

Peroxidase activity of hemoglobin and heme destruction in the presence of hydrogen peroxide and CT-DNA

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Abstract: The aim of this study was to investigate the peroxidase activity of Hb with different concentrations of hydrogen peroxide and compare it with hypochlorous acid effect on Hb. Hypochlorous acid at higher concentrations decomposed Hb and heme, releasing free iron ion from the metal center. High concentrations of hydrogen peroxide switched the peroxidase activity of Hb towards the partial Hb and heme destruction. Heme alone was degraded showing that the Hb conformation and protein environment protects Hb from the distraction in the presence of highly increased hydrogen peroxide concentration that occurs as a result of oxidative stress. In the presence of CT-DNA acted inhibition of the peroxidase activity of Hb was observed signaling inhibited hydrogen peroxide consumption.

INTRODUCTION

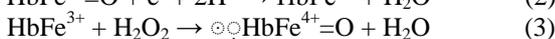
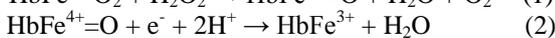
Hemoglobin (Hb) has an important role in human body as the oxygen-carrying metalloprotein, transporting oxygen from the lungs to the organs, cells, and tissues, where it releases O₂. The oxygenation and deoxygenation processes are highly controlled by the allosteric regulation with the relaxed (R) state between with the high oxygen affinity, and the tense (T) state having the low oxygen affinity, (Schechter, 2008) In addition to its mayor role as oxygen carrier, Hb also performs a number of other functions in biological systems. Hemoglobin is involved in nitric oxide and bicarbonate transport, neutrophile degranulation process, blood coagulation, regulation of a blood pressure, renal absorption, cellular oxidant detoxification and hydrogen peroxide catalytic process. As an example, deoxy-Hb can also show, under defined reaction condition, a nitrite reductase activity thus been involved in compensatory vasodilation in connection with nitric oxide regulation.

A heme (ferroprotoporphyrin IX) is a prosthetic group of a number of heme proteins and is noncovalently bonded with each subunit in the space created between the helices in the hydrophobic pocket. The protoporphyrin IX consists of four pyrrole rings linked in a heterocyclic ring system. The Fe(II) is coordinated to the four nitrogen groups of protoporphyrin. The nitrogen atom of proximal histidine is coordinated to the iron from the

bottom of the plane and the molecular oxygen binds to the iron on the opposite side.

Despite its significant biological role, under condition of oxidative stress, Hb can become toxic if it is released from erythrocytes. (Rifkind, Mohanty, Nagababu, 2015). This toxicity appeared to be caused by oxidative reactions catalyzed by Hb molecule. As a result, damage to surrounding tissues including biomolecules such as proteins, nucleic acids and lipids occurs (Alayash, Patel, Cashon, 2001). One of the reasons for the hemoglobin toxicity is its susceptibility to oxidation and autoxidation reactions. (Nagababu, Rifkind, 2004). In addition, in the presence of oxidative stress (i.g. high production of free radicals and hydrogen peroxide), heme metal center from hemoglobin is released in the surrounding environment generating more reactive oxygen species and, depending on the reaction condition, releasing free Fe(II). Free Fe(II) is sequestered and stored in ferritin through the tight control of ferritin synthesis in response to cellular iron status. Under extreme conditions, heme destruction is initiated, and excess of free iron is released in the surrounding environment, catalyzing the further formation of free radicals and amplifying host tissue damage (Nagababu, Chrest, Rifkind, 2003). Free Fe(II) can cause vasodilation and as result hypotension and metabolic acidosis will occurs.

Iron damage to mitochondria by lipid peroxidation pathway results in renal and hepatic damage. (Ong, Halliwell, 2004; Trinder, Fox Vautier *et al.*, 2002). The major source of hydrogen peroxide in the physiological system is the cellular respiratory burst that generates reactive oxygen species (ROS). This is oxygen dependent process where membrane-bound NADPH oxidase, catalyzes the reduction of molecular oxygen to the reactive oxygen intermediate, superoxide that in the presence of superoxide dismutase (SOD) produces hydrogen peroxide (H₂O₂). Other source of hydrogen peroxide productions in the endothelium are vascular NADPH oxidase, xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS) and mitochondrial overproduction of reactive oxygen species. Hb reacts with low H₂O₂ concentrations producing Compound II (○₂HbFe⁴⁺=O) (Reactions 1, 2, 3,).



Increased hydrogen peroxide concentration can occur in biological systems during various inflammatory events. Endogenous H₂O₂ produced by neutrophils also has an important role in microbial killing. Less is known that bacteria can also produce H₂O₂ that has an effect on host-microbial interactions. For example, streptococci, pneumococci and lactobacilli due to the structural components-flavoproteins, use molecular oxygen to convert to hydrogen peroxide. Catalase is not present in these microbes, allowing accumulation of hydrogen peroxide. It is known that *S. pneumoniae* infection causes increased production of H₂O₂, up to millimolar concentration (Loose, Hudel, Zimmer, *et al.* 2015; Pericone, Parck, Imlay, *et al.*, 2003).

Produced hydrogen peroxide then can serve as a substrate for the number of heme proteins or can be directly sequestered by Hb and incite the heme destruction and free Fe(II) release. Thus, knowing exact mechanism of heme release from hemoglobin and its activity in the presence of different concentrations of H₂O₂ is important since it can explain its determinant role in biological systems especially during the increased oxidative stress. Here we studied the reaction between human Hb (oxy-Hb) with increasing hydrogen peroxide concentrations using spectroscopic methods. We examined the reaction between heme and hydrogen peroxide using spectroscopic method and electrophoresis. Since hypochlorous acid (HOCl) is a strong oxidant and antimicrobial agent in the biological systems that can oxidized a number of biological molecules inflicting host tissue injury, we analyzed the reactions of Hb and heme with different HOCl concentrations. As control, Calf thymus DNA (CT-DNA) was used to test whether it can mitigate toxicity caused by degradation of Hb or heme.

EXPERIMENTAL

Chemicals

All chemicals used were of the highest purity grade and used without further purification. Sodium hypochlorite (NaOCl), heme, human hemoglobin (Hb), dimethylsulfoxide (DMSO), CT-DNA were obtained from Sigma Aldrich (St. Louis, MO, USA).

Absorbance measurements.

The absorbance spectra were recorded using Thermofisher Biosystems UV-visible spectrophotometer at 25°C, and in phosphate buffer at pH 7.2. Fixed concentrations of Hb and heme were prepared and increasing concentration of H₂O₂ added to the solution (10-400 μM). Absorbance spectra were then recorded from 300 to 700 nm. All solutions were prepared fresh before the absorbance measurements.

Agarose gel electrophoresis

Solutions containing the CT-DNA and the Hb were incubated in the presence or absence of H₂O₂ at room temperature. Aliquots were taken and loaded onto 1% agarose gel. Electrophoresis was carried out in TBE buffer (1 M Tris base, 1 M boric acid, and 0.05 M Na₂EDTA, pH 8). The staining of gel was done by ethidium bromide. DNA Step Ladder (50 bp) was used in experiment that contained 17 fragments consisting of 50 bp repeats from 50 to 500 bp, 100 bp repeats from 600 to 900 bp, and 1 kb repeats from 1 to 3 kb.

HOCl solution preparation.

Stock solution of HOCl was prepared by mixing 1 ml of sodium hypochlorite (NaOCl) aqueous solution to 40 ml of 154 mM NaCl. The value of the pH was set at 3 by addition of HCl solution. To convert all convert active chlorine species to OCl⁻, the volume 40 μl of 5M NaOH was added and the concentration of OCl⁻ was measured spectrophotometrically at 292 nm with molar extinction coefficient of 362 M⁻¹cm⁻¹. Since HOCl is very unstable, the stock solution was prepared just before the experiments, and stored on ice. All the dilutions were made from the stock solution by addition of appropriate volumes of 200 mM phosphate buffer, pH 7.2.

CT-DNA solution preparation

Stock DNA solutions were prepared by dissolving needed amount of DNA in 5 mM tris(hydroxymethyl)aminomethane (Tris) buffer, mixed with 50 mM NaCl, pH 7.2.

Hemoglobin solution preparation

Hb solution was prepared by dissolving 5 mg of lyophilized powder in 10 mL of 200 mM phosphate buffer pH 7.2 to prepare 7.9 μM solution. Further dilutions were made with the same phosphate buffer.

Heme solution preparation

Heme stock solution was prepared in DMSO and kept in the dark at 4°C. Diluted solutions were prepared by addition of a phosphate buffer, pH 7.2. The heme concentrations for each diluted solution was calculated by measuring absorbance at 404 nm using molar extinction coefficient of $170,000\text{M}^{-1}\text{cm}^{-1}$.

RESULTS AND DISCUSSION

The first spectra (Figure 1.) shows the changes in the spectra of Hb after addition of 40 μM H_2O_2 . The visible spectrum of Hb shows a typical Soret absorbance peak at 405 nm that is indication of a ferric (III) heme. The absorption spectra of pure Hb exhibits several electronic bands located at 279 nm that are the result of phenyl group of tryptophan and tyrosine residues. Another band at 349 nm (ϵ band) also known as Heme or Soret band is centered at 406 nm with two other bands at 540 and 570 nm (oxy band or Q-band) (Kristinsson, 2002).

It is well known that the appearance of strong Soret band (strongly allowed $p-p^*$ electron transition) at 406 nm in absorption spectra confirms Hb in its native form. This band is assigned to the heme group. Heme is deeply inserted in the hydrophobic pocket that is created from the protein's back bone through appropriate folding (Khan, Quigley, 2013).

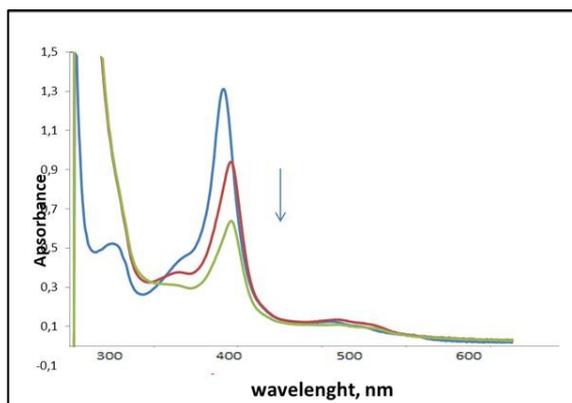


Figure1: The absorption spectra of Hb after addition of 40 μM hydrogen peroxide in phosphate buffer, pH=7.2

Any changes in Soret band are correlated to the changes in the heme microenvironment. After addition of H_2O_2 solution shift in Soret band and intensity was observed. Soret band shifted to 416 nm, but absorbance intensity was lowered. This can be explained by formation of Hb-Fe(IV)=O (Compound II) with a Soret band shifted to 418 nm and with less intensity due to the lower extinction coefficient. The solution of Hb was next incubated with H_2O_2 μM where final concentration of H_2O_2 was 400 μM and decrease in the absorbance was followed over time. (Figure 2).

Increasing concentration of H_2O_2 caused significant Hb heme decomposition that is confirmed by the loss of the intensity of the Soret peak. Incubation of Hb with 400 μM of H_2O_2 caused 70% decrease in the Soret band that after a time decayed indicating heme destruction due to the changes in the heme microenvironment that can be associated with protein aggregation and free iron release.

(Maitra, Andreana et al., 2011). As a control, the spectral changes of Hb in the presence of HOCl that can completely oxidize the heme structure of Hb were monitored over time.

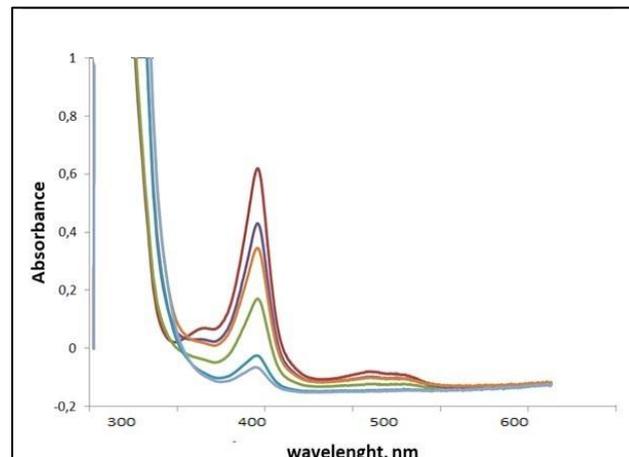


Figure2: The absorption spectra of Hb after addition of 400 μM hydrogen peroxide, in phosphate buffer, pH=7.2

As expected, after addition on a increments of HOCl (10, 20, 40, 80 μM), almost complete flattening of the Soret peaks was observed after 10 minutes indicating complete destruction of the heme structure in Hb (Figure 3.)

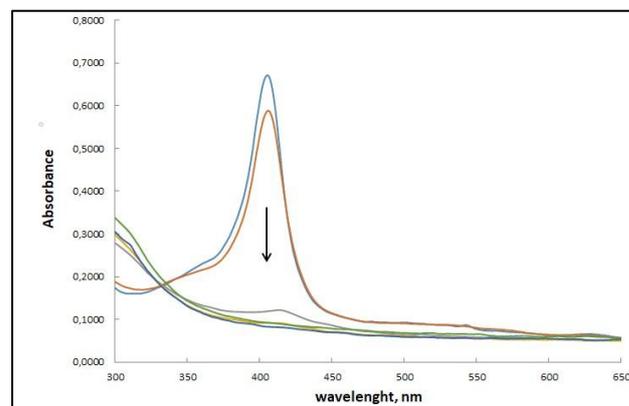


Figure 3: The absorption spectra of Hb obtained by titration with HOCl (5, 10, 20, 40, 80 μM)

Comparing the obtained spectra with ones where Hb changes were observed in the presence of H_2O_2 , it is clear that HOCl directly affect heme environment without formation of Compound II. Apparently HOCl directly cleave the covalent bonds of heme protoporphyrine with protein structure of Hb. Reaction of H_2O_2 with Hb follows peroxidase catalytic mechanism, thus giving the controlled decomposition of Hb. The peroxidase activity of Hb promotes the formation of free radicals and oxidative stress that leads to the modification of biomolecules. Interactions of Hb with CT-DNA were tested in the presence of H_2O_2 . Affinity of different forms of hemoglobin for double-stranded DNA was observed earlier (Tan, Cheng, Webber et al., 2005). More recently, a hemoprotein such as myeloperoxidase was detected to form MPO-DNA complexes in the process of NET-osis that is a characteristic response to severe inflammation. The

exact nature of the complex and its role in the defense system is still unknown. Data clearly showed the interactions between the heme proteins and the DNA double helix. Structurally, it is necessary for heme moiety to change the conformation in order to allowed nucleobase oxidation. The presence of H_2O_2 near the heme center and its role as a substrate for the heme center could block and control heme-DNA interaction. Figure 4 shows the absorption spectra of Hb after addition of 40 μM , in phosphate buffer, pH=7.2 with an addition of CT-DNA solution, 1 μM .

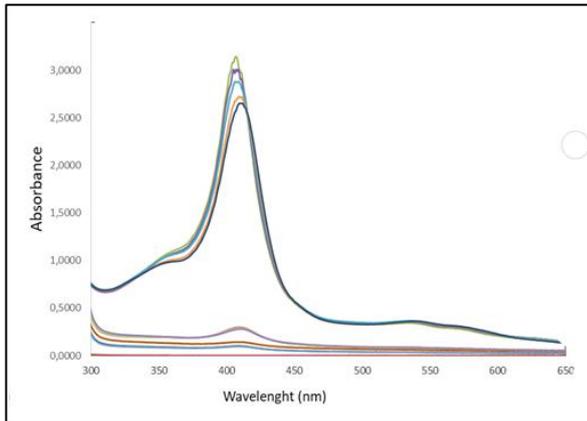


Figure 4. The absorption spectra of Hb after addition of 40 μM hydrogen peroxide, in phosphate buffer, pH=7.2 with an addition of ctDNA solution, 1 μM .

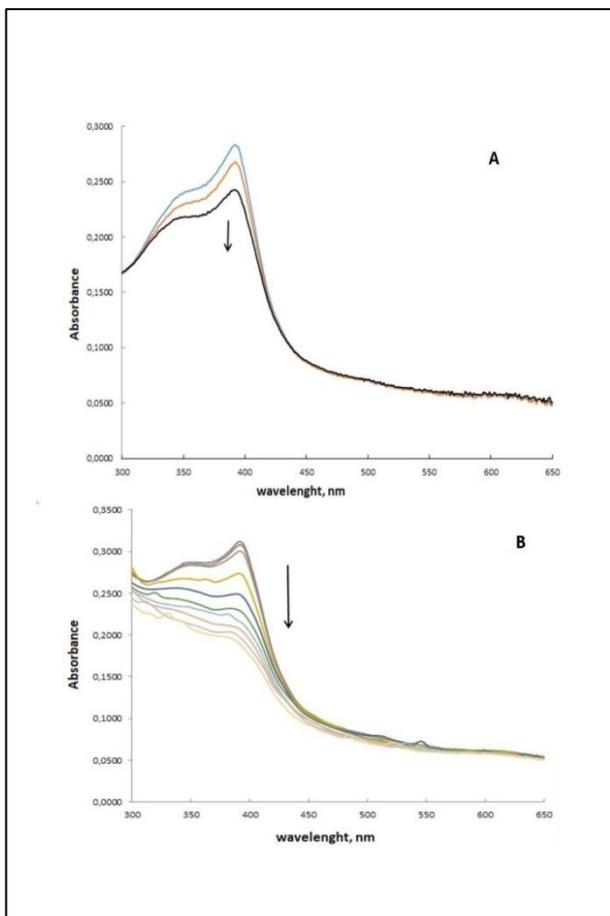


Figure 5. The absorption spectra of heme after addition of A.) 40 μM hydrogen peroxide, and B.) 400 μM hydrogen peroxide in phosphate buffer, pH=7.2

Upon addition of low concentration of H_2O_2 shift in Soret absorbance was observed concomitant with Compound II formation, however, intensity of the Soret absorbance was not significantly lowered when compared with the Soret peak in the absence of ctDNA. This clearly demonstrates that the heme of Hb does not consume H_2O_2 completely due to the blocked heme site. Clearly, molecule of CT-DNA acted as an inhibitor of the peroxidase activity of Hb. In order to test for the effect of the protein environment around heme structure, absorbance spectra of pure heme in the presence of H_2O_2 and HOCl were monitored and compared with those of Hb. (Figure5.)

Heme was completely decomposed upon addition of HOCl (data not shown). In reaction with H_2O_2 the decrease of the typical broad Soret band at 384 nm was observed. Addition of 400 μM of H_2O_2 lowered the absorbance even more and Soret band disappearance was observed indicating the heme decomposition. Clearly, the protein environment close to the heme pocket of Hb plays a role in the peroxidase activity of Hb.

Analysis of possible interactions between DNA and Hb that was incubated H_2O_2 was tested by agarose gel electrophoresis. (Figure 6).

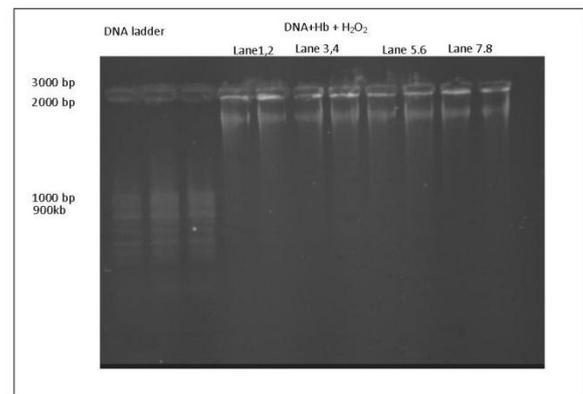


Figure 6. Gel electrophoresis showing the intensity band of ctDNA in the absence and presence of Hb with various hydrogen peroxide concentrations (lane1,2: 20 μM , lane 3,4: 40 μM , lane 5,6.:100 μM and lane 7.,8: 400 μM)

Lane 1 was untreated DNA alone, while lanes 2–4 ctDNA mixed with Hb and a different concentrations H_2O_2 (20, 40, 100 and 400 μM). Heme inhibited ctDNA fragmentation regardless of H_2O_2 concentrations confirming the possible interactions with the Hb and inhibition of its peroxidase activity. Possible aggregation of protein with ct DNA could explain electrophoretic behavior. In any case, the peroxidation activity of Hb was apparently inhibited.

CONCLUSIONS

The destruction of hemoprotein heme in any extent is a process that can be extremely toxic for surrounding environment. Free iron accumulation has the ability to participate in the production of free radicals via Fenton reaction. Hb reaction with H_2O_2 that follows the

peroxidase mechanism was not responsible for the complete heme destruction even at the higher concentration unlike highly reactive HOCl. Rather, Hb, in the presence of H₂O₂ showed formation of Compound II indicating classic peroxidase behavior. Mechanism of its peroxidase activity is highly controlled by the protein conformation rather than heme iron since heme alone was partially destructed by the increased in H₂O₂ concentration. CT-DNA partially blocked the peroxidase activity of Hb, with a possible formation of heme-DNA adduct. These interconnecting mechanisms indicate the highly controlled environment even in the cases of elevated oxidative stress and might act in a protective manner. Further studies are needed to decipher the exact nature of these interactions.

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Summary/Sažetak

Hemoglobin je protein sa hem centrom, tetramer koji se sastoji od četiri podgrupe proteinskih globina i četiri proteinske skupine hema. Najvažnija funkcija Hb je isporuka kisika iz pluća u periferno tkivo za stanični metabolizam. U uvjetima oksidativnog stresa mogući su načini uništavanja hema ili stvaranja drugih viših oksidativnih stanja. Cilj ove studije bio je istražiti aktivnost peroksidaze Hb s različitim koncentracijama vodikovog peroksida. Visoke koncentracije vodikovog peroksida preusmjerile su aktivnost peroksidaze Hb prema djelomičnom uništavanju Hb hema. Sam hem je degradiran, pokazujući da konformacija Hb i proteinsko okruženje štite Hb od distrukcije u prisustvu visoko povećane koncentracije vodikovog peroksida koja nastaje kao rezultat oksidativnog stresa.