Modeling the Reactions of Superoxide and Myeloperoxidase in the Neutrophil Phagosome

**IMPLICATIONS FOR MICROBIAL KILLING**[

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Neutrophils kill bacteria by ingesting them into phagosomes where superoxide and cytoplasmic granule constituents, including myeloperoxidase, are released. Myeloperoxidase converts chloride and hydrogen peroxide to hypochlorous acid (HOCl), which is strongly microbicidal. However, the role of oxidants in killing and the species responsible are poorly understood and the subject of current debate. To assess what oxidative mechanisms are likely to operate in the narrow confines of the phagosome, we have used a kinetic model to examine the fate of superoxide and its interactions with myeloperoxidase. Known rate constants for reactions of myeloperoxidase have been used and substrate concentrations estimated from neutrophil morphology. In the model, superoxide is generated at several mM/s. Most react with myeloperoxidase, which is present at millimolar concentrations, and rapidly convert the enzyme to compound III. Compound III turnover by superoxide is essential to maintain enzyme activity. Superoxide stabilizes at ~25 μM and hydrogen peroxide in the low micromolar range. HOCl production is efficient if there is adequate chloride supply, but further knowledge on chloride concentrations and transport mechanisms is needed to assess whether this is the case. Low myeloperoxidase concentrations also limit HOCl production by allowing more hydrogen peroxide to escape from the phagosome. In the absence of myeloperoxidase, superoxide increases to >100 μM but hydrogen peroxide to only ~30 μM. Most of the HOCl reacts with released granule proteins before reaching the bacterium, and chloramine products may be effectors of its antimicrobial activity. Hydroxyl radicals should form only after all susceptible protein targets are consumed.

Neutrophils kill micro-organisms by ingesting them into phagocytic vacuoles (phagosomes). Phagocytosis is accompanied by the activation of the NADPH oxidase, an enzyme complex that assembles in the phagosomal membrane and converts oxygen into the superoxide radical anion (O_2^-). O_2^- is generated at the external surface (i.e. inside the phagosome) with reducing equivalents supplied by intracellular NADPH. There are two apparently distinct mechanisms of killing, one requiring activation of the oxidase and the other involving antimicrobial peptides (1). Superoxide production is important for an effective antimicrobial defense, as illustrated by the recurrent infections seen in chronic granulomatous disease (2).

The specific mechanisms and species responsible for oxidative killing are the subject of continuing debate (3). The O_2^- produced by the oxidase undergoes dismutation to produce hydrogen peroxide (H_2O_2). At the same time, myeloperoxidase (MPO)² is released into the phagosome. MPO catalyzes the formation of hypochlorous acid (HOCl) from chloride and H_2O_2. It also oxidizes a wide range of reducing substrates to their corresponding radicals (4). HOCl is strongly microbicidal (1), has been detected in the neutrophil phagosome (5, 6), and is widely considered to be the major oxidative weapon of the neutrophil (1, 7). However, it is possible that sufficient HOCl may not be generated to kill ingested bacteria on its own (6, 8), and in view of the benign clinical consequences of hereditary deficiency (9), the importance of MPO has been questioned (10–12). Alternative explanations for antimicrobial activity, especially in MPO deficiency, include the build up of high concentrations of H_2O_2 that might kill directly or via formation of hydroxyl radicals (13, 14). Production of hydroxyl radicals (13) and singlet oxygen (15, 16) in secondary reactions of HOCl is possible. Ozone formation by neutrophils has also been suggested (17), although this proposal is controversial (18, 19). An alternative view is that O_2^- generation has an electrogenic function of bringing potassium ions into the phagosome to increase ionic strength and aid solubilization of granule proteins. According to this mechanism, oxidants are incidental to the killing process (11, 12, 20). However, this mechanism has been challenged in recent publications (21, 22).

To understand oxidative killing, knowledge of the fate of O_2^- and reactions of MPO in the phagosomal environment is required. MPO cycles through redox intermediates that undergo a complex array of reactions (Fig. 1). Oxidation of most substrates (including phenolic compounds, nitrite and ascorbate) occurs via a 1-electron step involving compounds I and II. Chloride undergoes a 2-electron oxidation by compound I. This reaction is inhibited by formation of compound II

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The abbreviations used are: MPO, myeloperoxidase; SOD, superoxide dismutase.
or the \( O_2^- \) adduct, compound III (4, 23). Superoxide is able to react with all the redox intermediates of MPO in ways that can enhance or inhibit chlorination (4, 24). It converts native MPO to compound III (25, 26); it reacts with compound II (23, 24) and, analogous to horseradish peroxidase (27), also with compound I. There is also evidence for a reaction with compound III (28). \( H_2O_2 \) also reacts with compounds I and II (29, 30). How MPO acts in the phagosome will depend on the interplay of these reactions.

The phagocytosing neutrophil spreads over the surface of the targeted bacterium, creating a very narrow space where oxidants are released and killing occurs (31–33). Very little extracellular medium is taken up (34), and the major constituents are released granule contents. This creates an environment with very high concentrations of granule proteins, including MPO, into which \( O_2^- \) is released at a high rate. These conditions are impossible to mimic in pure enzyme systems and difficult to investigate directly as this requires probes that distinguish the fate of oxidants in the phagosome. In the model, \( O_2^- \) is generated by the electrogenic effect of \( O_2 \) (42, 43). We have also assumed that charge compensation for the electrogenic effect of \( O_2^- \) production is met primarily by proton transport associated with activation of the oxidase, and we have assumed initial values and considered the impact of variations. The model of \( O_2^- \) is generated at a rate determined from measured rates of oxygen uptake by neutrophils, and subsequent reactions of \( O_2^- \) and its dismutation product \( H_2O_2 \) are followed. These include losses by diffusion as well as interactions with the redox intermediates of MPO. The volume of the phagosome and the total concentration of MPO are deduced from morphological data plus direct analyses of neutrophil MPO content. Where data are not available, we have assumed initial values and considered the impact of variations. The model has been used to assess the following: 1) the likely concentrations of \( O_2^- \) and \( H_2O_2 \) and the redox state of MPO; 2) critical determinants of the breakdown route of the \( O_2^- \) generated and the efficiency of HOCl production; and 3) the fate of HOCl and likelihood of it forming secondary products such as chloramines, hydroxyl radicals, and singlet oxygen.

**EXPERIMENTAL PROCEDURES**

*Construction of a Kinetic Model of the Phagosome*—The kinetic model of the phagosome considers rates of \( O_2^- \) generation and disappearance and includes the MPO redox transformations shown in Fig. 1. These and all the other reactions considered are shown in Table 1. Modeling was carried out using the Simulink feature of Matlab (The Math Works Inc, Natick, MA).

**Phagosome Volume**—Setting reactant concentrations requires knowledge of the phagosome volume. Killing of most micro-organisms takes place within minutes of phagocytosis (5, 35, 36) when degranulation but little phagosomal swelling occurs. For our standard conditions (Table 2), we used a volume of \( 1.2 \times 10^{-15} \) liters (1.2 \( \mu \)m\(^3\)). This is equivalent to a 0.25-\( \mu \)m space between a 1-\( \mu \)m diameter bacterium and the phagosomal membrane and includes the volume of the granules that are released. The neutrophil contains \( \approx 1500 \) azurophil granules of 0.3 \( \mu \)m diameter and twice as many specific granules of half that diameter (31, 37, 38). Assuming maximum phagocytosis of \( \approx 20 \) particles (5) is accompanied by 80% degranulation, granule contents will comprise almost half the phagosome volume. These dimensions will vary somewhat depending on the size and number of micro-organisms ingested but are consistent with electron micrographs of ingested bacteria (31, 32, 38).

**Myeloperoxidase Concentration**—Neutrophils contain 30 pmol (2.5 \( \mu \)g) of MPO per 10\(^6\) cells (39). With 80% degranulation into 20 phagosomes, this gives a concentration of 1 mM.

**Chloride**—The phagosomal chloride concentration has recently been estimated as 73 mM for neutrophils in medium containing 122 mM chloride (40). For initial modeling we have used 100 mM (the same as in extracellular fluid) and then assessed the impact of variations.

**Oxygen Consumption Rate and Site of Superoxide Generation**—When neutrophils phagocytose particles, oxygen is reduced on the phagosomal membrane and \( O_2^- \) is released into the lumen, with very little detected in the medium (41). This continues over a period of minutes during which killing takes place. We have assumed that oxygen consumption occurs at a constant rate, based on the maximum uptake by 10\(^6\) cells being 3–4 nmol/min (5, 35). This was observed with 15–20 particles ingested per cell and corresponds to about \( 3 \times 10^{-18} \) mol of oxygen per phagosome/s or with a phagosome volume of \( 1.2 \times 10^{-15} \) liters at 2.5 mM/s. This is a net rate, as oxygen is regenerated from the \( O_2^- \) and \( H_2O_2 \) is formed. As it is a measured value, it takes into account any limitation in oxygen availability because of diffusion. We have assumed that oxidase activity is not limited by NADPH availability. A more complex analysis would be required to test the effects of rapid oscillations in NADPH concentration as observed by Petty and co-workers (42, 43). We have also assumed that charge compensation for the electrogenic effect of \( O_2^- \) production is met primarily by proton transport associated with activation of the oxidase, and this provides sufficient protons for \( O_2^- \) dismutation and maintenance of pH (22, 44).

**Myeloperoxidase Reactions**—When killing occurs, the phagosomal pH is 7.4–7.8 (45), so we have used rate constants measured at neutral pH. The majority of these are known or can be estimated from literature data (Table 1). The rate constants for compounds I and II reacting with peroxidase substrates vary depending on the substrate. For most substrates, \( k_6 \) is between \( 10^9 \) and \( 10^7 \) M\(^{-1}\) s\(^{-1}\); values of \( k_8 \) are less and vary more widely (46). Good substrates (high \( k_6 \), e.g. tyrosine) and poor substrates that are potential inhibitors (low \( k_6 \), e.g. tryptophan and nitrite) have been modeled.

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3 A. J. Kettle, R. F. Anderson, M. B. Hampton, and C. C. Winterbourn, manuscript in preparation.
**Homogeneity of Phagosome Environment—** We have assumed that reactions take place in a homogeneous milieu. This is a simplification, as \( \text{O}_2^- \) is generated on the membrane, degranulation occurs at discrete sites, and MPO can bind to bacterial surfaces. The calculated diffusion distance\(^4\) for \( \text{O}_2^- \) varies depending on the concentration and redox state of MPO but is at least 1 \( \mu \text{m} \). This is substantially greater than the width of the phagosome (0.25 \( \mu \text{m} \)), so any concentration gradient for \( \text{O}_2^- \) should be slight. As \( \text{H}_2\text{O}_2 \) is generated from \( \text{O}_2^- \), it should be generated throughout the phagosome. If the MPO were in its native form and evenly distributed, the diffusion distance of \( \text{H}_2\text{O}_2 \) would be \( \sim 0.2 \mu \text{m} \).

Association of MPO with the surface of ingested bacteria has been observed microscopically (47). This could be a way of directing HOCl for effective killing (1). MPO has been shown to bind to bacterial surfaces. The calculated diffusion distance\(^4\) for \( \text{O}_2^- \) is therefore equal to the sum of \( R \) plus these rates.

For consumption of external \( \text{H}_2\text{O}_2 \) by 10\(^6\) cells/ml, the following relationship as shown in Equation 1 holds,

\[
\frac{d[\text{H}_2\text{O}_2]_{\text{out}}}{dt} = k_{\text{obs}}[\text{H}_2\text{O}_2]_{\text{out}} = \text{Influx} - \text{Efflux} = \frac{P \times A \times 10^{-3}([\text{H}_2\text{O}_2]_{\text{out}} - [\text{H}_2\text{O}_2]_{\text{in}})}{V} \quad (\text{Eq. 1})
\]

\(^4\) The diffusion distance of any reactant is given by \( I = \sqrt{(D/2k)} \), where \( D \) is the diffusion coefficient (1–2 \( \times 10^{-5} \) cm\(^2\)/s for most small ions) and \( 2k \) is the sum of \( k_{\text{substrate}} \) [substrate] for all the reactions of the reactant. For \( \text{O}_2^- \) assuming it reacts with MPO (1 \( \mu \text{m} \)) and it is all in the native form (\( k = 2 \times 10^9 \text{M}^{-1} \text{s}^{-1} \)) or dismutates, then \( I = 1 \mu\text{m} \), increasing to \( 2 \mu\text{m} \) with 90% compound III. If there is no MPO and the steady state concentration of \( \text{O}_2^- \) is 100 \( \mu\text{M} \), \( I \) increases to \( \sim 10 \mu\text{m} \). For \( \text{H}_2\text{O}_2 \), assuming it reacts with MPO (1 \( \mu\text{M} \)), \( k = 2.6 \times 10^6 \text{M}^{-1} \text{s}^{-1} \), and it is all ferric enzyme, \( I = 0.2 \mu\text{m} \); or if the enzyme is 90% compound III, \( I = 0.7 \mu\text{m} \). For HOCl, when thiols and methionines (50 \( \mu\text{M} \), \( k = 3 \times 10^9 \text{M}^{-1} \text{s}^{-1} \)) are available, \( I = 0.03 \mu\text{m} \) and if amines (50 \( \mu\text{M} \), \( k = 5 \times 10^9 \text{M}^{-1} \text{s}^{-1} \)) are the main target, \( I = 0.1 \mu\text{m} \).
where \( V = 10^{-3} \text{ liter} \), \( k_{\text{abs}} \) (s\(^{-1}\)) is the first order rate constant for \( \text{H}_2\text{O}_2 \) consumption, and \( A \) is the total surface area of 10\(^6\) cells (52). Using a mean neutrophil diameter of 8.5 \( \mu \text{m} \) and a surface area of 456 \( \mu \text{m}^2 \) per cell (53), \( A = 4.6 \text{ cm}^2 \) per 10\(^6\) cells. Assuming [\( \text{H}_2\text{O}_2 \)]\(_{\text{out}} \ll [\text{H}_2\text{O}_2]_{\text{in}} \), we can ignore [\( \text{H}_2\text{O}_2 \)]\(_{\text{in}} \), and this simplifies to Equation 2.

\[
P = \frac{k_{\text{abs}} \times V \times 10^3}{A} = 13 \times 10^{-4} \text{ cm.s}^{-1} \quad \text{(Eq. 2)}
\]

For comparison, values of \( 6 \times 10^{-4}, 2 \times 10^{-4}, \) and \( 16 \times 10^{-4} \text{ cm.s}^{-1} \) have been determined for erythrocytes, Jurkat cells, and \textit{Escherichia coli}, respectively (52, 54).

The rate of loss of \( \text{H}_2\text{O}_2 \) into the neutrophil cytoplasm from a single phagosome with surface area \( A \) (cm\(^2\)) and volume \( V \) (liters) of \( 1.2 \times 10^{-15} \) (see Ref. 52) is given by Equations 3 and 4,

\[
\frac{d[\text{H}_2\text{O}_2]}{dt} = \frac{P \times A}{V} \times 10^{-3} \times ([\text{H}_2\text{O}_2]_{\text{phagosome}} - [\text{H}_2\text{O}_2]_{\text{cytoplasm}})
\]

where

\[
k_{\text{H}_2\text{O}_2} = \frac{P \times A}{V} \times 10^{-3} \text{ s}^{-1} \quad \text{(Eq. 4)}
\]

It is reasonable to assume that once the \( \text{H}_2\text{O}_2 \) has crossed the phagosome membrane, it is scavenged within the cytoplasm so that [\( \text{H}_2\text{O}_2 \)]\(_{\text{cytoplasm}} \ll [\text{H}_2\text{O}_2]_{\text{phagosome}} \) and can be ignored. Assuming a phagosome diameter of 1.5 \( \mu \text{m} \), \( A = 7 \times 10^{-8} \text{ cm}^2 \) and \( V = 1.2 \times 10^{-15} \text{ liters} \). Substituting these values and \( P \) from above gives \( k_{\text{H}_2\text{O}_2} = 70 \text{ s}^{-1} \).

We have also considered \( \text{H}_2\text{O}_2 \) consumption by the bacterium, using appropriate values for \( P \) and \( A \). However, in view of the small bacterial volume, it cannot be assumed that [\( \text{H}_2\text{O}_2 \)]\(_{\text{in}} \ll [\text{H}_2\text{O}_2]_{\text{phagosome}} \) and two way diffusion across the membrane is likely. Therefore, although the bacterium would consume some \( \text{H}_2\text{O}_2 \), it would have much less impact than the neutrophil on the phagosomal \( \text{H}_2\text{O}_2 \) concentration.

Membranes have very low permeability to \( \text{O}_2^- \). We have used a permeability constant (\( P \)) of \( 2 \times 10^{-6} \text{ cm.s}^{-1} \) as determined for phospholipid vesicles (55), giving \( k_{\text{O}_2^-} = 0.1 \text{ s}^{-1} \).

**RESULTS AND DISCUSSION**

**Modeling Oxidative Reactions in the Phagosome**—Each simulation begins with oxygen being reduced to \( \text{O}_2^- \) at a given rate and with MPO present in its native form. Under the standard conditions defined in Table 2 and with the reactions described in Table 1, \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) build up within seconds to steady state concentrations where the rates of production and consumption are the same (Fig. 2A and Table 3). The \( \text{H}_2\text{O}_2 \) concentration is only 2 \( \mu \text{M} \), whereas the \( \text{O}_2^- \) concentration stabilizes at 25 \( \mu \text{M} \). HOCl is produced at a constant rate of 134 \( \mu \text{M} \text{.min}^{-1} \) (Fig. 2B), which is 89% of the maximum achievable if it accounted for all the oxygen consumed. Most of the MPO (93%) is converted to compound III, the remainder is native enzyme and its chloride complex, with <1% compound II. The \( \text{O}_2^- \) generation rate is 5.2 \( \mu \text{mol.s}^{-1} \). This flux is remarkably high when expressed as a concentration, and it reflects the very narrow space into which the \( \text{O}_2^- \) is released. \( \text{O}_2^- \) is generated at slightly more than twice the oxygen consumption rate of 2.5 \( \mu \text{mol.s}^{-1} \), indicating that a small amount does not dismutate to oxygen and \( \text{H}_2\text{O}_2 \) but is oxidized by MPO compound I (see below).

Experimental validation of the parameters in the model would ideally be performed using conditions resembling those in the phagosome. However, it is not possible to study the high MPO concentrations and \( \text{O}_2^- \) generation rates needed to do this. Therefore, we tested the model experimentally by measuring HOCl production from an \( \text{O}_2^- \)-generating system plus MPO and chloride in the presence and absence of SOD. As shown (supplemental Fig. S1), rate constants used in the phagosome model gave reasonable agreement with these data.

| TABLE 2 |
| --- |
| **Standard conditions selected for testing model** |
| **Parameter** | **Value** |
| Diameter of bacterium | 1 \( \mu \text{m} \) |
| Diameter of phagosome | 1.5 \( \mu \text{m} \) |
| Phagosome volume | \( 1.2 \times 10^{-15} \text{ liter} \) |
| Oxygen consumed per phagosome | \( 3 \times 10^{-14} \text{ mol/s (2.5 mM/s) }\) |
| Myeloperoxidase per phagosome | \( 1.2 \times 10^{-15} \text{ mol (1 mM) }\) |
| Chloride concentration (assumed to stay constant) | 100 mM |
| Membrane permeability to superoxide | \( 2 \times 10^{-6} \text{ cm/s }\) |
| Membrane permeability coefficient for hydrogen peroxide | \( 13 \times 10^{-4} \text{ cm/s }\) |
| No other MPO substrate present | |
Varying Physical Features and Oxygen Consumption Rate—The standard conditions of the model include estimates for several parameters. It is important to identify those that are critical outcome determinants and those that are not. Increasing the phagosome volume or varying membrane permeability to H₃O₂ has little impact (Table 3). The increase in efficiency of HOCl production seen if the membrane is impermeable to H₃O₂ indicates that under the standard conditions about 6% escapes the MPO and is lost by diffusion. Loss of O₂⁻ through the membrane has a negligible impact on the O₂⁻ concentration even if its permeability is increased 100-fold (not shown). This trapping of O₂⁻ in the phagosome explains why its concentration gets so high. Varying the rate of oxygen consumption causes parallel changes in the concentrations of O₂⁻ and H₂O₂ but has only a minor effect on the efficiency of HOCl production (Table 3).

Varying MPO Concentration—In the model, MPO consumes H₂O₂ and is necessary for HOCl production. More surprisingly, it is also the major consumer of O₂⁻. This occurs primarily via MPO-catalyzed dismutation, with the enzyme cycling between the ferric form and compound III (Mechanism A, r₁₀ + r₁₂).

\[ 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \]

**MECHANISM A**

O₂⁻ and H₂O₂ concentrations both increase with decreasing MPO concentration, with O₂⁻ remaining the higher (Table 3). HOCl production decreases, mainly because more H₂O₂ evades the MPO and escapes from the phagosome. Compound III remains close to 90%. The MPO concentration used for the standard conditions is probably an upper limit, because degranulation is not instantaneous and inactivation of MPO occurs over time (56). However, the MPO concentration can be decreased 10-fold and still give about half-maximum HOCl production.

Relevance to MPO Deficiency—Hereditary MPO deficiency occurs in 1 in 2000–4000 people in the general population (57). It is rarely associated with infection, although MPO-deficient neutrophils do kill some organisms poorly (1, 58). It is generally considered that they have alternative, less potent, MPO-independent oxidative killing mechanisms. The MPO-deficient neutrophil can be mimicked by modeling the phagosome with no MPO present. In this situation, all the O₂⁻ breaks down by spontaneous dismutation. This is much slower than with MPO present, and the O₂⁻ concentration increases to above 100 μM (Table 3). The majority of the H₂O₂ diffuses out of the phagosome into the cytoplasm and only a modest 30 μM accumulates. Any consumption by the bacterium would decrease this further.

It is usually assumed that in MPO deficiency, H₂O₂ accumulates to high concentrations and kills either directly or via secondary products such as hydroxyl radicals produced within the bacterium (14). This is not supported by the model. Greater than millimolar H₂O₂ concentrations need to be added to kill most bacteria in cell-free systems (59), and bacteria in the phagosome would be exposed to much lower concentrations than this. Our findings highlight the possibility that O₂⁻ itself may contribute to killing. Although O₂⁻ is usually discounted as benign (60), its protonated form (HO₂⁻) has been shown to penetrate *E. coli* and inactivate fumarase (61). Estimated O₂⁻ generation rates in the phagosome are 10⁶ times faster and achieve much higher concentrations of O₂⁻ than have been tested experimentally. Under these conditions, sufficient HO₂⁻ may be present (pKₐ 4.8) to be damaging.

Dependence on Chloride Availability—Chloride concentration is set to remain constant at 100 mM in the standard model. Lowering this concentration decreases HOCl production as O₂^- competes more effectively for compound I, but even with 20 mM chloride the reaction is still 73% efficient (Table 3). The decline in HOCl production is more marked, however, at lower MPO concentrations.

When there is no chloride, MPO still consumes the majority of the H₂O₂ and O₂⁻, and their steady state concentrations scarcely change. Although O₂⁻ removal is still predominantly by mechanism A under these conditions, the superoxide/hydrogen peroxide oxido-reductase activity of MPO (Mechanism B, r₃ + r₁₃ + r₁₄) comes more into play.

\[ H_2O_2 + 2O_2^- + 2H^+ \rightarrow O_2 + 2H_2O \]

**MECHANISM B**

TABLE 3
Simulated oxidant reactions in the neutrophil phagosome and effect of varying reactant concentrations

| Variations from standard conditions | [O₂⁻] | [H₂O₂] | MPO redox state (% compound III) | HOCl production (% O₂ consumed) |
|------------------------------------|-------|--------|--------------------------------|---------------------------------|
| Standard conditions               | 25    | 2.0    | 93                             | 89                              |
| 3-Fold larger phagosome           | 23    | 2.0    | 93                             | 89                              |
| 5-Fold faster efflux of H₂O₂      | 25    | 1.6    | 93                             | 72                              |
| No efflux of H₂O₂                 | 25    | 2.1    | 92                             | 95                              |
| 2-Fold higher O₂ consumption      | 48    | 3.9    | 93                             | 85                              |
| 2-Fold lower O₂ consumption       | 13    | 1.0    | 94                             | 91                              |

| Myeloperoxidase                    |       |        |                                |                                 |
|------------------------------------|-------|--------|--------------------------------|---------------------------------|
| 2-Fold less MPO                    | 45    | 3.7    | 93                             | 81                              |
| 10-Fold less MPO                   | 91    | 13     | 91                             | 51                              |
| Plus no H₂O₂ efflux                | 91    | 22     | 89                             | 82                              |
| No MPO                             | 112   | 33     | 0                              | 0                               |
| Plus no H₂O₂ efflux                | 112   | 200    | 0                              | 0                               |

| Chloride                           |       |        |                                |                                 |
|------------------------------------|-------|--------|--------------------------------|---------------------------------|
| 20 mM Cl⁻                          | 25    | 2.1    | 93                             | 75                              |
| Plus 10-fold lower MPO             | 92    | 14     | 87                             | 32                              |
| No Cl⁻                             | 27    | 2.8    | 86                             | 0                               |

*Standard conditions are given in Table 1.*
If chloride is consumed by MPO in the model and is not replenished, HOCl production rapidly ceases within a minute. Although chloride is regenerated when HOCl oxidizes substrates such as thiols and methionines, it would be depleted if long lived chloramines and other chlorinated products such as chlorotyrosine are formed. It would also decline if any HOCl leaves the phagosome. As discussed below, chloride-depleting reactions are likely to occur and ongoing production of HOCl in the phagosome should therefore require a continuous supply of chloride.

Measurements of chlorination of phagocytosed probes (5, 6) imply that chloride is present in the neutrophil phagosome. Some chloride would enter with the extracellular fluid taken up during phagocytosis and be diluted, probably to about a half, with released granule contents. Granules are packed with proteins anchored to a negatively charged sulfated proteoglycan matrix (62, 63), so their chloride content could be low. Recent studies with a chloride-sensitive probe indicated an intraphagosomal chloride concentration of about 60% that of the medium (40), which is consistent with such a dilution. These studies were carried out in the presence of azide and so did not assess the impact of MPO activity. Neutrophil cytoplasm contains 80 mM chloride (64), and agonists such as tumor necrosis factor or phorbol 12-myristate 13-acetate cause rapid efflux through specific chloride channels (65, 66). If these channels were activated in the phagosomal membrane in response to particle ingestion, they could provide an ongoing supply of chloride for HOCl production. More information on chloride distribution in the neutrophil is needed to assess whether it limits HOCl production.

Reactions of Hydrogen Peroxide with Myeloperoxidase—The main reaction of H$_2$O$_2$ with MPO in the phagosome is with the native enzyme, and this combined with reactions of compound I with chloride or O$_2^-$ keeps the H$_2$O$_2$ concentration very low. Although H$_2$O$_2$ reacts with compounds I and II, these reactions are not favored in the phagosome model and can be eliminated without affecting any outcome (not shown). Only in the absence of chloride does the catalase activity (29) of MPO (mechanism C) become significant and contribute (along with mechanism B) to H$_2$O$_2$ removal. However, with chloride present, these reactions are very minor, and the model does not support the proposal (12, 67) that the prime role of MPO in the phagosome is as a catalase (Mechanism C, $r_3 + r_4$).

$$2\text{H}_2\text{O}_2 \rightarrow \text{O}_2^- + 2\text{H}_2\text{O}$$  
**MECHANISM C**

Reactions of Superoxide and the Turnover of Compound III—Modeling clearly shows that under phagosomal conditions, O$_2^-$ is broken down primarily by MPO; it converts most of the enzyme to compound III, and it competes with chloride to influence HOCl production. The extent to which O$_2^-$ affects MPO reactivity depends on the values assigned to the rate constants of its reactions with the redox intermediates of the enzyme. Experimental determination of these values is difficult because the complexity of the interactions does not allow each to be studied in isolation. Therefore some of the values obtained are estimates with upper and lower limits (Table 2). Standard conditions in the model used mid-range values, and we examined the sensitivity of the system to variations within these ranges.

Varying the rate of reaction of O$_2^-$ with ferric MPO within the measured range had little effect (not shown). Reaction with compound III ($r_{13}$) is crucial for MPO to remain functional. Otherwise all the enzyme becomes trapped as in compound III the O$_2^-$ concentration rises, and negligible HOCl is produced (Table 4). No alternatives to O$_2^-$ for turning over compound III are apparent. Dissociation ($r_{14}$) is too slow, and reactions with H$_2$O$_2$ or compound II cannot be substituted. However, a slow reaction with O$_2^-$ is sufficient; a 10-fold decrease in rate, although giving 99% compound III, still allows more than half-maximal HOCl production (Table 4). This rate is too low to fit our experimental data (Table 2) or the findings of Wever and co-workers (28), and it is therefore unlikely that turnover of compound III is a major constraint for HOCl production.

The model is insensitive to a 3-fold increase or a 10-fold decrease in the rate of reaction of O$_2^-$ with compound II ($r_{14}$), which is well beyond the measured limits (not shown). The reaction of O$_2^-$ with compound I ($r_{13}$) occurs in competition with chloride. Decreasing $k_{13}$ to the lower limit of measured values has little impact, but at the upper limit, competition starts to decrease the efficiency of HOCl production (Table 4). This becomes more marked at lower chloride concentrations, particularly if the MPO concentration is also low and the O$_2^-$ concentration consequently higher.

**Alternative Myeloperoxidase Substrates**—If other MPO substrates are present in the phagosome, they are oxidized and decrease the efficiency of HOCl production to an extent that depends on their reaction rate with compound I (tyrosine $\sim$ tryptophan $<\text{nitrite} < \text{serotonin}$; see Table 1) and on their concentration relative to chloride (Fig. 3A). These reactions only impact HOCl production if there is continuous replenish-

### TABLE 4

| Variations from standard conditions | [O$_2$] | [H$_2$O$_2$] | MPO redox state (% compound III) | HOCl production (% O$_2$ consumed) |
|-----------------------------------|--------|-------------|---------------------------------|-------------------------------------|
| Standard conditions               | 25     | 2.0         | 93                              | 89                                  |
| No reaction of O$_2^-$ with III   | 112    | 33          | 100                             | 1.1                                 |
| 2-Fold increase in $k_{12}$       | 14     | 1.1         | 88                              | 52                                  |
| 10-Fold decrease in $k_{12}$      | 90     | 12          | 99                              | 94                                  |
| 2.5-Fold decrease in $r_{13}$ ($O_2^-$ and compound I) | 25     | 2.0         | 94                              | 92                                  |
| 2-Fold increase in $r_{12}$ ($O_2^-$ and compound I) | 25     | 2.0         | 93                              | 85                                  |
| Plus 20 mM chloride               | 26     | 2.1         | 92                              | 61                                  |
| Plus 10-fold less MPO             | 92     | 13          | 90                              | 44                                  |
| Plus 20 mM chloride and 10-fold less MPO | 93     | 14          | 85                              | 20                                  |
impact. However, nitrite or tryptophan, which react slowly with compound II, give an initial phase of efficient HOCl production, until ~98% of the MPO is converted to compound II. Turnover of this form is slow, and HOCl production is strongly curtailed. The initial burst is shorter for nitrite because of its higher $k_{1,3}$, but tryptophan reacts more slowly with compound II and eventually causes greater inhibition. As shown for nitrite (Fig. 3B), these substrates have more impact with less MPO. Because the MPO is inhibited, only small amounts of substrate are consumed, and inhibitory concentrations could be sustained. This situation could arise in the phagosome if nitrite is present or if tyrosyl peptides that convert MPO to compound II (69) are produced during protein digestion. Without $O_2^\ast$ production, the outcome would be inhibition of MPO activity and HOCl production. As proposed previously (4), one important reason for neutrophils generating $O_2^\ast$ as against $H_2O_2$, directly could be to allow their MPO to remain functional in such situations.

### Impact of Superoxide Scavenging

**—Superoxide dismutase (SOD)** on the surface or in the periplasm of many bacteria is a well recognized virulence factor and endows resistance to host phagocytes (70, 71). Furthermore, attachment of SOD to *S. aureus* inhibits oxidative killing by neutrophils by ~30%. This implies a direct role of $O_2^\ast$ in killing. This could occur via direct reactions of the high steady state concentration of $O_2^\ast$ as noted above or, as proposed previously (36), by recycling compound II and maintaining MPO activity. The effect of SOD in the phagosome model depends on its concentration. At 1 $\mu M$ it decreases the $O_2^\ast$ concentration from 26 to 2 $\mu M$ but does not influence $O_2^\ast$ interacting with MPO. Higher SOD concentrations (above 40 $\mu M$) allow compound II to accumulate and nitrite or tryptophan to inhibit HOCl production. Maximal inhibition of killing of *S. aureus* was observed with $\sim 2 \times 10^{-19}$ mol of bound SOD per bacterium (36), which corresponds to about 200 $\mu M$ in the phagosome and is in the range where it could influence MPO activity.

**—HOCl** reacts rapidly with a wide variety of biological molecules. It also reacts with $O_2^\ast$ to generate hydroxyl radicals and with $H_2O_2$ to generate singlet oxygen (Table 5). These reactions have been proposed as the source of the small amounts of hydroxyl radicals detected in neutrophils (72) and in a proposed mechanism for ozone generation from singlet oxygen (17). If no proteins or other biological targets for HOCl are included in the phagosome model, hydroxyl radicals are major products. In contrast, extremely little singlet oxygen is generated because of the slower reaction rate with $H_2O_2$ and its low concentration. However, the phagosome is packed with granule proteins. Based on the estimated concentrations and rate constants in Table 5, these will be major targets. Modeling shows that cysteines and methionines are almost exclusively lost first, followed by amine groups and disulfides (Fig. 4). Only then are hydroxyl radicals formed. Direct reactions of HOCl with tyrosine or tryptophan residues are less favored. If there is ongoing protein release from either the neutrophil or the ingested bacterium, 1% of the estimated reactive protein groups in Table 5 is sufficient to maintain hydroxyl radical production at <1%.

![FIGURE 3. A, simulation of inhibition of HOCl production by peroxidase substrates in competition with chloride. The system was modeled with each peroxidase substrate at 50 $\mu M$ and set to remain constant during the simulation, with chloride constant at either 100 $\text{mm}$ (black bars) or 20 $\text{mm}$ (gray bars). B, simulation of the time course of HOCl production if the initial NADPH oxidase were hydrogen peroxide rather than superoxide. a, standard conditions of superoxide generation as shown in Table 3; b, standard conditions but with direct generation of hydrogen peroxide; c, 50 $\mu M$ nitrite with standard MPO concentration; d, 50 $\mu M$ nitrite with 20% MPO concentration; e, 50 $\mu M$ tryptophan with standard MPO concentration. Simulations with 50 $\mu M$ tyrosine or serotonin, with standard or 20% MPO concentration, gave curves indistinguishable from b.](image)
TABLE 5
Additional reactions and concentrations considered for examining the fate of HOCI

| Reaction                                                                 | \( k_{\text{obs}} \) M\(^{-1}\) s\(^{-1}\) | Ref. |
|--------------------------------------------------------------------------|---------------------------------|------|
| \( O_2^- + \text{HOCI} \rightarrow O_3^- + \text{Cl}^- + \text{OH} \) | \(7 \times 10^3\) | 87   |
| \( \text{H}_2\text{O}_2 + \text{HOCI} \rightarrow \text{O}_3^- + \text{H}_2\text{O} + \text{HCl} \) | \(1.5 \times 10^3\) | 88   |
| \( \text{HOCI} + \text{Pr-SH or Pr-met} \rightarrow \text{products} \) | \(3 \times 10^4\) | a    |
| \( \text{Pr-met} = 50 \text{ mM} \)                                      |                  |      |
| \( \text{HOCI} + \text{Pr-NH}_2 \rightarrow \text{Pr-NH}_2^- + \text{H}_2\text{O} [\text{Pr-NH}_2] \) | \(5 \times 10^3\) | b    |
| \( [\text{Pr-NH}_2] = 150 \text{ mM} \)                                  |                  |      |
| \( \text{HOCI} + \text{Pr-SS-Pr} \rightarrow \text{products} \) | \(1 \times 10^7\) | 90   |
| \( \text{Pr} = \text{Thr or Arg residues} \rightarrow \text{products} \) | 50               | 90   |
| \( [\text{Pr} + \text{Arg}] = 150 \text{ mM} \)                        |                  |      |

a Values of \( >10^7 \text{ M}^{-1}\text{s}^{-1}\) (89) with 60-fold faster rates than for taurine (92) and 3.8 and \(3.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\) for methionine and GSH, respectively (90), have been determined.

b Data are determined for taurine. Values obtained for other amino acids were similar or up to 4-fold less (92) or between \(5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\) for lysyl residues and \(1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\) for \(\alpha\) amino groups (90).

FIGURE 4. Reactions of hypochlorous acid in the phagosome model. Modeling was carried out under the standard conditions described in Tables 1 and 2 with the concentrations of targets and reactions in Table 5.

Based on the concentrations and reactivities in Table 5, the diffusion distance of HOCI can be calculated as 0.03 \(\mu\text{m}\) when it can react with protein thiols and methionines and 0.1 \(\mu\text{m}\) for amines. Both are less than the width of the phagosome. Therefore, HOCI should react close to its site of generation. As already noted, association of MPO with the bacterial surface could direct some HOCI to the bacterium, but most of the HOCI should be generated by unbound MPO throughout the phagosomal space. A majority of this will react with granule proteins before reaching the bacterium. Initial reactions with methionine and cysteine could be considered as scavenging, but further oxidation generates protein chloramines. These have some cytotoxic action, and break down to give aldehydes, ammonia, and probably ammonia chloramine, which are more bactericidal (73, 74). MPO-dependent aldehyde formation has been detected in neutrophil phagosomes (75), and as suggested in these early papers, aldehyde and chloramine products may be major effectors of the antimicrobial action of HOCI. The findings of Segal and co-workers (67) that granule proteins at phagosomal concentrations protect bacteria against HOCI cannot be taken as evidence that neutrophils do not use HOCI to kill, as they used only enough HOCI to oxidize a small fraction of the methionine and cysteine residues.

A striking inference from these simulations is that HOCI generated in the phagosome would oxidize susceptible groups on degranulated proteins within a short time. These proteins are required to degrade ingested material, and it is unlikely that oxidation could be sustained without inactivation. Granule enzymes, including MPO, are inactivated by HOCI (76), and the NADPH oxidase contains thiols that could be oxidant-sensitive (77). Oxidant production, therefore, may need to be transient. A possible scenario is that a burst of HOCI production initiates the process of microbial killing and digestion. Production could terminate as a result of intrinsic mechanisms that deactivate the NADPH oxidase complex (78) or through oxidative inactivation of the oxidase and/or MPO. HOCI formation could become limited because chloride was consumed, with MPO then catalyzing breakdown of the \(O_2^-\) and \(H_2O_2\) generated by continuing oxidase activity. Experimentally, oxygen uptake by individual phagocytosing neutrophils is short lived (35, 45); the oxidative burst is extended in MPO deficiency (1), and MPO becomes inactivated following phagocytosis (56, 79).

Conclusions—In the conventional view of oxidative killing, particle ingestion induces neutrophils to release \(O_2^-\) and granule constituents into the phagosome. \(O_2^-\) breaks down spontaneously to \(H_2O_2\), that reacts with MPO and nonlimiting chloride to generate HOCI, and this kills the bacterium directly. \(H_2O_2\) builds up to high concentrations, particularly in MPO deficiency where it may contribute to alternative killing mechanisms. Modeling has revealed several unexpected features that contradict this image. Equally important, it has highlighted where the full picture cannot be understood until critical information is obtained from further experimentation.

The model has provided robust predictions for aspects of oxidant behavior in the phagosome. \(O_2^-\) will be generated at a high flux, and most will react with the high concentration of MPO present. Turnover of compound III by \(O_2^-\) prevents all the MPO becoming trapped in this inactive form. By cycling between the native enzyme and compound III, MPO functions essentially as a superoxide dismutase and maintains a 6-fold lower \(O_2^-\) concentration than achievable through spontaneous dismutation. This mechanism operates regardless of the chloride concentration and may be important for removal of excess products of the respiratory burst. Reaction of \(O_2^-\) with compound II maintains MPO activity should reversible inhibitors be present.

\(O_2^-\) cannot escape from the phagosome and reaches \(\sim 25 \mu\text{m}\). At this high concentration, it may undergo reactions that are seldom considered. It would also be an excellent trap for NO to localize peroxynitrite production. On the other hand, consumption by MPO maintains \(H_2O_2\), in the low micromolar range. In MPO deficiency, \(O_2^-\) concentrations rise substantially but diffusion out of the phagosome allows only modest \(H_2O_2\) accumulation. Some \(H_2O_2\) will enter the bacterium but directly bactericidal concentrations would not be reached.

It is possible for the majority of the oxygen consumed in the phagosome to be converted to HOCI, but the efficiency of this process is sensitive to variations in parameters about which we have limited knowledge. Low chloride, particularly...
in combination with a low MPO concentration, decreases HOCl production, and ongoing production requires replenishment of chloride consumed through HOCl escaping or undergoing chlorination reactions. Phagosomal chloride concentrations and transport mechanisms stand out as priorities for further investigation. More direct information on the efficiency of MPO release and the extent to which it becomes inactive is also needed.

The model has provided insight into likely reactions of HOCl in the phagosome environment. Some should be directed toward the ingested bacterium by MPO bound to the surface, in the phagosome environment. Some should be directed in the phagosome. Most of this HOCl should react with proteins released during degranulation and not reach the bacterium. Although it is possible that these reactions could inhibit microbicidal activity, it is more likely that they contribute via the generation of protein chloramines and secondary products such as aldehydes and ammonia chloramine. Hydroxyl radicals should become significant only when reactive groups (thiols, methionines, amines, and disulfides) are consumed, and singlet oxygen formation is not favorable. Ongoing production of HOCl should cause extensive oxidation and probable inactivation of released granule proteins. This is counterintuitive to the requirement for active degradative enzymes and raises the possibility that O2- or HOCl production in each phagosome is short lived and self-limiting. Experiments are now needed to test these predictions directly.

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