Oxidation of *Escherichia coli* Sulphydryl Components by the Peroxidase-Hydrogen Peroxide-Iodide Antimicrobial System

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Received for publication 9 February 1978

The chemical modification of bacterial components was studied following incubation of *Escherichia coli* with the peroxidase-hydrogen peroxide (H$_2$O$_2$)-iodide (I$^-$) antimicrobial system or with iodine (I$_2$). The oxidation of cell sulphydryls and the iodination of cell components were measured. Both the peroxidase system and I$_2$ oxidized sulphydryls. When the I$^-$ concentration in the peroxidase system was greater than 100 μM, the peroxidase system and I$_2$ were equivalent. That is, sulphydryl oxidation or killing per mole of H$_2$O$_2$ equaled that per mole of I$_2$. These results were consistent with peroxidase-catalyzed oxidation of I$^-$ to yield 1 mol of I$_2$ per mol of H$_2$O$_2$. Sulphydryls were oxidized to yield sulfenic acids and free I$^-$. With I$^-$ concentrations in the range of 10 to 100 μM, the amount of sulphydryls oxidized by the peroxidase system could exceed the amount of I$^-$. Because the oxidation of sulphydryls to sulfenic acids did not consume I$^-$, one I$^-$ ion could participate in the oxidation of many sulphydryls. With I$^-$ concentrations lower than 10 μM, complete oxidation of sulphydryls was not obtained. Incorporation of I$^-$ into iodinated derivatives of bacterial components partly depleted the system of I$^-$ and limited the formation of I$_2$. These results indicated that antimicrobial activity was due to peroxidase-catalyzed oxidation of I$^-$ to I$_2$, followed by I$_2$ oxidation of cell components. There was a direct relationship between sulphydryl oxidation and antimicrobial action. Although iodination of bacterial components accompanied sulphydryl oxidation, the amount of I$^-$ incorporation was not directly related to antimicrobial action. Also, incorporation of I$^-$ interfered with antimicrobial action at low I$^-$ concentrations.

In the preceding article (13), we proposed that the antimicrobial activity of the peroxidase-
H$_2$O$_2$-I$^-$ system was due to the oxidation of I$^-$ to I$_2$, followed by oxidation of cell components by I$_2$. Oxidation of cell components resulted in reduction of I$_2$ back to I$^-$, so that I$^-$ was not consumed. Therefore, one I$^-$ ion could participate in oxidation of many cell components.

$$\text{H}_2\text{O}_2 + 2 \text{H}^+ \xrightarrow{\text{peroxidase}} 2 \text{I}^- \xrightarrow{\text{oxidized cell components}} 2 \text{H}_2\text{O} + \text{I}_2$$

This reaction sequence implies that antimicrobial action and the oxidation of cell components are proportional to H$_2$O$_2$ and independent of I$^-$ concentration. However, two limitations to antimicrobial action were observed at low I$^-$ concentrations. Because *Escherichia coli* cells destroy H$_2$O$_2$, the bacteria competed effectively for H$_2$O$_2$ when the rate of oxidation of I$^-$ was low. This limitation could be overcome by increasing the peroxidase concentration or by adding more H$_2$O$_2$. An additional limitation was observed that could not be overcome by adding more peroxidase or H$_2$O$_2$. It was proposed that incorporation of I$^-$ into iodinated derivatives of bacterial components could deplete the system of I$^-$ and limit I$_2$ formation. To obtain direct chemical confirmation for these proposals, we have measured the oxidation of *E. coli* sulphydryl components and the incorporation of I$^-$ into bacterial components.

**MATERIALS AND METHODS**

The sources of enzymes and the methods for growth and harvest of *E. coli* incubation with I$_2$ or the peroxidase systems, and determination of inhibition of respiration or loss of viability were as described in the preceding article (13). *E. coli* cells were treated with ethylenediaminetetraacetic acid (EDTA) by the method of Leive (8) to yield EDTA-extracted cells and subjected to the procedure of Neu and Heppel (10) to yield osmotically shocked cells. Radioactive I$^-$ as Na[I]$^{125}$ (carrier-free) was obtained from Schwartz/Mann (Orangeburg, N.Y.) and was diluted to 5 mCi/ml with 1 μM NaI containing 0.1 mM sodium thiosulfate. [14C]thiourea (55.7 Ci/mol) was obtained.
from Amersham Corp. (Arlington Heights, Ill.), and 5,5'-dithiobis(2-nitrobenzoic acid) was obtained from Sigma Chemical Corp. (St. Louis, Mo.).

**Sulphydryl determination.** Sulphydryl content was measured by the reaction of the disulfide compound 5,5'-dithiobis(2-nitrobenzoic acid) with 1 mol of sulphydryls to yield 1 mol of the mixed disulfide and 1 mol of thio-2-nitrobenzoic acid (4). Two-milliliter portions containing 6 x 10⁸ cells per ml were diluted with 4 ml of a solution containing 0.1 M tris(hydroxymethyl)aminomethane, 10 mM EDTA, and hydrochloric acid to adjust to pH 8.0. Portions of 0.1 ml of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M potassium phosphate buffer, pH 7.0, and 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate were added. The mixtures were incubated at 37°C for 1 h, cooled to 0 to 5°C, and centrifuged at 18,000 x g for 10 min. Absorbance of the supernatants was measured at 412 nm. A molar extinction coefficient of 13,600 was assumed for 5-thio-2-nitrobenzoic acid (4).

**Sulfenyl determination.** Sulfenyl derivatives were determined by their reaction with [¹⁴C]thiourea to yield the radioactive mixed-disulfide derivative (11). Portions of 0.1 ml of cell suspensions were incubated at 37°C for 15 min with 0.9 mM [¹⁴C]thiourea and 1% (wt/vol) sodium dodecyl sulfate, and then 1 ml of cold 10% (wt/vol) trichloroacetic acid was added. The mixtures were filtered through nitrocellulose filters (Millipore Corp., Bedford, Mass.), and the filters were washed with 5% trichloroacetic acid. The filters were dissolved in scintillation fluid (2), and the radioactivity was determined in a liquid scintillation spectrometer.

**Iodination.** Incorporation of [¹²⁵I] into bacterial components was calculated from the amount of radioactivity removed from the suspension upon sedimentation of the bacteria by centrifugation. Reaction mixtures contained 25 µCi of [¹²⁵I] per ml and varying amounts of nonradioactive I⁻. The reaction mixtures were centrifuged for 10 min at 0 to 5°C, and then portions of the supernatant were taken and the radioactivity was determined.

### RESULTS

**Oxidation of cell sulphydryls.** Figure 1 shows the oxidation of E. coli cell sulphydryls by I₂. Loss of sulphydryls was proportional to I₂, with about 0.5 to 1 mol of sulphydryls oxidized per mol of I₂. Slightly smaller amounts of I₂ were required for complete loss of sulphydryls at 0 to 5°C than at 25°C. Also shown are results obtained with EDTA-extracted cells and osmotically shocked cells. These cells were partly depleted of sulphydryl components and had increased susceptibility to bactericidal action of the peroxidase system (15). Smaller amounts of I₂ were required for complete oxidation of sulphydryls of these sulphydryl-depleted cells. In other experiments, it was determined that the amount of I₂ required for complete oxidation of sulphydryls of each cell preparation was equal to the amount required for complete loss of viability.

Figure 2 compares sulphydryl oxidation with bactericidal action, measured after incubation of cells with lactoperoxidase, H₂O₂, and two concentrations of I⁻. With 1 mM NaI, loss of sulphydryls and loss of viability were complete. The amount of H₂O₂ required for complete oxidation of sulphydryls was equal to the amount required for complete loss of viability. Also, this amount of H₂O₂ was equal to the amount of I₂ required for complete sulphydryl oxidation and complete loss of viability (compare with Fig. 1). These

![Fig. 1. Oxidation of E. coli sulphydryls by I₂. The indicated amounts of I₂ were added to suspensions of intact cells (6 x 10⁸ cells per ml) at 25°C (○) or 0 to 5°C (●), of EDTA-extracted cells at 25°C (□), or of osmotically shocked cells at 25°C (■).](http://aac.asm.org/)

![Fig. 2. Comparison of sulphydryl oxidation with loss of viability. Intact E. coli (6 x 10⁸ cells per ml) were incubated at 0 to 5°C with 0.1 µM lactoperoxidase, the indicated amounts of H₂O₂, and either 1 mM NaI (○, □) or 10 µM NaI. Sulphydryl content (○, □) and viability (○, □) were measured.](http://aac.asm.org/)
results were consistent with oxidation of $I^-$ to yield 1 mol of $I_2$ per mol of $H_2O_2$.

With 10 $\mu$M NaI, the concentration of oxidized sulphydryls (52 $\mu$M) exceeded the concentration of $I^-$. Therefore, each $I^-$ ion participated in the oxidation of many sulphydryls. However, the oxidation of sulphydryls was not complete, and the amount of oxidized sulphydryls reached a plateau level. Similarly, bactericidal action reached a plateau level. No increase in sulphydryl oxidation or bactericidal action was obtained with further additions of lactoperoxidase or $H_2O_2$ or both.

**Oxidation of sulphydryls to sulfenic acids.**

Figure 3 shows that *E. coli* sulphydryl components were oxidized to sulfenyl derivatives. In other experiments, incorporation of iodine atoms into bacterial components was observed, but the incorporated iodine atoms were not released upon incubation with thiourea or the sulphydryl compound, dithiothreitol. Therefore, the sulfenyl derivatives formed in these experiments were sulfenic acids (R-S-OH) rather than sulfenyl iodide derivatives (R-S-I).

The yield of sulfenic acids reached a maximum at 120 to 150 $\mu$M $I_2$. This amount of $I_2$ was about equal to the amount required for complete sulphydryl oxidation and complete killing. The yield of sulfenic acids was lower than the amount of oxidized sulphydryls. This result may indicate that a portion of the sulphydryls was oxidized to other forms or that a portion of the sulfenic acids could not be measured by the method used, which required that the radioactive mixed-disulfide derivative be acid precipitable. When the amount of $I_2$ exceeded the amount required for complete oxidation of sulphydryls, the amount of sulfenic acids decreased. This loss of sulfenic acids presumably represents $I_2$ oxidation of sulfenic acids to sulfonic acids or other forms.

Figure 3 also shows the oxidation of *E. coli* sulphydryls to sulfenic acids by the lactoperoxidase system. With 1 mM NaI, results obtained with the peroxidase system were similar to those obtained with $I_2$. With 10 $\mu$M NaI, a lower yield of sulfenic acids were obtained. The yield did not increase or decrease as more $H_2O_2$ was added. No oxidation of sulphydryls or formation of sulfenyl derivatives was obtained in the absence of either lactoperoxidase or $H_2O_2$.

**Antimicrobial action, sulphydryl oxidation, and incorporation of $I^-$.**

Figure 4 compares inhibition of respiration, oxidation of sulphydryls, and incorporation of $I^-$ by the lactoperoxidase system. When the initial NaI concentration was 1 $\mu$M or lower, there was no inhibition and no oxidation of sulphydryls, regardless of lactoperoxidase or $H_2O_2$ concentrations. Nevertheless, about 30% of the $I^-$ present was incorporated into the bacteria. In the absence of either lactoperoxidase or $H_2O_2$, less than 2% of the $I^-$ was absorbed or accumulated by the bacteria at any $I^-$ concentration.

With initial NaI concentrations of 1 $\mu$M to 10 $\mu$M, the bacteria were killed when oxidized sulphydryls were present.

**Fig. 3. Oxidation of sulphydryls to sulfenic acids.**

Intact *E. coli* (6 x 10$^7$ cells per ml) were incubated at 0 to 5°C with the indicated amounts of $I_2$ (○) or with 0.1 $\mu$M lactoperoxidase, the indicated amounts of $H_2O_2$, and 2 mM NaI (△) NaI.

**Fig. 4. Comparison of inhibition of respiration, sulphydryl oxidation, and $I^-$ incorporation.**

Intact *E. coli* (6 x 10$^7$ cells per ml) were incubated at 0 to 5°C with 0.1 $\mu$M lactoperoxidase, 300 $\mu$M $H_2O_2$, and the indicated concentrations of NaI. Inhibition of respiration (○), sulphydryl oxidation (■), and incorporation of $^{125}I^-$ (△) were measured.
μM, inhibition of respiration and sulfhydryl oxidation were not complete. Adding more lactoperoxidase or H₂O₂ did not increase inhibition or sulfhydryl oxidation. In this range of I⁻ concentrations, the amount of oxidized sulfhydryls exceeded the amount of I⁻ present, indicating that each I⁻ ion participated in oxidation of many sulfhydryls. There appeared to be a direct relationship between oxidation of sulfhydryls and inhibition of respiration. In contrast, there was no correlation between the percent inhibition and percent incorporation of I⁻. At each I⁻ concentration, about 30 to 40% of the I⁻ present was incorporated into the bacteria. Also, the amount of oxidized sulfhydryls greatly exceeded the amount of incorporated I⁻.

Inhibition of respiration and oxidation of sulfhydryls were complete at an initial NaI concentration of about 30 μM. Nevertheless, the amount of incorporated I⁻ increased dramatically at higher I⁻ concentrations. For example, with 0.3 mM and 1 mM NaI, the amount of incorporated I⁻ was 84 μM and 190 μM, respectively. At these higher I⁻ concentrations, inhibition was complete regardless of the amount of incorporation, so that it was possible to obtain completely inhibited cells with either a small or large amount of incorporated I⁻. In other experiments, incorporation also increased when H₂O₂ exceeded the amount required for complete killing. Therefore, there was no correlation between antimicrobial action and the amount of incorporated I⁻.

Similar results were obtained when myeloperoxidase or horseradish peroxidase was used in place of lactoperoxidase. Also, similar results were obtained at 0 to 5°C or 37°C. Lowering the pH increased bactericidal action, sulfhydryl oxidation, and incorporation of I⁻ with all three peroxidases. The amount of oxidized sulfhydryls could exceed the amount of I⁻ regardless of pH or temperature.

**DISCUSSION**

A consistent, direct relationship was observed between antimicrobial action and oxidation of cell sulfhydryls. When oxidation of sulfhydryls was not complete, loss of viability was not complete. Cells partly depleted of sulfhydryl compounds were more susceptible to antimicrobial action. These quantitative correlations, as well as the ability of exogenous sulfhydryl compounds to partly reverse antimicrobial action (15), indicate an important role for sulfhydryl oxidation in peroxidase antimicrobial action.

Oxidation of cell sulfhydryls by the peroxidase-H₂O₂-I⁻ antimicrobial system was consistent with the equations:

\[ 2 \text{I}^- + \text{H}_2\text{O}_2 + 2 \text{H}^+ \xrightarrow{\text{peroxidase}} \text{I}_2 + 2 \text{H}_2\text{O} \]

\[ \text{I}_2 + \text{R} = \text{SH} + \text{H}_2\text{O} \rightarrow 2 \text{I}^- + \text{R} = \text{SH} + \text{H}_2\text{O} \]

Oxidation of I⁻ to I₂ was followed by I₂ oxidation of sulfhydryls to sulfenic acids and the reduction of I₂ to I⁻. The net result of these reactions was the peroxidase-catalyzed oxidation of cell sulfhydryls mediated by I₂, with I⁻ acting as a cofactor.

\[ \text{H}_2\text{O}_2 + \text{R} = \text{SH} \xrightarrow{\text{peroxidase}, \text{I}^-} \text{R} = \text{SOH} + \text{H}_2\text{O} \]

The amount of oxidized sulfhydryls was proportional to H₂O₂ and could exceed the amount of I⁻.

Oxidation of sulfhydryls to sulfenic acids may result from sulfinyl iodide formation, followed by hydrolysis (13).

\[ \text{I}_2 + \text{R} = \text{SH} \rightarrow \text{R} = \text{S} + \text{I}^- + \text{H}^+ \]

\[ \text{R} = \text{SH} + \text{H}_2\text{O} \rightarrow \text{R} = \text{SOH} + \text{I}^- + \text{H}^+ \]

Iodine atoms did not remain incorporated in the form of sulfinyl iodide derivatives. Iodinated tyrosine residues accounted for most of the incorporation of I⁻ (E. L. Thomas and T. M. Aune, unpublished results). Lowering the temperature mildly increased both antimicrobial action and sulfhydryl oxidation by I₂. Low temperature may favor I₂ oxidation of protein sulfhydryls over iodination of aromatic amino acid residues (3).

The ability of polymorphonuclear leukocytes to catalyze incorporation of I⁻ into an acid-precipitable form has been used as a measure of their antimicrobial potential (5, 7, 12). The high specific activity of radioactive I⁻ makes incorporation a very sensitive measure of peroxidase activity. Nevertheless, in the results presented here, incorporation of I⁻ accompanied peroxidase antimicrobial action but did not provide a direct measure of antimicrobial action. At low I⁻ concentrations, incorporation was obtained in the absence of killing. At high I⁻ concentrations, incorporation increased most dramatically when the amounts of H₂O₂ and I⁻ were higher than the amounts required for complete killing. These results support the conclusion that there may be no cause-effect relationship between iodination
and antimicrobial action within polymorphonuclear leukocytes (7).

Rather than contributing to antimicrobial action, incorporation of I− may interfere by depleting the system of I−. At low I− concentrations, depletion of I− appeared to limit I2 formation. Although I− was not completely depleted, the incorporation of I− as measured in this study may underestimate the depletion of I−. Transient accumulation of I2 or sulfenyl iodide derivatives could deplete I− completely. Incorporation of I− was measured after continued incubation and centrifugation, and the I− concentration may have been partly restored during these steps. A transient depletion of I− would halt I2 formation, and the excess H2O2 would be destroyed by the bacteria.

Results presented here do not indicate that sulphydryl oxidation alone accounts for antimicrobial action. Other biological components can be iodinated to yield unstable derivatives that decompose to yield I− and the oxidized component. For example, tryptophan residues are oxidized by I2 to yield an oxindole derivative and I− (1). In general, the reaction of I2 with protein sulphydryls is more rapid than reactions with other protein components and may go to completion before modification of other components becomes significant (3, 14). However, the arrangement of cell components may favor reaction with less-reactive components near the cell surface. Also, lactoperoxidase-catalyzed oxidation of I− can result in preferential iodination of exposed tyrosine residues at low I− concentrations (9). Therefore, certain microorganisms could have increased susceptibility to peroxidase antimicrobial action if essential components are exposed or otherwise unusually reactive.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant DE 04235 from the National Institute of Dental Research and Research Center grant CA 08480, Multidisciplinary Training grant CA 05176, and Cancer Center Support (CORE) grant CA 21765 from the National Cancer Institute, and by ALSAC.

We thank M. Morrison for helpful discussions and Kate Pera for technical assistance.

LITERATURE CITED


