay in the absorbance at the Soret band also occurred in this case, albeit slower than for hemoglobin. This absorbance change was accompanied by an appearance of a peak at 670 nm, reflecting an inactive enzymatic form (presumably a verdohemoprotein) of peroxidase

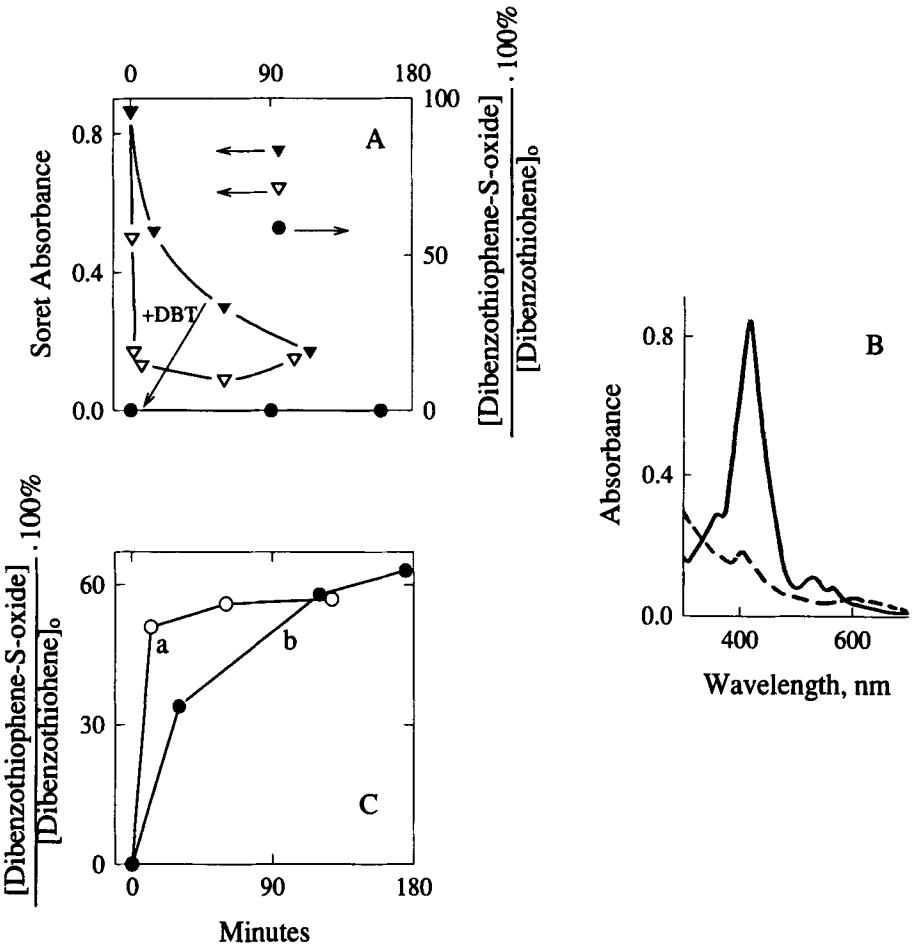

Fig. 5. Hemoglobin-catalyzed oxidation of dibenzothiophene and spectral characteristics of the protein. A. Absorbance at the Soret band in the presence of 10 (∇) and 0.4 (▼) m*M* hydrogen peroxide. Solid circles correspond to the time course of the S-oxide formation when dibenzothiophene was added 5 min after hydrogen peroxide. B. Spectrum of hemoglobin before (—) and 5 min after (---) addition of hydrogen peroxide. C. Oxidation of dibenzothiophene in the presence of 10 (a) and 0.4 (b) m*M* hydrogen peroxide. Experimental conditions: 2.5 μ*M* hemoglobin, 230 μ*M* dibenzothiophene, other conditions correspond to those in the Fig. 2.

(*17–19*). By analogy with the kinetic scheme suggested for horseradish peroxidase (*5,19*), we propose the mechanism of hemoglobin-catalyzed oxidation of dibenzothiophene that includes a concomitant inactivation of the enzyme by H_2O_2 (Fig. 6). According to this mechanism, the second molecule of hydrogen peroxide can also react with the protein, particularly if present in excess. In other words, a competition for compound I between dibenzothiophene and hydrogen peroxide can take place. Consequently, the concentration of the product and the turnover number of

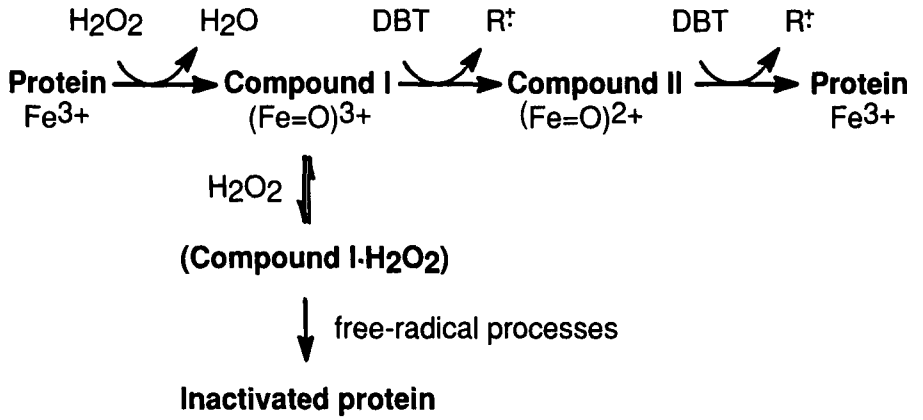

Fig. 6. Schematic representation of the mechanism of hemoglobin-catalyzed oxidation of dibenzothiophene by hydrogen peroxide.

the reaction depend on the concentration ratio dibenzothiophene:hydrogen peroxide:hemoglobin.

Hemoglobin Efficiency as a Function of Reaction Conditions

As mentioned above, the ratio of dibenzothiophene to hydrogen peroxide concentrations affects the reaction scheme depicted in Fig. 6. In mechanistic studies with horseradish peroxidase, it was found that the reducing substrate could stabilize the enzyme against inactivation by hydrogen peroxide (*19*). Therefore, we examined the hemoglobin-catalyzed oxidation of dibenzothiophene to dibenzothiophene-S-oxide as a function of the substrate concentration. The reaction was (necessarily) carried out at a high concentration of an organic solvent in order to increase 100-fold the solubility of the substrate. As seen in Fig. 7, curve a, the higher the concentration of dibenzothiophene, the more S-oxide is formed. The turnover number of the reaction (curve b) also increases, which means that the reaction accelerates and/or protein stabilization occurs. However, an increase in the dibenzothiophene concentration exceeds that for oxide, and, consequently, the maximal degree of conversion of dibenzothiophene decreases (Fig. 7, curve c). These data point to no substantial stabilization of hemoglobin by the reducing substrate in the system described.

The scheme in Fig. 6 explains a seemingly puzzling observation, depicted in Fig. 5C, that a 20-fold increase in H_2O_2 concentration, while dramatically accelerating the oxidation of dibenzothiophene, leads to no appreciable increase in the maximal product concentration. Our mechanism stipulates that not only the hemoglobin-catalyzed reaction but also the hemoglobin inactivation will be faster at higher hydrogen peroxide concentrations. Hence hemoglobin, while working faster, will have a shorter life time.

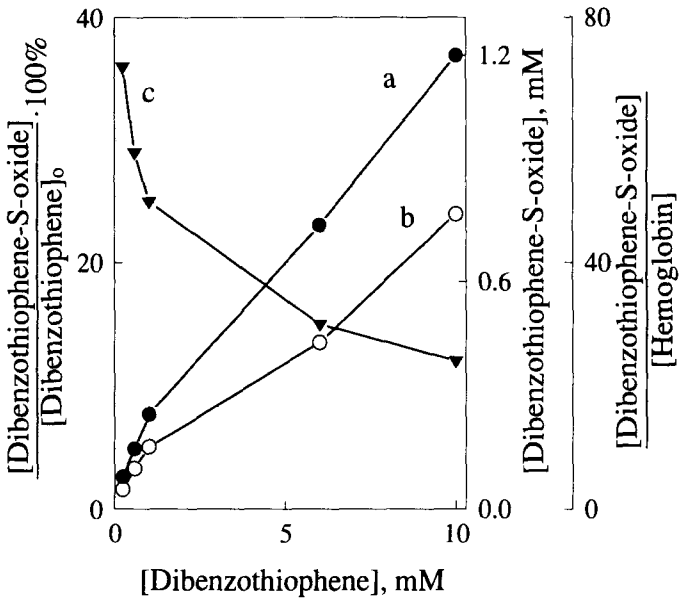

Fig. 7. Hemoglobin-catalyzed oxidation of dibenzothiophene as a function of the substrate concentration; (a) concentration of dibenzothiophene-S-oxide; (b) turnover number of the reaction ([Dibenzothiophene-S-oxide]/[Hemoglobin]); and (c) degree of conversion of dibenzothiophene ([Dibenzothiophene-S-oxide]/[Dibenzothiophene]$_0$). Experimental conditions: reaction medium is ethanol containing 0.13% of water; 25 μM hemoglobin (lyophilized from an aqueous solution of pH 4.1), 10 mM hydrogen peroxide, 30°C.

The proposed scheme (Fig. 6) also indicates that the way to shift the ratio between the productive route (horizontal line) and nonproductive route (vertical line) is to affect preferentially the pathways initiating at compound I. We experimentally tested this prediction.

Lowering the temperature should reduce the rates of the competing steps of the reaction to a different extent. With this in mind, we carried out the dibenzothiophene oxidation catalyzed by hemoglobin at two different temperatures, 7 and 30°C. It was found that when the temperature was reduced from 30 to 7°C, the maximal degree of dibenzothiophene oxidation increased three-fold: from 30 to 90% and from 5 to 15% at 1.5 and 0.25 μM hemoglobin, respectively. The turnover number of the reaction is 140 at 7°C as compared to 46 at 30°C.

It has been reported (*18,20*) that inactivation of horseradish peroxidase with hydrogen peroxide involves the participation of superoxide radicals. It is also known that tetranitromethane can act as a scavenger of superoxide radicals (*21,22*). Consequently, we examined the effect of tetranitromethane on the oxidation of dibenzothiophene catalyzed by hemoglobin. It was found that in the system depicted in Fig. 5C, curve a, addition of 0.02% of the scavenger indeed increased the maximal product yield from

56 to 92%. (Likewise, in the peroxidase system presented in Fig. 2, 0.02% tetranitromethane raised the yield of dibenzothiophene-S-oxide from 18 to 28%). In contrast, 0.1*M* mannitol or formate (known scavengers [23] of hydroxyl radicals) had no appreciable effect on the process.

It was also found that the pH of the reaction medium influenced the transformation of dibenzothiophene. For example, the maximal degree of oxidation at pH 5.2 was nearly twice as high as at pH 4.1 or pH 7.0 but almost 40% of the product at the last pH was the sulfone (otherwise, experimental conditions were the same as in Fig. 5C). Similarly, the nature of the organic cosolvent in this system significantly affected the maximal conversion of dibenzothiophene: this parameter varied from above 50% in 25% 1-propanol, ethanol, and acetonitrile to below 30% in 25% N,N-dimethylformamide, ethylene glycol, and tetrahydrofuran. Interestingly, 1 m*M* indole (a model of organic nitrogen in coal [24]) lowered a maximal yield of dibenzothiophene-S-oxide in 25% propanol from 56 to 7%. This observation suggests that coal components may affect (adversely or perhaps benefically) the hemoglobin-catalyzed desulfurization.

In future work, it should be important to determine whether the hemoglobin-catalyzed oxidation with hydrogen peroxide will be effective in lowering the organic sulfur content of a real (swollen) coal and what the operational characteristics and limitations of such a system will be.

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