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Catalytic scavenging of peroxynitrite by lactoperoxidase in the absence and presence of bicarbonate

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Abstract
The kinetics of the reaction of lactoperoxidase with peroxynitrite was studied under neutral and acidic pH. Lactoperoxidase catalyses peroxynitrite decay with the rate constant, k_c, increasing with decreasing pH. The values of k_c obtained at pH 7.1, 6.1 and 5.1 are $(1.9\pm0.1)\times10^6$, $(5.0\pm0.1)\times10^6$ and $(8.5\pm0.2)\times10^6$ $M^{-1}s^{-1}$, respectively. This tendency means that peroxynitrous acid is the species involved in the reaction with the catalytic centre of lactoperoxidase. Lactoperoxidase is also able to scavenge peroxynitrite in the presence of bicarbonate with the rate constant identical, within experimental error, to that measured in the absence of bicarbonate. It is thus concluded that $CO_3^{-\bullet}/{}^\bullet NO_2$ radicals formed in the system do not inactivate LPO. The mechanism of the catalytic scavenging of peroxynitrite by LPO is proposed. The physiological relevance of this reaction is discussed.

Keywords: *Bicarbonate, lactoperoxidase, peroxynitrite, stopped-flow spectrophotometry.*

Introduction

Lactoperoxidase (LPO) (~ 78 kDa [1]) is a member of mammalian heme peroxidases. Heme containing peroxidases catalyse one-electron oxidation of a wide range of substrates using hydrogen peroxide:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O_2 \quad (1)$$

$$\text{Compound I} + SH \rightarrow \text{Compound II} + S^\bullet \quad (2)$$

$$\text{Compound II} + SH \rightarrow \text{Peroxidase} + S^\bullet + H_2O \quad (3)$$

where compound I is an oxoferryl porphyrin π-cation radical, Fe(IV)=O(porphyrin$^\bullet$+), compound II is an oxoferryl intermediate, Fe(IV)=O and SH are substrates to be oxidized.

Heme peroxidases are also able to catalyse two-electron oxidation of some halide and (pseudo)halide (SCN^-) anions to the corresponding (pseudo)hypohalous acids using hydrogen peroxide. In this case only compound I is formed as a catalytic intermediate [2].

LPO is found in mammalian secretory fluids including milk, tears, saliva and airway mucus (recently reviewed in [3] and [4]). In each case LPO plays a role in antimicrobial defence by catalysing oxidation of thiocyanate to cytotoxic hypothiocyanous acid with the help of H_2O_2.

Peroxynitrite, the product of the diffusion controlled reaction between nitric oxide ($^\bullet NO$) and superoxide anion ($O_2^{-\bullet}$), is a strong oxidizing and nitrating agent. The participation of peroxynitrite in many pathological events has been reported [5]. Peroxynitrite has been also identified as a key cytotoxic effector of immune system cells towards invading bacteria and parasites [6]. The term peroxynitrite is used to refer to the sum of peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH). IUPAC recommended names are oxoperoxonitrate and hydrogen oxoperoxonitrate, respectively.

Peroxynitrite anion ($ONOO^-$) is stable in alkaline solution, but at physiological pH it undergoes protonation (pK 6.8). Peroxynitrous acid rapidly isomerizes to nitrate ($k=1.25\pm0.05$ s^{-1} at 25°C [7]), partially (~ 30%) via intermediate radical products, $^\bullet OH$ and

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$^{\bullet}NO_2$ [8,9], which may oxidize and/or nitrate biologically important molecules. However, most of peroxynitrite formed *in vivo* decays by direct reactions with CO_2 and proteins containing prosthetic groups or highly reactive cysteine residues [10]. These reactions may lead to changes in protein function. The reaction of peroxynitrite anion with CO_2, present in biological media at millimolar concentration, leads to the formation of $ONOOCO_2^-$ ($k=3\times10^4$ $M^{-1}s^{-1}$ at 24°C [11]) which rapidly undergoes homolysis to form $CO_3^{-\bullet}$ and $^{\bullet}NO_2$ with the yield of ~ 30% [12]. $CO_3^{-\bullet}/^{\bullet}NO_2$ radical pair possesses stronger nitration potential than $^{\bullet}OH/^{\bullet}NO_2$ radical pair formed from the isomerization of peroxynitrous acid. Due to potential cytotoxic effects mediated by peroxynitrite it is important to understand possible routes for peroxynitrite detoxification by naturally occurring compounds. It has been reported that some heme peroxidases, including LPO, catalytically scavenge peroxynitrite [13–16]. In the case of LPO the detailed kinetics of this reaction has not been presented yet. Here we show that LPO efficiently scavenges peroxynitrite in neutral as well as in acid solution both in the absence and in the presence of bicarbonate. The mechanism of the catalytic reaction is proposed.

Materials and methods

Materials

Lactoperoxidase from bovine milk (EC 1.11.1.7), lyophilized powder, $RZ=A_{412}/A_{280}$ of 0.8, was purchased from Sigma Co. (St. Louis, MO) and used as received. The concentration of LPO was determined spectrophotometrically at 412 nm using $\varepsilon_{412}=1.12\times10^5$ $M^{-1}cm^{-1}$ [17]. Sodium azide, sodium nitrite, hydrogen peroxide (30%) and guaiacol were also from Sigma. All others chemicals were of analytical grade. LPO compound II (LPOCII) was produced by mixing LPO with 5-fold excess of hydrogen peroxide. It was stable for ~ 10 min at room temperature.

Peroxynitrite was synthesized either by the ozonation of azide [18] or by the reaction of nitrite with hydrogen peroxide under acidic conditions [19]. In the first method, to reduce the content of unreacted N_3^-, ozonolysis was carried out for an additional 15 min after reaching the maximum concentration of $ONOO^-$ [20]. In the second method, unreacted hydrogen peroxide was removed from peroxynitrite solution by treatment with solid manganese dioxide. In both methods nitrite remained as a contaminant. The final concentration of peroxynitrite was 25–50 mM as determined spectrophotometrically using extinction coefficient $\varepsilon_{302=}1670$ $M^{-1}cm^{-1}$ [21]. The stock solutions of $ONOO^-$ were stored at –25°C and used within 3–4 weeks after synthesis. Nano-pure water from MilliQ (Millipore, Billerica, MA) was used throughout.

Azide and nitrite analysis

The concentration of azide in the peroxynitrite solutions was determined by the methemoglobin method [20]. It never exceeded 1% relative to peroxynitrite. The concentration of nitrite in the peroxynitrite solutions was measured by the Griess method [22] after peroxynitrite isomerization in an ice-cooled 25 mM phosphoric acid solution. Under these conditions, peroxynitrite exclusively isomerizes to nitrate. Nitrite contamination varied from 10–50% relative to peroxynitrite concentration. In order to determine the amount of nitrite formed from peroxynitrite decay, peroxynitrite was allowed to decay at the desired pH for 5 min and the total nitrite concentration in the sample was measured as described above. The concentration of nitrite formed from peroxynitrite decay was calculated by subtracting the amount of nitrite found as a contamination of peroxynitrite sample from the amount of total nitrite found in the experiment at a given pH.

Stopped-flow kinetic studies

The reactions of peroxynitrite with LPO were studied at pH ranging from 5.1–7.1. The SX-17 MV Applied Photophysic stopped-flow spectrophotometer with 1-cm cell and with a mixing time $<$ 1 ms was used throughout the study. LPO was dissolved in 0.2 M acetate or 0.2 M phosphate buffers and was bubbled with nitrogen in order to remove traces of CO_2. Peroxynitrite solutions were brought to the desired concentration by diluting the stock solution with 0.01 M NaOH. Equal volumes of solutions of peroxynitrite and enzyme were mixed in the cell and the reaction kinetics were followed at selected wavelengths between 265–450 nm. The concentration of peroxynitrite exceeded at least 10-fold the enzyme concentration. Replicate mixing was performed outside of the stopped-flow apparatus to measure the pH of the reaction mixture. The process of mixing increased the pH by 0.1 unit. In some experiments 20 mM sodium bicarbonate was added to the enzyme solution. To rule out any involvement of traces of free transition metal ions in peroxynitrite decay some experiments were performed in the presence of 0.1 mM diethylenetriaminepentaacetic acid (DTPA). All kinetic measurements were carried out at 23±0.5°C.

Pulse radiolysis study

Pulse radiolysis experiments were performed using the 6 MeV linear accelerator at the Institute of Applied Radiation Chemistry. Pulses of 7 ns delivering doses of 10–15 Gy were applied. A 1 cm optical path cell was used. Nitrogen dioxide radical was generated by radiolysis of aqueous solution of 0.1 M $NaNO_3$ and 0.1 M t-butanol saturated with Ar. Under such

conditions e_{aq}^- was converted into $^{\bullet}NO_2$ via the following reactions [23]:

$$e_{aq}^- + NO_3^- \rightarrow {}^{\bullet}NO_3^{2-} \quad (4)$$

$$^{\bullet}NO_3^{2-} + H_2O \rightarrow {}^{\bullet}NO_2 + OH^- \quad (5)$$

and $^{\bullet}OH$ radical was scavenged by t-butanol. Solutions of LPO with $NaNO_3$ and t-butanol were bubbled with Ar. Prior to pulse radiolysis experiments an appropriate amount of deaerated H_2O_2 was added to form LPOCII. Measurements were made within 10 min after mixing LPO with H_2O_2. The concentration of LPOCII exceeded the concentration of $^{\bullet}NO_2$ at least 5-fold.

Determination of LPO activity

The activity of LPO after the reaction with peroxynitrite was assayed as follows: enzyme at concentration of 1 μM was incubated at the desired pH with different amounts (50–400 μM) of peroxynitrite for 5 min. To assay the activity of LPO after the reaction of peroxynitrite in the presence of bicarbonate, the latter was added to the incubated solution at the concentration of 10 mM. Then the samples were diluted 100-fold and the activity was measured by the guaiacol method [24] at room temperature. Prior to the activity measurements, LPO solution was passed through a column containing Sephadex G25.

Statistics

All experiments were repeated at least in triplicate. Data shown in the figures are representative of all the corresponding experiments performed. The data are expressed as means±SD.

Results

We found that LPO retained full activity and unaltered absorption spectrum after incubation with peroxynitrite (50–400 μM) both in the absence and the presence of 10 mM of bicarbonate at investigated pH range (5.1–7.1).

The rates of the decay of peroxynitrite (100 μM) at three different pH in the absence or presence of LPO at 23°C were determined by following the absorbance changes at 265 nm (pH 5.1) or 302 nm (pH 6.1 and 7.1). We found that LPO catalysed peroxynitrite decay in a pH-dependent manner. The kinetics of peroxynitrite decay in the presence of LPO did not depend on the method of peroxynitrite synthesis nor on the presence of DTPA.

All recorded kinetic traces could be fitted with a single-exponential function. The observed peroxynitrite decay rate constants (k_{obs}) measured at different pHs were linearly dependent on the concentration of LPO (Figure 1). The values of k_c (the second-order reaction rate constant of peroxynitrite decay catalysed by LPO) obtained from the slopes of the plots increase with decreasing pH (Table I). The rates of the decay of peroxynitrite (100 μM) were also studied in the presence of compound II of LPO (1 μM) and the acceleration of peroxynitrite decay in the presence of LPO compound II was not observed (data not shown).

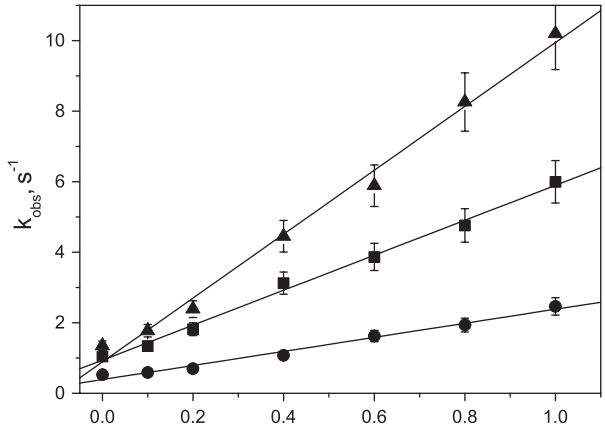

Figure 1. Plots of k_{obs} vs LPO concentration for the decay of peroxynitrite (100 μM) catalysed by LPO at: pH 5.1 (▲); pH 6.1 (■); and at pH 7.1 (•).

Considering the physiological relevance of the reaction between carbon dioxide and peroxynitrite we also studied LPO-mediated peroxynitrite decay in the presence of 10 mM of bicarbonate at pH 5.1 and 7.1. As shown in Figure 2, LPO accelerated the decay of peroxynitrite in a concentration-dependent manner. The values of k_c were identical, within experimental error, to those obtained in the absence of bicarbonate (Table I).

On the basis of spectrophotometric measurements, Floris et al. [25] have found that upon mixing LPO with peroxynitrite at pH 7.4 the formation of compound II followed by its conversion to the native enzyme is observed. We confirmed their observation. A decrease of the absorbance band at 412 nm, which is that of native LPO, was well correlated with a buildup of a new absorbance around 430 nm, characteristic for compound II of LPO (Figure 3). We were unable to observe kinetics of LPOCII formation when stoichiometric amounts of peroxynitrite and LPO were mixed, due to very low yield of LPOCII under such conditions. The yield of LPOCII increased with an increase of [peroxynitrite]/[LPO] ratio. In the longer time-scale the decay of LPOCII (430 nm) was observed parallel to the recovery of the native LPO

Table I. Catalytic rate constants (in 10^6 $M^{-1}s^{-1}$) for the LPO-mediated decay of peroxynitrite (100 μM) at various pH in the absence and presence of bicarbonate.

pH	No bicarbonate	10 mM bicarbonate
5.1	8.5±0.2	8.6±0.1
6.1	5.0±0.1	ND
7.1	1.9±0.1	2.0±0.1

ND, not determined.

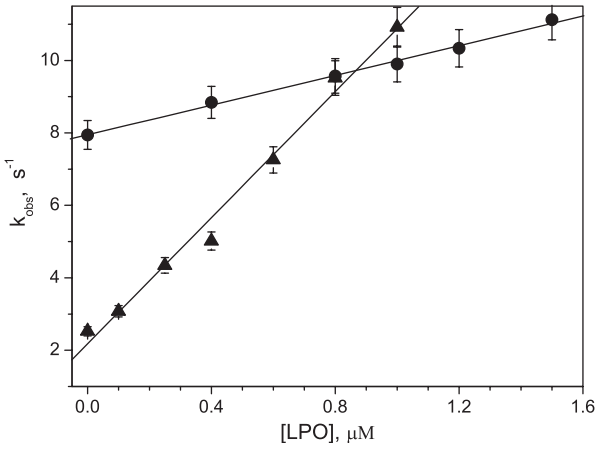

Figure 2. Plots of k_{obs} vs LPO concentration for the decay of peroxynitrite (100 μM) in the presence of 10 mM of bicarbonate and LPO at: pH 5.1 (▲) and at pH 7.1 (•).

(412 nm), both shown in Figure 4. The decay of compound II occurred in the same time scale as the decay of peroxynitrite (Figure 4).

To clarify the mechanism of LPOCII turnover in the reaction of LPO with peroxynitrite we studied the reaction of LPOCII with radiolytically generated $^{\bullet}NO_2$. The $^{\bullet}NO_2$ radical may be produced radiolytically by reaction of $^{\bullet}OH$ with a nitrite anion or by reaction of e_{aq}^- with a nitrate anion. LPOCII is inert toward NO_3^- but readily reacts with NO_2^- [26,27]. Therefore, the reaction of $^{\bullet}NO_2$ with LPOCII was investigated using NO_3^-/t-butanol system. The kinetics of the reaction of $^{\bullet}NO_2$ with LPOCII was studied by following the absorption decay at 430 nm, the maximum of absorbance of LPOCII. Absorbance increase at 412 nm (maximum of absorbance of LPO) was also observed. The kinetic traces could be fitted with a single exponential equation. The observed rate constants (k_{obs}) were linearly dependent on the concentration of LPOCII (Figure 5). From the slope of the plot the

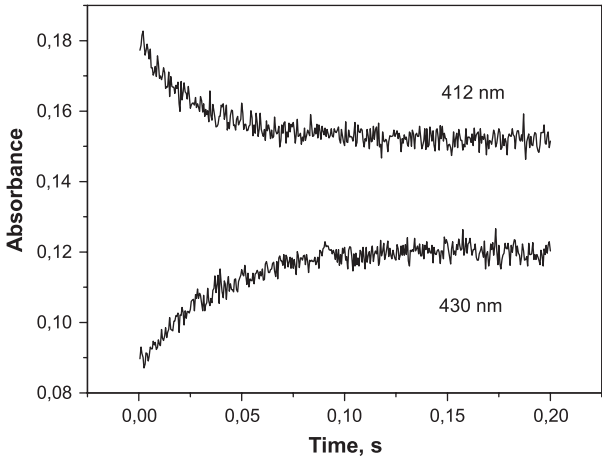

Figure 3. Time courses of the absorbance changes after mixing 18 μM peroxynitrite with 1.6 μM LPO in 0.1 M phosphate buffer at pH 7.1 observed in millisecond time scale: 412 nm (decay of LPO) and 430 nm (formation of LPOCII).

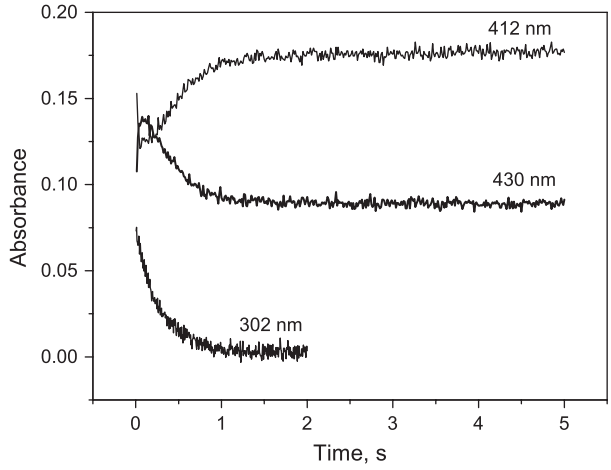

Figure 4. Time courses of the absorption changes after mixing 100 μM peroxynitrite with 1.5 μM LPO at pH 7.1: 302 nm (decay of peroxynitrite), 412 nm (recovery of native LPO preceded by its fast decay) and 430 nm (decay of LPOCII preceded by its fast formation). Both fast proceses are shown in Fig.3 at shorter timescale and for lower peroxynitrite concentration.

second order rate constant of the reaction of $^{\bullet}NO_2$ with LPOCII was calculated to be $(5.7\pm0.5)\times10^7$ $M^{-1}s^{-1}$.

It is known that at pH > 5 a spontaneous peroxynitrite disappearance occurs not only via isomerization ($ONOOH \rightarrow NO_3^- + H+$), but also via decomposition ($ONOOH + ONOO^- \rightarrow 2\ NO_2^- + O_2 + H+$) [28]. To find out whether LPO influences the ratio of $[NO_3^-]/[NO_2^-]$, we measured nitrite concentration after the decay of 100 μM peroxynitrite in the absence and in the presence of LPO. We found that a spontaneous peroxynitrite decomposition at 23°C resulted in the formation of 16±1, 21±2 and 25±2% of nitrite at pH 5.1, 6.1 and 7.1, respectively. These values fall well into the range reported earlier [28]. In the presence of LPO the amount of nitrite in the reaction mixture after peroxynitrite decay decreased to 3±1, 6±1 and 10±2% at pH 5.1, 6.1 and 7.1, respectively.

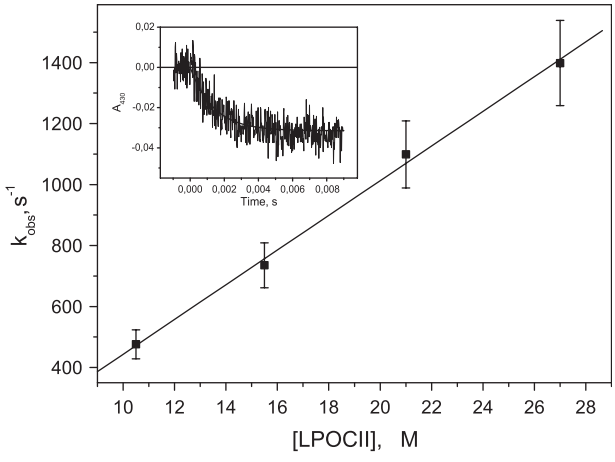

Figure 5. The pseudo first-order rate constant of LPOCII decay in the reaction with $^{\bullet}NO_2$ vs LPOCII concentration: Ar-saturated solution, 0.1 M $NaNO_3$, 0.1 M t-butanol, pH 7.0, dose 10 Gy. *Inset*: Kinetic trace of the decay of 15.5 μM LPOCII in the reaction with $^{\bullet}NO_2$ observed at 430 nm.

Discussion

We have shown above that the observed rate of the decay of peroxynitrite, being in excess towards LPO, increases linearly with the concentration of enzyme. This result suggests that LPO catalyses the decay of peroxynitrite. The second-order reaction rate constant, k_c, of peroxynitrite decay catalysed by LPO increases with decreasing pH, which indicates that peroxynitrous acid is the species involved in the reaction with the catalytic centre of LPO. The values of k_c for LPO are higher than those obtained for chloroperoxidase under similar reaction conditions [16]. It is worth noting that only some peroxiredoxins and glutathione peroxidase react more efficiently with peroxynitrite than LPO (the rate constants of their reactions with peroxynitrite are 7×10^7 and 8×10^6 $M^{-1}s^{-1}$, respectively, at pH 7.4 and 25°C [29,30]).

The values of the second-order rate constants of peroxynitrite decay catalysed by LPO are ca. two orders of magnitude higher than the rate constant of the reaction of peroxynitrite with CO_2. LPO concentration in some body fluids (up to 0.15 μM in human saliva and airway [31,32]) is much lower than that of CO_2 (~ 1.3 mM [33]). However, taking into account that at pH 5.1 only 2% of peroxynitrite exists as a peroxynitrite anion (reactant for CO_2) and that LPO reacts with peroxynitrous acid (98% of peroxynitrite is protonated at pH 5.1) it is conceivable that LPO may play a role in peroxynitrite detoxification when the local pH is lowered. There is increasing evidence that peroxynitrite is produced in airway diseases [34]. As LPO is secreted in the respiratory tract it may locally prevent some of the detrimental actions of peroxynitrite towards other proteins present even at submillimolar concentration (having in mind that maximal rate constant of peroxynitrite with amino acid residues it that with cysteine and it is of the order of 10^3 $M^{-1}s^{-1}$ [35]).

We have also shown that LPO is able to scavenge peroxynitrite in the presence of bicarbonate. Taking into account that bicarbonate is in equilibrium with CO_2 (pK_a=6.4 at 25°C [33]), up to 60% and up to 10% of peroxynitrite should react with LPO at pH 5.1 and 7.1, respectively, under our experimental conditions. The remaining part of peroxynitrite disappears in the reaction with CO_2. The values of the rate constants of peroxynitrite decay catalysed by LPO in the presence of bicarbonate (CO_2) are identical, within experimental error, to those measured in the absence of CO_2. It means that $CO_3^{\bullet-}$/$^{\bullet}NO_2$ radicals formed in the reaction of peroxynitrite with CO_2 do not influence the kinetics of the reaction of LPO with peroxynitrite. The assays of LPO activity after the reaction with peroxynitrite in the presence of bicarbonate confirm that these radicals neither modify the amino acid residues important for the catalytic activity of LPO nor damage the heme centre. It has been reported that for methemoglobin and metmyoglobin the values of the catalytic rate constants of peroxynitrite decay are three times larger in the presence of CO_2 than in the absence of CO_2 [36]. The authors have suggested that large concentration of bicarbonate (2.5-times higher than in our case) could cause conformational changes of the protein molecule which could lead to a faster reaction with peroxynitrite. Pietraforte et al. [37], however, have found that scavenging activity of methemoglobin vs peroxynitrite has been inhibited by CO_2.

It has been observed that for LPO, myeloperoxidase (MPO) and chloroperoxidase (CPO) only compound II is a detectable intermediate in the reaction with peroxynitrite [14–16,25,38]. The rate constant of the formation of compound II in the reaction of LPO with peroxynitrite reported in the literature varies from $8.0\pm0.2\times10^5$ $M^{-1}s^{-1}$ at pH 7.1 [15] to 3.3×10^5 at pH 7.4 [25] at room temperature. Two reaction mechanisms for LPO and MPO have been considered. Floris et al. [25] suggested the following reaction scheme:

$$\text{LPO/MPO} + \text{ONOOH} \rightarrow [\text{compound I} - NO_2^-] \quad (6)$$

$$[\text{compound I} - NO_2^-] \rightarrow \text{compound II} + {}^{\bullet}NO_2 \quad (7)$$

As they have not observed any intermediate they have proposed that reaction (6) is the rate determining step. Furtmuller et al. [38] have suggested that the rate determining step in the reaction of MPO with peroxynitrite is the formation of the transient MPO-peroxynitrito complex:

$$\text{MPO} + \text{ONOOH} \rightarrow \text{MPO-OONO}_2 \quad (8)$$

$$\text{MPO-OONO} \rightarrow \text{compound II} + {}^{\bullet}O_2 \quad (9)$$

The formation of compound II in the reaction of LPO, MPO or CPO with peroxynitrite implicates simultaneous generation of nitrogen dioxide (reactions (7) and (9)). The completion of the catalytic cycle of the peroxynitrite decay by heme peroxidases requires the presence of an efficient one-electron reductant in the system. Such reductant has not been unequivocally identified so far. In our earlier work we have excluded nitrite, a contaminant in the system, as a possible candidate to complete catalytic cycle of LPO-mediated peroxynitrite decay (the reaction of compound II with nitrite is too slow to contribute to the catalytic cycle of peroxynitrite decay) [15]. Here we have found that compound II of LPO does not accelerate peroxynitrite decay. Thus, peroxynitrite cannot be considered as a reductant of compound II either (although such a reaction is thermodynamically feasible [39]). A suggestion has been made [38,40] that $^{\bullet}NO_2$ is a likely candidate to be a reactant for compound II in the catalytic cycle of scavenging of peroxynitrite:

$$\text{LPOCII} + {}^{\bullet}NO_2 \rightarrow \text{LPO} + NO_3^- + \text{H+} \quad (10)$$

Our pulse radiolysis results demonstrate that $^{\bullet}NO_2$ radical rapidly reacts with LPOCII to re-form LPO.

Therefore this reaction most probably completes the catalytic cycle of LPO-mediated peroxynitrite decay. It has been demonstrated, by means of pulse radiolysis, that $^{\bullet}NO_2$ reacts with ferryl myoglobin and ferryl haemoglobin (analogues of peroxidase compound II), with the rate constants of the order of 10^7 $M^{-1}s^{-1}$ to form intermediate complexes which then rapidly dissociate to ferric protein and nitrate [41,42]. In the case of LPO we have not observed an intermediate complex.

The concentration of nitrite left after peroxynitrite decay in the presence of 1 μM LPO considerably decreases which means that LPO transforms peroxynitrite to nitrate. Decreased nitrite level after peroxynitrite decay has also been observed in the presence of methemoglobin and metmyoglobin [36,37].

In conclusion, our results confirm and extend previous studies demonstrating that LPO catalytically scavenges peroxynitrite at neutral and acidic pH. LPO reacts with peroxynitrite to form compound II and nitrogen dioxide which then react with each other to complete the reaction cycle. Experiments performed in the presence of bicarbonate show that $CO_3^{-\bullet}$/$^{\bullet}NO_2$ radicals formed in the system do not inactivate LPO. It is likely that LPO may be a part of the defense system against peroxynitrite in respiratory tract, particularly when local pH is lowered.

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