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pH-Dependent Regulation of Myeloperoxidase Activity

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Abstract—The balance between peroxidase and chlorinating activities of myeloperoxidase (MPO) is very important for the enhancement of antimicrobial action and prevention of damage caused by hypochlorite. In the present paper, the peroxidase and chlorinating activities have been studied at various pH values. The possibility of using neutrophil protein solution for the evaluation of MPO activity has been demonstrated. It is shown that at neutral pH MPO had higher affinity to peroxidase substrate guaiacol: at pH 7.4, chloride ions did not compete with guaiacol up to the concentration of 150 mM. At acidic pH, chlorinating activity of MPO dominates: only hypochlorite production can be detected at equal chloride and guaiacol concentrations of 15 mM. However, horseradish peroxidase does not exhibit any difference in activity in the presence of chloride ions even at acidic pH values. It was demonstrated by MALDI-TOF mass-spectrometry that the amount of hypochlorite produced is sufficient to modify phospholipids (with formation of Cl- and Br-hydrins and lyso-derivatives) only at acidic pH (5.0). Thus, in the presence of phenolic peroxidase substrate, MPO chlorinating activity can be displayed at acidic pH only. It can lead to elimination of hypochlorite production in normal tissues at neutral pH (7.4) and its enhancement in phagosomes where the pH range is 4.7-6.0.

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Myeloperoxidase (MPO) (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) is the main neutrophil enzyme, playing an essential role in protection against infection [1-4]. Beside its peroxidase activity, the enzyme also has a unique oxidizing activity towards halogenides (Cl^-, Br^-, I^-) and pseudohalogenides (SCN^-) that results in the formation of highly reactive hypohalogenites (OX^-) [1, 5-8]:

$$X^- + H_2O_2 \rightarrow OX^- + H_2O,$$

$$OX^- + H^+ \leftrightarrow HOX,$$

where X^- is a halogenide or pseudohalogenide.

A simplified mechanism of MPO action is shown in Fig. 1. The interaction between H_2O_2 and native ferriform of MPO heme leads to the formation of compound I (reaction *1*). This MPO form has very high oxidative activity and can undergo two further alternative reactions. The first one is two-electron reduction of compound I by halogenide to the native enzyme (reaction *2*), thus completing so-called halogenating cycle. The second is a sequential reduction to the native enzyme via formation of compound II followed by one-electron oxidation of a number of compounds (reactions *3* and *4*), which are the substrates for peroxidases (peroxidase cycle). In this way, the enzyme can display either halogenating or peroxidase activity [1, 5-13].

It is believed that chloride is a preferable substrate for compound I due to its high concentration *in vivo* (Cl^- concentration in blood plasma is 100-140 mM); therefore, the halogenating cycle is often called the chlorinating cycle [1, 11]. Nevertheless, it is established that MPO interacts effectively with other halogenides (Br^- and I^-), which are present in blood plasma at significantly lower concentrations (20-100 and 0.1-0.6 μM, respectively) [8].

Abbreviations: CTAB) cetyltrimethylammonium bromide; DEANOate) 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DTNB) 5,5′-dithio-2-nitrobenzoic acid; HRP) horseradish peroxidase; MALDI-TOF/MS) matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MCD) monochlorodimedone; MPO) myeloperoxidase; NP) neutrophil proteins; PAPC) 1-palmitoyl-2-arachidonoyl- *sn*-glycero-3-phosphocholine; POPC) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TNB) 5-thio-2-nitrobenzoic acid.

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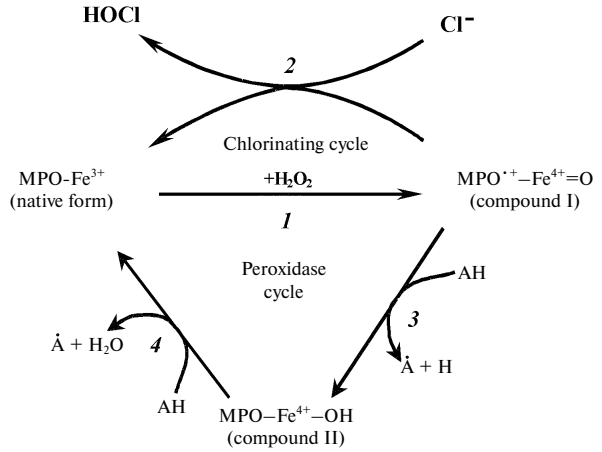

Fig. 1. MPO mechanisms showing the chlorinating and peroxidase cycles.

The substrates for the MPO peroxidase cycle are present in blood plasma and intracellular space in low concentrations, but their diversity (tyrosine, serotonin, ascorbate, urate, nitrite, β-ketones, and others) suggests that they provide competition to halogenides during the interaction with compound I [1, 9-14]. One of the main substrates for peroxidases is tyrosine, a natural amino acid found in many proteins. Its concentration in plasma is about 0.2 mM [10]. Moreover, tyrosyl radicals oxidize thiol groups or unsaturated lipids and undergo reduction to tyrosine [15], stabilizing its concentration.

Beside release of MPO, activation of neutrophils is also accompanied by secretion of inducible NO synthase. Active forms of nitrogen are known to influence MPO function, whereas such influence depends on many factors, especially on the ratio between the concentrations of H_2O_2 and an active nitrogen form itself [16-18]. Nitrite, one of the main products of NO metabolism, is a substrate for peroxidase. Its enzymatic oxidation results in the formation of nitrogen dioxide, which is an oxidant, and along with tyrosyl radicals can cause peroxidation of lipids [10, 14].

MPO activity dramatically depends on the pH of the medium. It is known that chlorinating activity increases with decrease in pH [6, 7]. However, the peroxidase activity of MPO or horseradish peroxidase (HRP) is practically independent of pH within the range of pH 5 to 8 [9, 19]. It has been shown that physiological concentrations of chloride do not affect the oxidation of L-tyrosine by myeloperoxidase at neutral pH [10]. On the other hand, the presence of peroxidase substrates can dose-dependently inhibit the formation of hypochlorite at neutral pH [5, 12]. It was also demonstrated [5] that a number of indole and tryptamine derivatives (which cause the accumulation of compound II in the presence of H_2O_2) also lead to the corresponding inhibition of chlorinating activity of MPO. The effective concentrations resulting in such inhibition are within the micromolar range at pH 7.4, but they increase by approximately two orders of magnitude as the pH decreases to 5.0.

Upon activation of neutrophils, MPO is released in the extracellular space from azurophilic granules. Hypochlorite, the product of reaction (1) catalyzed by this enzyme, can impact deleteriously on biologically important molecules and cells [2, 20-22]. How is this action directed? How is H_2O_2 formed as a result of phagocyte "oxidative burst" consumed? Not only the efficiency of cell response toward pathogens at acidic pH, but also deleterious impact of hypochlorite at neutral pH observed in phagosomes [2, 6, 7] are dependent on the ratio between peroxidase and chlorinating activities of MPO.

In the present work we have investigated the question of how peroxidase and chlorinating activities of MPO correlate under different conditions: normal (when biological liquids have as a rule neutral pH) and under cellular response, i.e., in phagosomes formed by neutrophils and having acidic pH. The possibility of using neutrophil proteins for the detection of MPO activity has been demonstrated. It has been established that the choice of substrate for the peroxidase or halogenating cycle depends on the pH of the medium. At neutral pH and in the presence of peroxidase substrates, MPO does not catalyze the production of hypochlorite, whereas at decreased pH all oxidative potential of compound I is directed to the production of hypochlorite. Moreover, it has been shown that the production of hypochlorite is regulated by active forms of nitrogen.

MATERIALS AND METHODS

Chemicals. All salts used in this work for the preparation of buffers, as well as NaCl, NaBr, citric acid, guaiacol (2-methoxyphenol), cetyltrimethylammonium bromide (CTAB), 2,5-dihydroxybenzoic acid, trifluoroacetic acid, Folin's reagent for determination of protein, and horseradish peroxidase were purchased from Sigma-Aldrich (USA). Other reagents used—taurine, methionine, monochlorodimedone (MCD), 5,5′-dithio-2-nitrobenzoic acid (DTNB), NaN_3—were from Fluka (Switzerland). Salicylhydroxamic acid and hydrazide 4-aminobenzoic acid were obtained through Aldrich Chemical Co. (USA); MPO from polymorphous nuclear leukocytes was from Planta Natural Products (Austria); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) were from Avanti Polar Lipids (USA). The NO donor 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEANOate) from Cayman Chemical Co. (USA) spontaneously produces 1.5 mol NO per mol of DEANOate with half lifetime of 16 min at 22-25°C.

Working solutions of H_2O_2 were prepared by diluting 30% stock solution (Merck, Germany). HOBr solution (10 mM) was freshly prepared by mixing equal volumes of 20 mM hypochlorite and 20 mM NaBr solutions *prior to* the experiment [23].

Neutrophils were isolated from fresh blood of healthy donors using heparin (10 units/ml) as an anticoagulant. Cells were isolated after sedimentation of erythrocytes (the blood was allowed to sediment at room temperature for 1 h) by centrifugation in a Ficoll–urographin density gradient (density of mixture 1.078 g/cm^3, centrifugation for 20 min at 400*g*), followed by lysis of remaining erythrocytes by distilled water and two times washing of neutrophils by Hanks' solution without Ca^{2+} and Mg^{2+} [24]. Cells were pelleted by centrifugation for 10 min at 400*g*. All procedures were performed in plastic ware at 4°C.

Cell lysis. After the last washing step the cells were resuspended in 10 mM phosphate buffer (pH 7.4) containing 0.5% CTAB [25]. The suspension was incubated at room temperature for 15-20 min and then frozen. The lysate was kept at –20°C until further use but not longer than for two months. After thawing, the cells were centrifuged for 15 min at 1500*g*. The supernatant containing the mixture of neutrophil proteins was collected. The pellet was resuspended in 1 ml of 10 mM phosphate buffer, pH 7.4, and then centrifuged again for 15 min at 1500*g*. The solution resulting from the combination of supernatants was dialyzed against 50 mM phosphate buffer, pH 7.4 (for 3 h against 500-fold excess of buffer and for 16 h against 1500-fold excess of buffer), to remove CTAB. The final solution of neutrophil proteins (further referred to as neutrophil lysate) was either used for the determination of MPO activity or purified for production of high molecular weight fraction of neutrophil proteins containing MPO (144 kD [1, 3]).

Production of high molecular weight fraction of neutrophil proteins. Proteins were separated by gel chromatography using Sephadex G-150 [26]. The column volume was 35-40 ml, the volume of applied lysate 1 ml, and elution rate 30 ml/h per cm^2. High molecular weight MPO fractions detected by development of color (λ_{max} 470 nm) in the peroxidase reaction with guaiacol substrate were collected, where the samples with the highest activity were selected. The protein concentration was determined by the Lowry method [27].

All spectrophotometric measurements described in this work were carried out for high molecular weight fraction of neutrophil proteins, which will be further referred to as neutrophil protein (NP) solution. The majority of experiments was also repeated for the neutrophil lysate and isolated MPO. If the results were different from that for NP solution, it is specifically mentioned below.

Determination of MPO activity. The chlorinating and peroxidase activities of MPO were determined in phosphate buffer with pH 7.4, 7.0, 6.0 (obtained by addition of NaOH to 50 mM NaH_2PO_4), or 5.0 (obtained by addition of citric acid to 50 mM Na_2HPO_4). All measurements were performed using a DU-65 spectrophotometer (Beckman, USA).

Peroxidase activity was measured using guaiacol substrate [28, 29]. Solution containing 50 mM phosphate buffer, 15 mM guaiacol, and 50 or 100 μM H_2O_2 was prepared. The addition of NP aliquot (as well as neutrophil lysate or MPO) to this solution resulted in the formation of polymerization products of oxidized guaiacol with absorbance at 470 nm (ε_{470} = 26,600 $M^{-1}\cdot cm^{-1}$). The specific enzyme activity was expressed as an amount of substrate (μmol) converted by 1 mg of enzyme per min (units/mg protein). HRP was used as a control. The extent of inhibition of peroxidase activity by chloride was characterized by the chloride concentration required for 2-fold inhibition of peroxidase activity (IC_{50}).

Chlorinating activity was measured using taurine [30] or MCD [30-32]. In the first case, 10 mM taurine was added to NP solution (as well to neutrophil lysate and MPO solution) diluted with 50 mM phosphate buffer, pH 7.4, up to the protein concentration of 10-15 μg/ml. The solution was pipetted into the reaction tubes, then 50 mM phosphate buffer, pH 7.4, (control) or NaCl was added up to the required concentration into each tube. The reaction was initiated by addition of 50 μM H_2O_2. The mixture was incubated for 10 min, after which another portion of 50 μM H_2O_2 was added. After 10 min, the tubes were cooled on ice, and the excess H_2O_2 was removed by addition of catalase (2 μg/ml). Then 25 μl of 1 mM solution of 5-thio-2-nitrobenzoic acid (TNB) was added, incubated for 5 min in the dark, and absorbance was measured at 412 nm (in the case of NP solution or isolated MPO) using extinction coefficient ε_{412} = 14,100 $M^{-1}\cdot cm^{-1}$. Formation of one DTNB molecule resulting from the interaction of two TNB molecules with taurine chloramine was taken into account by using a coefficient of 2. Interaction between hypochlorite (or hypobromite) and taurine leads to the formation of a stable product (chlorotaurine or bromotaurine), which is further registered by TNB. Such an approach allows accumulating taurine derivatives in solution during a long time (tens of minutes), thus providing an increase in sensitivity and accuracy of the method for determination of chlorinating activity. However, when using this method for determination of activity it should be kept in mind that the major amount of hypohalogenite is produced during the first 2-3 min after addition of H_2O_2, so there is no need to normal the activity to time. The chlorinating activity of protein solution in the reaction with taurine was characterized by the amount of hypohalogenite (μmol) formed in the solution for 10 min per mg protein.

TNB was prepared by addition of alkali to DTNB up to pH 12.0. The solution was incubated for 5 min at room temperature, and the pH was then lowered to 7.4 by addi-

tion of hydrochloric acid [24]. After addition of 1 mM EDTA, the solution was kept at 4°C and used during one week.

In those experiments where it was necessary to measure the MPO activity at acidic pH or to register the kinetics of hypochlorite formation, the method for determination of activity based on MCD was used [30-32]. To 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl and 100 μM MCD, the solution containing MPO was added so that the final protein concentration was 5 μg/ml. Then 100 μM H_2O_2 was added and the kinetics of the reaction was followed by the decrease in absorbance at 290 nm (ε_{290} = 19,000 $M^{-1}\cdot cm^{-1}$). The specific enzyme activity was expressed as the amount of substrate (μmol MCD) reacted with hypochlorite during 1 min per mg protein (units/mg protein).

Incubation of phospholipid liposomes with the MPO + H_2O_2 + Cl^- system. Multilayer liposomes were obtained from POPC or PAPC in 50 mM phosphate buffer, pH 5.0, containing 140 mM NaCl (or 0.1 mM NaBr) by dispersion of dry lipid film during 30 sec. The film was produced previously by evaporation of chloroform solution under vacuum. The liposomes (final lipid concentration ~0.03 mg/ml) were incubated for 40 min in the same buffer at room temperature in the presence of MPO (0.14 μM). The reaction was initiated by addition of 3 μM H_2O_2 to the reaction mixture, then additions were made every 3 min (n = 14). The number of control experiments was performed in the absence of enzyme or one of its substrates as well as in the presence of sodium azide (1 mM), an inhibitor of MPO, or methionine (1 mM) and taurine (1 mM), the quenchers of hypochlorite. At the end of incubation, the lipid material was extracted with chloroform–methanol (2 : 1 v/v).

Analysis of lipids by MALDI-TOF mass spectrometry. An aliquot of phospholipid extract (10-50 μl) was taken, and the organic solvent was evaporated *in vacuo*. Matrix solution (5 μl of 0.5 M 2,5-dihydroxybenzoic acid in methanol containing 0.1% trifluoroacetic acid) was added to the sample. The mixture (1 μl) was applied onto the MALDI target plate and dried under air.

MALDI-TOF measurements were performed in the positive ionization mode using a Voyager Biospectrometry Workstation (PerSeptive Biosystems, USA) equipped with a 337 nm pulsed nitrogen laser. The voltage was 20,000 V. The recorded signal was an average of 128 individual laser impulses. The mass range of interest was 450-1500 daltons.

Statistics. All values of enzyme activity are averages of three replicates (± standard deviation). The relative error for the determination of enzyme activity did not exceed 10% of the experimental value. The difference was examined statistically using Student's criterion and was considered to be significant when $p < 0.05$. The figures given in this work (except for Fig. 4) represent the results of a typical experiment among at least three replicates.

RESULTS

Peroxidase and chlorinating activity of neutrophil protein solution. Figure 2 shows the dependence of peroxidase activity of NP solution on the concentration of H_2O_2. As seen from the figure, NP activity increases with increase in H_2O_2 concentration up to 200 μM. Further increase in H_2O_2 concentration results in some decrease in activity. We obtained similar dependence of peroxidase activity on the concentration of H_2O_2 also for isolated MPO (data not shown) (see also [29]). Optimal H_2O_2 concentration used further in this work was 50 μM (or 100 μM), since higher H_2O_2 concentrations inhibit chlorinating activity of the enzyme [30]; moreover, they are not found *in vivo* [21].

Figures 3a and 3b illustrate the dependences of chlorinating and brominating activities of NP solution on the concentrations of Cl^- and Br^-, respectively. The measurements were carried out using a taurine-based method, when chlorotaurine or bromotaurine produced by NP solution during the 10 min reaction was registered. Chlorinating activity of NP solution is linearly proportional to the concentration of Cl^- in the concentration range of NaCl between 0 and 120 mM (Fig. 3a). Further increase in NaCl concentration does not result in significant change in the amount of hypochlorite formed. When Br^- is used as a substrate, the maximal enzyme activity is observed already at the concentration of substrate of 3 mM, thus demonstrating higher affinity of NP enzyme to bromide (Fig. 3b). In the absence of one of the MPO substrates (halogenide or H_2O_2), the formation of hypohalogenites was not detected (data not shown).

The difference between maximal amount of hypohalogenites formed by NP solution (0.8 and 0.5 μmol per

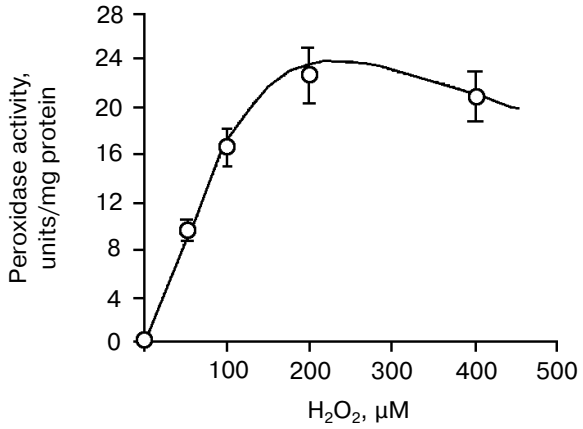

Fig. 2. Peroxidase activity of high molecular weight fraction of neutrophil proteins depending on concentration of H_2O_2. The peroxidase activity was measured by accumulation of guaiacol oxidation products (λ_{max} 470 nm) in solution containing 15 mM guaiacol and 1.5 μg/ml neutrophil proteins in 50 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of H_2O_2.

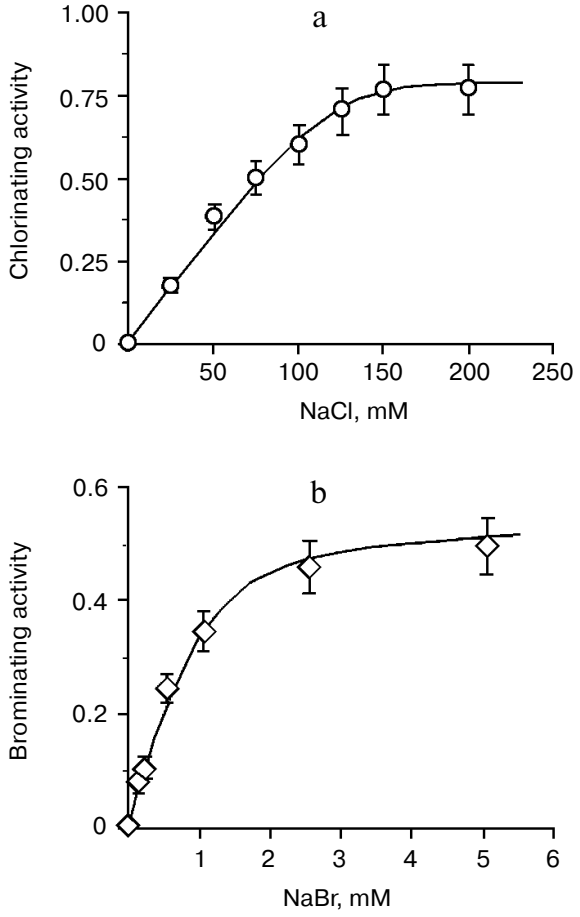

Fig. 3. Chlorinating (a) and brominating (b) activities of high molecular weight fraction of neutrophil proteins depending on concentration of chloride and bromide ions, respectively. The reaction was performed in 50 mM phosphate buffer, pH 7.4, containing 10 mM taurine and 15 μg/ml neutrophil proteins. The reaction was initiated by two additions of 50 μM H_2O_2 (with 10 min interval). The produced chlorotaurine and bromotaurine was measured using TNB. The activity was characterized by the amount of hypohalogenite (μmol) formed in the solution for 10 min per mg protein.

mg of protein during 10 min for hypochlorite and hypobromite, respectively) is most probably associated with higher reactivity of hypobromite compared to hypochlorite (for example, towards SH-groups in proteins [33]). As a result, some amount of hypobromite formed reacts with the reactive protein groups and does not contribute to the interaction with taurine. Moreover, if the dependence of amount of hypochlorite formed on the concentration of chloride in the case of neutrophil lysate (data not shown) practically matched that for the NP solution (Fig. 3a), then brominating activity of MPO in the neutrophil lysate (where the protein content is significantly higher than in NP solution) could not be registered using the taurine-based method at all.

Since the formation of hypohalogenites is a specific MPO function, the results indicate that both high molecular weight fraction of neutrophil proteins and neutrophil lysate used in this work contain active MPO.

Dependence of MPO activity on pH. Figure 4a shows the peroxidase activity of HRP and NP solution at different pH values. For visualization of this comparison, the enzyme activity is expressed as percentage of activity at pH 7.0, which is 260 ± 30 units/mg protein for HRP and 12 ± 2 units/mg protein for NP solution. Figure 4b illustrates chlorinating activity of MPO in NP solution at different pH values, measured as oxidation of MCD.

As seen from Fig. 4a, HRP activity is relatively stable in the investigated pH range, which is in agreement with data obtained previously [19]. However, the measurements demonstrated that the MPO activity depends on pH: peroxidase activity decreases twofold when the pH of

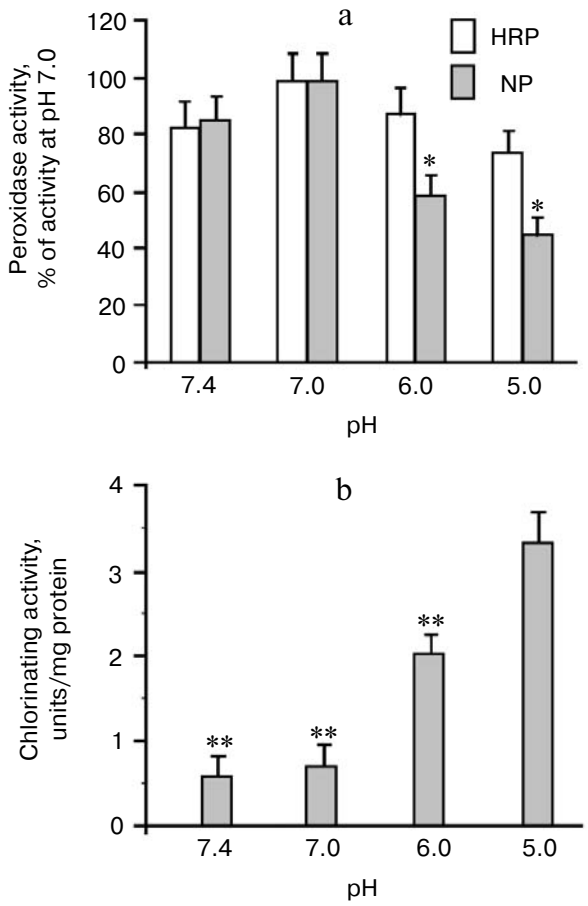

Fig. 4. Values of peroxidase (a) and chlorinating (b) activity for HRP and NP solution at different pH values. The peroxidase activity was measured by monitoring the oxidation of guaiacol (for conditions see the legend to Fig. 2, 50 μM H_2O_2). The chlorinating activity was measured using the MCD method. To NP solution (5 μg/ml) in 50 mM phosphate buffer containing 100 μM MCD and 100 mM NaCl, 100 μM H_2O_2 was added and the kinetics were measured by the decrease in absorbance at 290 nm. The results are presented as averages for three independent experiments: a) * $p < 0.05$ for the peroxidase activity value at pH 7.0; b) ** $p < 0.05$ for the chlorinating activity value at pH 5.0.

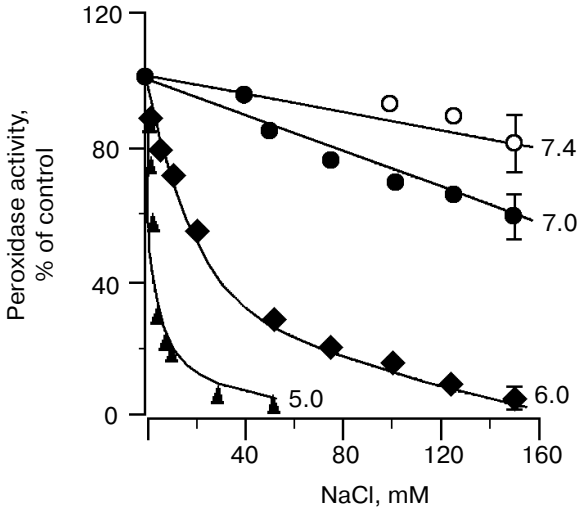

Fig. 5. Dependence of peroxidase activity of NP solution on concentration of chloride at different pH values. The activity values are expressed as percentage ratios to the activity in the control (in the absence of chloride) at the same pH value. The pH values are indicated by the numbers near the corresponding curves. The conditions for determination of activity are given in the legend to Fig. 2 (50 μM H_2O_2).

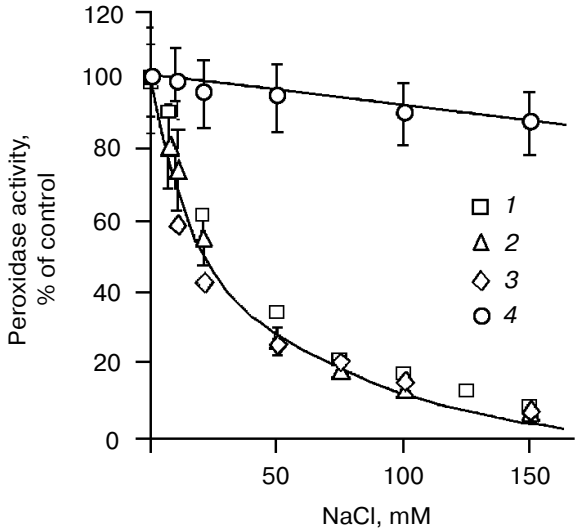

Fig. 6. Comparison of the effect of Cl^- on peroxidase activity of MPO of various purification degree and HRP at pH 6.0: *1*) supernatant of neutrophil lysate; *2*) NP solution; *3*) isolated MPO; *4*) HRP. Activity was measured as oxidation of guaiacol in the peroxidase reaction at pH 6.0. For conditions, see the legend to Fig. 2 (50 μM H_2O_2).

the medium changes from 7.0 to 5.0 (Fig. 4a). The chlorinating activity increases more than fivefold under the same conditions (Fig. 4b).

Influence of Cl^- on peroxidase activity of NP solution at different pH values. To investigate the influence of chloride (a substrate for the chlorinating cycle) on peroxidase activity of MPO in the NP preparation, we measured the peroxidase activity of NP at pH 5.0, 6.0, 7.0, and 7.4 in the presence of various chloride concentrations (Fig. 5). As can be seen, chloride decreases peroxidase activity of MPO dose-dependently, whereas the degree of this effect significantly depends on the pH of the solution. At pH 7.4 and 7.0, chloride weakly inhibits the oxidation of guaiacol by the enzyme. Physiological concentrations of NaCl (100-140 mM) almost completely inhibit the peroxidase activity of NP solution still at pH 6.0. The presence of 15 mM substrate of the chlorinating cycle at pH 5.0 is enough for complete switch of enzyme activity to production of hypochlorite, when the presence of 15 mM guaiacol (the substrate for peroxidase cycle) is completely ignored.

The table lists the IC_{50} values characterizing the degree of inhibition of peroxidase activity of MPO by chloride in the studied reactions at various pH values. It can be seen that decrease in pH results in dramatic decrease in chloride concentration, which leads to a twofold drop in peroxidase activity. It should be noted that the IC_{50} value for each pH value (within the experimental error) is the same for isolated MPO, NP solution, and neutrophil lysate.

Figure 6 presents the results that allow comparing the effect of chloride on peroxidase activity of MPO solutions of different degree of purification as well as on HRP at pH 6.0. It is evident that HRP activity at pH 6.0 decreases with increase in chloride concentration. However, these changes are not pronounced and account for just 12 ± 3% from the control value at NaCl concentration of 150 mM. Peroxidase activity of MPO of different degree of purity decreases dramatically as the concentration of chloride increases, whereas the dependences obtained in the case of isolated MPO, NP solution, and neutrophil lysate coincide within the experimental error.

IC_{50} values for inhibition of peroxidase activity by chloride of: neutrophil lysate, NP solution, and isolated MPO at different pH values. The experiment conditions are given in the legend to Fig. 2. The results are presented as averages of three independent replicates ± standard deviation

	IC_{50}, mM		
	pH 5.0	pH 6.0	pH 7.0
Neutrophil lysate	2.50 ± 0.35	25 ± 7	>150
NP solution	2.00 ± 0.30	32 ± 5	>150
Isolated MPO	1.75 ± 0.25	20 ± 4	>150

The results presented in Fig. 6 and the table suggest that registered peroxidase activity both in lysate and NP solution is associated mostly with MPO. It is necessary to note that peroxidase and chlorinating activities of neutrophil lysate stored at 4°C remained unchanged during 24 h. In the case of NP solution, the activity drops by 20% under these conditions, whereas the activity of isolated MPO solution decreases twofold.

Known peroxidase inhibitors such as salicylhydroxamic acid (0.1 mM) and 4-aminobenzoic acid hydrazide (0.05 mM) completely inhibited peroxidase activity of NP solution at all studied pH values both in the presence and absence of NaCl. However, known hypochlorite quenchers, taurine (10 mM) and methionine (1 mM), did not have any influence on measured peroxidase activity. Moreover, addition of either 25 μM HOCl or HOBr did not effect the measurements. This is a confirmation of the fact that the effect of NaCl is associated with the involvement of chloride in the chlorinating cycle, but not with generation of chemically active hypochlorite.

Modification of phospholipid liposomes by the MPO + H_2O_2 + Cl^- system. Figures 7 and 8 show mass spectra of extracts of phosphatidylcholine liposomes, both native and incubated in the presence of MPO + H_2O_2 + Cl^- for 40 min. The spectra of native phosphatidylcholines (spectra *1* in Figs. 7 and 8) contain two

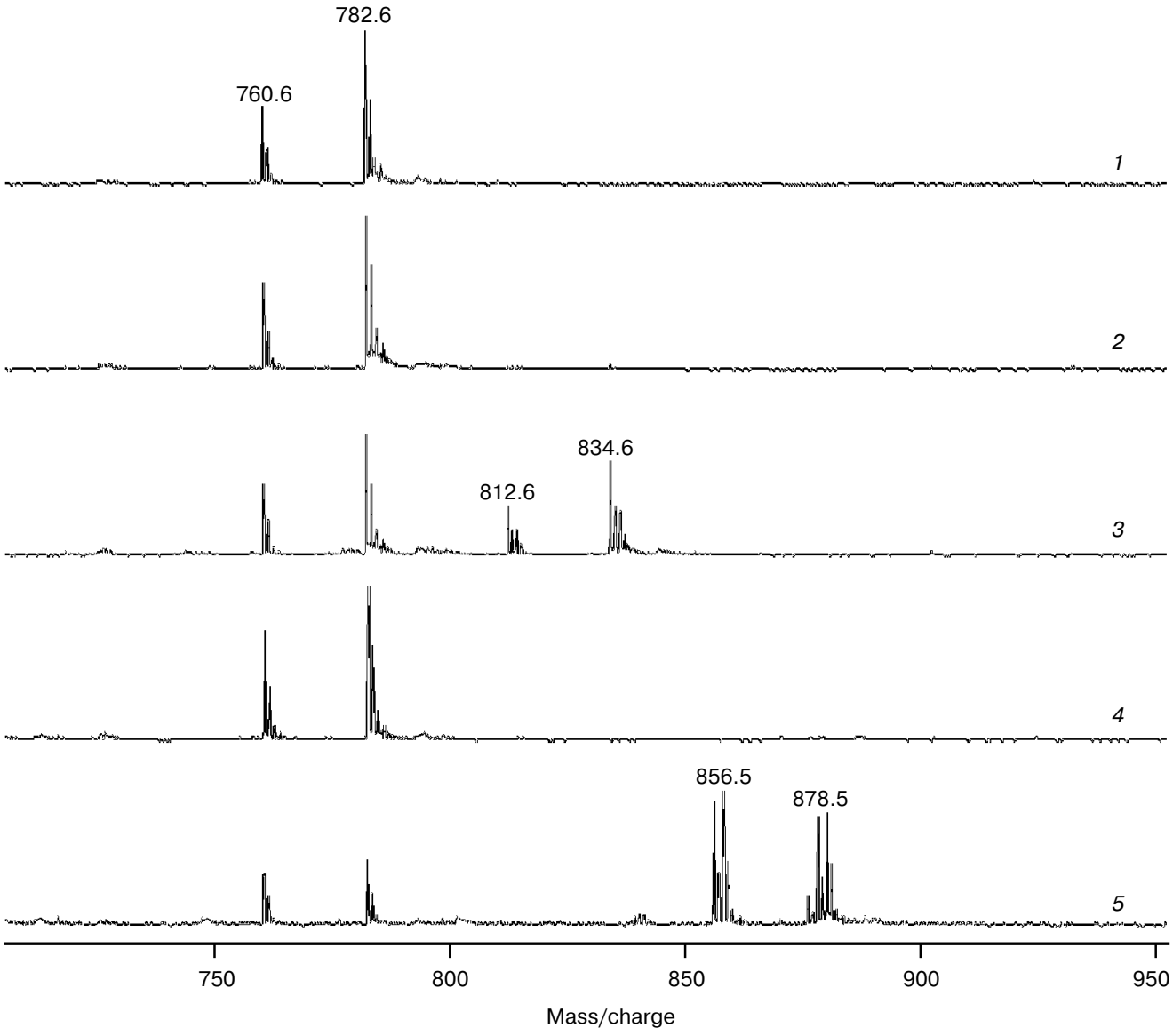

Fig. 7. Mass spectra of POPC liposome extract before (*1*) and after incubation in the presence of MPO + H_2O_2 + Cl^- (*2*, *3*) or MPO + H_2O_2 + Br^- (*4*, *5*) at pH 7.4 (*1*, *2*, *4*) or pH 5.0 (*3*, *5*). Incubation medium: 50 mM phosphate buffer, 140 mM NaCl or 0.1 mM NaBr, 30 μg/ml lipids, 0.14 μM MPO. The reaction was initiated by addition of H_2O_2 portions (3 μM with 3 min intervals) to the incubation medium. Conditions: incubation for 40 min, room temperature.

components differing in their molecular weight by 22 daltons and associated with ions formed by addition of a proton $[M_f + 1]^+$ or sodium ion $[M_f + 23]^+$ to phospholipid molecules. Besides this, there is a peak with a molecular weight of 551.1 present in all spectra and belonging (as previously shown [34]) to a derivative of the matrix.

When liposomes were produced from POPC, a mono unsaturated compound (Fig. 7), additional peaks were found in mass spectra after incubation for 40 min at pH 5.0 in the presence of the MPO + H_2O_2 + Cl^- system. Major peaks had *m/z* of 812.6 and 834.6, respectively, and belonged to H^+- and Na^+-adducts of the monochlorohydrin formed (Fig. 7, spectrum *3*). It is necessary to point out that the formation of chlorohydrins could be observed only at acidic pH values. Incubation of liposomes with the MPO + H_2O_2 + Cl^- system at pH 7.4 did not result in the emergence of any peaks different from those that were observed for native phospholipids (spectra *1* and *2*, Fig. 7). An analogous effect was registered for the incubation of liposomes formed by POPC with the MPO + H_2O_2 + Br^- system. High molecular weight peaks (*m/z* 856.5 and 878.5) in mass spectra of phospholipid extracts indicating the formation of H^+- and Na^+-adducts of POPC monobromohydrin (spectra *4* and *5*, Fig. 7) could only be observed at pH 5.0.

In the case of liposomes produced from polyunsaturated phosphatidylcholine, for instance PAPC, incubation with the MPO + H_2O_2 + Cl^- system at pH 5.0 for 40 min resulted in appearance of intense peaks at *m/z* 496.3 and 518.3, belonging to 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine) with the added proton or sodium ion, respectively (spectrum *3*, Fig. 8). Broadened peaks of low intensity with *m/z* higher than that for initial PAPC belong to multiple products of reaction between hypochlorite and carbon double bonds in PAPC [34-36]. It is important to note that the formation of PAPC destruction products occurred only at acidic pH. At pH 7.4, such products were practically not registered (spectrum *2*, Fig. 8).

If the liposomes were incubated in the absence of MPO (or at least one of its substrates, H_2O_2 or Cl^-), no

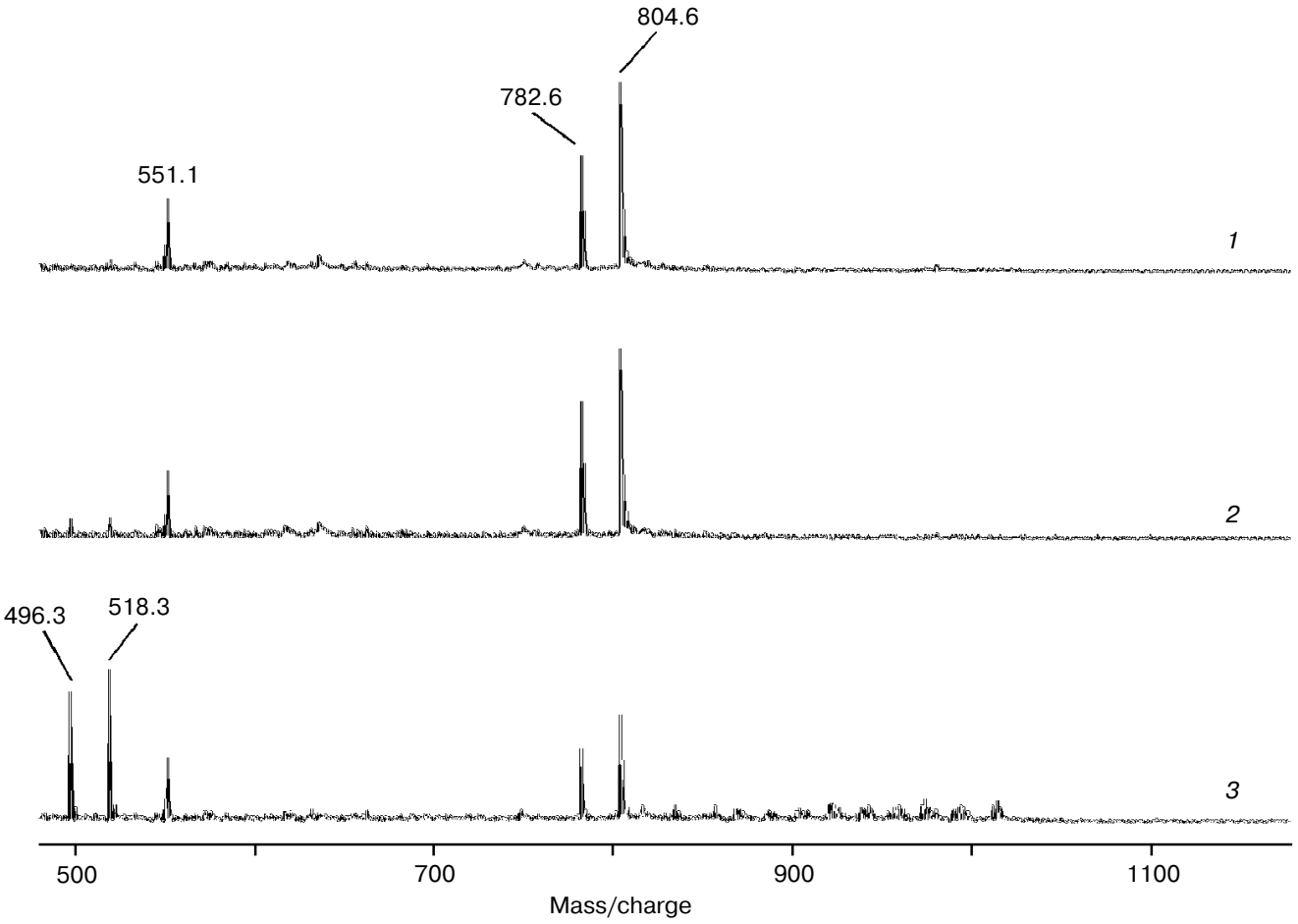

Fig. 8. Mass spectra of PAPC liposome extract before (*1*) and after incubation in the presence of MPO + H_2O_2 + Cl^- (*2*, *3*) at pH 7.4 (*1*, *2*) or pH 5.0 (*3*). Incubation medium: 50 mM phosphate buffer, 140 mM NaCl, 30 μg/ml lipids, 0.14 μM MPO. The reaction was initiated by addition of H_2O_2 portions (3 μM with 3 min intervals) to the incubation medium. Conditions: incubation for 40 min, room temperature.

molecular peaks different from those for the native phospholipids were observed in the mass spectrum. Addition of hypochlorite quenchers such as taurine (1 mM) or methionine (1 mM) as well as sodium azide (an inhibitor of MPO) to the incubation medium completely prevented the formation of chlorohydrins or lyso-derivatives of phospholipids. The data indicate that the formation of not only chloro-derivatives, but also lysophosphatidylcholines at pH 5.0 occurs due to the interaction between unsaturated phospholipids and hypochlorite formed in the course of the MPO catalyzed reaction.

Influence of active forms of nitrogen on halogenating activity of MPO. Figure 9 shows the dependence of production of hypochlorite and hypobromite by myeloperoxidase at pH 7.4 on the concentration of the NO donor DEANOate. Preliminary incubation of DEANOate for 30 min results in accumulation of active forms of nitrogen (first NO is produced, then it is quickly converted to NO_2^- under aerobic conditions), which are able to influence the MPO activity [16-18]. As seen from Fig. 9, halogenating activity of MPO decreases dramatically with the increase in DEANOate concentration in the incubation medium. Micromolar concentrations of nitrogen derivatives are sufficient for the inhibition of both chlorinating and brominating activity of NP solution at pH 7.4. Such an effect can be caused either by direct interaction between active forms of nitrogen and halogenites formed (HOCl and HOBr), or by the ability of active forms of nitrogen (in particular, NO and NO_2^-) to be substrates for the peroxidase cycle [16, 17], shifting the equilibrium to the formation of compound II (see the scheme in Fig. 1), which in turn leads to a decrease in halogenating activity of compound I.

It was shown previously [37] that HOCl indeed reacts with NO_2^-, but the rate of this reaction is at least 10 times lower than the rate of the reaction between hypochlorite and amino groups. Available data [38] indicate that hypochlorite does not react with NO_2^- when there is a large excess of taurine (10 mM). We have confirmed this by a specially designed experiment, where hypochlorite (25 μM) was added to the buffer solution (either containing DEANOate at the concentration of 20 or 50 μM or not, previously incubated for 30 min) immediately after addition of taurine (10 mM). The measurements with TNB substrate demonstrated that the amounts of chloramines formed are equal both in experimental (containing DEANOate) and control samples. This is an indication that hypochlorite does not interact with NO_2^- under our experimental conditions. Consequently, it can be assumed that the observed effect is mainly associated with the involvement of active forms of nitrogen in the reactions of the peroxidase cycle of MPO [16, 17].

However, it should be noted that addition of DEANOate did not have any effect on the peroxidase activity of NP solution measured with guaiacol, since the phenolic substrate was in excess (15 mM).

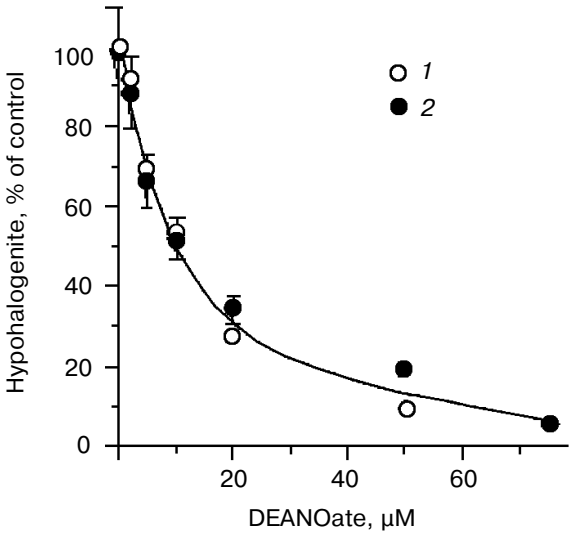

Fig. 9. Dependence of chlorinating (*1*) and brominating (*2*) activities of NP solution on the concentration of NO donor DEANOate. DEANOate solution (100 μM) was preincubated with 50 mM phosphate buffer, pH 7.4, for 30 min and then added to NP solution (15 μg/ml, in the same buffer containing 100 mM NaCl (*1*) or 2 mM NaBr (*2*) and 10 mM taurine) up to the required concentration of the NO donor. Control: sample without DEANOate. The reaction was initiated by two additions of 50 μM H_2O_2 (10 min interval). The amount of chlorotaurine product was measured using the TNB method.

DISCUSSION

Myeloperoxidase is a unique enzyme, which (besides the oxidation of a large number of compounds in the peroxidase cycle) is capable of catalyzing two-electron oxidation of chloride with the formation of hypochlorite. The latter can oxidize and modify lipids, proteins, carbohydrates, nucleotides, and other biologically important molecules [14, 22, 33, 39-43]. The ratio between peroxidase and chlorinating MPO activities is significant for directed synthesis of hypochlorite at the sites of inflammation and injury. In the present work, we have shown that the pH of the medium is an important factor for the regulation of this ratio. MPO concentration in plasma is some tens of nM [42]. Therefore, both substrates for peroxidase reaction and chloride are in a large excess towards the enzyme, what is also true for our experiments. In the neutral pH range (7.0-7.4), the enzyme works mainly via the peroxidase cycle. Even at high concentrations of the substrate of the chlorinating reaction (exceeding the concentration of the peroxidase substrate by an order of magnitude, for instance 150 mM NaCl and 15 mM guaiacol), there is only a slight difference in peroxidase activity compared to the control (60% of the control value at pH 7.0 and 70% at pH 7.4, see also Fig. 5). Decreasing pH to 6.0 leads to the complete inhibition of peroxidase activity by physiological concentration of chloride ion (100-140 mM). The concentration of

NaCl of 15-20 mM is sufficient for inhibition of peroxidase activity at pH 5.0. It was also shown previously [5] that the substrates for the peroxidase reaction inhibit chlorinating activity of MPO, whereas the concentration required for complete inhibition of activity is 100 times higher at pH 5.0 than at pH 7.4. Hence, the oxidizing ability of compound I at acidic pH is directed mostly to the oxidation of chloride. Contrary to that, the main working enzyme pathway at neutral pH is the oxidation of peroxidase substrates. It can be assumed that such alterations of enzyme activity are associated with conformational changes in the MPO active site with the change in pH [44].

Normally, the pH of biological fluids is close to 7, and therefore the secretion of MPO in the extracellular space cannot result in substantial production of hypochlorite. It was shown for a number of cells that MPO binds to glycoproteins and cellular receptors on the membrane surface [41, 45]. The interaction between MPO and low density lipoproteins (LDL) is assumed to be mediated by apoB. The main MPO substrates in this case are amino acids, for instance, tyrosine residues. For this reason, one should, first of all, expect oxidative modification of proteins (for instance, formation of tyrosyl and thiyl radicals [9, 10, 14, 15, 43]) in the presence of MPO at neutral pH. The radicals formed as a result of peroxidase activity of MPO display high oxidizing ability; however, they are characterized by lower redox potential than hypochlorite (for example, redox potential for the tyrosyl radical/tyrosine pair is 0.9 V, whereas the redox potential of the $HOCl/Cl^-$ pair is 1.29 V at pH 7.0) [15, 36]. The presence of antioxidants serving as intracellular substrates for the peroxidase cycle of MPO or as radical acceptors (ascorbate, urate, thiol groups, glutathione) [1, 15] as well as recombination of radicals [10, 15, 46] dramatically decrease the negative consequences of peroxidase activity of MPO.

During cellular response when the pH of formed phagosomes decreases to 4.7-5.5 and a sufficient amount of H_2O_2 due to the activation of NADP(H) oxidase is produced, MPO predominantly catalyzes the oxidation of chloride by the two-electron mechanism. It was shown in our experiments that even small amounts of NaCl (much lower than intracellular NaCl amount) result in the inhibition of peroxidase activity at pH 5.0. NaCl present in the biological fluids at the concentration of 140 mM completely inhibits the peroxidase activity of MPO at pH 6.0. As a control, we used HRP. The activity for this enzyme differs insignificantly at pH 7.0 and 5.0. Moreover, the influence of NaCl on enzyme activity is not dramatic and can be also caused by alterations in ionic strength of the solution or by the weak ability of HRP to catalyze the reaction of hypohalogenite formation at acidic pH [47].

Therefore, a strong oxidant (hypochlorite) is produced only when it is required to fight against infections. Normally, the production of hypochlorite is not desirable since it can cause significant damage to macromolecules. The catalysis of hypochlorite production is minimized in the presence of the substrates for peroxidase activity of MPO.

Modification of unsaturated lipids is one of the consequences of deleterious action of hypochlorite. It was shown previously using the MALDI-TOF mass spectrometry technique, that hypochlorite added to phospholipids liposomes is able to modify the acyl chains of lipids, resulting in the formation of chlorohydrins and lysoderivatives [48-50]. However, it was proven that MPO-induced destruction of unsaturated phosphatidylcholines can only be observed in acidic media (pH 5.0). At neutral pH, the amount of produced hypochlorite was not sufficient for the modification of phospholipids (Figs. 7 and 8).

It is known that atherosclerotic tissues are characterized by acidic pH due to the impaired diffusion of oxygen [39]. Moreover, they contain some amount of hydroperoxides, which can be reduced by MPO leading to compound I. Hence MPO present in atherosclerotic plaques [51] can be a main reason for oxidation and modification of lipids resulting from the production of hypochlorite.

It is necessary to mention that the majority of experiments in this work were carried out using NP fraction obtained by the separation of a total protein mixture on a Sephadex G-150 column. A number of experiments were repeated for the neutrophil lysate as well as for the isolated MPO. In the majority of cases, the results coincide. This is important since the agreement of the results obtained for the neutrophil lysate, NP solution, and MPO indicated that the peroxidase and chlorinating activity belongs specifically to MPO. The results demonstrate the possibility of using the neutrophil lysate for the investigation of not only peroxidase [52, 53] but also chlorinating activity of MPO, when the enzyme preparation protocol can be significantly simplified and the system adjusted to *in vivo* conditions.

This work also shows the inhibiting action of active forms of nitrogen on the chlorinating activity of MPO. Under our experimental conditions, inhibition of hypochlorite formation due to nitrosylation of the enzyme heme cannot have any appreciable effect on MPO activity, since nitrogen oxide weakly binds to ferriheme under aerobic conditions [16]. As a rule, inhibition of chlorinating activity at neutral pH is due to the interactions between active forms of nitrogen as peroxidase substrates with compound I and II.

The results indicate that in the presence of peroxidase substrate MPO is able to work efficiently by the halogenation cycle only at acidic pH.

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