Membrane Association of the Carbon Monoxide Oxidation System in Rhodopseudomonas gelatinosa†

BASSAM T. WAKIM AND ROBERT L. UFFEN*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101

Received 16 August 1982/Accepted 22 October 1982

A comparison of the distribution of CO oxidation activity between soluble and particulate protein fractions obtained after disruption of CO-grown Rhodopseudomonas gelatinosa 1 by French pressure cell breakage and osmotic lysis of spheroplasts suggested that, in situ, the enzyme complex was associated with the cell membrane. An improved, strictly anaerobic method is given for spectrophotometric measurement of CO oxidation activity based on the carbon monoxide:methyl viologen oxidoreductase reaction.

A variety of different aerobic and anaerobic bacteria have been reported to metabolize CO, and although this oxidation activity usually occurs adventitiously, a few microorganisms have been reported to use the gas for growth (2-6, 8, 10-13). Rhodopseudomonas gelatinosa 1 (11, 12), a phototrophic microorganism, exhibited a requirement for CO for growth under anaerobic, dark conditions in which cells oxidized the energy-yielding substrate according to the equation

$$\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2 \quad (\Delta G^° = -4.8 \text{ kcal} \quad (-20.1 \text{ kJ/mol of CO}).$$

Based on composite reports of studies examining the properties of the CO oxidation reaction, it appears that in most microbial systems the CO-binding oxidation complex is found in the cell cytosol (4, 12, 13). Nevertheless, Cyponka et al. (2) and Kim and Hegeman (6) speculated that a membrane association could exist. Experiments were thus undertaken to examine this possibility, since a membrane association of the protein could help to explain the difficulty we encountered in obtaining reproducible CO oxidation activity from R. gelatinosa 1.

Strain 1 was grown without light under strictly O2-free conditions in a liquid medium supplemented with 0.1% (wt/vol) Trypticase (BBL Microbiology Systems) as previously described (11). Growth occurred in 1.5 liters of sterile reduced medium under O2-free pure CO gas inside a 3-liter Erlenmeyer flask. Trace amounts of O2 were removed from the CO (chemically pure grade, 99.5%; Matheson Scientific Inc., Joliet, Ill.) by passage through a solution with reduced methyl viologen (MV) dissolved in 5 mM Tris. (The MV trapping agent was reduced as necessary by adding a few crystals of sodium dithionite.) During incubation at 30°C in a water bath shaker rotating at about 140 rpm, a slow stream of the CO gas substrate was introduced into the culture fluid with a fritted-glass gas dispersion tube. Once the culture reached an optical density of about 1.0 at 640 nm (representing a density of 3.6 × 10^9 cells per ml or 0.7 mg of cell protein per ml) under the continuous-gassing growth condition, cells were collected under argon gas by centrifugation at 4°C (11) in screw-cap centrifuge tubes and then stored at -70°C under argon. All subsequent studies with strain 1 cells were performed under O2-free conditions (11, 12).

For the experiments, frozen cells were suspended in Tris-hydrochloride buffer, disrupted, and fractionated under the different conditions described below. CO oxidation activity in the cellular fractions was assayed spectrophotometrically, based on MV reduction at 578 nm as used in other anaerobic CO oxidation systems (8, 12). All assay mixtures for CO:MV oxidoreductase activity, however, were conveniently prepared in cuvettes inside an anaerobic glove chamber (Coy Manufacturing Co., Ann Arbor, Mich.) and contained 2.0 mM MV and 0.15 mM dithiothreitol (DTT) in 0.1 M 2(N-cyclohexylamino)ethanesulfonic acid buffer (pH 9.0). Reaction cuvettes were sealed with a serum bottle stopper, removed from the anaerobic chamber, and then bubbled for 3 to 5 min at room temperature with a stream of O2-free CO gas with a hypodermic needle inserted through the serum bottle stopper. Because the anaerobic chamber assured reliable anaerobic conditions, it was not necessary to titrate the MV reaction mixture with sodium dithionite to remove trace amounts of O2 (8, 12) before measuring enzymic CO

† Michigan Agricultural Experiment Station journal article no. 10546.
TABLE 1. Presence of malate dehydrogenase and CO oxidation activity in fractions from R. gelatinosa 1 cells grown under CO in the dark after French pressure cell breakage

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Malate dehydrogenase</th>
<th>CO oxidation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/100 mg of protein</td>
<td>% Yield</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>40.2</td>
<td>100</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>40.0</td>
<td>99.6</td>
</tr>
<tr>
<td>Particulate</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>material</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Measured by the method of Alefounder and Ferguson (1).
* Low-speed centrifugation (27,000 × g, 20 min, 4°C).
* High-speed centrifugation (184,000 × g, 60 min, 4°C).

oxidation activity. Protein samples were introduced anaerobically with a hypodermic syringe into the CO-saturated assay solution (final volume, 1.0 ml), and the CO oxidation activity was calculated from the extinction coefficient of MV (ε_{528} = 9.7 mM$^{-1}$ cm$^{-1}$) according to the equation in which 2 mol of MV is reduced per mol of CO oxidized (unpublished data). One unit of activity was the amount of enzyme needed to utilize 1 μmol of substrate per min. Protein was estimated by the method of Lowry et al. (7), with bovine serum albumin used as the standard.

Earlier attempts to obtain a cell fraction from CO-grown R. gelatinosa 1 with reproducibly high CO oxidation activity were discouraging, since a variety of different methods used to obtain cell preparations produced widely varying recoveries, specific activities, and soluble and particulate fraction distribution of CO oxidation activity. These inconsistent results finally became more understandable with improved experimental conditions, and a comparison between different methods of cellular disruption showed that the CO oxidation system was associated with cell membrane material.

The distribution of CO oxidation activity in protein fractions from CO-grown cells after French pressure cell breakage is shown in Table 1. Strain 1 cells were suspended in 30 mM Tris-hydrochloride buffer (pH 8.0) containing 0.3 mM DTT and 0.6 M sucrose and disrupted by a single passage through a French pressure cell (16,000 lb/in$^2$, 4°C). After cell breakage and low-speed centrifugation (Table 1), the supernatant solution contained 44 U of activity per 100 mg of protein. (Although this total activity yield was somewhat low, the distribution of the enzyme proteins between the different cell fractions was representative of other experiments.) When the crude protein solution was fractionated by ultracentrifugation (Table 1), 88% of the CO oxidation activity remained in the soluble protein fraction. Under improved experimental conditions, using both sucrose and DTT in the suspending solution, the apparent loss in CO oxidation activity between the combined soluble protein and particulate material fractions was only about 9%. The data on the occurrence of CO oxidation activity in comparison with results in the same experiment for malate dehydrogenase, the soluble enzyme protein (1) used as an internal reference, suggested that the CO oxidation complex existed in the strain 1 cell cytosol. Nevertheless, at least 3.2% of the CO oxidation activity remained with the particulate material (Table 1). Since this was observed in repeated experiments and the amount of activity was almost eightfold greater than that of malate dehydrogenase in the same particulate fraction, it could represent a specific membrane associa-

TABLE 2. Presence of malate dehydrogenase, succinate dehydrogenase, and CO oxidation activity in fractions prepared from spheroplasts of R. gelatinosa 1 grown under CO in the dark

<table>
<thead>
<tr>
<th>Fraction and procedure</th>
<th>Malate dehydrogenase</th>
<th>Succinate dehydrogenase</th>
<th>CO oxidation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/100 mg of protein</td>
<td>% Yield</td>
<td>U/100 mg of protein</td>
</tr>
<tr>
<td>Low-speed centrifugation (5,000 × g, 4°C, 20 min)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soluble periplasmic protein</td>
<td>175</td>
<td>100</td>
<td>2.6</td>
</tr>
<tr>
<td>Particulate material</td>
<td>130</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Osmotic shock plus low-speed centrifugation (5,000 × g, 4°C, 20 min)</td>
<td>45</td>
<td>25</td>
<td>2</td>
</tr>
</tbody>
</table>

* Measured by the method of Oelze and Kamen (9). Low succinate dehydrogenase activity was consistently obtained on three separate trials with CO-grown cells.
tion. We examined this by using osmotic lysis of spheroplasts as a more gentle method of obtaining CO oxidative activity.

For preparing spheroplasts from frozen or freshly harvested CO-grown R. gelatinosa, cells were washed twice at 4°C in anaerobic 30 mM Tris-hydrochloride buffer (pH 8.0) with 0.3 mM DTT. Enough washed strain 1 cells were then added to the solution as before, but the solution also contained 0.6 M sucrose plus 1 mg of chicken egg white lysozyme (IUB no. 3.2.1.17; Worthington Biochemical Corp., Freehold, N.J.) per ml to reach an optical density of 2.0 at 640 nm (representing 7.2 × 10⁹ cells per ml or about 1.4 mg of cell protein per ml). During 30 min of gentle stirring at 30°C, more than 80% of the rod-shaped cells changed to spheroplasts, as judged by microscopic observation. Spheroplasts in the particulate material after low-speed centrifugation of the lysozyme-treated cell suspension (Table 2) were suspended in cooled 20 mM Tris-hydrochloride buffer (pH 8.0) with DTT and stirred vigorously for 20 min at 4°C. Cytoplasmic proteins released by osmotic lysis of the spheroplasts were obtained in the supernatant solution after low-speed centrifugation (Table 2). Enzymic analyses showed that 85% of the CO oxidation activity remained with the particulate material. Only 15% of the CO oxidation activity was detected in the soluble cytosol fraction, and none appeared to be released with the periplasmic fluid during spheroplast formation (Table 2). In accord with these data and with the results when strain 1 malate dehydrogenase and succinate dehydrogenase were used as internal controls for soluble and particulate enzyme protein activity (1, 9), respectively, the CO oxidative system in strain 1 after osmotic lysis indeed appeared to be membrane bound. However, the association seemed to be loose, as treatment of cells by French pressure cell breakage released most of the CO oxidation activity into the soluble protein fraction (Table 1).

An in situ membrane association of CO oxidative activity in R. gelatinosa strain 1 was not entirely unexpected, since the system seemed to operate as a principal anaerobic respiratory energy-yielding reaction in ATP regeneration (unpublished data). A similar membrane association might likewise occur in other microbes, especially in those bacteria in which the oxidation reaction can support a meaningful cell growth response (2, 3, 5, 6, 12). As in our earlier studies, however, this involvement might be overlooked if only one method of cell disruption is used. Finally, we observed that preparations of the membrane-associated CO oxidation system of R. gelatinosa strain 1 are stable for more than 1 year under argon at −70°C with no loss in CO:MV oxidoreductase activity. It is believed that this stability will make purification of the anaerobic CO oxidation system possible in its membrane association and thereby facilitate studies examining the physical properties and mechanism of action of the complex enzymic system.

This work was supported by grant PCM 7918135 from the National Science Foundation.

LITERATURE CITED