# Inactivation of Hemoglobin by Hydrogen Peroxide and Protection by a Reductant Substrate

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**Abstract:** Inactivation and degradation of hemoglobin were examined in the presence of hydrogen peroxide and guaiacol. Hemoglobin is inactivated upon exposure to hydrogen peroxide. The inactivation and degradation of hemoglobin are two correlated processes. The presence of reducing substrate in addition to hydrogen peroxide partly or completely protected the hemoglobin from inactivation. A reaction mechanism is proposed, in which two competitive routes exist for Compound Ⅱ of hemoglobin; one catalytic and one inactivating. [Life Science Journal. 2006; 3(1):52 – 58] (ISSN: 1097 – 8135).

**Keywords:** hemoglobin; hydrogen peroxide; guaiacol; inactivation

## 1 Theory

Hemoglobin (Hb) is the major heme protein of red blood cells and is responsible for the transport of oxygen to the tissues. The function of Hb depends upon the ability of ferrous iron in the heme group to bind and release oxygen. Despite its principle role as an oxygen-carrier, the Hb molecular possesses different enzymatic activities (Giardina, 1995).

Hb has been reported as able to oxide aniline (Mieyal, 1976), lipids (Yoshida, 1994), dibenzothiophene (Wu, 1994), N-heterocycles (Ortiz, 1992), styrene (Ortiz, 1985) and o-phenylenediamine (OPDA) (Zhang, 2000; Liu, 2000). Zhang et al (2000) had reported that the catalytic effectiveness of methemoglobin (metHb) with OPDA as a substrate was the highest comparing with the other mimic of peroxidase such as hemin, βCD-hemin and MnTCPP. Moreover, metHb had a higher catalytic activity than the horseradish peroxidase (HRP) if their catalytic effectiveness is expressed on a molar basis rather than in terms of unit weight (Zhang, 2000). As with HRP (Maria, 2002), lignin peroxidase (Mylrajan, 1990), manganese peroxidase (Timofeevski, 1998), lactoperoxidase (Huwiler, 1986), and other peroxidases, hemin is inactivated by an excess of hydrogen peroxide ($H_2O_2$) or organic hydroperoxides in the absence of reducing substrate (Zheng, 2001). The substrate inactivation of peroxidases leads to the modification of the heme prosthetic group and probably to the formation of a verdohemoprotein as a final product (Rodriguez-López, 1997). The inactivation mechanism of peroxidases, however, has not yet been clearly elucidated.

The knowledge and understanding of Hb inactivation and reactivation kinetics and its related mechanisms would offer new possibilities in improving the potential and effective use of this pseudoenzyme in such diverse and broad areas. As the oxidant substrate of peroxidase, $H_2O_2$ is also an inactivation agent of this enzyme (Kathy, 1994). The reaction of $H_2O_2$ with Fe(Ⅱ) Hb (oxyHb and deoxyHb) and Fe(Ⅲ) Hb results in the formation of ferrylhemoglobin (ferrylHb) and oxoferrylhemoglobin (oxoferrylHb), respectively (Kathy, 1994; Giuliv, 1994). Both are strong oxidizing agents. The formation of heme-derived products that are covalently cross-linked to the globin molecule has been reported during the reaction of hemeproteins with $H_2O_2$ and trichlorobromomethane (Osawa, 1996; Osawa, 1994). The reaction of Hb with a molar excess of $H_2O_2$ will lead to the degradation of heme, the release of iron (Gutteridge, 1986), and the formation of two fluorescent products (Enika, 1998). As a mimetic enzyme of HRP, hemin can also be inactivated during the catalytic process for the oxidation degradation of it (Zheng, 2001; Lissi, 1994). But the inactivation of Hb during its catalytic reaction process

has not been reported.

The main objective of this work was to give a kinetic and mechanistic study of Hb inactivation during the reaction of Hb with the guaiacol/$H_2O_2$ system.

## 2 Materials and Methods

### 2.1 Materials

MetHb (bovine) from Shanghai Institute of Biochemistry (Shanghai, China) was used without further purification. Guaiacol was purchased from Sigma. The stock solution of guaiacol was prepared by dissolved 116 mg guaiacol in 400 $\mu$L dioxane and 500 $\mu$L double distilled deionized water, the working solution was prepared by dissolve the stock solution to appropriate concentration. Hydrogen peroxide solutions were prepared by appropriate dilution of the 30% solution with double distilled deionized water (standardized by titration with $KMnO_4$). All other reagents were of the highest available grade and all solutions were prepared in 0.1 M citric-$Na_2HPO_4$ buffer (pH 5.0) and all experiments were carried out at room temperature (25 ± 1°C) unless stated otherwise.

### 2.2 Apparatus

UV-Vis measurements were performed on a UV-1601 rapid scan spectrophotometer (Shimdzu, Japan) using 1 cm light path quartz cuvette. A 420A pH meter (Orion Research Inc. USA) was used.

### 2.3 Determination of the heme bleaching of Hb

The reaction was carried out in 3 mL 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0) at 25°C, containing 1 mM Hb and different concentration of $H_2O_2$ (~1 mM), in the presence or absence of 0.4 mM benzidine. After 30 min of reaction, the absorbance at 410 nm was recorded.

### 2.4 Rate of inactivation of Hb

The inactivation of Hb was carried out at 25°C in 1-ml incubations of 0.1 M citric-$Na_2HPO_4$ buffer containing the enzyme (37 $\mu$M). Two types of incubation were performed: (a) incubation in the presence of 10 mM $H_2O_2$ and (b) incubation in the presence of 5 mM guaiacol. At specified time intervals, 30 $\mu$L aliquots of the incubation mixtures were transfer to cuvetts containing 3 mL of an assay mixture composed of 5 mM guaiacol and 1.0 mM $H_2O_2$ in 0.1 M citric-$Na_2HPO_4$ buffer (pH 5.0). the peroxidase activity was measured by the increase in the absorbance at 470 nm, which is characteristic for the guaiacol oxidation product tetra-guaiacol ($\varepsilon_{470} = 5570\ M^{-1}cm^{-1}$) (Alexander, 2000).

### 2.5 Determination of the product accumulated at the end of the reaction

The reactions were realized in 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0). The media, with a final volume of 3 mL, were incubated at 25°C, Hb, guaiacol and $H_2O_2$ were added, the concentration of each in turn being varied while the remaining two were kept constant. Thus, because the final values of $A_{470\ nm}$ showed a good stability, it was possible to estimate maximum values for the product accumulated at the end of each reaction. The experiments were run a minimum of three times.

### 2.6 Iron release from Hb during the inactivation reaction

Hb (10 $\mu$M) was incubated with the different concentrations of $H_2O_2$ in the presence and absence of guaiacol in 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0) at 25°C for 30 min. Free iron was measured by the Ferrozine method (Cater, 1971). Briefly, a 0.45-ml sample taken from the reaction mixture was mixed with 50 ml of 100% (W/V) trichloroacetic acid. Then, 0.5 ml of 0.02% ascorbic acid in 0.1 N HCl was added, the system was incubated for 5 min at room temperature, and 0.4 ml of ammonium acetate (10%) and 0.1 ml of Ferrozine solution (75 mg of Ferrozine and 75 mg of neocuproine in 25 ml water) were added. After an additional incubation for 5 min at room temperature, the color developed was measured at 562 nm. The iron concentration was calculated using a millimolar extinction coefficient of 27.9 $mM^{-1}cm^{-1}$.

## 3 Results

### 3.1 Heme degradation of Hb mediated by $H_2O_2$

$H_2O_2$ can mediated the heme degradation of various hemoproteins (Gutteridge, 1986; Jose, 2000). Mixing of Hb with $H_2O_2$ results in a red shift in the maximal absorbance of Hb from 405 nm to 409 nm as observed previously (Hai-cheng, 2002), probably due to the formation compound Ⅰ, and a rapid decline in peak absorbance in the Soret region indicating the degradation of the heme prosthetic group (Figure 1). The rate constants for heme destruction and the loss of catalytic activity differed (Figure 2). After five minutes of incubation with $H_2O_2$, the Hb lost 78% of its activity (Figure 2), 83% of the heme has been destroyed, according to the Soret band (Figure 2). The heme destruction process therefore appeared to precede the inactivation process.

The residual enzymatic activity was checked after incubation of Hb in media containing: (a) $H_2O_2$ or (b) guaiacol. Figure 2 showed a different behavior for both kinds of incubation. After 10 min

of incubation of Hb and $H_2O_2$, the activity loss of Hb was 93.5%, but for guaiacol, the activity loss of Hb was only 4.8%. Therefore, the loss in Hb activity did not depend on the guaiacol; instead, $H_2O_2$ was the inactivation agent.

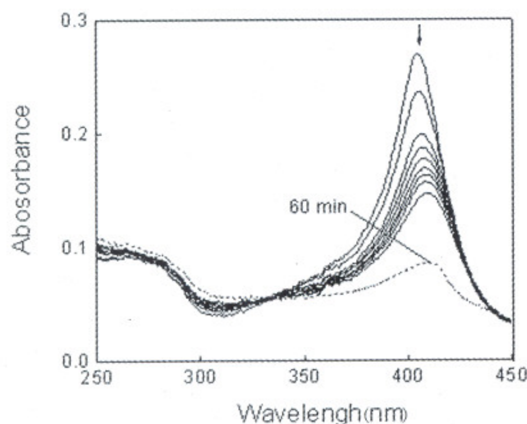



**Figure 1.** Hb degradation by $H_2O_2$. Hb (0.5 μM) was mixed with 10 mM $H_2O_2$ in citric-$Na_2HPO_4$ buffer (pH 5.0), and incubated at 25°C. Absorption spectra (250 – 450 nm) were taken at 1.0 min intervals starting immediately after mixing (9 upper continuous traces) and then after 60 min (dotted trace) at 250 – 450 nm. Heme prosthetic group destruction as indicated by disappearance of the Soret band, and the arrow indicates the direction of change.

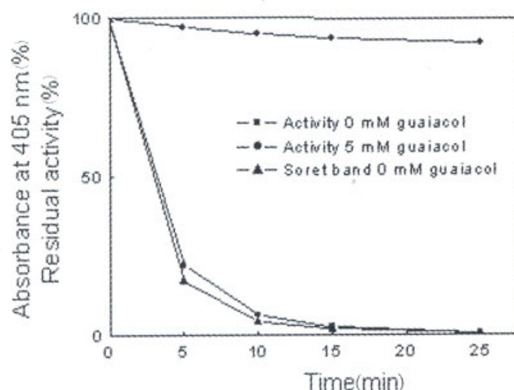



**Figure 2.** Time-dependent inactivation and heme degradation of Hb. 37 μM Hb was incubated with 10 mM $H_2O_2$ or 5 mM guaiacol. At specified time intervals, 30 μL aliquots of the incubation mixtures were transfer to cuvetts containing 3 ml of an assay mixture composed of 5 mM guaiacol and 1.0 mM $H_2O_2$ in 0.1 M citric-$Na_2HPO_4$ buffer (pH 5.0). The peroxidase activity was measured by the increase in the absorbance at 470 nm. As for the heme degradation, at specified time intervals, the absorbance of Hb at 405 nm was determined using the completely degraded Hb solution (with the same concentration of Hb incubated with large excess of $H_2O_2$ at 25°C for 2 h) as control.

To corroborate the heme destruction results, the iron release was measured using Ferrozine method (Figure 3). Incubation of Hb with various concentrations of $H_2O_2$ for 30 min resulted in the release of iron detected in the reaction mixture in a dose dependent manner (Figure 3). After 30 min incubation in the presence of 100 mM $H_2O_2$, 47% of iron present in the protein was released and could be detected as protein-free iron in the supernatant solution, after protein precipitation. No soluble iron was detected in control samples incubated without $H_2O_2$ and extracted in the same manner as the experimental samples. In contrast, negligible iron was released from Hb in the presence of 5 mM guaiacol.

Figure 4 showed the Hb degradation mediated by $H_2O_2$ and the protection role of benzidine. 0.17 mM $H_2O_2$ caused about 87% of the Soret band loss of Hb. Addition of 0.4 mM benzidine strongly inhibited this process.

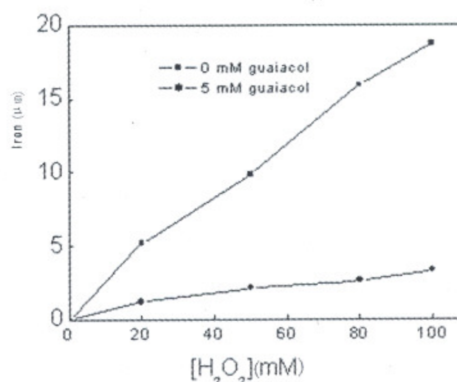



**Figure 3.** Iron release from Hb mediated by $H_2O_2$ in the presence and absence of guaiacol. Hb (10 μM) was incubated with the indicated concentrations of $H_2O_2$ in the presence and absence of guaiacol in 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0) at 25°C for 30 min in a total volume of 1.0 mL. The concentration iron was determined by the Ferrozine method.

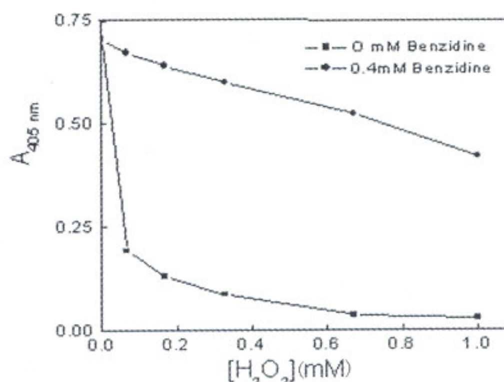



**Figure 4.** Heme bleaching of Hb by excess $H_2O_2$ in the presence and absence of benzidine, 1.0 μM Hb was incubated with different concentration of $H_2O_2$ in the presence and absence of 0.4 mM benzidine for 30 min, then the absorbance at 405 nm was determined.

### 3.2 Effect of $H_2O_2$ on the product accumulated in the end of the reaction

The Hb inactivation may also be demonstrated by measuring the product accumulated at the end of reaction. Figure 5A showed the time courses of guaiacol oxidation for a range of variable $H_2O_2$ concentrations. The product accumulated at the end of the reaction was obtained in a shorter reaction time when the $H_2O_2$ concentration was higher. Figure 5B showed that as the concentration of $H_2O_2$ was increased, the values of the absorbance of the product decreased. This confirmed the inactivating nature of the $H_2O_2$.

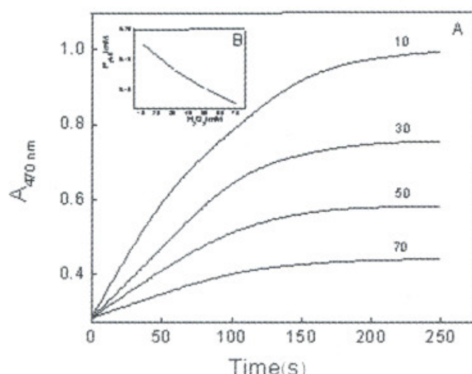



**Figure 5.** Relationship between the product accumulated at the end of the reaction and $H_2O_2$ concentration. (A) Time-courses of guaiacol oxidation for a range of variable $H_2O_2$ concentrations. The reaction mixture contained 0.25 $\mu$M Hb, 10 mM, guaiacol; 10,30,50,70 mM $H_2O_2$ respectively. The reaction was followed as $\triangle A_{470}$ nm vs. time. (B) The values of product accumulated at the end of the reaction (Pyield), obtained from (A), were plotted vs. $H_2O_2$ concentration.

### 3.3 Effect of guaiacol on the product accumulated in the end of the reaction

Figure 6 showed a plot of the absorption of product vs. the concentration of guaiacol. Keeping the Hb concentration constant, it could be observed that lower $H_2O_2$ concentrations produce higher yield of the product for each $H_2O_2$ concentration. When the guaiacol concentration was increase, the yield of the product also increaseed.

These results confirm the inactivating nature of the $H_2O_2$ and also showed the protective behavior of guaiacol towards Hb: this protective behavior increaseed as its concentration was increased.

### 3.4 Effect of Hb on the product accumulated in the end of the reaction

The plot of concentration of product vs. concentration of Hb when both guaiacol and $H_2O_2$ concentrations are kept constant. Figure 7 showed a linear trace, indicating that Hb inactivation did not depend on any product of the reaction. The slope of this straight line was related to the partition ratio ($r = [P_{yield}]/[Hb]$), which was the number of turnover carried out by one active site of the enzyme before its inactivation. This parameter remained constant for the total range of Hb concentrations used (Figure 7). It should be pointed out that the value of $r$ depended on the [guaiacol]/[$H_2O_2$] ratio used (Figure 6).

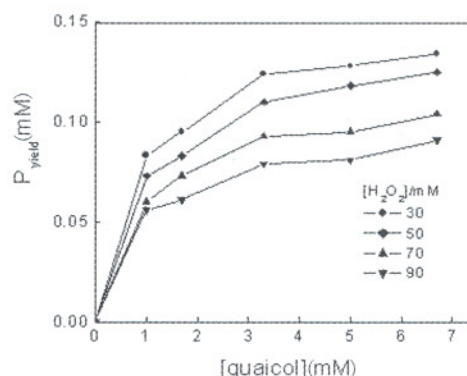



**Figure 6.** Concentration of reaction product accumulated at the end of the reaction as a function of the initial guaiacol concentration. Different concentrations of $H_2O_2$ were also assayed. The media contained 0.25 $\mu$M Hb in 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0). Each product absorption value was estimated as in Figure 5.

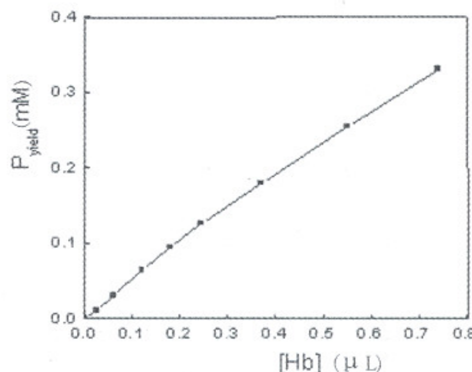



**Figure 7.** Concentration of product accumulated at the end of the reaction as a function of Hb concentration. The media contained 5 mM guaiacol and 30 mM $H_2O_2$ in 0.1 mM citric-$Na_2HPO_4$ buffer (pH 5.0). The product concentration was estimated as Figure 5.

## 4 Discussion

It is well established fact, and it has been known since the 1950s, that Hb possesses various pseudoenzymatic activities and is able to catalyze the oxidation of a variety of compounds (Mieyal, 1985; Grisham, 1991). It has considerable peroxidase activity (Everse, 1994), thus, Hb is able to mimic the enzymatic activities of a variety of other heme enzymes. Particular attention has been paid to ability of this protein to catalyze the oxidation of

aromatic substrates, including polycyclic aromatic hydrocarbons (Ortiz-Leon, 1995).

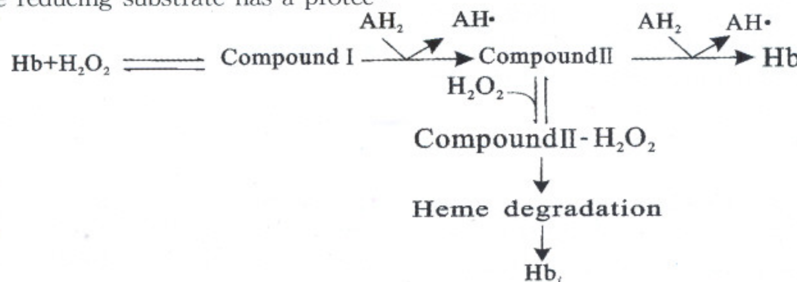
Over 20 years ago, Brown (1976) analyzed the biliverdin isomers produced by the $H_2O_2$ oxidation of various hemoproteins and suggested that the amino acid residues adjacent to the methylene bridges of the porphyrin ring might protect these sites against $H_2O_2$ oxidation. As shown in Figure 1 and Figure 2, Hb is inactivated by treatment with $H_2O_2$, and the heme prosthetic group is degraded. In the case of peroxidases, $H_2O_2$ inactivation leads to the modification of the heme prosthetic group to form a verdohaemoprotein as a final product (Mylrajan, 1990). Two principal mechanisms have been proposed for the inactivation of peroxidases by $H_2O_2$. One mechanism involves the reaction of Compound Ⅱ with $H_2O_2$ in the absence of a reducing substrate to form Compound Ⅲ. If Compound Ⅲ is a peroxyiron(Ⅲ) porphyrin free radical, it should be considered a highly reactive intermediate. Because of the proximity of the uncoupled electron to the porphyrin ring, any electron transfer from the ferrous state to an extra $H_2O_2$ moiety would generate a hydroxyl radical, which could in turn react with the heme group to produce irreversible inactivation. An alternative mechanism involves a reaction of Compound I with an excess of $H_2O_2$ in the absence of reducing substrate to form an irreversibly inactivated verdohaemoprotein. In this mechanism, Compound Ⅲ is proposed to be a superoxide-anion-generating system that has a protective effect against inactivation. The inactivation mechanism for peroxidases, however, has not been clearly elucidated at present (Cai, 1992; Hiner, 1995; Rodriguez-López, 1997).

In this work, we have shown that turnover-induced inactivation of Hb and peroxide-induced heme destruction are two correlated processes. Heme destruction appears to precede inactivation process (Figure 2). In the absence of reducing substrates and at high $H_2O_2$ concentrations, Hb is inactivated in a time- and $H_2O_2$- concentration dependent process. The reducing substrate has a protective effect on the $H_2O_2$ mediated Hb degradation. This protective effect of reducing substrate has also been described for types of peroxidase other than Hb, such as HRP (Arnao, 1990) and the lignin-degrading peroxidase (Wariishi, 1989).

It has been reported that ferrylHb, corresponds to peroxidase Compound Ⅱ, can withdraw one electron from the reducing sbustrate. In the absence of reducing substrate, ferrylHb would oxidize $H_2O_2$ to produce superoxide and metHb, and the superoxide generated in the heme pocket can oxidize the tetrapyrole rings, leading to the degradation of heme, the release of iron (Enika, 2000). But oxoferrylHb, corresponds to peroxidase Compound Ⅰ, can react with $H_2O_2$ in the absence of reducing substrates to produce metHb and oxygen (Arnao, 1990), which cannot mediated heme degradation. Our experimental results showed that the heme degradation and inactivation of Hb are two correlated process, so we can conclude that the $H_2O_2$ mediated the inactivation of Hb through the reaction of $H_2O_2$ with Compound Ⅱ, not with Compound Ⅰ.

Arnao (1990) has reported that $H_2O_2$ mediated the peroxidase inactivation by reaction with Compound Ⅰ base on the founding that the number of catalytic cycles given by the enzyme before its inactivation is a function of a constant set and the [ABTS]/[$H_2O_2$] ratio, in which the number of turnover is linearly with the [ABTS]/[$H_2O_2$] ratio. Our results show that the number of catalytic cycles given by Hb before its inactivation is a function of the [guaiacol]/[$H_2O_2$] ratio, but not linearly with it (Figure 4), so the mechanism of Hb inactivation mediated by $H_2O_2$ is different from that of peroxidase.

The results presented in this paper suggest the need to include a Hb inactivation step (Hbi) in the usual schemes for reactions catalyzed by Hb. In consequence, and according to Arnao (Arnao, 1990) a mimimum scheme for the Hb/$H_2O_2$/guaiacol system could be:




**Scheme 1.** Possible catalytic and inactivation pathways of Hb. $AH_2$ is the reducing substrate such as guaiacol, ABTS, benzidine and so on.

The expression (Compound Ⅱ-$H_2O_2$) represents an intermediate complex in the pathway, which leads to the heme degradation of Hb and thus the inactivation of it. As can be seen in Scheme 1, a competition for Compound Ⅱ between guaiacol and $H_2O_2$ is established, which protects Hb as long as [guaiacol]/[$H_2O_2$] ratio is high.

We show in this paper that $H_2O_2$ itself is the inactivating agent, and that the heme degradation and inactivation of Hb are two correlated process. We also show that the reducing substrates are necessary for the catalytic turnover of the Hb and to protect the Hb from inactivation.

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