Myeloperoxidase Shows Selective Bacterial Binding

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ABSTRACT

Background: Myeloperoxidase (MPO) exerts potent oxidative microbicidal action against a broad spectrum of Gram-negative and Gram-positive bacteria.

Methods: MPO binding to bacteria was qualitatively observed and photographically documented. Binding was quantified by dithionite-reduced minus oxidized (R-O) difference spectral analysis of *Staphylococcus aureus* and *Streptococcus viridans* exposed to MPO, then washed with normal saline (NS), and then resuspended in NS. For increased sensitivity of detection, a luminol-based luminescence method was constructed for Scatchard analysis of MPO binding. For comparison, fungal chloroperoxidase (CPO) binding activities were also tested. The Scatchard-derived MPO- and CPO-bound/free (B/F) ratios, affinity constants (K_{ass}), and binding sites per microbe were determined for *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Streptococcus viridans*.

Results: MPO showed strong binding to *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. Binding to *Streptococcus viridans* was undetected. By R-O difference spectral analysis, the direct binding of 20 pmol MPO to 2.1 x 10^9 *Staphylococcus aureus* (~5700 MPO molecules/bacterium) was demonstrated. By Scatchard analysis the MPO B/F ratio was 14.6 for *Staphylococcus aureus* and 0.4 for *Streptococcus viridans*; the CPO B/F ratio was 0.1 for *Staphylococcus aureus* and 0.2 for *Streptococcus viridans*.

Conclusions: MPO avidly binds to the Gram-negative and some Gram-positive bacteria tested, but not to *Streptococcus viridans*. As a member of the lactic acid bacteria family, *Streptococcus viridans* is cytochrome-deficient, and as such, its metabolic products, ie, lactic acid and H_2O_2, provide optimal acid pH and substrate for MPO oxidation of Cl^- to OCl^-, an established microbicidal agent. Singlet oxygen ($^1O_2$*) is produced when OCl^- reacts with an additional H_2O_2. This potent metastable oxygenating agent has a µs lifetime that restricts reaction to the proximity of its generation. As such, low MPO binding protects *Streptococcus viridans* from its metabolic product H_2O_2, while high MPO binding to other bacteria provides *Streptococcus viridans* with a competitive advantage in establishing its niche in the normal flora.

INTRODUCTION

Myeloperoxidase (MPO) exerts potent and broad-spectrum killing activity against Gram-negative and Gram-positive bacteria, as well as yeast and fungi.[1] Although MPO can catalyze classical peroxidase activity (ie, the dehydrogenation of dye [eg, guaiacol] to a colored product), it is the haloperoxidase activity of MPO that is responsible for microbe killing. In addition to hydrogen peroxide, an appropriate halide cofactor is required for bacterial and fungal killing.[1]

We have observed that MPO binds to bacteria and that the extent of binding was bacterium dependent. The following report documents the selective binding properties of MPO with regard to Gram-negative and Gram-positive bacteria and yeast.

METHODS

Haloperoxidases

Porcine myeloperoxidase (pMPO) was produced by Exoxemis, Inc. The absorbance extinction coefficient (ε) at 430 nm (ε_{430nm}) for pMPO is reported in the literature to be 91 $mM^{-1}cm^{-1}$ (also 95 $mM^{-1}cm^{-1}$).[2,3] This was used to calculate the pMPO values presented in the abstract. However, based on weight, absorbance spectrum of pure pMPO, and alkaline pyridine spectrum, the actual ε_{430nm} value of pMPO is about twice as large (ie, 182 $mM^{-1}cm^{-1}$). The purity of the pMPO was determined by the RZ (purity number), ie, the ratio of 430 nm to 280 nm absorbance. The $A_{430/280}$ RZ for the pMPO used in the binding experiments was 0.7, indicating about 88% purity.[4] Fungal (*Caldariomyces fumago*) chloroperoxidase (CPO) was purchased from Sigma Chemical Company. The 400 nm to 280 nm absorbance ($A_{400/280}$) ratio for CPO was 0.8. Spectrophotometric measurements were performed on a DW2000 UV-visible spectrophotometer (SLM Instruments, Inc.).

Microbes

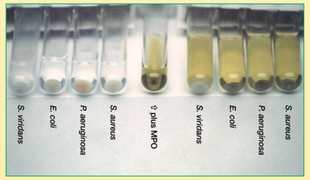
The bacteria *Staphylococcus aureus*, *Streptococcus viridans*, *Escherichia coli*, and *Pseudomonas aeruginosa* were grown for about 16 hours in trypticase soy broth (TSB) at 35°C. The yeast *Candida albicans* was grown for about 16 hours in Sabouraud's dextrose agar (SDA) at 35°C. The cultures were centrifuged at 3000 rpm for 15 minutes and the supernatants discarded. The microbial pellets were resuspended with sterile 0.85% NS. The washed microbes were resuspended and diluted with NS to an absorbance of 0.1 at a wavelength of 540 nm, ie, about 10^8 bacteria colony forming units (CFU) per mL and about 10^7 yeast CFU per mL.

Chemiluminescence (CL) measurement of MPO activity

The acid-optimum halide-dependent CL activity of pMPO was measured using H_2O_2 as oxidant, Br^- as halide, and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) as the chemiluminigenic substrate at pH 5. This acid CL method is absolutely halide-dependent and measures haloperoxidase activity, as opposed to classical peroxidase activity.[5] The CL of a 100-µL aliquot of each tested MPO dilution was measured with a LB950 luminometer (Berthold Instruments). Two injectors were employed. The first injected 300 µL of 150 µM luminol in 50 mM acetate buffer at pH 5 containing 5 mEq/L Br^-. Twenty-second CL measurements were taken following injection of 300 µL of 16.7 mM H_2O_2 by the second injector.

RESULTS I

Figure 1.



Visual Evidence of MPO Binding

We have observed that most bacterial suspensions exposed to pMPO show dark staining of the centrifuged pellet as illustrated in the photograph in Figure 1.

Each bacterial suspension, prepared as described in Methods, was centrifuged. The supernatant was discarded, and the bacterial pellet was resuspended in NS. The wash steps were repeated and the bacterial pellets were then resuspended in NS or NS plus an equivalent volume of pMPO.

A tube containing 2 mg/mL pMPO without bacteria is shown in the center of the photograph in Figure 1. This quantity and volume of pMPO was added to each bacteria suspension followed by mixing and centrifugation to pellet the bacteria. The untreated bacteria pellets are shown in the tubes to the left and the MPO-treated bacteria pellets are shown in the tubes to the right in Figure 1.

RESULTS II

Dithionite-reduced Minus Oxidized (R-O) Difference Absorbance Spectroscopy

The degree of pMPO binding to various bacteria was measured by R-O difference absorbance spectroscopy.[6] After measuring the absorbance spectrum of untreated (oxidized) pMPO, a few grains (slight excess) of sodium dithionite (hydrosulfite) were directly added to the cuvette, and the dithionite-reduced MPO absorbance spectrum was measured.

A 1.05 mg/mL solution of pure ($A_{430/280nm}$ RZ = 0.80) pMPO was used for the spectra of Figure 2. As illustrated, dithionite reduction causes the Soret band of pMPO to shift from 430 nm to 475 nm. Based on the concentration, the spectra, and a molecular weight estimate of 145 kD, the oxidized extinction coefficient (ε) at 430 nm (ε_{430nm}) is estimated to be about 182 $mM^{-1}cm^{-1}$ for pMPO, or about twice the amount reported in the literature.[3,4]

Subtracting the oxidized absorbance from the dithionite-reduced absorbance yields the unique R-O difference absorbance spectrum for pMPO as illustrated in Figures 3-5. At 475 nm the R-O absorbance extinction coefficient (ε_{475nm}) is calculated to be about 157.8 $mM^{-1}cm^{-1}$.

The bacteria tested were grown, centrifuged, washed with NS, and turbidimetrically adjusted to about a 10^6/µL stock suspension. Bacterial counts were determined by serial-dilution plate colony counts. pMPO was added to the bacterial suspension and mixed gently for about 30 minutes and then centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and saved for quantification of free pMPO. The pellet was washed by resuspension in 5 equivalent volumes of NS, mixed for 10 minutes, and again centrifuged. The supernatant was removed and the pellet was resuspended to the original volume with NS.

In order to compensate for the turbidimetric fluctuations and other complexities and to better quantify pMPO binding, the R-O difference absorbance measurements at 449 nm and 500 nm were averaged, and this average was subtracted from the 475-nm measurement value. The concentration of bacterial-bound pMPO was calculated by dividing this adjusted R-O difference A_{475nm} by a ε_{475nm} of 157.8 $mM^{-1}cm^{-1}$. The same procedure was used to calculate the free pMPO remaining in the supernatant postcentrifugation. These values were used to calculate the pMPO-bacteria B/F ratio as an index of binding.

Despite limitations, this spectroscopic approach provides objective quantitative evidence of pMPO binding to bacteria. Note that the microbe-bound pMPO persists following centrifugation, removal of supernatant, resuspension in 5 equivalent volumes of NS, recentrifugation, removal of supernatant NS wash, and resuspension to original volume with fresh NS. Spectral analysis quantifies the visual observation that pMPO binding is significant and selective. Consistent with direct observation, pMPO binding to *Pseudomonas aeruginosa* is especially large, and pMPO binding to *Streptococcus viridans* is especially small.

Figure 2a.

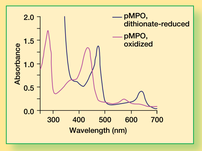
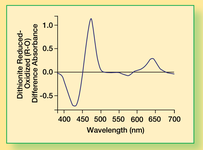


Figure 2b.



RESULTS II (cont)

Figure 3.

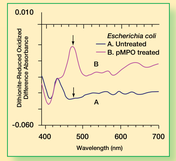


Figure 4.

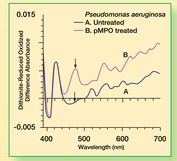
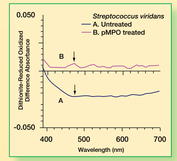


Figure 5.



Figures 3 and **4** illustrate the R-O difference absorbance spectra of untreated and pMPO-treated *Escherichia coli* and *Pseudomonas aeruginosa* suspensions, respectively. As illustrated in the figures, both bacteria are rich in cytochromes and have their own complex R-O spectra prior to pMPO treatment. The prosthetic group of MPO has heme *a* (cytochrome *a*) character and a unique spectrum with a maximum R-O positive difference at about 475 nm (indicated by arrows in the figures). Note that the R-O spectra of untreated *Escherichia coli* and *Pseudomonas aeruginosa* show little activity in this area of the spectrum, thus allowing a window for measurement of MPO. Bacterial-bound MPO is quantified as the large increases in R-O difference absorbance at A_{475nm} for both *Escherichia coli* and *Pseudomonas aeruginosa* following pMPO treatment.

Figure 5 illustrates the R-O difference absorbance spectra of untreated and pMPO-treated *Streptococcus viridans*. The same procedure was applied as described for Figures 3 and 4. Note that the untreated *Streptococcus viridans* spectrum is relatively less complicated than the spectra of untreated *Escherichia coli* or untreated *Pseudomonas aeruginosa*. This observation is consistent with *Streptococcus viridans* being a lactic acid bacteria, which are not capable of synthesizing cytochromes.

Table 1.

Microbe	CFU/µL	Bound pMPO, pmol/mL	Free pMPO, pmol/mL	B/F ratio	Bound pMPO, molecules/microbe
Escherichia coli	6,400,000	108.50	152.54	0.71	10,210
Pseudomonas aeruginosa	810,000	22.44	20.72	1.08	16,685
Streptococcus viridans	5,320,000	16.63	184.05	0.09	1882

Table 1. In Table 1, the number of molecules of pMPO bound per mL was estimated by multiplying the moles (pmol = 10^{-12} mole) of bound pMPO per mL by the Avogadro constant (6.022 x 10^{23} mol^{-1}). Dividing the number of molecules of bound pMPO per mL by the number of bacteria per mL yields the number of molecules of pMPO bound per bacteria.

RESULTS III

CL-based Scatchard Analysis of pMPO and CPO Microbe Binding

A highly sensitive CL method was developed for specifically measuring haloperoxidase activity. Both free and microbe-bound pMPO and CPO activities in the pmol to sub-pmol range were measured as acid-optimum, halide-dependent CL activity as described in Methods. The results were used for Scatchard analysis of pMPO and CPO binding affinity.[7]

Serial 1.5[n] or 2[n] dilutions of pMPO and CPO were prepared and added to each suspension: *Streptococcus viridans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. The concentration of pMPO or CPO was varied and the number of microbes was held constant. One volume of pMPO dilution, CPO dilution, or NS was added to 1 volume of microbial suspension. After gentle mixing and 30 minutes incubation at 23°C, the preparation was centrifuged (15,000 rpm for 5 minutes). The supernatant was decanted and saved for CL testing, and the pellet was resuspended with an equivalent volume of NS and recentrifuged. This wash supernatant was discarded and the microbe pellet was resuspended to its original volume in NS for CL testing.

Figure 6.

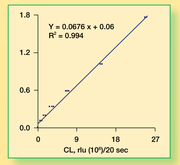


Figure 6 shows the plot of CL, expressed as relative light units (rlu) x 10^6 per 20 seconds versus the pmol quantity of pMPO tested. The actual concentration of pMPO was established spectrophotometrically as described in Results II. Precision was assessed by triplicate measurements. The linearity was tested by serial dilution of stock pMPO. At very high dilutions, some loss of pMPO due to polystyrene surface binding was observed. CL-pMPO linearity was tested with each microbe-binding experiment. The relationship of pMPO to CL was essentially linear, as shown in Figure 6, but slightly better by power fit. The global R^2 for eight runs was 0.9817 by linear fit ($y = 0.065x + 0.066$) and, slightly better, 0.9837 by power fit ($y = 0.1057x^{0.855}$).

RESULTS III (cont)

Figure 7.

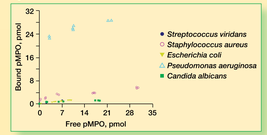
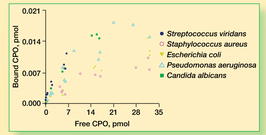


Figure 8.



Figures 7 and **8** plot the CL activities expressed as pmol of free pMPO or CPO versus pmol of microbe-bound pMPO or CPO, respectively. With regard to equivalent quantities of free haloperoxidase, pMPO binding to all microbes tested was greater than CPO binding by several orders of magnitude. Compare the ordinate values for Figures 7 and 8. Consistent with visual observation, and the R-O difference absorbance experiments described in Results II, pMPO binding to *Pseudomonas aeruginosa* was particularly high.

Figure 9.

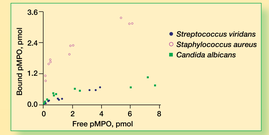


Figure 10.

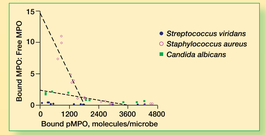


Figure 9 explodes the lower left portion of Figure 7 to focus attention on free versus microbe-bound relationship of pMPO to *Streptococcus viridans*, *Staphylococcus aureus*, and *Candida albicans*.

Figure 10 shows the replot of these data where the abscissa is bound pMPO expressed as molecules (moles multiplied by the Avogadro constant) of pMPO per microbe (molecules divided by the number of microbes), and the ordinate is the ratio of microbe-bound pMPO to free pMPO. Binding can be considered according to the Rosenthal-Scatchard relationship:

Free XPO + Microbe ↔ Microbe-Bound XPO
$\therefore K_{ass}$ = [Microbe-Bound XPO]/[Free XPO][Microbes]
$\therefore$ B/F = Microbe-Bound XPO/Free XPO = K_{ass}[Microbe]

The curves drawn in Figure 10 estimate the affinity constants (K_{ass}) for the 3 microbes. Extrapolating the slope to its intercept with the abscissa estimates the number of pMPO binding sites per microbe. *Staphylococcus aureus* shows a higher affinity for pMPO than does *Candida albicans*, and *Streptococcus viridans* shows relatively poor binding affinity.

CONCLUSIONS

- pMPO selectively binds to microorganisms as demonstrated by direct visual observation, R-O difference absorbance spectroscopy, and CL-based Scatchard analysis
- pMPO showed strong binding to most of the bacteria tested, including pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*
- pMPO binding to *Streptococcus viridans* was very weak in comparison to the other microbes tested
- Although pMPO binding to *Candida albicans* was weaker than observed for most bacteria, it was stronger than observed for *Streptococcus viridans*
- Except for *Streptococcus viridans*, pMPO-microbe binding was magnitudinally greater than CPO-microbe binding, based on CL-Scatchard analysis

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