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Exploitation of the unusual thermodynamic properties of human myeloperoxidase in inhibitor design

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Abstract

Myeloperoxidase plays a fundamental role in oxidant production by neutrophils. It uses hydrogen peroxide and chloride to catalyze the production of hypochlorous acid (HOCl), which contributes to both bacterial killing and oxidative injury of host tissue. Thus, MPO is an interesting target for anti-inflammatory therapy. Here, based on the extraordinary and MPO-specific redox properties of its intermediates compound I and compound II, we present a rational approach in selection and design of reversible inhibitors of HOCl production mediated by MPO. In detail, indole and tryptamine derivatives were investigated for their ability to reduce compounds I and II and to affect the chlorinating activity of MPO. It is shown that these aromatic one-electron donors bound to the hydrophobic pocket at the distal heme cavity and were oxidized efficiently by compound I (k_3), which has a one-electron reduction potential of 1.35 V. By contrast, compound II ($E^{\circ\prime}$ of the compound II/ferric couple is 0.97 V) reduction (k_4) was extremely slow. As a consequence compound II, which does not participate in the halogenation cycle, accumulated. The extent of chlorinating activity inhibition (IC_{50}) was related to the k_3/k_4 ratio. The most efficient inhibitors were 5-fluorotryptamine and 5-chlorotryptamine with IC_{50} of 0.79 μM and 0.73 μM and k_3/k_4 ratios of 386,000 and 224,000, respectively. The reversible mechanism of inhibition is discussed with respect to the enzymology of MPO and the development of drugs against HOCl-dependent tissue damage.

Keywords: Myeloperoxidase; Chlorination activity; Inflammation; Tissue damage; Reduction potential; Inhibitor

1. Introduction

The heme enzyme myeloperoxidase (MPO) is one of the major player in the first line of the unspecific immune defense system, responsible for the microbicidal activity within human neutrophils. This enzyme is packed inside the cytoplasmic azurophilic granules, in relatively high concentration up to 5% of the dry weight of the cell [1]. At present it is open to debate whether pathogens are killed predominantly by means of oxygen-dependent mechanisms via myeloperoxidase-catalysed halogenation, as was the traditional point of view [2], or via oxygen-independent mechanisms, involving bactericidal proteins such as lysozyme and lactoferrin and proteases such as elastase. Albeit recent findings [3] suggesting proteases to play a dominant role in neutrophil antimicrobial activity, it is evidently not the only mechanism and does not explain the large amount of evidence implicating direct function of myeloperoxidase derived oxidants and reactive oxygen species (ROS) in microbial killing [4]. Despite its indispensable role in microbial killing, myeloperoxidase is implicated in a growing number of diseases like atherosclerosis, rheumatoid arthritis, lung cancer and many more [5]. Immunoreactivity and typical MPO oxidation products were even detected in brains of patients diagnosed having Alzheimer's disease [6–8] and in the central nervous system in multiple sclerosis (MS) patients [9].

MPO as the prominent generator of reactive oxidizing species in neutrophils uses hydrogen peroxide (H_2O_2) and

Abbreviations: MPO, myeloperoxidase; LPO, lactoperoxidase; HRP, horseradish peroxidase; $E^{\circ\prime}$, standard reduction potential; MCD, monochlorodimedon

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halides (X^-) to produce hypohalous acids (HOX) (Reactions (1) and (2)). Reaction takes place preferentially with Cl^- because of its high concentration in body fluids (100–140 mM [10]) compared to other halides. Hypochlorous acid (HOCl) is the most powerful neutrophil oxidant. It is extremely cytotoxic and reacts readily with most biological molecules [4] thereby also promoting inflammatory tissue damage caused by neutrophils [11]:

$$\mathrm{MPO(III)} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{compound\ I} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{compound\ I} + \mathrm{X^-} + \mathrm{H^+} \xrightarrow{k_2} \mathrm{MPO(III)} + \mathrm{HOX} \quad (2)$$

Scavengers of HOCl have been suggested as potential inhibitors of inflammatory tissue damage [12]. However, they are of limited use because the extreme and indiscriminate reactivity of HOCl would require high concentrations of scavengers to prevent oxidation of biological targets. A more feasible approach is to develop specific inhibitors of MPO. Several anti-inflammatory drugs inhibit MPO [13–17], however in most cases the mechanism of inhibition is unclear. By using the H_2O_2-electrode Kettle and Winterbourn [18] assessed the ability of a variety of anti-inflammatory drugs to inhibit HOCl formation mediated by MPO, showing that many of them promote the formation of compound II, which is a redox intermediate that does not participate in the halogenation cycle. Salicylhydroxamic acid is oxidized by MPO in preference to chloride and prevents binding of chloride and hydrogen peroxide [19,20]. Hydrazines ($RNHNH_2$) and hydrazides ($RCONHNH_2$) are general suicide substrates of peroxidases causing irreversible destruction of heme prosthetic groups [21–23]. At the moment 4-aminobenzoic acid hydrazide (ABAH) is the most potent inhibitor of hypochlorous acid production mediated by MPO [23,24].

Here, we introduce a rational approach in developing inhibitors of myeloperoxidase that will dampen inflammation without destroying the enzyme and precipitating irreversibly its physiological role. Recently, the standard reduction potentials ($E^{\circ\prime}$) of all relevant redox couples of myeloperoxidase have been published [25–27]. Table 1 summarizes these $E^{\circ\prime}$ values in comparison to other heme peroxidases. It clearly demonstrates some MPO-specific peculiarities, namely the significant difference between $E^{\circ\prime}$ (compound I/compound II) and $E^{\circ\prime}$ (compound II/ferric MPO). Since in other human peroxidases like lactoperoxidase (LPO) the corresponding $E^{\circ\prime}$ values are very similar we decided to exploit these MPO-typical thermodynamic properties in selecting reversible inhibitors. Based on recent investigations about the role of indole derivatives as one-electron donors in the peroxidase cycle (Reactions (1), (3) and (4))

$$\mathrm{compound\ I} + \mathrm{AH} \xrightarrow{k_3} \mathrm{compound\ II} + \mathrm{A^\bullet} \quad (3)$$

$$\mathrm{compound\ II} + \mathrm{AH} \xrightarrow{k_4} \mathrm{compound\ II} + \mathrm{A^\bullet} + \mathrm{H_2O} \quad (4)$$

of MPO [28,29] we tested the following hypothesis: indole and tryptamine derivatives with redox potentials following the correlation $0.97\ \mathrm{V} \ll E^{\circ\prime}(\mathrm{A^\bullet}, \mathrm{H^+/AH}) < 1.35\ \mathrm{V}$ should be excellent competitors of chloride for compound I and efficiently reduce compound I to compound II which is outside the halogenation cycle (i.e. Reactions (1) and (2)). But based on the much lower oxidation capacity of MPO compound II these donors will not be able to mediate compound II reduction. As a consequence compound II will accumulate and the chlorination activity is inhibited. Since serotonin (5-hydroxytryptamine) with $E^{\circ\prime}$ ($\mathrm{A^\bullet}$, $\mathrm{H^+/AH}$) = 0.65 V is an excellent electron donor of both compound I and compound II [30], it is evident that structural aspects like access and finally binding of tryptamine derivatives to both redox intermediates would not have a strong impact on these reactions.

In the present paper we show that this approach is very promising. We could measure actual bimolecular rate constants of Reactions (3) and (4) using indole, 5-nitroindole, 5-cyanoindole, tryptamine, 5-fluorotryptamine, 5-hydroxytryptamine and 5-chlorotryptamine as electron donors and determined their ability to inhibit hypochlorous acid formation. The mechanism of inhibition is shown and discussed with respect to the rational design of MPO inhibitors in future.

2. Materials and methods

2.1. Materials

Highly purified myeloperoxidase of a purity index > 0.86 (A_{430}/A_{280}) was purchased from Planta Natural Products (http://www.myeloperoxidase.com). Enzyme

Table 1
Standard reduction potentials (E'°) of all relevant redox couples of heme peroxidases

Redox couple	Reduction potentials (E'°)				
	Myeloperoxidase	Eosinophil peroxidase	Lactoperoxidase	Horseradish peroxidase	*Arthromyces ramosus* peroxidase
Compound I/ferric MPO	1.16 [25]	1.10 [25]	1.09 [35]	0.883[a]	0.949[b]
Compound I/compound II	1.35 [26]	n.d.	1.14 [35]	0.898 [36]	0.915 [37]
Compound II/ferric MPO	0.97 [26]	n.d.	1.04 [35]	0.869 [36]	0.982 [37]

n.d.: not determined.
[a] Calculated from reference [36].
[b] Calculated from reference [37].

concentration was determined spectrophotometrically by using a molar extinction coefficient of 91000 M^{-1} cm^{-1} [31].

Hydrogen peroxide was obtained as a 30% solution. After dilution the concentration was determined by absorbance measurement at 240 nm by using an extinction coefficient of 39.4 M^{-1} cm^{-1} [32].

5-Chlorotryptamine hydrochloride was purchased from Lancaster synthesis, England. Other indole and tryptamine derivatives (indole, 5-nitroindole, 5-cyanoindole, tryptamine, 5-fluorotryptamine hydrochloride, 5-hydroxytryptamine hydrochloride (serotonin)) and all other chemicals were from Sigma Aldrich Chemical Co. at the highest grade available.

Stock solutions of indole and tryptamine derivatives were prepared freshly daily. Indole, 5-cyano-, and 5-nitroindole had to be stored in pure ethanol in dark flasks due to solubility and stability reasons. All solutions have been diluted before each measurement with 100 mM aqueous buffer to a final ethanol concentration of 4% (v/v) in all assays. Experiments were performed at 25 °C and ionic strength of 100 mM. The following buffers were used: 100 mM citric acid/NaOH, pH 5.0, and 100 mM KH_2PO_4/Na_2HPO_4, pH 7.0.

2.2. Methods

2.2.1. Transient-state kinetics

All kinetic measurements were performed using a temperature-controlled stopped-flow apparatus (model SX-18 MV) from Applied Photophysics (UK) with either a diode-array detector (Applied Photophysics PD.1) or a monochromator attached to the stopped-flow machine. In a typical sequential-mixing stopped-flow experiment the enzyme solution (2 μM heme) was premixed with a 10-fold excess of hydrogen peroxide. After a delay time of 20 ms, compound I (50% hypochromicity at 430 nm) was formed and allowed to react with varying concentrations of substrate in 200 mM phosphate buffer (pH 7.0). The reactions were followed at the Soret maximum of compound II (456 nm). To determine the kinetics of reduction of compound II, 4 μM enzyme solution was premixed with 40 μM hydrogen peroxide and 3.6 μM homovanillic acid. After a delay time of 40 s, compound II was allowed to react with varying concentrations of substrates in 100 mM phosphate buffer (pH 7.0). Reactions were followed at 456 nm (disappearance of compound II) or 430 nm (formation of ferric MPO). Alternatively, compound II formation and reduction was followed in one measurement starting with the addition of the electron donors to compound I. The resulting biphasic curves at 456 nm showed the initial formation of compound II (exponential increase at 456 nm) and then its subsequent transition to ferric MPO (exponential decrease at 456 nm).

Reactions were analyzed by fitting the monophasic time traces using the single-exponential equation, provided by the Applied Photophysics software program. From the slope of the linear plot of the k_{obs} values versus substrate concentrations, the apparent second-order rate constant was obtained for each substance by linear least-squares regression analysis. At least three determinations (2000 data points) of pseudo-first-order rate constants (k_{obs}) were performed for each substrate concentration (pH 7.0, 25 °C) and the mean value was used in the calculation of the second-order rate constant.

2.2.2. Myeloperoxidase-catalyzed chlorination of monochlorodimedon

Monochlorodimedon (MCD) is often used to study the chlorinating activity of MPO. The enol form of MCD reacts very fast with HOCl, producing dichlorodimedon [33], which results in a decrease in absorbance at 289 nm. A 1 mL solution contained 200 nM MPO in 100 mM phosphate buffer pH 7.0 (or 20 nM MPO in 100 mM citric acid/NaOH buffer, pH 5.0), 100 mM chloride, 100 μM MCD in the absence or presence of various inhibitor concentrations. Reaction assays were started with 100 μM hydrogen peroxide and carried out using a conventional diode-array spectrophotometer (Zeiss Specord S-10) equipped with a temperature controller at 25 °C.

2.2.3. Dose-response curves

The activity of MPO was assayed either with the MCD assay or by using the hydrogen peroxide electrode (see below). The initial rates were determined by drawing a tangent to the initial linear part of the curve for MCD chlorination or H_2O_2 loss. A rectangular hyperbola was fitted to the dose-response curves for each inhibitor using non-linear regression (SigmaPlot, Jandel Scientific, San Rafael, CA, U.S.A.). From that equation, the concentration of inhibitor that decreased HOCl production by 50% (IC_{50}) was calculated.

2.2.4. Polarographic measurements of H_2O_2 utilization

Activity of MPO was measured in the presence of Cl^- (100 mM) and Br^- (20 mM), respectively by continuously monitoring hydrogen peroxide concentration polarographically, using a platinum electrode covered with a hydrophilic membrane and fitted to an Amperometric Biosensor Detector 3001 (Universal Sensors Inc., U.S.A.). At pH 7.0, the applied electrode potential was 0.65 V. The electrode filling solution was prepared freshly every day and the H_2O_2 electrode was calibrated against known concentrations of hydrogen peroxide. All reactions were performed in a thermally jacked tube at 25 °C and started by addition of 200 nM MPO at pH 7.0 or 20 nM MPO at pH 5.0. Typically, a 5 mL solution contained 50 μM hydrogen peroxide in 100 mM phosphate buffer, pH 7.0, or 100 mM citric acid/NaOH buffer, pH 5.0, at 25 °C. Reactions were investigated in the absence and presence of various inhibitor concentrations.

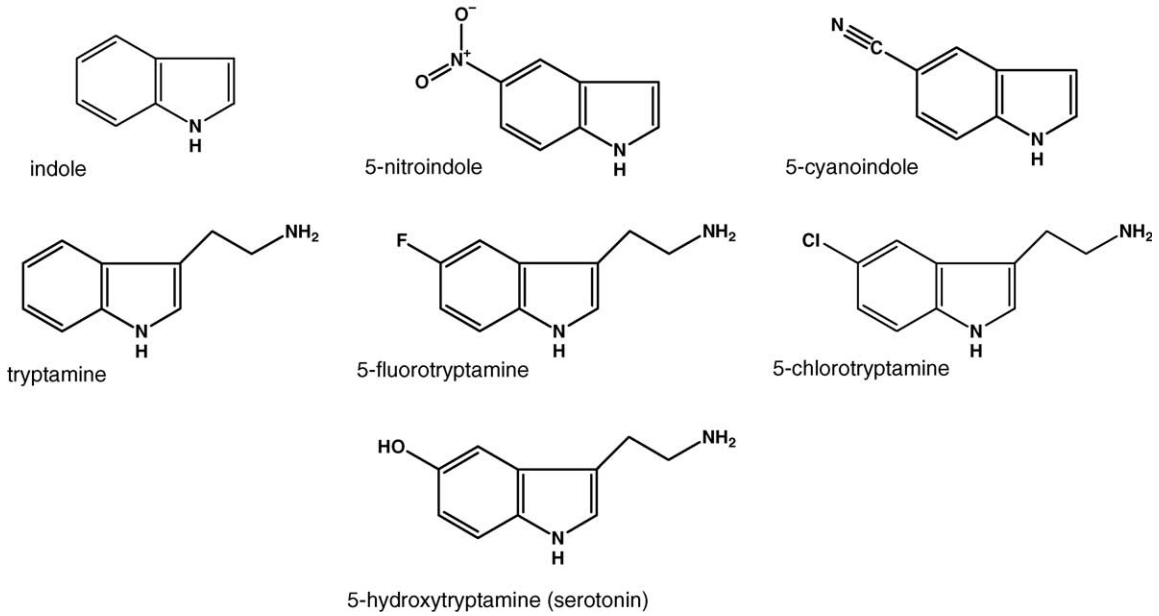

Fig. 1. Structures of indole and tryptamine derivatives.

3. Results

In the first part of this study the reactivity of three indoles (indole, 5-nitroindole, 5-cyanoindole) and four tryptamines (tryptamine, 5-fluorotryptamin, 5-chlorotryptamin and 5-hydroxytryptamin (serotonin) with the redox intermediates compound I and compound II of human myeloperoxidase was investigated. These aromatic donors (Fig. 1) were selected because almost all of the standard one-electron reduction potentials are known (Table 2). The sequential-mixing stopped flow technique had to be used because of the instability of the redox intermediates of myeloperoxidase. The Soret maximum of ferric MPO is at 430 nm and upon addition of H_2O_2 (i.e. compound I formation) a hypochromicity of about 50% is observed. The first spectrum in Fig. 2A represents the spectral features of compound I. In the presence of one-electron donors it is reduced to compound II, which has its Soret band at 456 nm and another maximum at 629 nm. A direct conversion of compound I to compound II exhibits distinct isosbestic points at 435, 489, 585 and 657 nm.

3.1. Compound I reduction

Compound II formation (k_3) was followed at 456 nm (Fig. 2B). All indole and tryptamine derivatives were very efficient in reduction of compound I (Fig. 2B). All reactions were monophasic and the time traces could be fitted to a single-exponential function (Fig. 2B). The obtained pseudo-first-order rate constants were linearly dependent on the concentration of the electron donor and allowed calculation of the second-order-rate constants (k_3) from the corresponding plots (Fig. 2C). Though the redox potentials of these substituted indoles and tryptamines, which are clearly related to Hammett coefficients for the substituents [34] (Table 2), vary between 0.65 and 1.23 V,

Table 2
Bimolecular rate constants of the reactions between compounds I (k_3) and II (k_4) of human myeloperoxidase with indole and tryptamine derivatives

Substrate	$E^{\circ\prime}$ (V)	k_3 (M^{-1} s^{-1})	k_4 (M^{-1} s^{-1})	k_3/k_4	Chlorination inhibition by 50% in μM	
					pH 7	pH 5
Indole	0.97 [34]	$(5.5 \pm 0.3) \times 10^6$	$(1.6 \pm 0.09) \times 10^3$	3440	12.3	250
5-Nitroindole	1.20 [38]	$(2.5 \pm 0.08) \times 10^5$	(78 ± 6)	3205	29	581
5-Cyanoindole	1.23 [38]	$(3.0 \pm 0.2) \times 10^5$	(53 ± 10)	5660	13.2	135
Tryptamine	1.015 [38]	$(8.6 \pm 0.4) \times 10^6$	$(5.2 \pm 0.2) \times 10^2$	16538	0.77	122
5-Fluorotryptamine	n.f.	$(1.7 \pm 0.1) \times 10^7$	(44 ± 1)	386364	0.79	25.8
5-Chlorotryptamine	n.f.	$(1.5 \pm 0.06) \times 10^7$	(67 ± 5)	223881	0.73	61.8
5-Hydroxytryptamine (serotonin)	0.65 [39]	$(1.7 \pm 0.1) \times 10^7$ [30]	$(1.4 \pm 0.1) \times 10^6$ [30]	12	–	–
Tyrosine [29]	0.93	$(7.7 \pm 0.1) \times 10^5$	$(1.6 \pm 0.6) \times 10^4$	48	–	–

The one-electron reduction potentials of the donors at pH 7 ($E^{\circ\prime}$) are included (with references in brackets). n.f.: not found in the literature. For details concerning sequential stopped-flow experiments see Section 2. The table also includes IC_{50} values, reflecting the effect of these substances on the chlorination activity of MPO measured with the monochlorodimedon assay at pH 5.0 and 7.0, as well as the k_3 and k_4 values for tyrosine, which was used to demonstrate the reversible character of inhibition. For details see Section 2.

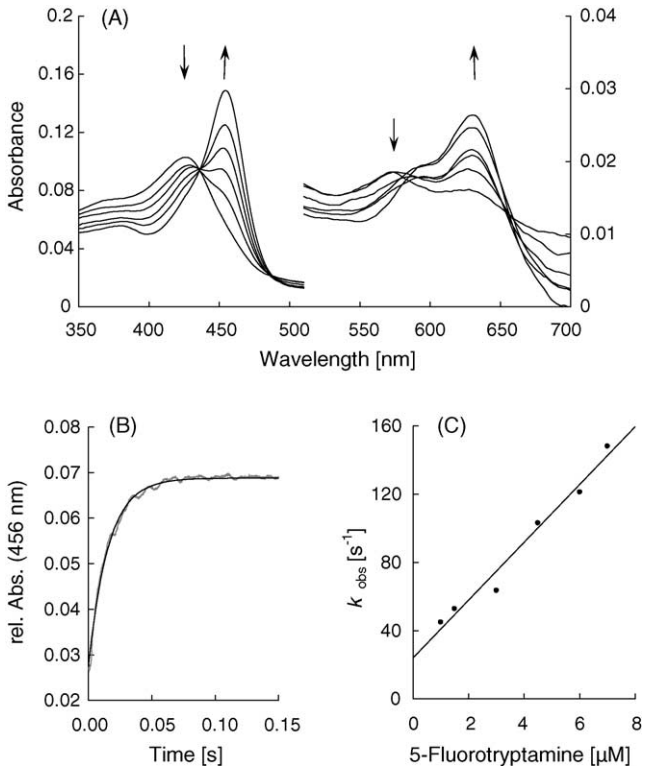

Fig. 2. Reaction of compound I of myeloperoxidase (MPO) with 5-fluorotryptamine. Experiment was carried out in the sequential-mixing stopped-flow mode. (A) Spectral changes upon reaction of 2 μM MPO compound I with 10 μM 5-fluorotryptamine showing a direct monophasic transition to compound II (buffer: 100 mM phosphate buffer pH 7.0). For details see Section 2. Arrows indicate absorbance changes. The first spectrum was taken 1.3 ms after mixing, subsequent spectra at 3.8, 6.4, 8.9, 14.1 and 24.3 ms. (B) Time trace and single-exponential fit of the reaction between 1 μM MPO compound I and 3 μM 5-fluorotryptamine, followed at 456 nm (absorbance maximum of compound II). Conditions as in (A). (C) Pseudo-first-order rate constants k_{obs} for compound I reduction plotted against 5-fluorotryptamine concentrations.

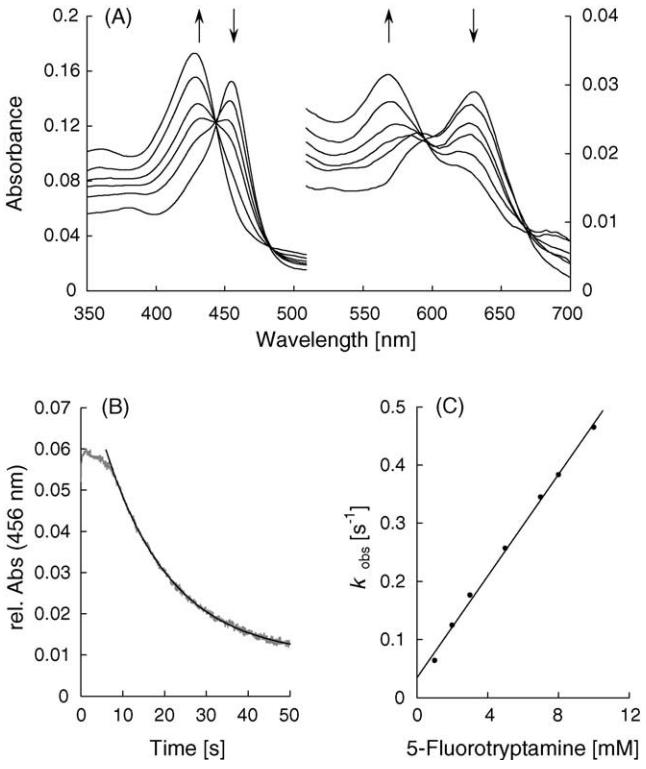

Fig. 3. Reaction of MPO compound II with 5-fluorotryptamine. (A) Spectral changes of compound II reduction back to ferric MPO. Starting from 2 μM MPO compound I, addition of 1 mM fluorotryptamine leads to complete formation of compound II after 1.15 s (first spectrum), subsequent spectra at 6.42, 12.86, 16.73, 21.18 and 49.26 s (buffer: 100 mM phosphate buffer, pH 7.0). Arrows indicate absorbance changes. (B) Time trace and single-exponential fit of compound II reduction to ferric MPO followed at 456 nm. For details see Section 2. (C) Pseudo-first-order rate constants k_{obs} for compound II reduction plotted against 5-fluorotryptamine concentrations.

the differences in the calculated k_3 values are comparatively small. The rates of compound I reduction varied only by a factor of 70 and tryptamine derivatives were more efficient than indole derivatives. This clearly underlines, that MPO compound I is extremely competent in oxidizing one-electron donors.

3.2. Compound II reduction

Regarding the calculated rates of the reactions between MPO compound II and these donors (k_4), the situation is completely different. With the exception of serotonin, k_4 of all donors was much smaller than k_3. Only the unsubstituted donors (indole and tryptamine) could reduce compound II in micromolar concentrations within several seconds. With all other substrates millimolar concentrations had to be added in order to monitor the formation of ferric MPO. Nevertheless compound II was reduced in a one-electron process with clear isosbestic points at 443, 485, 595 and 669 nm (Fig. 3A) and the time traces exhibited a typical single-exponential behaviour (Fig. 3B). Typically, the enzyme cycled (see lag phase in Fig. 3B), because a 10-fold excess of H_2O_2 was necessary for compound II formation. With the exception of serotonin this steady-state phase was seen with all donors. Thus, MPO compound II reduction was also followed by conventional spectrophotometry as described by Kettle and Candaeis for the oxidation of tryptophan, which is also a very poor electron donor for compound II [28]. Compound II was formed by adding an excess of H_2O_2 to ferric MPO and after addition of (nanomolar) monofunctional catalase to get rid of H_2O_2, the substrates were added and the resulting time trace was fitted to a single-exponential curve. Similar results as with the stopped-flow method were obtained within experimental error. In all cases the k_{obs} values strongly depended on the donor concentration (Fig. 3C).

With the exception of serotonin, the differences between k_3 and k_4 were significant. The observed k_3/k_4 ratios were 3440 (indole), 3205 (5-nitroindole), 5660 (5-cyanoindole), 16,540 (tryptamine), 386,360 (5-fluorotryptamine) and 223,900 (5-chlorotryptamine), respectively. Assuming that the binding site of these aromatic donors to compounds I and II is identical, thermodynamic and not kinetic factors seem to be responsible for the observed reactivities. This is clearly

underlined by comparison with serotonin ($E^{\circ\prime} = 0.65$ V), which is one of the best electron donors of both compound I and compound II [30] with a k_3/k_4 ratio of 12.

3.3. Effect of indole and tryptamine derivatives on hydrogen peroxide consumption and chloride oxidation by myeloperoxidase

In the second part of this study we determined the ability of these substances to inhibit the MPO mediated oxidation of chloride. Fig. 3C shows the effect of increasing concentrations of 5-fluorotryptamine on the H_2O_2 consumption of the system MPO/H_2O_2/chloride at pH 7.0. At higher concentrations the time traces are typically biphasic with a first slow phase followed by a second phase of fast H_2O_2 consumption. With increasing indole or tryptamine concentrations the slope of the first phase decreased and its length increased (3 μM 5-fluorotryptamine ≈ 3 min 5 μM 5-fluorotryptamine > 5 min), whereas the slope of the second phase fully corresponded to that of hydrogen peroxide consumption in the MPO/H_2O_2/chloride system in absence of these aromatic substances. Similar effects were observed in the MCD assay (not shown). In each case the slope of the first slow phase was used in calculation of inhibition and for each substrate the IC_{50} was determined from its dose-response curve, such as shown in Figs. 4A (pH 7.0) and 4B (pH 5.0). Table 2 summarizes the IC_{50} values at pH 7.0 and 5.0. Generally, the substrates were excellent inhibitors of myeloperoxidase at pH 7.0, with the exception of serotonin (see below). The IC_{50} values of 5-fluoro- and 5-chlorotryptamine were determined to be 0.79 and 0.73 μM, respectively, at pH 7.0. The indoles were moderately effective in inhibiting the chlorination activity of MPO.

Spectral steady-state investigations revealed that the dominating redox intermediate of the system MPO/H_2O_2/chloride was compound II even in the presence of very low concentrations of substituted tryptamines. This strongly suggests that these substances promote the accumulation of compound II and fits well with the (i) k_3/k_4 ratios calculated from the stopped-flow experiments, (ii) the observed lag-phase when these electron donors were added to compound II (formed by an excess of H_2O_2), and (iii) the biphasicity of the time traces monitored in the H_2O_2 electrode and MCD chlorination assays. In order to confirm this reversible character of inhibition, we either added tyrosine (not shown), ascorbate (not shown) or bromide (Fig. 4D) to the system. In these cases H_2O_2 consumption and chlorination of MCD was re-enhanced. Tyrosine [29] and ascorbate [40] are known to be a good electron donors of MPO compound I and bromide is a much better two-electron reductant of compound I compared with chloride [41]. In both cases ferric MPO was recovered enabling the enzyme to cycle.

At pH 5.0, the effect of the indole and tryptamine derivatives on the consumption of H_2O_2 or chlorination

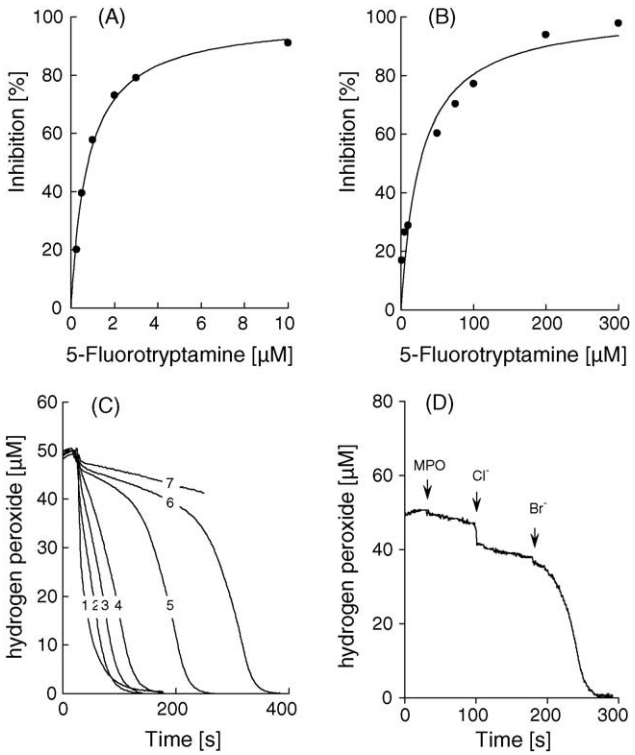

Fig. 4. Dose-response curves, representing the effect of 5-fluorotryptamine on the chlorination activity of myeloperoxidase at pH 7.0 (A) and pH 5.0 (B) determined with the monochlorodimedon assay as described in Section 2. (C) Inhibition of H_2O_2 consumption in the absence (1) and presence of 500 nM (2), 1 μM (3), 2 μM (4), 3 μM (5), 4 μM (6) and 5 μM (7) 5-fluorotryptamine. Conditions: 50 μM H_2O_2 and 100 mM chloride in 100 mM phosphate buffer, pH 7.0. (D) H_2O_2 consumption after adding 200 nM MPO (start), 100 mM Cl^-, and 20 mM Br^- as indicated by arrows. Assay conditions: 50 μM H_2O_2, 50 μM 5-fluorotryptamine in 100 mM phosphate buffer pH 7.0.

of MCD mediated by the MPO/H_2O_2/chloride system dramatically decreased. As a consequence the IC_{50} values increased significantly (Table 2), which also strengthens the hypothesis that the observed inhibition of chlorination at pH 7.0 is reversible, since it is well known that the bimolecular rate constant of the reaction between compound I and chloride is about two-orders of magnitude higher at pH 5.0 compared to pH 7.0 [41].

As has been outlined above, serotonin is an excellent electron donor of both compound I and II. At pH 7.0 serotonin enhances the chlorination of MCD in the MPO/H_2O_2/chloride system. In Fig. 5A the effect of increasing concentrations of serotonin on the chlorination of MCD is shown. By contrast, at pH 5 both a stimulating and inhibitory effect was observed depending on the serotonin concentration in the MCD assay. Based on the well known effect that in the MPO/H_2O_2/chloride system compound II accumulates with time due to compound II formation from compound I mediated by H_2O_2 [42], the stimulating effect at pH 7.0 can be explained by the efficient reduction of compound II by serotonin, thus recovering native MPO that participates in the halogenation cycle. At decreasing pH compound II formation becomes less important since the

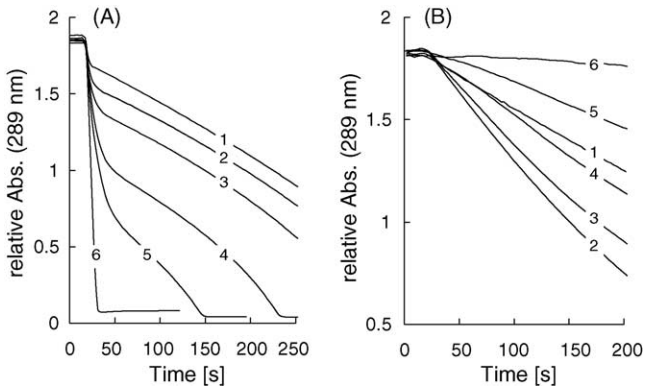

Fig. 5. Influence of serotonin (5-hydroxytryptamine) on the chlorination activity of MPO. Chlorinating activity of MPO was followed by using the monochlorodimedon assay. (A) 200 nM MPO, 100 μM MCD, 100 mM chloride in 100 mM phosphate buffer, pH 7.0 in the absence (1) and presence of 300 nM (2), 500 nM (3), 800 nM (4), 1 μM (5) and 5 μM (6) serotonin. Reaction was started upon addition of 100 μM H_2O_2. (B) 20 nM MPO, 100 μM MCD, 100 mM chloride in 100 mM citric acid/NaOH buffer, pH 5.0, in the absence (1) and presence of 10 μM (2), 100 μM (3), 200 μM (4), 300 μM (5) and 500 μM (6) serotonin.

rate of the two-electron reduction of compound I exceeds that of the H_2O_2-mediated compound II formation [42]. Since chloride, H_2O_2 and serotonin are competitors for compound I, differences in their concentrations can lead to different pseudo-first-order rate constants and as a consequence a stimulatory or an inhibitory effect on the HOCl formation can be observed. This again, fully underlines the reversible effect of these aromatic donors on the chlorination activity of MPO.

4. Discussion

Here we have shown that indole and tryptamine derivatives are excellent reversible inhibitors of human myeloperoxidase when they exhibit a one-electron reduction potential $E^{\circ\prime}(A^{\bullet}, H^{+}/AH) > E^{\circ\prime}$ (compound II/ferric MPO) = 0.97 V. Generally, tryptamines were more effective in inhibition of chlorination than indoles and this strongly correlated with the determined k_3/k_4 ratios (Table 2). 5-Fluorotryptamine and 5-chlorotryptamine were the most effective chlorination inhibitors of this study.

It is reasonable to assume that the overall topology of the actual MPO binding site for aromatic substrates as well as the substrate channel is identical in compound I or compound II reaction. From the crystal structure of native MPO and its complex with salicylhydroxamic acid [42] it can be concluded that there are no significant conformational differences between the active site regions of the complex and the native enzyme (with the exception that three water molecules from the distal heme cavity were displaced by binding of salicylhydroxamic acid). Indoles have been shown to bind also to peroxidases [29] and this paper clearly demonstrated that the binding and oxidation of tryptamine derivatives by MPO compound I is very effective. This is best illustrated by the determined rate constants of the reactions between MPO compound I and tryptamine, 5-fluorotryptamine, 5-chlorotryptamine and 5-hydroxytryptamine, which varied only by a factor of two (!). These findings strongly suggest that the hydrogen bonding ability of the hydroxyl group in serotonin does not favour binding of serotonin to MPO compound I in comparison with the halogen-substituted derivatives.

However, only serotonin (5-hydroxytryptamine) is an excellent peroxidase substrate. Due to $E^{\circ\prime} = 0.65$ V it is also an ideal electron donor for compound II and guarantees a high turnover of MPO in its peroxidatic cycle (Reactions (1), (3) and (4)). In the peroxidase cycle reduction of compound II is rate-limiting (since $k_1 > k_3 > k_4$). The higher k_4 is, the higher is the enzyme turnover and the rate of H_2O_2 consumption. Exchanging the electron-donating substituent (–OH) by electron-withdrawing substituents (–Cl, –F) will increase the redox potential of the corresponding tryptamines and, as a consequence, disqualify them to act as peroxidase substrate. Though the actual reduction potentials of 5-fluorotryptamine and 5-chlorotryptamine are unknown, it is reasonable to assume that the positive correlation between $E^{\circ\prime}(A^{\bullet}, H^{+}/AH)$ and the corresponding k_3/k_4 ratios found for indoles is also valid for the tryptamine derivatives. The determined k_3 values of the various donors ($0.65\ V \leq E^{\circ\prime} \leq 1.23\ V$) varied by a factor of only 70 [ranging from $2.5 \times 10^5\ M^{-1}\ s^{-1}$ (5-cyanoindole) to $1.7 \times 10^7\ M^{-1}\ s^{-1}$ (serotonin)], whereas the determined k_4 values varied by a factor of 32,000 [ranging from $44\ M^{-1}\ s^{-1}$ (5-fluorotryptamine) to $1.4 \times 10^6\ M^{-1}\ s^{-1}$ (serotonin)]. This reflects the MPO-specific significant difference in the one-electron oxidation capacity of compound I ($E^{\circ\prime} = 1.35$ V) and compound II ($E^{\circ\prime} = 0.97$ V). In other mammalian heme peroxidases these differences are much smaller and in plant-type peroxidases the reduction potentials of compound I and compound II are reported to be even similar (Table 1). In any case, MPO compound I is by far the strongest oxidizing redox intermediate known in heme peroxidases, which is an excellent precondition in designing reversible inhibitors. It guarantees specificity since even substrates with very positive reduction potentials are oxidized by MPO compound I at rates that exceed the rate of chloride oxidation (i.e. $2.4 \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.0, [41]), whereas other peroxidases or heme enzymes will not be able to use them as substrates. As has been shown in this investigation, even at 100 mM chloride concentration, micromolar concentrations of these substances could compete successfully and promoted the formation of compound II, which is a very poor oxidizing agent of these donors. As a consequence compound II accumulates ($k_1 > k_3 \gg k_4$), which diverts MPO from its chlorination cycle.

In effect these indole and tryptamine derivatives are very poor peroxidase substrates of MPO. However, tyrosine, ascorbate and bromide overcame this by their facile reduction of either compound II or compound I back to the native

enzyme, which underlines the reversible character of this inhibition. Under conditions where compound II is readily recycled, production of indole or tryptamine radicals will occur at a significant rate at sites of inflammation and the fate of these products is still unknown. That the efficiency of these reversible inhibitors of the chlorination activity of MPO could be diminished has been demonstrated by the effect of tryptophan on the hypochlorous acid formation by human neutrophils [28]. The IC_{50} values increased from 5 μM (effect with the pure enzyme) to 80 μM, since physiological substrates like ascorbate, tyrosine, urate or superoxide could reduce compound II enabling the enzyme to cycle though it is not fully understood whether and when all of these reactions operate in vivo in the neutrophils (e.g. the superoxide flux is high only at the beginning of phagocytosis and ascorbate will be oxidized efficiently by HOCl).

Summing up, we could demonstrate that exploiting the peculiar redox properties of MPO and selecting aromatic inhibitors with a high reduction potential ($0.97\ V \ll E^{o\prime}(A^{\bullet}, H^{+}/AH) < 1.35\ V$) efficiently diverts MPO from its chlorination cycle as a consequence of a high k_3/k_4 ratio. This will provide a rational basis for designing new inhibitors as potential (anti-inflammatory) drugs that will dampen HOCl-mediated oxidation reactions and tissue injury without totally precipitating MPOs role in unspecific immune defense.

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