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Hemoglobin, Horseradish Peroxidase, and Heme-Bovine Serum Albumin as Biocatalyst for the Oxidation of Dibenzothiophene

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ABSTRACT

Hemoglobin, horseradish peroxidase, and bovine serum albumin incubated heme-catalyzed the oxidation of dibenzothiophene into sulfoxide in the presence of hydrogen peroxide. This reaction was carried out in an aqueous buffer containing 25% of water-miscible organic solvents. The observation of this transient state of hemoproteins during sulfoxidation showed heme degradation. None of the compounds usually involved in a classical peroxidative activity mechanism were detected. Furthermore, this activity did not appear to be based on a Fenton-type reaction. The highest degrees of sulfoxidation were obtained with hemoglobin. Under the best conditions of reaction, 100% of dibenzothiophene were converted into dibenzothiophene sulfoxide by hemoglobin. Heat-denatured hemoproteins did keep their sulfoxidation activity. With hemoglobin, a k_{cat} of 0.22 min^{-1} was determined. Nearly the same values were obtained with heat-denatured hemoglobin and bovine serum albumin-adsorbed heme. With horseradish peroxidase, only 4% of conversion was attained. This percentage could be slightly increased by using a less pure peroxidase or heat-denatured peroxidase.

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Index Entries: Hemoglobin; peroxidase; heme-BSA; dibenzothiophene; heat-denatured hemoprotein; heme-dependent oxidation; aqueous-organic media; peroxidase activity; reactive oxygen species.

Abbreviations: Heme, iron protoporphyrin IX regardless of the oxidation and ligation state; Hb, hemoglobin; HRP, horseradish peroxidase; DBT, dibenzothiophene; SOD, superoxide dismutase; BSA, bovine serum albumin; ABTS, 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMF, *N,N*-dimethylformamide; Hemin, chloroprotoporphyrin IX iron (III); HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid.

INTRODUCTION

Many studies are carried out to determine the influence of various peroxidases, oxidases, or monooxygenases on organic sulfur xenobiotics oxidation. Moreover, enzymes, such as peroxidases and monooxygenases, are supposed to be involved in microbial degradation of asphaltenes components (*1,2*). The chemical regio- and stereoselective sulfide oxidation is a difficult task, and few chemical methods exist. Therefore, numerous and interesting studies are investigated to characterize new catalysts (such as hemoproteins) and their mechanisms. Peroxidases, which are heme-containing enzymes, catalyze the oxygenation of organosulfur compounds (*3–7*).

Hemoglobin (Hb) and myoglobin, which normally transport oxygen, catalyze peroxidative and monooxygenative reactions with H_2O_2 or oxygen and a reducing substrate.

The most used biological probes are alkyl aryl sulfides and thiophenic derivatives, which are not very soluble in aqueous medium. Their solubilization needs a cosolvent in reaction medium. Klyachko and Klibanov (*8*) have shown that in the presence of water-miscible organic solvent, hemoglobin was much more efficient than HRP for dibenzothiophene (DBT) oxidation. Nevertheless, they proposed a classical peroxidative mechanism.

Mechanistic studies have established that the ferric enzyme reacts with H_2O_2 to give compound I, a two-electron oxidized species in which the heme is oxidized to a ferryl porphyrin radical cation ($P^+.Fe^{IV} = O$). In classical peroxidase reactions, sequential electron abstraction from two substrates reduces the porphyrin radical cation first to the ferryl porphyrin ($Pe^{IV} = O$) species known as compound II and, subsequently, to the ferric resting state (*9*).

It is a known fact that ferrylHb ($Fe^{IV} = O$) is formed by reaction of MetHb with H_2O_2. The reaction of these globins with H_2O_2 produces the ferryl species ($Fe^{IV} = O$) and a protein radical, an analog to compound II of peroxidase (*10,11*).

Under the experimental conditions used by Klyachko and Klibanov, the protein was denatured by the solvent and by the high peroxide Hb concentration. This prompted us to study DBT sulfoxidation with HB and horseradish peroxide (HRP) in a more detailed way. In the present article, we will examine the hemoproteic catalyst evolution during the sulfoxidation. During the sulfoxidation reaction, we can observe an almost complete degradation of hemoproteins. In the reaction medium, no iron ions were involved. Various experiments were carried out so as to compare heme catalytic power in different proteic environments.

EXPERIMENTAL PROCEDURES

Materials

HRP (types II and VI), bovine Hb, superoxide dismutase (SOD), catalase, bovine serum albumin (BSA), human serum albumin (HSA), hydrogen peroxide, and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma (St. Louis, MO). DBT (99%), DBT sulfone (97%), and bovine hemin were obtained from Aldrich.

The commercial Hb was purified by gel filtration. Solutions of Hb in acetate buffer, 0.05*M*, pH 5.0, were eluted twice through a closed system containing a Sephadex G-75 column (bed volume 74 mL, Pharmacia). Purified Hb was controlled by SDS-PAGE, and no other protein was detected. The same degrees of DBT sulfoxidation were obtained by using commercial Hb whether purified or not.

All solutions were prepared from distilled deionized water by a milli-Q water-purification system. All experiments were run at least twice.

Spectrophotometric analyses were performed on a Hewlett-Packard 8452A diode array spectrophotometer. Concentrations were determined by using the following extinction coefficients: HRP, at 403 nm, $\epsilon = 1.02\ 10^5 M^{-1}$/cm (*12*), Hb, at 522 nm, $\epsilon = 3.0\ 10^4 M^{-1}$/cm (*13*); H_2O_2, at 240 nm, $\epsilon = 39.4 M^{-1}$/cm (*14*), and stock solutions were determined by iodometric titration. To follow HRP and Hb spectral states, spectral scans were carried out from 700 to 300 nm every minute for 2 h.

Catalytic Oxidations

Typical procedures were run using a mixture of 25% of organic solvent and 75% of buffer. HRP or Hb at various concentrations were first stirred in aqueous acetate buffer solution, 0.05*M*, pH 5.0. Sulfoxidation by Hb was tested at various pHs, and it was found that the maximum degree of sulfoxidation was at pH 5.0. Organic solvent at a final amount of 25% was needed to solubilize and dilute DBT at 300 μM. A stock solution of DBT at 30 m*M* was prepared in *N,N*-dimethylformamide (DMF). The concentration of DMF in the final solutions was always negligible (activity of HRP with ABTS was tested and did not change when 1% of

DMF was added in the reaction medium). The reactions were carried out at room temperature and started by addition of various amounts of hydrogen peroxide. When heme-BSA was used as a catalyst, a stock solution of hemin, 1 m*M*, was always prepared daily in alkaline aqueous solution of NaOH, 0.1*M*.

Initial Rate

The formation of DBT sulfoxide was linear for the first 25 min. At low catalyst concentrations, a lag time of about 5 min was observed. This lag time decreased as catalyst concentration increased. Two-milliliter aliquots of the reaction mixture were withdrawn at appropriate incubation times, and 200 μL of catalase (0.8 mg/mL) were added to stop the reaction. Reaction products were then directly quantified by HPLC, taking the dilution into account.

Product Analysis

The HPLC analysis was carried out on a μbondapak C_{18} column, 125 Å, 3.9 × 300 mm, at a solvent flow rate of 2 mL/min with a detector set at 255 or 280 nm (equipment from Waters associates). DBT and the corresponding sulfoxide and sulfone were separated by elution with a gradient of acetonitrile-water as described previously (*8*). Retention times were: DBT 11.6 min; DBT sulfoxide 3.7 min; DBT sulfone 5.8 min. The identity of the products was confirmed by GC/MS, they were extracted into methylene chloride, and the solvent was removed on a rotator evaporator. The GC/MS analyses were performed on an SSQ 710 Finnigan instrument equipped with a fused silica capillary column with a bonded OV 1710 (Cp Sil 19CB) liquid phase and spitless injector (*15*).

Peroxidase Activity Assay

The ABTS oxidation rates, using either pure acetate buffer or 25% of 1-propanol acetate buffer, were determined with 1 mL of 5 m*M* ABTS and 1 mL of 1 m*M* H_2O_2. The reaction was started by addition of 20 μL of various HRP concentrations. Peroxidase activity was estimated as the increase of absorbance at 414 nm/min at 25°C. The same conditions were used to determine the influence of other organic solvents on the peroxidase activity or to test the activity of the catalyst after storage in 99% of different organic solvents.

RESULTS AND DISCUSSION

In order to understand why Hb was able to catalyze the oxidation of DBT by H_2O_2 with a much higher efficiency than HRP, we studied degrees of DBT conversion into DBT sulfoxide under various conditions by vary-

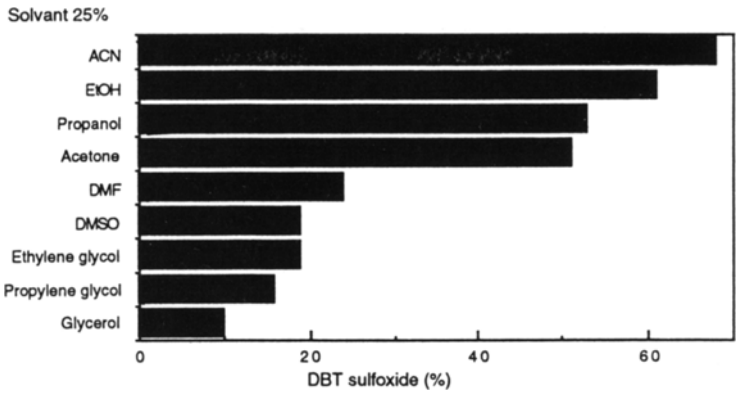

Fig. 1. Degrees of DBT sulfoxidation by Hb with various organic solvents. The reaction mixture contained 25% solvent tested in acetate buffer, pH 5.0; 2 μM Hb, 300 μM DBT, and 300 μM H_2O_2.

ing H_2O_2 and protein concentrations in different kinds of solvents. All reactions were completed within 4 h. The HPLC analysis revealed another product, DBT sulfone, which almost never exceeded 1%.

Control mixtures without protein or H_2O_2 did not show any DBT sulfoxide or any other product formation. When DBT sulfoxide was incubated with Hb and H_2O_2, under the same conditions, the concentration of DBT sulfoxide did not change, and no formation of any other products was observed.

Under the experimental conditions used by Klyachko and Klibanov (*8*), we obtained the same results: 18 and 100% of DBT conversion for the HRP type II and Hb, respectively. These maximum degrees of conversion were obtained in 25% of 1-propanol, 300 μM of DBT, 25 μM of protein (100 μM of heme), and 10 mM of H_2O_2. In these conditions, Hb was degraded by propanol and the high peroxide concentration, and moreover with Hb, total conversion corresponded only to three turnovers.

In order to avoid secondary reactions owing to the presence of contaminating proteins in the commercial peroxidase, HRP type II was replaced by HRP type VI, which is a purer commercial preparation containing a majority of isoenzyme C. Unfortunately, with HRP type VI, degrees of conversion did not exceed 4%.

Solvent Involvement

The solvent involvement was studied for Hb and HRP with several solvents. Reactions were carried out in a mixture containing 25% of cosolvent, 75% of acetate buffer with 0, 0.3 or 1 mM H_2O_2, 2 μM Hb or HRP, and 300 μM DBT. Reaction mixtures were continuously stirred. No apparication of DBT sulfoxide was recorded when H_2O_2 was omitted. Percentages of sulfoxidation with Hb are given in Fig. 1 with 300 μM H_2O_2.

Degrees of oxidation were good in propanol, acetone, acetonitrile, and ethanol.

With HRP, DBT conversion did not exceed 4%, whatever the solvent tested. We checked that HRP was not denatured by these solvents, since its activity with ABTS—a classical substrate for HRP—remained high when using these solvents.

In 100% aqueous acetate buffer, results were very approximate, because the detection was limited by the low solubility of DBT. In the presence of 3 μM DBT and 0.0625 μM Hb, it was necessary to use 30 mM of H_2O_2 to oxidize around 30% of DBT,

UV-Visible Spectral Studies

UV-visible spectra were followed to study the hemoprotein interactions with the solvent and the two substrates (H_2O_2, DBT). To investigate the possibility that DBT might be an electron donor substrate for HRP and Hb—like the substrate oxidized in a classical peroxidative reaction —we looked for UV-visible spectral changes on incubation of the proteins with DBT. The spectra of native HRP (ferric state) did not change in the presence of 1-propanol, ethanol, or when DBT was added; by contrast, when HRP or chloroperoxidase was incubated with some alkyl aryl sulfides, significant changes were observed (*7*). Whereas the spectrum of Hb in 25% of ethanol was not changed, conversely, hemichromes were formed when MetHb was incubated in 25% of 1-propanol. Brown MetHb solutions (ferric state) gave a bright red color when 1-propanol was added. As is shown in Fig. 2(A), the spectra revealed a shift of the peak maximum from 500 to 535 nm, but no change in Soret band. The presence of hemichromes was confirmed by adding sodium dithionite to the solution. We obtained the characteristic spectrum of hemochromes with peaks at 430, 525, and 560 nm. Hemichromes are the first step of the denaturation of Hb (*16*), but it was recorded that it is a reversible denaturation. Indeed, spectra of MetHb and hemichromes were recorded after 10-fold dilution in 100% of acetate buffer. The two solutions gave a superimposeable spectrum characteristic of the MetHb.

The spectral changes in Soret band with Hb/H_2O_2 are shown in Fig. 2(B). The spectra recorded during incubation of Hb with H_2O_2 revealed a decrease in absorbance in the visible and Soret bands. This decrease was owing to the degradation of the prosthetic heme group, and no appearance of ferrylHb was recorded. Hb slowly disappeared with apparent first-order kinetics. Plots of log absorbance vs time indicated a half-life for Hb of 32 min at concentrations from 0.625 to 2 μM with 300 μM of H_2O_2. Similar degradation was obtained with HRP/H_2O_2 without compound I or compound II formation.

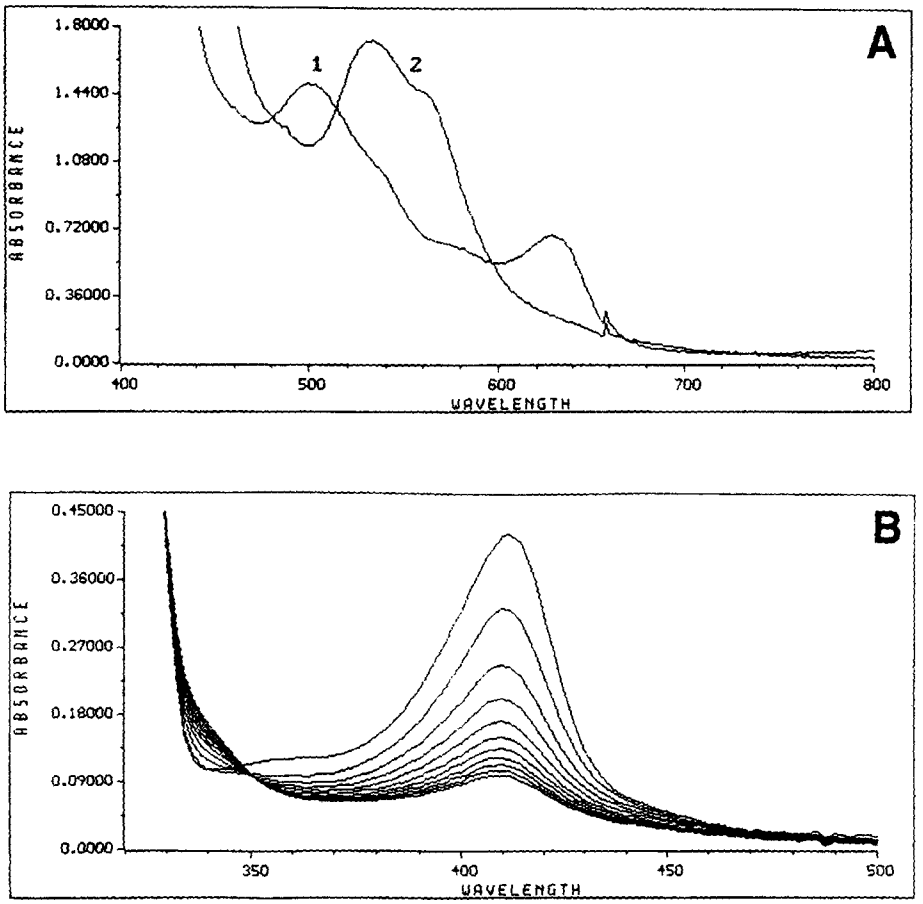

Fig. 2. (**A**) Absorption spectra of Hb solution (20 μM). Stock solution of Hb was diluted in 100% of acetate buffer to give MetHb (1), or in a mixture containing 75% of acetate buffer and 25% of 1-propanol, to give hemichromes (2). (**B**) Spectrophotometric changes accompanying the reaction of 1 μM Hb with 300 μM H_2O_2 and 300 μM DBT. Spectra have been recorded each 10 min during 2 h. Soret maximum is still at 408 nm.

Spectral Changes During Turnovers

In order to detect the formation of ferryl species, we worked on one catalytic cycle. An equimolar concentration of H_2O_2 was added to the proteins incubated in a mixture of 75% of acetate buffer and 25% of organic solvent (propanol for HRP and ethanol for Hb):

1. Without any exogenous electron donor;
2. With 75 μM or DBT; or
3. With 75 μM of ascorbate, which is a good electron donor.

Reactions were followed spectrophotometrically. When H_2O_2 was added to peroxidase without any electron donor, a mixture of compound I and compound II was formed. Nearly total peroxidase was autoreduced after 1 h of reaction. The same results and the same autoreduction rate was recorded in the presence of DBT. Conversely, when ascorbate was added as a substrate, total peroxidase returned immediately to a ferric state. Ascorbate is known to reduce the ferryl species of hemoproteins to a ferric state efficiently (*17*).

The same reactions were investigated with Hb in 25% ethanol in which Hb remained in native ferric state (MetHb). When an equimolar concentration of H_2O_2 was added, MetHb gave a majority of ferrylHb. Afterward, a fraction of ferrylHb returned to MetHb, whereas the remainder gave a mixture of ferrylHb and some hemichromes. This behavior occurred with or without DBT. When ascorbate was added, total ferrylHb was immediately reduced to MetHb. We came to the conclusion that the DBT sulfoxidation is not a classical peroxidase mechanism (formation of compound I, followed by formation of compound II, and returned to the native enzyme state, as was observed previously with HRP and some alkyl aryl sulfides) (*7,18*).

Role of Tertiary Structure?

We compared the degrees of sulfoxidation between Hb or HRP, and these same proteins denatured by heat. During 30 min at 100°C, tertiary structures of the proteins were damaged. The presence of an absorbing peak in the Soret band revealed that the himinic moiety was not damaged. The stock solution was precipitated by heat, but could be dissolved again by dilution in the final reaction medium. Hb degraded by heat showed the same degrees of DBT oxidation. An increase in the degrees of oxidation from 4 to 10–20% was recorded with heat-denatured HRP (type VI). Experiments with heat-denatured HRP showed lower reproducibility. Moreover, when H_2O_2 as added by several steps in large amounts (H_2O_2/DBT = 60), DBT conversion reached 60% (Fig. 3), whereas HRP was denatured with these H_2O_2 concentrations. These results raised the question: what is really catalyzing the DBT sulfoxidation? As hemoprotein, be it native, heat-denatured, or solvent-denatured, it always showed the same activity.

Is Hb a Fenton Reagent?

The reaction of MetHb with H_2O_2 caused the formation of $OH^{\bullet}$ and release of iron ions from the proteins. It was shown that the $OH^{\bullet}$ formation was inhibited by compound like deferoxamine. Indeed, it was suggested that the peroxidase activity of Hb was mediated by iron ions released from the protein rather than by Hb itself (*19–21*). Indeed, we examined whether the observed sulfoxidation was catalyzed by heme itself or by iron ions present in the reaction medium or released from Hb.

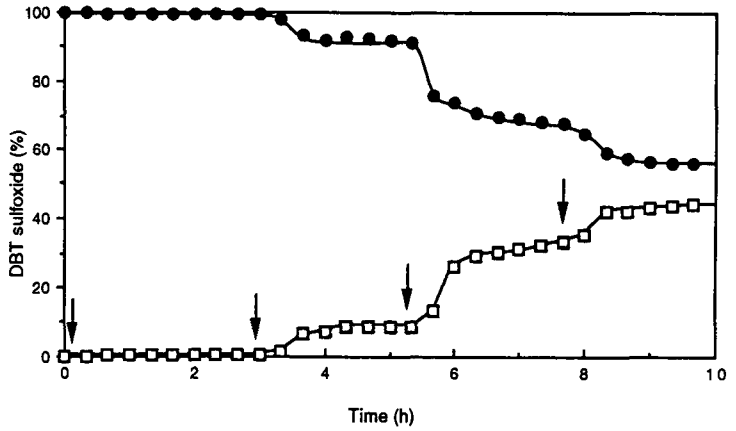

Fig. 3. Oxidation of DBT (●) to DBT sulfoxide (□) by 25 μM HRP. The reaction mixture contained 25% 1-propanol in acetate buffer, pH 5.0; 300 μM DBT and 5 mM of H_2O_2 were added several times as indicated by the arrows.

The conversion of DBT to DBT sulfoxide by Hb (2 μM) in the presence of various iron chelators or scavengers of reactive oxygen species was recorded with 300 μM DBT and 0, 0.3, 0.6, and 1.2 mM H_2O_2. When the reaction stopped, the ratio of DBT oxidation products was calculated by HPLC analysis, and the Hb degradation was measured by spectrophotometric analysis in Soret band. A control experiment with Hb gave 50% of oxidation of DBT and a complete degradation of Hb. The addition of EDTA (1.5 mM), DTPA (1.5 mM), or SOD (0.01 mg/mL) in the reaction mixture had no effect on the degree of sulfoxidation, or on the heme degradation. When $FeCl_3$ (10 μM)-EDTA (10 μM) was added to the reaction mixture, a 10% increase of DBT oxidation was recorded. In the presence of deferoxamine mesylate (0.2 mM), a protection of Hb from denaturation caused by H_2O_2 and a decrease of some several percents of the degree of sulfoxidation at 300 μM of H_2O_2 were recorded, but there was no effect at higher H_2O_2 concentrations. In the presence of catalase (0.01 mg/mL), total inhibition of sulfoxidation and Hb degradation was recorded. The peroxide was more rapidly consumed by catalase than it was used for the oxidation of DBT. These results show that chelators and SOD have little influence on DBT sulfoxidation and on protein degradation. Traces of iron and superoxide ions are not responsible for the sulfoxidation. Furthermore, ethanol, which is known to be a scavenger of $OH^{\bullet}$, was a good solvent for DBT oxidation.

Therefore, several experiments reported in Table 1 were performed. No apparition of DBT oxidation product was detected when Hb was substituted by Fe^{3+} or Fe^{2+}. With HRP type VI, there was a competition between the sulfoxidation reaction and the denaturation reaction, since the peroxidase used peroxide for its self-degradation. H_2O_2 acted as a suicide substrate as described previously (22). An increase in the percen-

Table 1
Conversion of DBT to DBT Sulfoxide by Various Catalysts[a]

Reaction mixture containing 25 μM of	Percentage recovery of DBT sulfoxide
Hb	85 (+15% of sulfone)
HRP	5
BSA	0
$FeSO_4$ ± BSA	0
$FeCl_3$ ± BSA	0
Heme	5–11
HRP + BSA	10–20
Heme + HRP	10
Heme + BSA	70–80 (+1% of sulfone)

[a] 25 μM of each catalyzing species were incubated for a day with H_2O_2 10 mM, DBT 300 μM in acetate buffer 75%, and 1-propanol 25%.

tage of DBT oxidation was observed when BSA was added to the reaction mixture containing the peroxidase. BSA played the role of protector from degradation by H_2O_2. This could explain why HRP type II has a better efficiency than type VI. Peroxidase is probably protected by the proteins contaminating the commercial preparation. Heme alone was not very efficient. The DBT oxidation was considerably increased when BSA or HSA was incubated with heme, since a proteic environment should be necessary for heme to activate the reaction. It has been observed, for example, as far as lipid peroxidation is concerned, that heme binding protens could enhance, decrease, or completely inhibit the catalytic reactivity of heme, depending on their affinity for heme and type of interaction (*23*). Previous studies have shown that human serum albumin has one high-affinity binding site and additional low-affinity sites for heme (*24,25*). We also observed an insolubilization of HRP, BSA, and Hb at high concentrations that occurred during the sulfoxidation process; this could be owing to protein crosslinking caused by H_2O_2 and indicative of reactive oxygen species (*26*).

Experiments with Hb, HRP, and Heme-BSA

Comparison between the behavior of Hb, heat-denatured Hb, and heme-BSA was performed. Experiments with BSA denatured by heat were not done, because BSA coagulated when it was heated. The number of sulfoxidation turnovers was computed vs heminic species concentration, showing that 1 mol of Hb contains 4 mol of hemes. Figure 4 shows that in all the three cases, heme was not stoechiometrically consumed. Therefore, the sulfoxidation of DBT by these heminic species is actually a catalysis. The highest number of turnovers was obtained in all cases when 1 μM heme concentration was used. We found nearly 30 turnovers

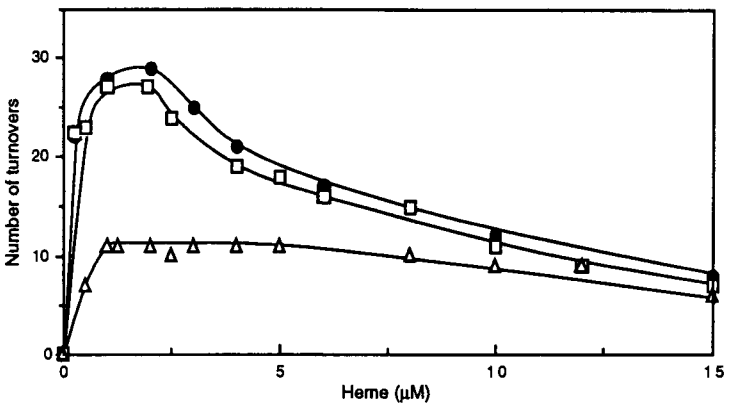

Fig. 4. Number of turnovers of the DBT sulfoxidation vs heme concentration of Hb (●); heat-denatured Hb (□) and heme-BSA (△). The reaction mixture contained 25% 1-propanol in acetate buffer, pH 5.0, 300 μM DBT, and 300 μM H_2O_2. An equimolar concentration of BSA was always added to heme.

and Hb and heat-denatured Hb, and nearly 10 for heme-BSA, in the presence of 300 μM DBT and 300 μM H_2O_2. These numbers dropped under unity for HRP (type VI) regardless of what H_2O_2 and HRP concentrations may have been added. Taking the examples of Fig. 3, we found out that when H_2O_2 was added step by step using very low DBT/HRP and DBT/H_2O_2 ratios, the number of turnovers was around 6 at the end of the reaction.

Degrees of conversion of DBT to DBT sulfoxide conversion vs H_2O_2 concentration were then studied at various concentrations of Hb or heme-BSA. Under the reaction conditions explained in Fig. 5, the production of DBT sulfone never exceeded 0.05%. Several comments can be drawn from these experiments. First of all, the shape of the graphs for Hb and heme-BSA was nearly the same. Degrees of conversion increased when H_2O_2 concentration was increased. The maximum efficiency was attained with lower heme concentration at low H_2O_2 concentrations. The degrees of conversion also increased with the increasing Hb concentrations, except at low H_2O_2 concentrations. In fact, the degrees of conversion depended on the DBT/H_2O_2/catalyst ratio (DBT was not inhibitor of the reaction). We found out that the reaction stopped long before all the DBT was oxidized at low Hb concentrations and started again when fresh Hb was added to the reaction mixture. We looked for the limiting product of the reaction either with Hb or with heme-BSA. A second addition of H_2O_2, DBT, or BSA had no effect, but the reaction started again when Hb, heme, or heme-BSA was added. These observations led us to the conclusion that DBT oxidation is limited by the degradation of heme, be it covalently or noncovalently bonded to a protein. This raises the question whether Hb and heme in a proteic environment act as enzymatic or chemical catalysts.

In order to explain this phenomenon, a kinetic study was started. A comparison of the initial rates was done for HRP (type VI), native Hb, heat-denatured Hb, and heme-BSA. Rate measure conditions were explained

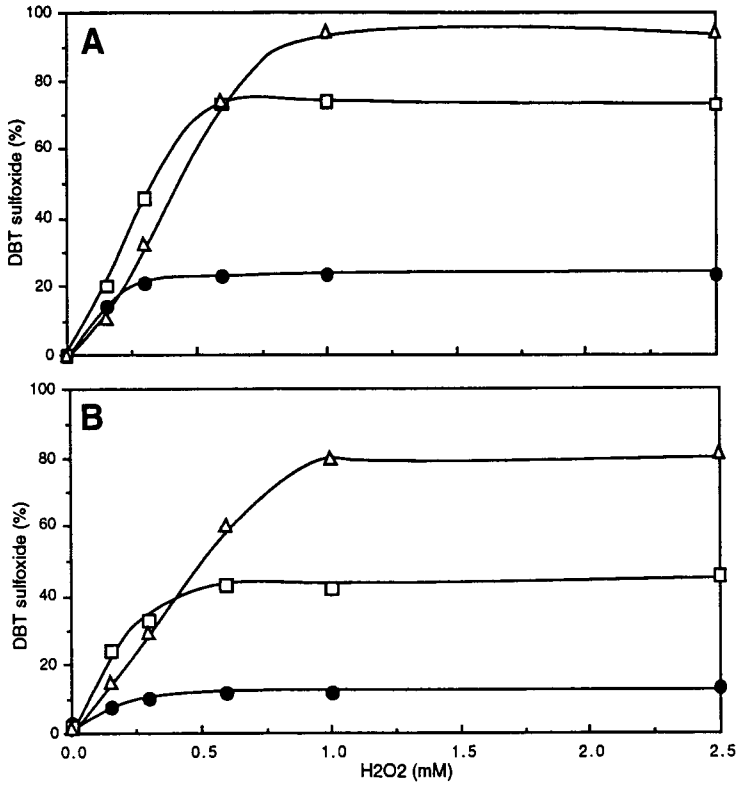

Fig. 5. Degrees of DBT sulfoxidation to the corresponding sulfoxide by Hb (**A**) and heme-BSA (**B**) vs H_2O_2 concentration. The reaction mixture contained 25% 1-propanol in acetate buffer, pH 5.0, 300 μM DBT, and the following heme concentration: 2.5 (●), 10 (□), and 25 (△) μM.

and commented on in the experimental procedure. In the presence of 300 μM H_2O_2 and 300 μM DBT, initial rates were proportional to native Hb, heat-denatured Hb, and heme incubated with BSA for heme concentrations up to 10 μM. The rate values were nearly the same for these three catalysts. For HRP in these conditions, the degrees of conversion were so small and the initial rate so slow, that it was difficult to calculate initial rates; as rough examples, 0.015 and 0.020 μM of DBT were oxidized/min by 1 μM of HRP and heat-denatured HRP, respectively.

The k_{cat} for the oxidation of DBT was 0.22 min^{-1} with Hb, 0.21 min^{-1} with heat-denatured Hb, and 0.16 min^{-1} with heme-BSA.

CONCLUSION

Native Hb, heat-denatured Hb, and heme-BSA all catalyze the DBT sulfoxidation with 100% conversion, whereas DBT oxidation was limited to 4% with HRP (type VI) under the same conditions.

The present study indicates that only when covalently or noncovalently bonded to a protein does heme catalyze the reaction (BSA may substitute the globine, heme may not be substituted by Fe^{3+}). Proteins, such as BSA, with a relatively low affinity for heme enhanced heme-dependent DBT sulfoxidation. Heme in native Hb or heat-denatured Hb is a much better catalyst of H_2O_2-DBT sulfoxidation than HRP. The poor efficiency of HRP as a catalyst for this reaction can be explained by the steric requirements imposed by HRP, its heme edge, or the fact that heme iron is probably inaccessible to DBT. This would explain why heat-denatured HRP is slightly more efficient an HRP in the native state.

The sulfoxidation is limited by heme and protein denaturation by H_2O_2. It was observed that DBT did not act as an electron donor substrate that would protect the hemoprotein from the denaturation by the peroxide, as in a normal peroxidase cycle. These data also demonstrate that DBT sulfoxidation is neither a Fenton-type reaction nor a classical peroxidase reaction. A proteic environment is needed for heme to catalyze the reaction, probably because it increases the solubility and the availability of heme.

Nevertheless, other experiments are necessary: to determine the involvement of the cosolvent and the importance of the proteic part and to improve the activity and stability of Hb, which would be an interesting biocatalyst because it is easily available.

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