Localization of Tetramethylphenylenediamine-Oxidase in the Outer Wall Layer of Neisseria meningitidis

I. W. DEVOE* AND J. E. GILCHRIST

Macdonald Campus of McGill University, Department of Microbiology, Montreal, Quebec, Canada HOA 1CO

Received for publication 23 June 1976

A highly active tetramethylphenylenediamine-oxidase has been found in association with the cell wall blebs, evaginations of the outer wall membrane, of Neisseria meningitidis. Isolated wall blebs consumed oxygen in the presence of ascorbate-tetramethylphenylenediamine but not in the presence of succinate, whereas cell envelope preparations are active on both substrates. The ratio of succinate dehydrogenase/tetramethylphenylenediamine-oxidase activities in preparations of envelopes was approximately 100 times that in isolated wall blebs, indicating that the outer membrane preparations were highly purified.

Kovacs's (11) oxidase test for the presence of "cytochrome oxidase" is now used routinely as a taxonomic tool for the neisseriae. The oxidase reaction was first introduced by Gordon and McLeod (6) as an aid in the identification of gonococci. When dimethyl-p-phenylenediamine (1 to 1.5%) was poured over an agar plate containing gonococcal colonies, a pink color developed within seconds, followed by purple and eventually black. The tetramethyl form, N,N',N',N'-tetramethyl-p-phenylenediamine (TMPD) of the compound was found to be less toxic and more active and was introduced by DeVoe and Gilchrist and Ellingworth et al. (5).

Subsequent investigations into the use of this compound as a taxonomic tool have shown that other genera also exhibit high TMPD oxidation rates, e.g., Pseudomonas, Azotobacter, Aeromonas, Rhizobium (D. N. McQuilty, O. Marucci, and P. Jurtshuk, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, p. 152, p. 170), Bran-hamella, and Moraxella (1). On the other hand, the activity of whole cells of other aerobic bacteria, e.g., the Enterobacteriaceae and most gram-positive bacteria, is relatively very low with respect to TMPD oxidation.

The purpose of the study we present here was to investigate TMPD-oxidase activity of Neisseria meningitidis and to determine the location of such activity in the cell envelope. We present evidence for a TMPD-oxidase located in the outer cell wall layer. Furthermore, this enzyme can be released into the surrounding medium by the manipulation of the ionic environment.

MATERIALS AND METHODS

Organism. Group B N. meningitidis (SDIC) was Naval Medical Research Unit no. 1, obtained from the Neisseria repository, University of California, Berkeley. Cells were maintained and grown as described by DeVoe (2). This strain readily dissociates into both a rough and smooth colonial type. Only the smooth strain was used here.

Fractionation of cell components. Procedures for the removal and isolation of cell wall blebs were described by DeVoe and Gilchrist (3). After the removal of cell wall blebs (Fig. 1), whole cells were further treated by suspension in tris(hydroxymethyl)aminomethane (Tris)-buffered saline-citrate (0.05 M Tris-hydrochloride, pH 7.5, 60 mM NaCl, 30 mM Na_, citrate (TBSC), see Fig. 1) at 37°C (30 min), followed by centrifugation (20,000 x g, 20 min). Supernatant fluids were cleared of residual blebs by centrifuging twice (100,000 x g, 1 h), followed by centrifugation at 180,000 x g (3 h). The detailed protocol for fractionation of cell wall blebs is presented in Fig. 1.

Protein determination. Proteins were determined by the method of Lowry et al. (12) after the treatment of samples with 1.0 M NaOH for 5 min.

Enzyme assays. Glucose-6-phosphate dehydrogenase activity (10) was used here as a marker for leakage of soluble cytoplasmic material from whole cells.

Succinate dehydrogenase (SDH), a membrane-bound enzyme, was used as a cytoplasmic membrane marker. Activity of SDH was determined by the method of Kasahara and Anraku (8). One unit of SDH was that amount of enzyme required to reduce 1 nmol of 2,6-dichlorophenolindophenol (DCPIP) per min.

TMPD-oxidase activity was determined by two methods. (i) Activity was measured as an absorbance increase at 520 nm (A520) (Gilford spectrophotometer model 240) at 37°C in a 3-ml volume (1-cm cuvette) containing the test sample, 1 mM TMPD, 0.1 M Tris-hydrochloride, pH 7.5, 0.15 M NaCl. All activities reported are corrected for autooxidation of TMPD itself in the absence of the test sample. All activities are based on initial rates taken from the first 0.5 min of the reaction. One unit of TMPD-oxidase was defined as that amount of enzyme re-
Cells in growth medium

10,000 \times g, 5 min

Pellet (whole cells)  Supernatant fluid (discard)

(1) Suspend in a balanced salt solution
(2) Blender treatment (2 s)
(3) 20,000 \times g, 20 min

Pellet (whole cells)  Supernatant fluid (cell wall blebs)

(1) 20,000 \times g, 20 min
(2) Remove supernatant fluid (repeat 2 ×)

Pellet (whole cells)  Supernatant fluid (discard)

100,000 \times g, 1 h

Pellet (washed blebs)  Supernatant fluid

(1) Suspend in TBSC
(2) 37°C, 30 min
(3) 100,000 \times g, 1 h

RESULTS

Cell wall blebs, membranous evaginations of the outer membrane of meningococci, have been observed in abundance on all strains of meningococci analyzed by this laboratory (3, 4). The removal of these blebs is a relatively simple operation involving blending for 2 s. Such treatment does not otherwise damage whole cells nor does it affect their viability (3). The easy removal and isolation of these outer membrane vesicles provides a useful tool for analysis of outer cell wall components without having to resort to prolonged fractionation of cell

required to produce an increase in A_{520} of 0.1 unit/min under the above conditions.

(ii) TMPD-oxidase activity was also measured by oxygen consumption (YSI oxygen monitor, Yellow Springs Instrument Co., model 53) in an assay system containing the test sample, 1.5 mM ascorbate, 1 mM TMPD, 0.05 M MgCl_{2}, 0.1 M Tris-hydrochloride, pH 7.5, and 0.15 M NaCl (37°C). One unit of TMPD-oxidase by the second method was that amount of enzyme required to reduce 1 ng-atom of oxygen/min. In experiments where KCN was used as an inhibitor, the final concentration was 1 mM. Sodium succinate where used was 30 mM final concentration.

Cell envelope preparations. Cells suspended in TBSC (Fig. 1) were broken by light sonic oscillation by subjecting cells to three treatments for 10-s intervals with alternate cooling of both probe and sample in an ice bath to avoid loss of enzyme activity due to heat. Cell-free extracts were centrifuged at 5,000 \times g for 10 min (4°C). All SDH activity in supernatant fluids was sedimented with particulate envelope fractions after centrifugation at 100,000 \times g for 1 h.

FIG. 1. Flow diagram for the isolation of cell wall blebs of the meningococcus. Balanced salt solution: 0.15 M NaCl, 2 mM KCl, 0.2 mM MgCl_{2}, Tris-hydrochloride, pH 7.5. The cation concentrations of this solution are identical with those in spent Mueller-Hinton broth (Archibald and DeVoe, unpublished data).
envelopes into inner and outer membranes. In our experiments on TMPD-oxidase, the first indication that oxidase activity might be found in the outer cell wall was the decrease in oxidase activity associated with whole cells after blebs had been removed (Table 1).

Surface blebs released 20% of their protein upon transfer from a balanced salt solution (0.15 M NaCl, 2 mM KCl, 0.2 mM MgCl, Tris-hydrochloride, pH 7.5; the cation concentrations are identical with those spent in Mueller-Hinton broth [Archibald and Devoe, unpublished data]) to TBSC, a solution devoid of Mg$^{2+}$. As blebs originate by evagination of the outer membrane itself, one can deduce that 20% of the outer wall protein is also released upon similar transfer of whole cells to TBSC. Such protein loss occurred without cell lysis as evidenced by retention by the cells of 100% activity of the soluble cytoplasmic enzyme glucose-6-phosphate dehydrogenase (data not presented). Whole cells, less blebs, suspended in TBSC did, however, lose 60% of their TMPD-oxidase activity (Table 1). Only a small fraction of the lost oxidase activity was recoverable in the supernatant fluids, indicating that the oxidase was either denatured on the cell or released in an inactive form (Table 1). Although more than half of the oxidase activity had been lost when cells were transferred from balanced salts to TBSC, the rate of respiration in cells with succinate as substrate remained essentially unchanged. These results were taken as confirmatory evidence that the cytoplasmic membranes had not been grossly damaged by TBSC, i.e., that the respiratory chain was intact.

Many aerobic bacteria appear oxidase negative by the routine TMPD assay on colonies, but such organisms do have active oxidase(s) in their isolated cytoplasmic membranes measurable by the use of ascorbate-TMPD. It was conceivable in our experiments that by using whole cells we might not be measuring the total TMPD-oxidase activity, especially if some enzyme was situated in the cytoplasmic membrane so as to be accessible only from inside of the cell. If such were the case, light sonic oscillations of whole cells should make additional oxidase available to TMPD. When this hypothesis was tested, we routinely found a nearly twofold increase in TMPD-oxidase activity (Table 1). However, the rate of oxygen consumption in these same cells using succinate as substrate was somewhat less after sonic treatment (400 and 240 ng-atoms of oxygen consumed/min per 10$^{10}$ cells, respectively), indicating that the membrane had indeed been broken up but that most of the respiratory chain itself had remained intact. Although the respiratory chain as a whole was not stimulated by light sonic oscillation, it is possible that such treatment did not expose additional oxidase to TMPD, as we had proposed, but it may have stimulated TMPD-oxidase itself. Our results do not rule out either of these possibilities.

To determine whether oxidase was located in the outer membrane of the cell wall, blebs from this layer were isolated (Fig. 1) and tested for their ability to oxidize TMPD (Table 2) and also for their ability to reduce O$_2$ in the presence of ascorbate-TMPD and succinate (Table 3). The specific activity of oxidase in the blebs was relatively high by either method, but it is significant that oxygen was consumed with succinate as the substrate only in envelope fractions (Table 3). Both the oxidase activity associated with envelope preparations and that associated with wall blebs was completely inhibited by CN$^{-}$ or by boiling of these preparations (Table 3). The boiling was a necessary control treatment not only to implicate an enzyme in the oxidation reactions but also to rule out the rapid nonenzymatic oxygen consumption that could have occurred if certain free metal ions, such as those of iron or copper, were bound to the cell surface materials.

### Table 1. TMPD-oxidase activity of Neisseria meningitidis

<table>
<thead>
<tr>
<th>Description</th>
<th>TMPD-oxidase activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells with attached blebs</td>
<td>127</td>
</tr>
<tr>
<td>Whole cells after removal of blebs</td>
<td>109</td>
</tr>
<tr>
<td>Whole cells after suspension in TBSC (assayed in balanced salt solution)</td>
<td>40</td>
</tr>
<tr>
<td>TBSC supernatant fluid after removal of cells</td>
<td>0.2</td>
</tr>
<tr>
<td>After light sonic oscillation of whole cells suspended in TBSC (assayed in balanced salt solution)</td>
<td>71</td>
</tr>
</tbody>
</table>

$^a$ $A_{120}$ of 0.1 unit/min per 10$^{10}$ cells.

### Table 2. TMPD-oxidase activity in the outer wall membrane (blebs) and in material released from whole cells assayed in balanced salt solution

<table>
<thead>
<tr>
<th>Description</th>
<th>TMPD-oxidase activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated wall blebs</td>
<td>36.4</td>
</tr>
<tr>
<td>Blebs after washed in TBSC</td>
<td>41.2</td>
</tr>
<tr>
<td>Material washed off blebs with TBSC</td>
<td>50.0</td>
</tr>
<tr>
<td>Material washed off whole cells with TBSC</td>
<td>3.2</td>
</tr>
<tr>
<td>Supernatant fluid (180,000 × g, 3 h) of material washed off whole cells with TBSC</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

$^a$ $A_{120}$ of 0.1 unit/min per mg of protein.
The results (Table 3) indicate that cell envelope preparations, TMPD-oxidase activities to be similar in the membrane of the cell. However, the purity of the TMPD-oxidase of this portion of the total oxidase was negatively determined by centrifugation. Furthermore, these vesicles which contained both the cytoplasmic membrane and the outer membrane of this meningococcus (3). It is conceivable that the cell wall oxidase represents one terminal oxidase of a branch respiratory pathway, and that there may exist intermediate electron carriers in the periplasm which could transfer electrons from the cytoplasmic membrane to a terminal oxidase in the outer membrane of the wall. Knowles et al. (9) have found a soluble c-type cytochrome in the periplasm of a marine pseudomonad. Such a soluble electron carrier in the periplasm of meningococcus could act as an electron shuttle.

DISCUSSION

The data presented show that the outer membrane of the cell wall contains a highly active TMPD-oxidase. A portion of this enzyme(s) can be readily separated from isolated outer membrane by suspending these vesicles in TBSC, a solution devoid of Mg++. The enzyme "washed off" in this manner is not soluble, however, but is sedimented at 180,000 x g in 3 h. It is difficult to conceive of an oxidase located in or on the outer membrane of the cell wall that could be a terminal electron acceptor for the respiratory chain, which is itself located in the cytoplasmic membrane. This seems even more unlikely in view of the physical distance between the cytoplasmic membrane and the outer membrane of this meningococcus (3). It is conceivable that the cell wall oxidase represents one terminal oxidase of a branch respiratory pathway, and that there may exist intermediate electron carriers in the periplasm which could transfer electrons from the cytoplasmic membrane to a terminal oxidase in the outer membrane of the wall. Knowles et al. (9) have found a soluble c-type cytochrome in the periplasm of a marine pseudomonad. Such a soluble electron carrier in the periplasm of meningococcus could act as an electron shuttle.

Relatively little is known, as yet, about bacterial oxidases, but information that is accumulating indicates that respiratory chains are branched in some organisms, with separate branches having terminal oxidases with individual properties (7, 13, 14).

ACKNOWLEDGMENTS

I am indebted to J. M. Ingram for his valuable suggestions and informative discussions.

This work was supported by grants from the National Research Council of Canada, The Defense Research Board of Canada, and the Faculty of Graduate Studies and Research of McGill University.
LITERATURE CITED


