L-Cysteine Oxidase Activity in the Membrane of Neisseria meningitidis

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Among the L-amino acids, only L-cysteine was oxidized by isolated washed membranes of group B Neisseria meningitidis SD1C. The cysteine oxidase in the membrane obeyed Michaelis-Menten kinetics and was heat labile. The pH optimum for the maximum velocity of the reaction was 9.8. Specific activity of the enzyme increased as cell growth progressed through the exponential phase toward the stationary phase of growth. The enzyme activity was markedly sensitive to inhibition by metal chelators, but was resistant to inhibitors of terminal oxidases with the exception of cyanide. All known cytochromes in the membrane, except b₆₃₃, were reduced with L-cysteine. The additive nature of L-cysteine oxidase and succinate oxidase activities suggests that an unidentified oxidase is involved in the oxidation of cysteine.

The intermediary metabolism of sulfur-containing amino acids, and cysteine and cystine in particular, is complex, and many aspects still remain obscure. In animal tissues the oxidation of L-cysteine is enzymatically catalyzed by an oxygenase which forms cysteine sulfinic acid, the key intermediate in cysteine metabolism (17, 23, 25, 29). In bacterial systems, however, very little is known about cysteine metabolism. Recent work on Proteus and Corynebacterium has provided evidence for both soluble and membrane-bound L-amino acid oxidases which catalyze the oxidative deamination of a wide spectrum of L-amino acids, including L-cysteine (5, 6, 9, 22). Tauber and Russell (28) have shown the presence in neisseriae of cysteine oxidase which is involved in the conversion of cysteine to cystine. Although the function of this enzyme in the amino acid metabolism of the genus Neisseria is not understood (20), it has been established that all strains of gonococci and some strains of meningococci have an absolute requirement for cysteine for growth (4). This is in contrast to the apparent inhibitory effect of cysteine on most other bacteria grown aerobically (3).

Our present study reveals the enzymatic nature of the cysteine reaction and demonstrates the involvement of the respiratory chain and possibly an alternate oxidase in the process.

MATERIALS AND METHODS
Organism. The group B Neisseria meningitidis strain, SD1C, was obtained from the Neisseria Repository, Naval Medical Research Unit no. 1, University of California, Berkeley. Maintenance of both stock and working cultures was described previously (7), as were routine examinations for strain purity. This strain readily dissociates into rough and smooth colonial types (8). Only the smooth strain was used in this study.

Cell growth and fractionation. The growth of cells was described previously (33). Cells were harvested at 10,000 × g, 20 min (4°C). Cells in cold 0.05 M Tris (Trizma Base, Sigma Chemical Co., St. Louis, Mo.) plus 0.05 M MgCl₂ (pH 7.5) were broken by high-pressure (12,000 to 18,000 lb/in²) extrusion from a precooled French pressure cell. Whole cells and large debris were removed from the remaining cell-free extract by centrifuging at 19,000 × g for 20 min. The supernatant extract was centrifuged at 180,000 × g (mean force) for 3 h in a Beckman L-65 ultracentrifuge with a 60Ti rotor to separate the particulate membranous fraction (pellet) from the supernatant one. The particulate membranous fraction was twice suspended in and centrifuged (180,000 × g, 2 h) from 0.06 M potassium phosphate or Tris buffer (pH 7.5).

Oxidase assay. Oxygen consumption was measured by a Rank polarographic oxygen cell (Rank Bros., Bottisham, Cambridge, England). All assays were carried out at 37°C in an air-saturated buffer. Assays carried out at pH 7.5 were in 0.05 M potassium phosphate buffer or 0.05 M Tris buffer, whereas universal buffer (2) was used for assays at pH 9.8 and for studies of the pH optimum. The final concentration of L-cysteine in a typical run was 25 mM. The resulting activities were corrected for the negligible endogenous respiration and autooxidation of L-cysteine. Inhibitors, when applied, were added 1 min before the addition of the substrate. Any effects of inhibitors on the autooxidation of L-cysteine were taken into account as well. Ascorbate plus N,N,N',N'-tetramethyl-p-phenylene-diamine (TMPD) oxidase activity was measured as described in detail elsewhere (32). All substrates and inhibitors were freshly prepared. Water-insoluble inhibitors were dissolved in dimethyl formamide, which was shown to have no adverse effect on the assay.
system during the time course of the assay. Oxidase activities were expressed as nanomoles of O₂ consumed per minute per milligram of protein at 37°C at a specific pH.

**Difference spectra.** Difference spectra (650 to 400 nm) were carried out at room temperature with a Perkin-Elmer Ultraviolet-Visible Double-beam Spectrophotometer, model 555. Reduced-minus-oxidized difference spectra and reduced-plus-CO-minus-reduced difference spectra were obtained in anaerobic cuvettes as previously described (33), using L-cysteine as the reducing agent. Other inhibitors, when used, were added in small amounts until no further changes were observed in the spectra.

Miscellaneous assays. An amino acid analysis was carried out by using a Beckman 120C Amino Acid Analyser (Beckman Instruments, Inc., Montreal, Quebec). Protein was estimated by the method of Lowry et al. (18) with bovine serum albumin as the standard.

**Chemicals.** All salts, including potassium cyanide, were reagent grade (Fisher Scientific Co., Fairlawn, N.J.). Salicylhydroxamate was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). TMPD was purchased from Eastman Organic Chemicals (Rochester, N.Y.). All other reagents were obtained from Sigma Chemical Co.

**RESULTS**

Cell-free extracts of *N. meningitidis* consumed oxygen on L-cysteine (Table 1). When cell-free extracts were fractionated into membranous and supernatant fractions, nearly all activity was associated with the membranous fraction of the cell. In contrast to the L-amino acid oxidases reported in other bacteria (5, 28), the one in meningococcal membranes exhibited a relatively limited specificity. Of the amino acids tested, only L-cysteine, d-cysteine, and cysteamine produced detectable oxygen consumption (Table 2).

The specific activity (Table 3) of the L-cysteine oxidase increased approximately fourfold over the course of growth from the early exponential phase to the stationary phase of growth (Fig. 1). There was an initial rapid increase in activity as cells entered the midexponential growth phase. Such activity remained relatively constant throughout the remainder of exponential growth, but rose sharply as cells entered the stationary phase. This pattern could reflect the decreasing dissolved oxygen in the medium as meningococci progress toward the stationary phase (1). In most experiments cells were grown to the stationary phase of growth to take advantage of the higher specific activity in these preparations.

There was the possibility that the oxidation of cysteine was nonenzymatic and due solely to a heavy-metal-catalyzed oxidation. The inactivity of cell-membrane preparations that had been

**Table 1. Intracellular distribution of L-cysteine oxidase in meningococcus at stationary phase of growth**

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Sp act*</th>
<th>% of total activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>19.8</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant fraction (180,000 × g, 3 h)</td>
<td>6.8</td>
<td>3</td>
</tr>
<tr>
<td>Membranous fraction</td>
<td>28.1</td>
<td>83</td>
</tr>
</tbody>
</table>

*Activities are expressed as nanomoles of O₂ consumed per minute per milligram of protein at 37°C (pH 7.5).
boiled (5 min) or those treated with Triton X-100 (a detergent which solubilizes the cytoplasmic membrane; 24) was taken as evidence that the oxidation of cysteine was enzymatic. Moreover, the oxidase activity was saturable and obeyed Michaelis-Menten kinetics with a \( K_m \) of \( 1.4 \times 10^{-3} \) M. This \( K_m \) is in close agreement with other bacterial sulfur amino acid oxidases (9).

The activity of the oxidase increased considerably with increasing pH, with a maximum activity at pH 9.8 and 50% activity at pH values of 8.4 and 10.0. For comparison, the pH optimum for the ascorbate-TMPD oxidase of the membrane was approximately 7.5, with 50% activity slightly below pH 6.0 (the lowest pH used) and at pH 8.4. Therefore, the high optimum pH for cysteine oxidase was unique to that reaction and not a general property of cell oxidases. The high pH optimum for this cysteine oxidase is in agreement with the reports of sulfur amino acid oxidases in other bacteria (6, 23, 29).

In an earlier report on the cysteine oxidase of Neisseria, Tauber and Russell (28) concluded that the product of the cysteine-oxidase reaction was cystine. Our results are consistent with their conclusion. Cysteine sulfinate, a key intermediate of mitochondrial L-cysteine dioxygenases (17, 23, 25, 29), was not detected as a product of the reaction by several methods (17, 25): thin-layer chromatography, amino acid analysis, or column separation of the reaction products (data not shown). It was not possible to determine directly whether \( \text{H}_2\text{O}_2 \) was the product of the reduction of oxygen because of the very high catalase activity in the membranous fraction of the cell-free extract. However, an excess of horseradish peroxidase added to the reaction mixture (data not shown) changed the reaction rate by less than 10%, indicating that \( \text{H}_2\text{O}_2 \) was not a major product of the reaction.

The direct involvement of the respiratory chain has been proposed for other bacterial amino acid oxidases (9, 22). We examined the possibility that the electrons from cysteine are carried along the respiratory chain to oxygen. The spectrum in Fig. 2 shows that the major cytochromes for this bacterium are reduced by L-cysteine. The L-cysteine-reduced-minus-air-oxidized spectrum was similar to what we obtained previously with dithionite as the reducing agent for the membrane cytochromes (32, 33). Notable in the spectrum was the broad peak at 600 nm, suggestive of the reduction of an \( \alpha \)-type cytochrome, and a sharp peak at 552 nm corresponding to a cytochrome \( c \). A deep trough at 450 nm indicative of a strong reduction of flavoprotein suggests that electrons from L-cysteine enter the electron transport chain at the

![Fig. 1. Growth of N. meningitidis SD1C in Mueller-Hinton broth.](http://jb.asm.org/)

![Fig. 2. L-Cysteine-reduced minus air-oxidized difference spectrum (25°C) of the envelope preparation of the meningococcus. Vertical bar represents 0.04 absorbance units for Soret curve and 0.2 absorbance units for the inset. Samples contained 20 mg of protein.](http://jb.asm.org/)
flavoprotein level in a manner similar to those from succinate and NADH in this organism (32). Positions of shoulders in the absorption region were especially apparent in the expanded spectra (Fig. 2, insert). Such shoulders correspond to the previously reported cytochromes (33) $b_{550}$, $b_{567}$, and $c_{547}$ in the bacterium. These results suggest that L-cysteine oxidation can proceed via the respiratory chain, including an L-cysteine flavoprotein.

Carbon monoxide binds to cytochrome oxidases only when they are in the reduced state. Therefore, the L-cysteine-reduced-plus-CO-minus-L-cysteine-reduced difference spectrum should permit the identification of the oxidases reduced by L-cysteine. The spectrum in Fig. 3 indicates that more than one cytochrome oxidase is involved in cysteine oxidation. The Soret peak at 417 nm and the absorption trough at 559 nm are strongly suggestive of cytochrome $o$, whereas the Soret peak at 430 nm and the trough in the area of 590 nm indicate that an $a$ cytochrome is reduced. These two oxidases have been reported previously for this organism (32, 33).

It was conceivable that endogenous respiration in the membrane preparation, separate from any attributable to L-cysteine, could itself result in the reduction of cytochromes, giving false results from CO binding in the presence of L-cysteine. This possibility was ruled out when it was determined that there was no detectable endogenous oxidase activity in the washed membrane preparations, and, moreover, the cytochromes remained oxidized in the absence of L-cysteine.

The study was continued with the use of inhibitors of the electron transport chain (Table 4). The iron chelators o-phenanthroline and salicylhydroxamate were both effective inhibitors of L-cysteine oxidase activity, which is in agreement with the reports of cysteine oxidation in other systems (9, 17, 23). That o-phenanthroline was effective at the flavoprotein level of the transport chain is seen in the lack of reduction by L-cysteine in the L-cysteine-plus-o-phenanthroline-minus-air-oxidized spectrum (Fig. 4). The disappearance of trough at 450 nm indicates that the inhibition by o-phenanthroline prevented the reduction of the flavoprotein itself (12).

Interesting results were obtained when the terminal oxidase inhibitors cyanide, azide, and hydroxylamine were tested for their effects on the oxidation of cysteine (Table 4). Whereas strong inhibition was obtained with cyanide, a finding in agreement with reports on other bacteria (9, 17, 26, 29), the cystine oxidase activity was only slightly affected by azide or hydroxylamine. Although the cytochrome oxidases $o$ and $a$ of this organism have different sensitivities to azide (32), the 150 mM azide, which was unable to inhibit the enzymatic oxidation of cysteine, completely inhibited the oxidation of succinate,
NADH, or ascorbate-TMPD via the meningococcal electron transport chain (32). Hydroxylamine (5 mM) produced 80 to 100% inhibition of ascorbate-TMPD, succinate, or NADH oxidase activities (32), but had little or no effect on cysteine oxidation at the same concentration. Therefore, one must conclude that the electrons from cysteine have access to the electron transport chain used by succinate and NADH, but there exists, in addition, a separate oxidase that is sensitive to cyanide but relatively insensitive to azide and hydroxylamine. If cyanide inhibition were at the terminal oxidase level, then one would expect that the cytochrome difference spectrum for cysteine-reduced-plus-cyanide-minus-air-oxidized would be identical to the cysteine-reduced-minus-air-oxidized spectrum shown in Fig. 2. When the spectrum of the cyanide-poisoned system was determined, the scan tracing (not shown) was identical to that in Fig. 2, indicating that the effect of cyanide was indeed at the terminal oxidase level, which is in agreement with the findings of others (29). The nature of the alternative oxidase pathway in meningococci is the subject of an ongoing investigation.

Another piece of evidence suggesting that there is an alternative, as yet undefined, pathway for electrons from cysteine is the failure of antimycin A and 2-n-heptyl-4-hydroxyquinoline-n-oxide (HQQNO) to significantly inhibit cysteine oxidase activity. These agents, used at concentrations strongly inhibitory for NADH and succinate oxidases (32), inhibited cysteine oxidase activity less than 20%.

Since the above results suggested that oxidation of L-cysteine involved an additional oxidase separate from the terminal oxidases previously reported for succinate, NADH, or ascorbate-TMPD, we tested the possibility that oxidase activities in the presence of multiple substrates might be additive. Figure 5 shows that this was indeed the case. The combined activities due to succinate and L-cysteine were similar regardless of the order of substrate addition (Fig. 5A and B). In the presence of azide, succinate oxidation was completely inhibited, whereas L-cysteine oxidase was not affected (Fig. 5B and C). This latter was taken as additional evidence suggestive of a separate oxidase for the oxidation of cysteine. It appears that cysteine oxidation may proceed not only by an azide-resistant oxidase but, from the difference spectra and inhibitor studies above, also via the α and o oxidases used by succinate and NADH.

DISCUSSION

In studies on pathogenesis a knowledge of vital nutrients for disease-producing bacteria is an important consideration. Although most of the meningococci and all of the gonococci tested have an absolute requirement for cysteine (4), little is known about cysteine metabolism in these important human pathogens. Tauber and Russell (28) reported their findings of a cysteine oxidase and cysteine desulphhydrase in meningococci, but neither of the reactions associated with these enzymes was studied in any detail.

The membrane-bound cysteine oxidase we have studied here has a narrow range of specificity. Among the 20 common L-amino acids tested, it catalyzed only the enzymatic oxidation of cysteine. Such findings clearly distinguish this enzyme from the cysteine oxidase (dioxygenase) in animal tissues, which leads to the production of cysteine sulfinate (17, 23, 25, 29), or the broad-spectrum L-amino acid oxidases in other bacteria (5, 6, 9, 22). Such bacterial amino acid oxidases catalyze the deamination of amino acids to their respective keto-acids (5, 6, 9, 22). The cysteine oxidase activity in meningococci was heat labile, was inactivated in Triton X-100, and obeyed Michaelis-Menten kinetics of enzyme-catalyzed reactions.

The electrons from cysteine appear to have ready access into the respiratory electron transport chain so that cysteine may be oxidized via cytochrome oxidases α and o. The reduction of the normal spectrum of cytochromes by cysteine and the ability of cysteine to reduce cytochrome oxidases α and o (33) supports this conclusion.
Furthermore, the inability of flavoprotein or cytochromes to be reduced in the presence of o-phenanthroline, known to inhibit electron transport at the flavoprotein level, suggests that electrons from cysteine enter the respiratory chain at an early stage.

As the result of recently reported studies from this laboratory, a branched respiratory electron transport chain has been proposed for the meningococci (32). To this branched chain we have added additional information which is consistent with our findings presented here (Fig. 6). Any inhibition of electron flow in the respiratory chain results in the shunting of electrons from cysteine to an alternate oxidase. This alternative oxidase, unlike the cytochrome oxidases of the respiratory chain, is resistant to hydroxylamine and azide. Multiple oxidases are well documented in bacteria (15, 30) and in certain mitochondrial systems of eucaryotic cells (13). The additive nature of succinate and cysteine activities in meningococcal membranes lends support to the alternative oxidase hypothesis as does the insensitivity of cysteine oxidase to antimycin A and 2-n-heptyl-4-hydroxyquinoline-n-oxide, which act between b and c cytochromes.

The physiological role of the cysteine oxidase in meningococci is not known. The conversion of cysteine to cystine occurs nonenzymatically in the presence of oxygen and heavy metals. A recent report on a membrane-bound oxidase catalyzing the oxidation of glutathione to glutathione disulfide presents evidence suggesting that the conversion is involved in the translocation of the substrate across the membrane (27). Since the oxidation of cysteine to cystine is analogous to the oxidation of glutathione to glutathione disulfide, it seems reasonable that the oxidation of cysteine may also be part of a translocating process. Alternatively, one can speculate that the mere oxidation of cysteine to cystine via the respiratory chain may serve no other function than to translocate protons from the cytoplasm to the outside of the cell, thereby increasing the proton motive force needed to drive oxidative phosphorylation required for growth and maintenance under conditions where other substrates become limiting. A similar proposal has been put forward for glutathione oxidation, as well (21).

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Fig. 6. Proposed scheme for the L-cysteine oxidation in the electron transport chain on the meningococcal envelope preparation. HOQNO, 2-n-Hexyl-4-hydroxyquinoline-n-oxide; SHAM, salicylhydroxamate; UbQ, Ubiquinone.

LITERATURE CITED


