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Reactions of Peroxynitrite and Nitrite with Organic Molecules and Hemoglobin

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Abstract—In this work, the reactions of nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) with organic molecules as well as with hemoglobin (Hb) were examined and the potential interference with the detection of hydrogen peroxide and Hb was investigated. $ONOO^-$ at low concentrations (35-140 μM) induced a concentration-dependent oxidation of *o*-phenylenediamine and guaiacol, and this process can be improved by the addition of Hb in a concentration-dependent manner. This enhancing effect of Hb was possibly due to the formation of such highly reactive species as ferrylHb during the reaction of $ONOO^-$ and Hb. NO_2^- also oxidized the aromatic amine *o*-phenylenediamine, but its efficiency was much lower than that of $ONOO^-$. A 300-fold excess of NO_2^- over hydrogen peroxide inhibited the oxidation of Pyrogallol Red mediated by hydrogen peroxide and Hb, which was due in part to the reaction of NO_2^- with Hb ferryl species compound I and compound II and the phenoxyl radical. These data suggest that $ONOO^-$ and NO_2^- can interfere with the detection of hydrogen peroxide. The overestimation or underestimation of the hydrogen peroxide detected is dependent upon the organic molecule utilized for detection and the relative rate of NO_2^-, superoxide, and $ONOO^-$ generation.

Key words: peroxynitrite, nitrite, hemoglobin, *o*-phenylenediamine

Hydrogen peroxide, the product of the reactions catalyzed by a large number of oxidases in cellular oxygen metabolism, can be formed either by the two electron reduction of oxygen in the presence of the corresponding oxidase enzyme or from the dismutation of O_2^- by superoxide dismutase (SOD) [1]. It is significant in chemical, biological, clinical, and many other fields. Elevated production of O_2^- and H_2O_2 can overwhelm the antioxidant capacity of the cell, which can then result in oxidation of key cellular components and cell death. Oxidative stress has been considered as the etiological factor for pathologic conditions such as ischemia–reperfusion injury, neurodegenerative disorders, and inflammatory cell-mediated tissue injury [2]. Therefore, measuring changes in the concentration of H_2O_2 has been critical in assessing the contribution of reactive oxygen species (ROS) in the pathology of several experimental models. At physiological pH, H_2O_2 is uncharged; hence, it can diffuse outside cells where it can be detected by oxidizing a target organic molecule. Because H_2O_2 at concentrations that have been measured in cells (10-100 μM) is not capable of oxidizing these organic molecules, peroxidase [3] and hemoglobin (Hb) [4, 5] are usually employed to catalyze the oxidation. Several compounds have been utilized for the detection of H_2O_2 by the peroxidase and Hb catalyzed reaction. The most commonly used organic compounds are 1,2-phenylenediamine (OPDA) [5, 6], hydroxyphenylacetic acid (HPA) [7], guaiacol [8, 9], phenol red [10], and 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) [11].

Endothelial, inflammatory, and other cell types, in addition to forming ROS, under proper stimulation release active nitrogen species such as peroxynitrite ($ONOO^-$) and nitrite (NO_2^-) [12, 13]. Recently, $ONOO^-$ chemistry has become of considerable interest as a result of increasing evidence for the role of $ONOO^-$ in the development of oxidative damage in various pathologies [14]. $ONOO^-$ is formed at a near diffusion controlled rate by the reaction of NO with O_2^- (a byproduct of cellular respiration), which is very likely to occur even with the presence of SOD in physiological concentrations [15] due to its high second-order rate constant of about 10^{10} $M^{-1}\cdot sec^{-1}$ [16]. Although these precursors are relatively unreactive, $ONOO^-$ is a powerful oxidizing and nitrating agent that can react with biological substances such as protein [17], nucleic acid [18], lipids [19], car-

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bonate [20], and tyrosine [21] and other phenolics [22] in cells and tissues. NO_2^- is the major end product of the NO· metabolism. Thus, local NO_2^- concentrations in cells are mostly correlated to the extent of NO· production and can reach micromolar levels [23, 24]. NO_2^- has recently been shown to be oxidized to NO_2 by peroxidases in the presence of H_2O_2 [25]. This reaction system can nitrate tyrosine and is currently considered as the major alternative to $ONOO^-$-mediated tyrosine nitration *in vivo* [26]. We hypothesized that the simultaneous release of $ONOO^-$ and NO_2^- interferes with the detection of H_2O_2. Moreover, the presence of a heme protein such as Hb may facilitate the reaction between NO_2^- and H_2O_2 as well as the reactivity of $ONOO^-$ [27] and, therefore, cause yet another interference with the measurement of H_2O_2. This hypothesis is also consistent with the finding that $ONOO^-$ oxidized luminol [28], dihydrorhodamine 123 [29], and 2′,7′-dichlorodihydrofluorescein [30] without requiring peroxidase or metal catalysis, and the oxidation of luminol by agonist-stimulated endothelial cells was due to the formation of $ONOO^-$ [31]. Furthermore, Panus et al. [32], using the dimerization of HPA to measure the release of H_2O_2 from endothelial cells, found that as much as 50% of the fluorescence was independent of peroxidase. Because H_2O_2 alone did not result in a significant fluorescence after reacting with HPA, the authors suggested the release of a stronger oxidant such as $ONOO^-$ may be responsible for the peroxidase-independent oxidation of HPA. Therefore, we examined the ability of $ONOO^-$ and NO_2^- to oxide organic molecules in the presence or absence of Hb and H_2O_2.

MATERIALS AND METHODS

Chemicals. OPDA (Shanghai Chemical Agent, Inc., China) was sublimed before use. Guaiacol, diethylenetriaminepentaacetic acid (DTPA), Pyrogallol Red (PR), and sodium nitrite were obtained from Sigma (USA) and were used without further purification unless stated otherwise. Bovine methemoglobin (metHb) from Shanghai Biochemical Institute (Shanghai, China) was used without further purification. H_2O_2 solutions were prepared by appropriate dilution of 30% solution with double distilled deionized water (standardized by titration with $KMnO_4$). All other reagents were of the highest grade available. $ONOO^-$ was synthesized by reaction of 0.6 M NO_2^- with 0.7 M H_2O_2 at pH 13 and characterized according to the method reported previously [33]. Excess H_2O_2 was removed by passage through a column of manganese dioxide. The desired concentration of $ONOO^-$ was prepared daily by the dilution of the stock solution in aqueous 0.5-1.0% NaOH and this solution was kept in an ice bath (less than 10% of $ONOO^-$ had decomposed over 8 h). $ONOO^-$ was stored at −20°C and the concentration of $ONOO^-$ was determined spectrally in 0.1 M NaOH at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}\cdot cm^{-1}$) immediately prior to each experiment [34]. After 3-5 days at room temperature, the $ONOO^-$ had completely decomposed; this solution was called "decomposed $ONOO^-$" and was used in blank experiments.

Ferrylhemoglobin (ferrylHb) solutions were prepared according to the method reported previously [35]. The ferrylHb solution was diluted with buffer (0.1 M phosphate buffer, pH 7.4) to the required concentration and used within 10 min.

The concentration of metHb and ferrylHb was determined spectrophotometrically by measuring the absorbance at 560, 577, and 630 nm as described by Winterbourn and using the following equations derived from the corresponding molar extinction coefficients of each species [36]:

$$[\text{metHb}] = 79A_{560} + 42A_{577} + 319A_{630};$$

$$[\text{ferrylHb}] = 96A_{560} + 55A_{577} + 47A_{630}.$$

Absorption spectra were measured on a TU-1800pc UV/Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, China) and Cary 100 spectrophotometer (Varian, USA) using 1 cm light path quartz cuvette. All pH values were measured with a pH S-301 digital ion meter.

Procedure. All reactions were performed in 0.1 M potassium phosphate buffer, pH 7.4 (PBS), unless stated otherwise, containing 0.1 mM DTPA and followed spectrally in a UV-Vis spectrophotometer equipped with a temperature controlled stirring module. The yield of OPDA oxidation was determined at 418 nm. The yield of guaiacol oxidation was measured by the increase in the absorbance at 470 nm, which is characteristic for the guaiacol oxidation product tetra-guaiacol ($\varepsilon_{470} = 5570\ M^{-1}\cdot cm^{-1}$ [8, 9]). The oxidation of PR was determined by the decrease in the absorbance at 542 nm [37]. The experiments were run a minimum of three times.

RESULTS

Oxidation of OPDA by $ONOO^-$ and NO_2^-. As can be seen from Fig. 1, addition of $ONOO^-$ to OPDA in 0.1 M PBS (pH 7.4) and NO_2^- to OPDA in 0.2 M KCl-HCl buffer solution (pH 2.0) caused the oxidation of OPDA in a concentration-dependent manner, and NO_2^--caused oxidation was much less than that of $ONOO^-$. Addition as much as 100-fold molar excess of NO_2^- over OPDA did not cause detectable absorbance change of the reaction system at neutral pH. The yield of OPDA oxidation by $ONOO^-$ was increased by the presence of Hb (Fig. 2), and this enhancement was dependent on the concentration of Hb (Fig. 3). It was interesting to find that when the reaction mixture was bubbled with N_2 for 30 min before

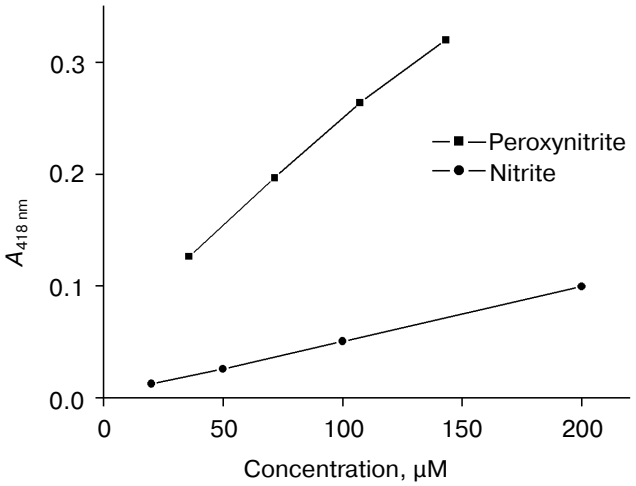

Fig. 1. Oxidation of OPDA by different concentrations of $ONOO^-$ and NO_2^-. The reaction was carried out in a test tube containing 1.0 mM OPDA and 0.1 mM DTPA, then different amounts of $ONOO^-$ and NO_2^- were added to give a final volume of 3 ml (0.1 M PBS, pH 7.4, was used for $ONOO^-$ and 0.2 M KCl-HCl buffer, pH 2.0, was used for NO_2^-). After 60 min of reaction at 25°C, the pH was adjusted to pH 7.4 using 10 M NaOH or 12 M HCl, and the absorbance at 418 nm was determined against a duplicate without addition of $ONOO^-$ and NO_2^-.

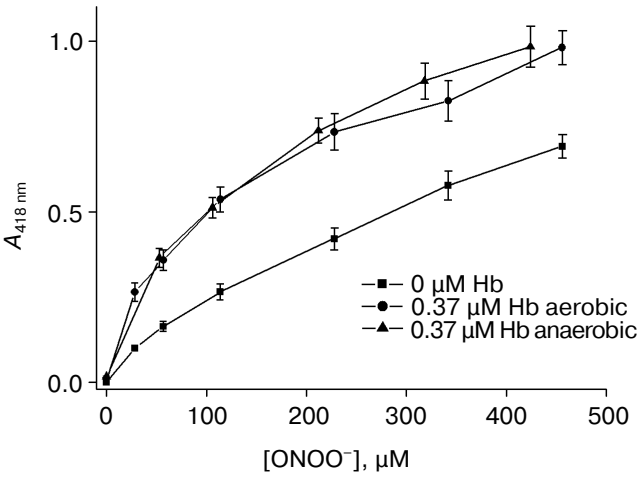

Fig. 2. Oxidation of OPDA by $ONOO^-$ in the presence or absence of Hb. The reaction was carried out as described in Fig. 1 except for the presence or absence of 0.37 μM Hb. Anaerobic conditions were obtained by bubbling the reaction mixture with N_2 for 30 min before the reaction. The absorbance at 418 nm was determined against a duplicate without addition of OPDA when in the presence of Hb.

the reaction, the yield of OPDA oxidation by $ONOO^-$ was slightly increased in the presence of Hb compared with that which was not bubbled with N_2. The decrease in the yield of OPDA oxidation by $ONOO^-$ in the presence of Hb and air may be attributed to O_2 or CO_2 dissolved in the reaction mixture.

Effect of carbon dioxide on oxidation OPDA by $ONOO^-$ in the presence of Hb. The effect of CO_2 on the oxidation of OPDA by $ONOO^-$ in the presence or absence of Hb was studied to determine whether CO_2 decreased the oxidation of OPDA by $ONOO^-$ in the presence of Hb. An effect of CO_2 was detected when 1 mM CO_2 was incorporated into the system by adding 60 μl $NaHCO_3$ (1.25 M) to the reaction mixture before the addition of $ONOO^-$ [38] giving the final concentration of 25 mM $NaHCO_3$. The experimental results are shown in Fig. 4. The yield of OPDA oxidation by $ONOO^-$ in the absence of Hb was enhanced by adding $NaHCO_3$ to the reaction mixture. But oxidation of OPDA by $ONOO^-$ in the presence of Hb was depressed by adding $NaHCO_3$ to the reaction mixture before reaction. Since oxygen is an oxidant, so we can conclude that the decreased yield of OPDA oxidation by $ONOO^-$ in the presence of Hb under air was attributed to carbon dioxide dissolved in the reaction mixture (Fig. 2).

Oxidation of guaiacol by $ONOO^-$. $ONOO^-$ can also oxidize guaiacol to tetra-guaiacol in a concentration-dependent manner (Fig. 5). The yield of tetra-guaiacol during guaiacol oxidation was determined by its characteristic absorbance at 470 nm [8, 9]. Figure 5 shows that the yield of tetra-guaiacol is increased in the presence of Hb. This is due to the formation of the highly reactive species ferrylHb during the reaction of metHb with $ONOO^-$, and which was confirmed subsequently.

Effect of NO_2^- on the oxidation of Pyrogallol Red (PR). The effect of NO_2^- on the oxidation of PR by H_2O_2 and Hb was tested. As can be seen from Fig. 6, addition of 0.3 μM Hb and 0.1 mM H_2O_2 to 50 μM PR in 0.1 M PBS (pH 7.4) caused the bleaching of PR, the presence of

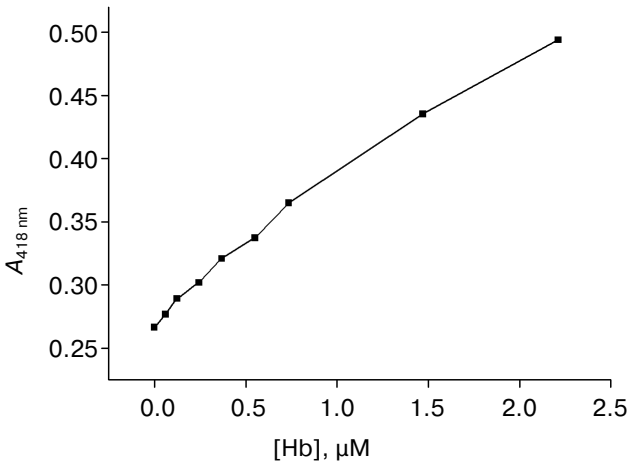

Fig. 3. Effect of Hb on the oxidation of OPDA by $ONOO^-$. The reaction was carried out as described in Fig. 1 except for the presence of variable concentrations of Hb. The absorbance at 418 nm was determined against a duplicate without adding OPDA. The concentration of $ONOO^-$ was 168 μM.

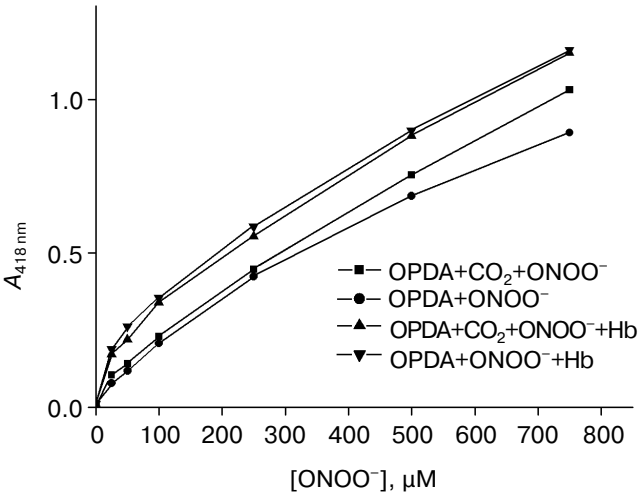

Fig. 4. Effect of carbon dioxide on oxidation of OPDA by $ONOO^-$ in the presence of Hb. The reaction was carried out as described in Fig. 2 except for the presence or absence of 25 mM $NaHCO_3$. Hb concentration was 0.37 μM. The absorbance at 418 nm was determined against a duplicate without adding of OPDA when in the presence of Hb.

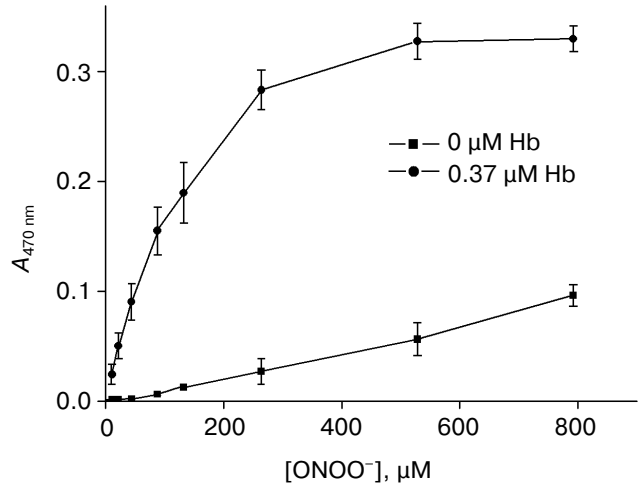

Fig. 5. Oxidation of guaiacol by $ONOO^-$ in the presence or absence of Hb. The reaction was carried out in 0.1 M PBS (pH 7.4) containing 0.1 mM DTPA and 1.0 mM guaiacol at 25°C in the presence or absence of 0.37 μM Hb, then different concentration of $ONOO^-$ were added to give a final volume of 3 ml. After 60 min of reaction at 25°C, the pH was adjusted to pH 7.4 using 10 M NaOH or 12 M HCl, and the absorbance at 470 nm was recorded against a duplicate without adding guaiacol when Hb was added.

NO_2^- in 300-fold excess over H_2O_2 resulting in the inhibition of the PR oxidation by H_2O_2 and Hb (Fig. 7).

Reaction of NO_2^- with H_2O_2 and Hb. Figure 8 shows the spectral changes of Hb after addition of NO_2^- or H_2O_2. Upon application of a stoichiometric amount of H_2O_2, the Soret band shifts from 400 to 419 nm and its intensity decreases dramatically. The spectra of Hb in Fig. 8 are identical to the electronic spectra of Hb-Compound II (Fig. 8*c*) [39]. Hb-NO_2^- is formed by the reaction of NO_2^- with Hb (Fig. 8*b*).

The reaction of Hb-Compound II with NO_2^- results in the formation of Hb or Hb-NO_2^- adduct when NO_2^- is in 300-fold excess of Compound II (Fig. 9). The spectral changes taking place during this reaction were monitored by rapid-scan spectroscopy between 390 and 460 nm. Upon mixing of a large excess of NO_2^- with Hb-Compound II at pH 7.4, the absorbance maximum of the Soret band shifted from 419 nm (Compound II, spectrum *a* in Fig. 9) to 405 nm (metHb at pH 7.4, spectrum *b* in Fig. 9). We found no evidence for a reaction between NO_2^- and H_2O_2 in 0.1 M PBS, pH 7.4. So, it is possible that NO_2^- can inhibit H_2O_2-Hb-mediated oxidation of organic substrates by reacting with ferrylHb species—Compounds I and II—which are necessary for the oxidation.

Reaction of guaiacol and NO_2^- with ferrylHb. Evidence for the formation of ferrylHb was obtained by the identification, after addition of Na_2S, of iron(II) sulfohemoglobin. This species has an intense absorbance band at 620 nm and is formed specifically from the reaction of ferrylHb with Na_2S [40, 41]. Na_2S was first added to a ferrylHb solution at pH 7.4, and later changes were observed in the UV-Vis spectrum after 30 min incubation at room temperature. In four separate experiments, 0, 163, 326, or 651 μM $ONOO^-$ was added to this solution. In all three cases, a new absorbance band with a maximum around 620 nm appeared after 30 min incubation at room temperature (Fig. 10), and the absorption intensity was dependent on $ONOO^-$ concentration. So ferrylHb formed during the reaction of metHb with $ONOO^-$.

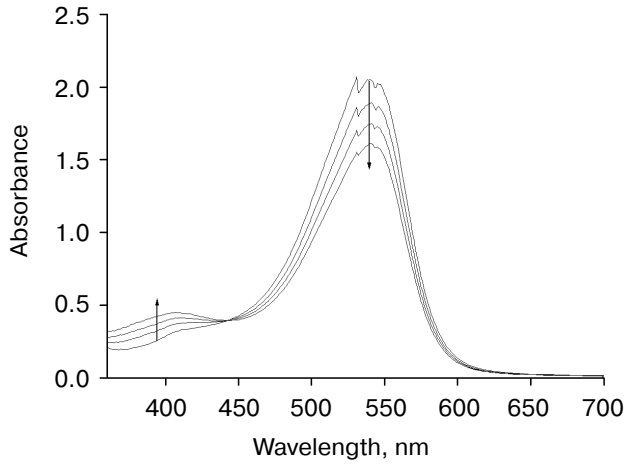

Fig. 6. Bleaching of Pyrogallol Red (PR) by H_2O_2 and Hb. The reaction was carried out in 0.1 mM PBS (pH 7.4) containing 0.1 mM DTPA and 50 μM PR at 25°C; 0.1 mM H_2O_2 and 0.37 μM Hb were added to start the reaction. The spectra in the range 350-700 nm were scanned at 10 min intervals.

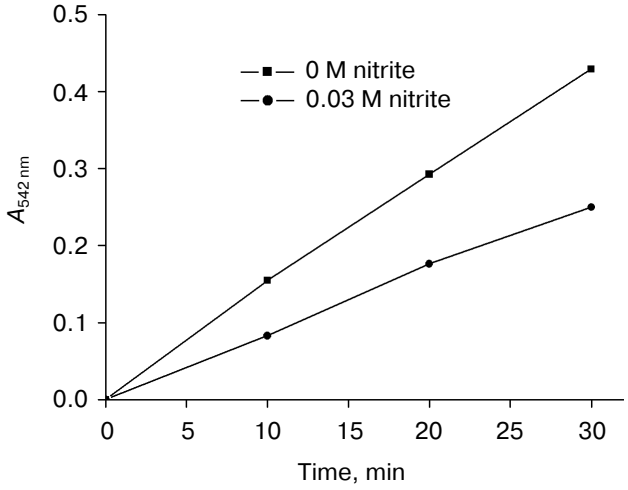

Fig. 7. Bleaching of PR by H_2O_2 and Hb in the absence or presence of NO_2^-. The reaction was carried out as described in Fig. 6 except for the presence of 0.03 M NO_2^-. The absorbance decrease was measured at 542 nm at different time intervals.

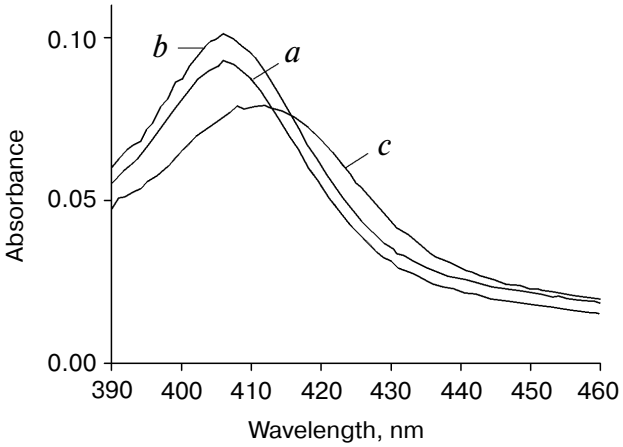

Fig. 8. Soret and visible optical absorption spectra of 0.3 μM Hb (*a*), Hb-NO_2^- (*b*) formed by reaction of 100 μM NO_2^- with 0.3 μM Hb, and Compound II (*c*) formed by the addition of 0.1 mM H_2O_2 to 0.3 μM Hb in 0.1 M PBS (pH 7.4) with 0.1 mM DTPA at 25°C.

Figure 11 shows the decay of ferrylHb (3.4 μM) preformed with an excess of H_2O_2. FerrylHb alone was rather stable, and the decay was moderately enhanced in the presence of NO_2^- or guaiacol, indicating that both compounds are able to react with ferrylHb and reduce ferrylHb to metHb.

DISCUSSION

The strong oxidant $ONOO^-$, formed by activated inflammatory cells and agonist stimulated endothelial cells [12-15], has been found to oxidize several organic molecules and to nitrate free or protein tyrosine residues and other phenolics [22]. Our experimental results show that $ONOO^-$ can oxidize OPDA along without the participation of peroxidase or Hb (Fig. 1), and Hb can increase the yield of guaiacol (Fig. 5) and OPDA oxidation by $ONOO^-$ in a concentration-dependent manner (Figs. 2 and 3). MetHb has been shown to react with the protonated form of $ONOO^-$, peroxynitrous acid (ONOOH), with a rate constant 4-fold faster than H_2O_2 [42]. It has

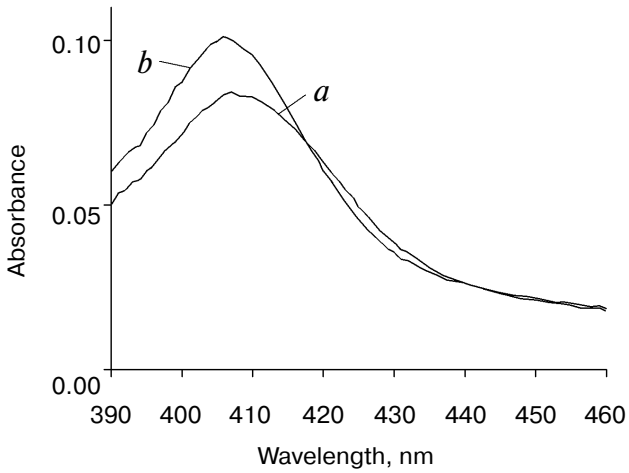

Fig. 9. Reaction of NO_2^- with Hb-Compound II. Addition of H_2O_2 to the Hb-NO_2^- resulted in the formation of Compound II (*a*) and addition of NO_2^- in excess to Compound II resulted in the formation of Hb-NO_2^- (*b*). All spectra were recorded in 0.1 M PBS (pH 7.4) with 0.1 mM DTPA at 25°C.

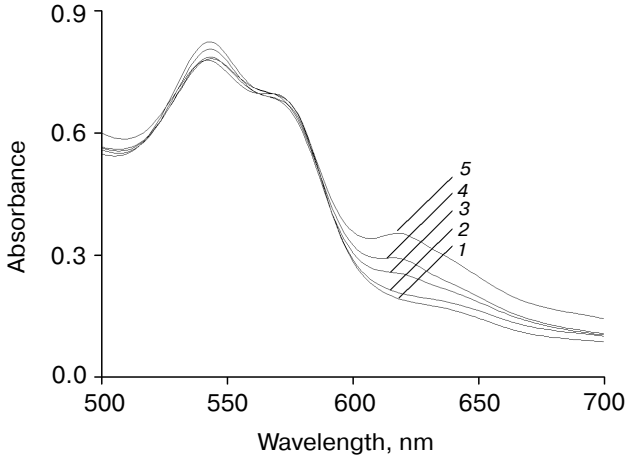

Fig. 10. UV-Vis spectra of 100 μM metHb in 0.1 M Na_2HPO_4-citrate buffer at pH 7.4 after addition of Na_2S and $ONOO^-$ and incubation for 30 min at 25°C: *1*) 25 μM metHb; *2*) 25 μM metHb and 200 μM Na_2S; *3*) 25 μM metHb, 200 μM Na_2S, and 163 μM $ONOO^-$; *4*) 25 μM metHb, 200 μM Na_2S, and 326 μM $ONOO^-$; *5*) 25 μM metHb, 200 μM Na_2S, and 651 μM $ONOO^-$.

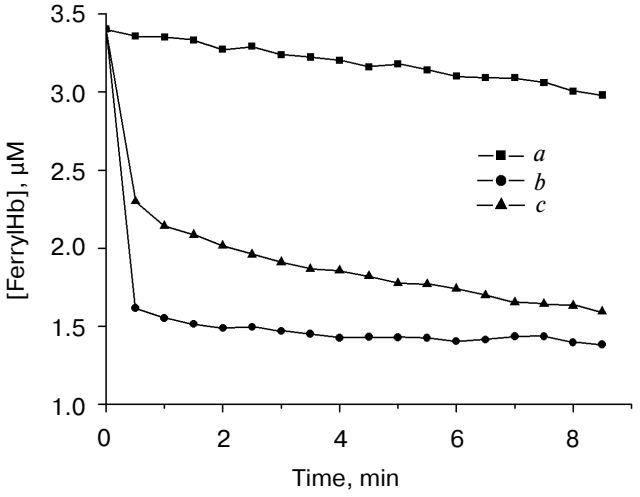

Fig. 11. Time course of ferrylHb reduction to metHb. Decay of ferrylHb (3.4 μM) in 0.1 M Na_2HPO_4-citrate buffer (pH 7.4) with 0.1 mM DTPA (*a*) and after addition of 580 μM sodium nitrite (*b*) or 2 mM guaiacol (*c*). Spectra were measured every 30 sec against a duplicate without adding Hb. FerrylHb concentration was determined as described under "Materials and Methods".

been proposed the reaction of $ONOO^-$ with metHb can form a complex that could be viewed as a radical pair between $^{\cdot}NO_2$ and Fe(IV)=O-globin [43], leading to Fe(III)-hemoglobin and nitrate as end products. Fe(IV)=O-globin is perferryl heme that is highly unstable and can be further rapidly bleached by the reaction products [42, 44]. In the absence of other organic molecule, a fast bleaching of perferryl heme can be performed by NO_2^-, a known peroxidase substrate, leading to the formation of nitrate and metHb as end products [44]. In the presence of another organic molecule, $^{\cdot}NO_2$ and Fe(IV)=O-globin can oxidize and nitrate the organic molecule [42, 44]. Thus the enhancing effect of Hb on the oxidation of an organic molecule is probably due to the formation of highly unstable intermediate of perferryl heme during the reaction of $ONOO^-$ with metHb. In our experimental results, the formation of ferrylHb was confirmed during the reaction of metHb and $ONOO^-$ (Fig. 10). NO_2^- and guaiacol can quickly bleaching the ferrylHb, leading to the formation of metHb (Fig. 11), and NO_2^- can inhibit the PR oxidation by H_2O_2 and Hb (Fig. 7). These observations suggest that the reaction of $ONOO^-$ with heme proteins generate a strong oxidizing species like ferrylHb, and it is possible that NO_2^- can inhibit H_2O_2-Hb-mediated oxidation of organic substrates by reacting with ferrylHb species Compound I and II, which are necessary for the oxidation of organic molecules. Thus, $ONOO^-$ and NO_2^- can interfere with the determination of H_2O_2 and Hb when organic substrates are used for detection.

$ONOO^-$ can rapidly react with CO_2 to yield 1-carboxy-2-nitrosodioxide ($ONOOCO_2^-$) [45], a stronger nitrating agent than $ONOO^-$ [20]. The life-time of $ONOOCO_2^-$ is too short to cross biological membranes [46], but it has been suggested that the radicals produced in its decay process, $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ [47], increase the nitration of tyrosine residues and one electron oxidations [48]. This is well consistent with our experimental results that CO_2 increased the yield of OPDA oxidation by $ONOO^-$ in the absence of Hb. It has been reported that CO_2 decreases the conversion of OxyHb to metHb mediated by $ONOO^-$ [34], proceeding via an intermediate ferryl complex, which, in a second step, reacts further with $ONOO^-$ to yield metHb [41]. It was interesting to find in our experiment that CO_2 decreased the yield of OPDA oxidation by $ONOO^-$ in the presence of Hb. We infer that this is due partly to CO_2 competing with Hb to react with $ONOO^-$, so as to decrease the ferrylHb formation during the reaction of Hb with $ONOO^-$.

Local NO_2^- concentrations in tissues are linked to the amounts of $NO^{\cdot}$ produced. Indeed, except for nitrate generated from the reaction of $NO^{\cdot}$ with oxyhemoglobin (oxyHb), NO_2^- is the major end product of $NO^{\cdot}$ metabolism. NO_2^- concentrations have been shown to reach micromolar levels [23, 24]. The reaction between high valency forms of heme proteins (Compounds I and II) and NO_2^- has recently attracted the interest of a large number of research groups. It has been shown that these reactions can generate NO_2 and it has repeatedly been proposed that they may represent an important pathway for tyrosine nitration *in vivo* [25]. In particular, the myeloperoxidase (MPO) catalyzed oxidation of NO_2^- is considered as the most significant alternative to $ONOO^-$-mediated nitration of tyrosine under physiological conditions [26]. The rate constants for the reaction of NO_2^- with MPO-, lactoperoxidase- (LPO), and horseradish peroxidase- (HRP) Compound II have recently been measured. NO_2^- has been shown to enhance the reactivity of LPO, MPO, and HRP towards compounds such as tyrosine by nitrating them [45]. This rate enhancement is thought to occur because tyrosine reacts more rapidly with $NO_2^{\cdot}$, generated from the oxidation of NO_2^- by LPO-Compounds I and II, than with LPO-Compound II [49]. In the present work, the reaction of NO_2^- with OPDA was studied. At neutral pH, no detectable absorption changes were observed during the reaction of NO_2^- with OPDA, but at pH 2.0, we can determine the oxidation of OPDA by NO_2^- in a concentration-dependent manner (Fig. 1), which demonstrated that HNO_2 has higher reactivity toward OPDA. The effects of NO_2^- on the oxidation of PR by H_2O_2 and Hb was also tested. The experimental results showed that NO_2^- in excess over H_2O_2 appears to inhibit the oxidation of PR (Fig. 7). The inhibition of PR oxidation may be due to the reaction of NO_2^- with ferrylHb species—Compounds I and II—which are necessary for the oxidation of organic substrate. Therefore, NO_2^- may terminate the oxidation of organic molecules and appear to act as an antioxidant. However, in order for NO_2^- to act as an antioxidant it must be pres-

ent in a several-fold excess over other oxidants. Thus, the relative rates of NO_2^- and H_2O_2 may be critical in determining the outcome of biological oxidations.

Overall, these data suggest that H_2O_2 measured from cells and tissues in continuous assays can be underestimated or overestimated if NO_2^- or $ONOO^-$ are generated simultaneously. Because H_2O_2 released in the cell media is relatively unreactive, a more reliable assay to measure H_2O_2 is to add the cell media to a solution containing peroxidase and a detector molecule as suggested [50]. The oxidation of PR by $ONOO^-$ and not by NO_2^- or H_2O_2 in the absence of Hb may be useful in detecting $ONOO^-$ formation from cells and tissues [37]. The multifaceted reactivity of heme-containing proteins necessitates a careful examination of the products formed in order to fully understand their roles in the oxidation of biomolecules.

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