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

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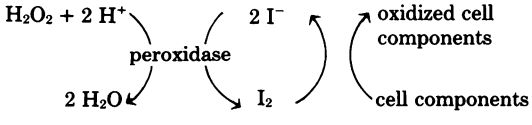
The chemical modification of bacterial components was studied following incubation of *Escherichia coli* with the peroxidase-hydrogen peroxide (H_2O_2)-iodide (I^-) antimicrobial system or with iodine (I_2). The oxidation of cell sulfhydryls and the iodination of cell components were measured. Both the peroxidase system and I_2 oxidized sulfhydryls. When the I^- concentration in the peroxidase system was greater than 100 μM, the peroxidase system and I_2 were equivalent. That is, sulfhydryl oxidation or killing per mole of H_2O_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_2O_2. Sulfhydryls were oxidized to yield sulfenic acids and free I^-. With I^- concentrations in the range of 10 to 100 μM, the amount of sulfhydryls oxidized by the peroxidase system could exceed the amount of I^-. Because the oxidation of sulfhydryls to sulfenic acids did not consume I^-, one I^- ion could participate in the oxidation of many sulfhydryls. With I^- concentrations lower than 10 μM, complete oxidation of sulfhydryls 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 sulfhydryl oxidation and antimicrobial action. Although iodination of bacterial components accompanied sulfhydryl 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_2O_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.



This reaction sequence implies that antimicrobial action and the oxidation of cell components are proportional to H_2O_2 and independent of I^- concentration. However, two limitations to antimicrobial action were observed at low I^- concentrations. Because *Escherichia coli* cells destroy H_2O_2, the bacteria competed effectively for H_2O_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_2O_2. An additional limitation was observed that could not be overcome by adding more peroxidase or H_2O_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* sulfhydryl 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 $Na^{125}I$ (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. [^{14}C]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.).

Sulfhydryl determination. Sulfhydryl content was measured by the reaction of the disulfide compound 5,5'-dithiobis(2-nitrobenzoic acid) with 1 mol of sulfhydryls to yield 1 mol of the mixed disulfide and 1 mol of 5-thio-2-nitrobenzoic acid (4). Two-milliliter portions containing 6×10^8 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 × *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 [^{14}C]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 [^{14}C]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 $^{125}I^-$ into bacterial components was calculated from the amount of radioactivity removed from the suspension upon sedimenting the bacteria by centrifugation. Reaction mixtures contained 25 μCi of $^{125}I^-$ 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 sulfhydryls. Figure 1 shows the oxidation of *E. coli* cell sulfhydryls by I_2. Loss of sulfhydryls was proportional to I_2, with about 0.5 to 1 mol of sulfhydryls oxidized per mol of I_2. Slightly smaller amounts of I_2 were required for complete loss of sulfhydryls at 0 to 5° than at 25°C. Also shown are results obtained with EDTA-extracted cells and osmotically shocked cells. These cells were partly depleted of sulfhydryl components and had increased susceptibility to bactericidal action of the peroxidase system (15). Smaller amounts of I_2 were required for complete oxidation of sulfhydryls of these sulfhydryl-depleted cells. In other experiments, it was determined that the amount of I_2 required for complete oxidation of sulfhydryls of each cell preparation was equal to the amount required for complete loss of viability.

Figure 2 compares sulfhydryl oxidation with bactericidal action, measured after incubation of cells with lactoperoxidase, H_2O_2, and two concentrations of I^-. With 1 mM NaI, loss of sulfhydryls and loss of viability was complete. The amount of H_2O_2 required for complete oxidation of sulfhydryls was equal to the amount required for complete loss of viability. Also, this amount of H_2O_2 was equal to the amount of I_2 required for complete sulfhydryl oxidation and complete loss of viability (compare with Fig. 1). These

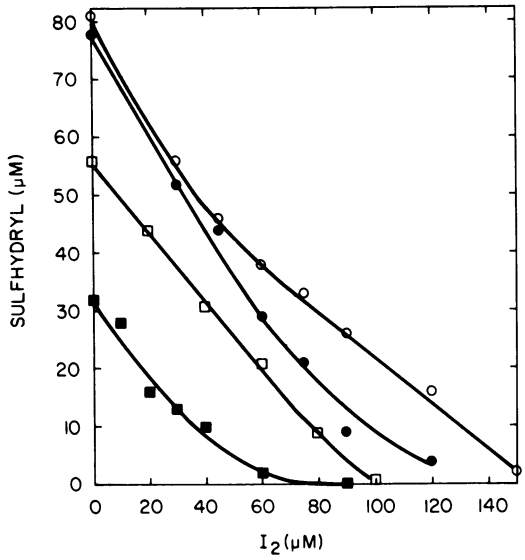


FIG. 1. *Oxidation of E. coli sulfhydryls by I_2. The indicated amounts of I_2 were added to suspensions of intact cells (6×10^8 cells per ml) at 25°C (○) or 0 to 5°C (●), of EDTA-extracted cells at 25°C (□), or of osmotically shocked cells at 25°C (■).*

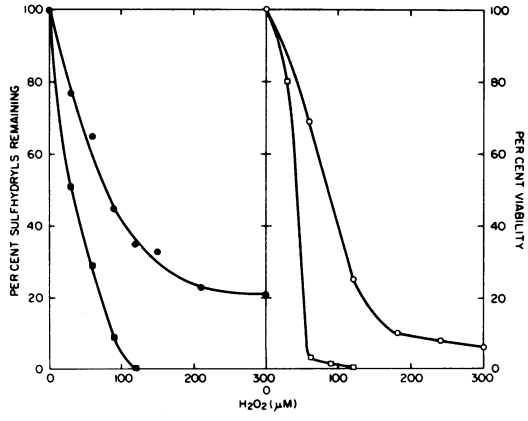


FIG. 2. *Comparison of sulfhydryl oxidation with loss of viability. Intact E. coli (6×10^8 cells per ml) were incubated at 0 to 5°C with 0.1 μM lactoperoxidase, the indicated amounts of H_2O_2, and either 1 mM NaI (●, ○) or 10 μM (■, □) NaI. Sulfhydryl content (●, ■) and viability (○, □) were measured.*

results were consistent with oxidation of I^- to yield 1 mol of I_2 per mol of H_2O_2.

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

Oxidation of sulfhydryls to sulfenic acids. Figure 3 shows that *E. coli* sulfhydryl 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 sulfhydryl 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 μM I_2. This amount of I_2 was about equal to the amount required for complete sulfhydryl oxidation and complete killing. The yield of sulfenic acids was lower than the amount of oxidized sulfhydryls. This result may indicate that a portion of the sulfhydryls 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 sulfhydryls, 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* sulfhydryls 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 μM 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 sulfhydryls or formation of sulfenyl derivatives was obtained in the absence of either lactoperoxidase or H_2O_2.

Antimicrobial action, sulfhydryl oxidation, and incorporation of I^-. Figure 4 compares inhibition of respiration, oxidation of sulfhydryls, and incorporation of I^- by the lactoperoxidase system. When the initial NaI concentration was 1 μM or lower, there was no inhibition and no oxidation of sulfhydryls, 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 μM to 10

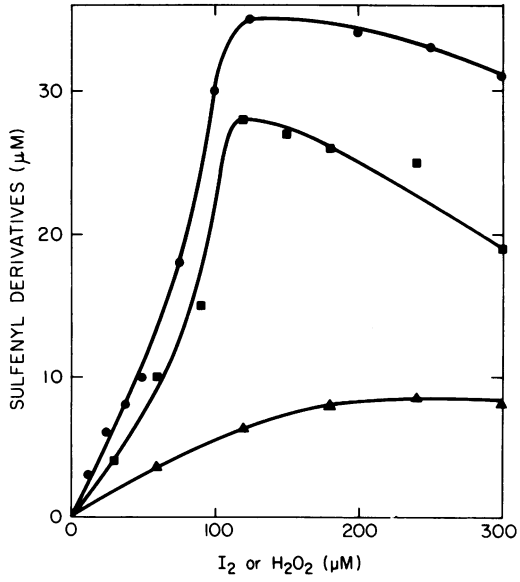


FIG. 3. *Oxidation of sulfhydryls to sulfenic acids. Intact E. coli (6 × 10⁸ cells per ml) were incubated at 0 to 5°C with the indicated amounts of I_2 (●) or with 0.1 μM lactoperoxidase, the indicated amounts of H_2O_2, and 1 mM (■) or 10 μM (▲) NaI.*

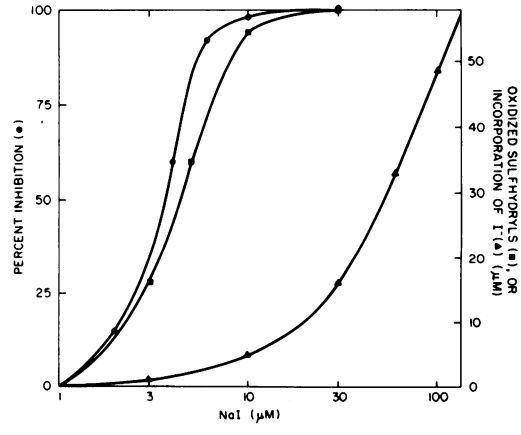


FIG. 4. *Comparison of inhibition of respiration, sulfhydryl oxidation, and I^- incorporation. Intact E. coli (6 × 10⁸ cells per ml) were incubated at 0 to 5°C with 0.1 μM lactoperoxidase, 300 μM H_2O_2, and the indicated concentrations of NaI. Inhibition of respiration (●), sulfhydryl oxidation (■), and incorporation of $^{125}I^-$ (▲) were measured.*

μM, inhibition of respiration and sulfhydryl oxidation were not complete. Adding more lactoperoxidase or H_2O_2 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 H_2O_2 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-H_2O_2-I^- antimicrobial system was consistent with the equations:

$$2\,I^- + H_2O_2 + 2\,H^+ \xrightarrow{\text{peroxidase}} I_2 + 2\,H_2O$$

$$I_2 + R{=}SH + H_2O \rightarrow 2\,I^- + R{=}S{=}OH + 2\,H^+$$

Oxidation of I^- to I_2 was followed by I_2 oxidation of sulfhydryls to sulfenic acids and the reduction of I_2 to I^-. The net result of these reactions was the peroxidase-catalyzed oxidation of cell sulfhydryls mediated by I_2, with I^- acting as a cofactor.

$$H_2O_2 + R{=}SH \xrightarrow{\text{peroxidase, } I^-} R{=}SOH + H_2O$$

The amount of oxidized sulfhydryls was proportional to H_2O_2 and could exceed the amount of I^-.

Oxidation of sulfhydryls has previously been proposed as the bactericidal mechanism of I_2 (6). Sulfhydryls are essential to the activity of many enzymes, bacterial electron transport, and membrane transport systems. However, partial loss of sulfhydryls does not necessarily result in loss of viability. For example, osmotic shock causes a significant loss of sulfhydryl components without loss of viability (15). It is likely that only a portion of cell sulfhydryls, such as protein sulfhydryls, are essential to viability, but that all cell sulfhydryls are oxidized by I_2.

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

$$I_2 + R{=}SH \rightarrow R{=}S{=}I + I^- + H^+$$

$$R{=}S{=}I + H_2O \rightarrow R{=}S{=}OH + I^- + H^+$$

Iodine atoms did not remain incorporated in the form of sulfenyl 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 slightly increased both antimicrobial action and sulfhydryl oxidation by I_2. Low temperature may favor I_2 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 H_2O_2 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 I_2 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 I_2 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 I_2 formation, and the excess H_2O_2 would be destroyed by the bacteria.

Results presented here do not indicate that sulfhydryl 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 I_2 to yield an oxindole derivative and I^- (1). In general, the reaction of I_2 with protein sulfhydryls 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

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