Cytochrome o as a Terminal Oxidase and Receptor for Aerotaxis in Salmonella typhimurium

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Cytochrome o was the only oxidase of the electron transport system that was present in exponentially growing Salmonella typhimurium ST1. Identification of cytochrome o was made by the (CO-reduced)-minus-(reduced) difference spectra and by the photochemical action spectrum of the relief, by light, of CO-inhibited respiration. Cytochrome o also functioned as the receptor for chemotaxis to oxygen (aerotaxis). The concentration of oxygen that elicited the maximum response for aerotaxis (0.7 μM) was similar to the Kₘ for respiration (0.74 μM), and both aerotaxis and respiration were blocked by 5 mM KCN.

Aerotaxis is the migrational response of microorganisms to a gradient of oxygen (for reviews see references 26 and 27). The accumulations of bacteria around an air bubble (11) and at the edge of a cover glass (4) were among the first aspects of microbial behavior to be studied, but it was only recently that the biochemical mechanism of aerotaxis was systematically investigated. Links (Ph.D. thesis, Rijksuniversiteit, Leiden, The Netherlands, 1955) and Clayton (6) proposed that tactic responses to oxygen and other chemical attractants were triggered by a decrease in the rate of metabolism or the energy supply to the motor apparatus. Bacteria entering a hypoxic region experience decreased respiration and oxidative phosphorylation, and they were assumed to respond to the resulting fall in intracellular ATP concentration by changing swimming direction. Adler (1, 2) subsequently demonstrated that there are specific receptors for chemoeffectors and that chemotaxis is independent of metabolic utilization of the effectors. As a result of the studies by Adler and other investigators, the Links-Clayton hypothesis was largely discarded (3, 14, 29a).

It is now evident that previous concepts of chemotaxis were oversimplified. Taylor et al. (29) demonstrated a distinction between the mechanism of chemotaxis to sugars and amino acids and the mechanism of taxis to oxygen and alternative electron acceptors for the respiratory chain. The former is independent of the respiratory chain, whereas the latter appears to be mediated by respiration-induced changes in the proton motive force. Thus, for the limited class that includes aerotaxis and electron acceptor taxis, the Links-Clayton hypothesis has some validity, although two concepts in the original presentation are incorrect. The proton motive force, and not ATP, mediates the response, and the bacteria are attracted by oxygen rather than “shocked” by anoxia (16, 29).

Many of the recent studies of aerotaxis have been with Salmonella typhimurium, for which the concentration of oxygen that elicits half the maximum aerotactic response (K₀₅₅) was reported to be ca. 0.4 μM as estimated from a dose-response curve (16). However, no cytochrome oxidase with a Kₘ in that range has been reported for S. typhimurium. The only reported terminal oxidase is cytochrome d (9), which has a Kₘ of 0.02 μM in Escherichia coli (24). In this study the Kₘ for respiration in S. typhimurium was shown to be similar to the K₀₅₅ for aerotaxis, and cytochrome o was identified as the terminal oxidase in exponentially growing cells. A preliminary account of these investigations has been presented at the 72nd Annual Meeting of the American Society of Biological Chemists, June 1981, St. Louis, Mo. (B. L. Fandrich and D. J. Laszlo, Fed. Proc. 40:1637, 1981).

MATERIALS AND METHODS

Bacterial strain and growth conditions. The strain used in this study was S. typhimurium ST1, which was derived in the Koshland laboratory by selecting S. typhimurium LT2 for improved chemotaxis (19). Aerotaxis in strain ST1 was similar to aerotaxis in strain LT2 (unpublished data). Cells were grown aerobically in Vogel and Bonner medium E (30) with glycerol (1% [vol/vol]) as the carbon source or anaerobically in the same medium supplemented with KNO₃ (30 mM) or potassium furmarate (30 mM) as the electron acceptor.

Aerotaxis assay. Aerotaxis was assayed by a method similar to that of Laszlo and Taylor (16). Bacteria were washed twice and suspended in medium E with glucose. A 2-μl drop was spread over a 6-mm diameter on a microscope slide, and the slide was inserted into a gas flow cell with nitrogen flow. After 1 min of anaerobiosis, the flow cell was ventilated with various oxygen-nitrogen mixtures. The smooth-swimming response of the bacteria to the oxygen was observed. The duration of the response was defined as the period from the beginning of the smooth response to the time when tumbling was restored in 50% of the cells.

Kinetics of respiration. The Kₘ of the terminal oxidase was determined by the method of Rice and Hempfling (24). Although this method uses a Clarke-type oxygen electrode to measure oxygen uptake in a closed reaction vessel, the problem of the hysteresis of the electrode is avoided by allowing the electrode to stabilize at zero oxygen concentration and then adding minimal amounts of oxygenated medium (8). The time course of oxygen depletion was recorded on a strip chart recorder, the oxygen concentrations at 1-s intervals were tabulated, and the Kₘ for the terminal oxidase was calculated with a Texas Instruments TI990 computer.

Difference spectroscopy. Cells were grown in 12-liter batches in a bench-top fermentor (model SF-116; New Brunswick Scientific Co., Inc.), washed twice, and resus-
FIG. 1. Double-reciprocal plot of respiration in *S. typhimurium* ST1. The *K*<sub>m</sub> for respiration was determined by the procedure of Rice and Hempfling (24). A 170-ml reaction vessel (Fleaker; Corning Glass Works) was placed in a water jacket (30°C) over a magnetic stirrer and filled with strain ST1, which was suspended in medium E (30) with glucose at an optical density of 0.02 at 600 nm. The capped vessel was rendered anaerobic by respiration of the cells and flushing with nitrogen. Oxygen was added by injection of 1 to 2 ml of air-saturated buffer into the vessel. The time course of oxygen uptake was recorded by using a Clarke-type oxygen electrode (Transidyne General) and a Chemical Microsensor oxygen meter (Transidyne General). The concentration of oxygen at 2-s intervals was determined from the recording, and velocities and a Lineweaver-Burk plot of the data were obtained by using a Texas Instrument TI F5990 computer (24).

patched in buffer containing 50 mM N-Tris-(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (pH 7.0) and 50 mM KCl. The protein concentration was determined by the biuret procedure (17), and the cell suspension was diluted to a final concentration of 10 mg of protein per ml. (Dithionite-reduced)-minus-(H<sub>2</sub>O<sub>2</sub>-oxidized) and (CO-reduced)-minus-(reduced) difference spectra were obtained by using a dual-beam spectrophotometer (model DW-2a; American Instrument Co.). Anaerobic cuvettes (American Instrument Co.) with a 1-cm light path were used for studies at room temperature, and a low-temperature attachment with a cuvette with a 2-mm light path was used for spectra at 77 K. The amounts of cytochromes *b*<sub>1</sub>, *d*, and *o* were quantified by using the extinction coefficients of Jones and Redfearn (13) and Daniel (7).

Photochemical action spectra. Cells were grown to mid-exponential phase, washed, and resuspended in medium E. Photochemical action spectra were determined on whole cell suspensions by using the procedure of Edwards et al. (10). This essentially consists of measuring the variation of oxygen tension of the respiring medium by using an oxygen electrode (a microelectrode made of platinum and silver) as a function of the wavelength of the probing dye laser. The monochromatic light from the dye laser (model no. 490; Coherent Radiation) pumped by an argon ion laser (model no. 96; Lexel Corp.) is tuned by using an intracavity birefringent filter. Relief of CO-inhibited respiration was measured as a rate increase from steady-state respiration in the dark. Determinations were made at room temperature.

RESULTS

If the aerotaxis receptor is the terminal oxidase of the respiratory chain, the *K*<sub>m</sub> for respiration should be similar to the *K*<sub>m</sub>,<sub>s</sub> defined as the concentration of oxygen that elicits a half-maximal response in aerotaxis. The rate of respiration was determined as a function of oxygen concentration by computer analysis of the time course of oxygen consumption by *S. typhimurium* ST1 in a closed vessel (24). The *K*<sub>m</sub> of the terminal oxidase of the respiratory chain was 0.74 μM in exponentially growing cells (Fig. 1). This *K*<sub>m</sub> is similar to the *K*<sub>m</sub> (0.2 μM) for respiration in *E. coli* (24). For the studies of aerotaxis, a temporal assay was used in which a predetermined stepwise increase in oxygen concentration was imposed on *S. typhimurium* ST1, and the motility was observed under the microscope (16). The smooth-swimming response to the increase in oxygen concentration was timed and analyzed as a function of oxygen concentration. The *K*<sub>m</sub> for aerotaxis was ca. 0.7 μM (Fig. 2). Both respiration and aerotaxis were blocked by 5 mM KCN.

The studies of respiration and aerotaxis utilized *S. typhimurium* from the early exponential phase of growth (the optical density at 600 nm was 0.2 to 0.3). The (CO-reduced)-minus-(reduced) difference spectrum of similar whole cells at 23°C included a major peak at 417 nm, a trough at 432 nm, and minor peaks at 570 and 537 nm (Fig. 3). The peaks corresponded in position to those of the Soret, α, and β bands, respectively, of cytochrome *o* of *E. coli* (23). There

![FIG. 2. Double-reciprocal plot for aerotaxis in *S. typhimurium* ST1. The bacteria were suspended in medium E (30) with glucose, and a 2-μl drop was placed in a gas flow cell, as described in the text. The flow cell was ventilated with nitrogen for 1 min, and then a predetermined oxygen-nitrogen mixture was introduced into the cell. The behavioral response of the bacteria was observed in the microscope. Timing of the response commenced when the bacteria accelerated after exposure to oxygen and ended when 50% of the bacteria resumed random swimming.](http://jb.asm.org/)
was no absorption peak at 625 or 590 nm, suggesting the absence of cytochromes d and a₁ (12).

The oxidase present in exponentially growing S. typhimurium ST1 was confirmed as cytochrome o by a photochemical action spectrum of the relief by light of CO-inhibited respiration (Fig. 4). A drop of cell suspension around a platinum microelectrode was held in a gas atmosphere of CO-O₂ in a 60:20 ratio. Illumination of the cells by laser light resulted in relief of CO inhibition, detected as a rapid increase in respiration rate with time (10). When the light was switched off, CO became rebound to the oxidase, and respiration was gradually inhibited, resulting in a gradual increase in oxygen tension. Maximum relief of CO inhibition was observed at 570 nm, which is the λ_max of cytochrome o in E. coli (10). The wavelengths (568 to 603 nm) of laser light used in this experiment encompass the useful range of the rhodamine 6G dye. Measurements below 568 nm were not practical, but the relief of CO inhibition was consistently greater at 570 nm than at 568 or 569 nm. Cytochrome a₁ (λ_max = 592) has been discussed as a possible oxidase in E. coli (10, 22) but was not present in S. typhimurium from early-exponential-growth phase (Fig. 4).

Aerotaxis involves a functional electron transport system in S. typhimurium and is observed in cells grown either aerobically or anaerobically (16). Therefore, it was of interest to determine which cytochromes, in addition to cytochrome o, are present in S. typhimurium and are potentially involved in aerotaxis. Low-temperature reduced-minus-oxidized difference spectra were obtained with whole cells of S. typhimurium grown under four different conditions: (i) aerobically grown, harvested in the exponential phase; (ii) aerobically grown, harvested in the stationary phase; (iii) anaerobically grown with fumarate; and (iv) anaerobically grown with nitrate. Low-temperature (CO-reduced)-minus-(reduced) spectra of the same samples were also prepared. The resulting spectra (data not shown) were similar to published spectra from E. coli grown under similar conditions (12). Cytochrome o contributes to the oxidized-minus-reduced absorption peaks at 556, 536, and 432 nm (5, 12). Additional b-type cytochromes (b_{556} and b_{562}) were present in exponential-phase aerobic cells, and absorption maxima were observed at 556 and 562 nm.

The cytochrome composition changed with the growth conditions (Table 1). Cytochrome d (625 nm) was induced in stationary-phase aerobic cells and in cells grown anaerobically in the presence of fumarate or nitrate. A red shift in the cytochrome d spectrum was often observed in S. typhimurium grown anaerobically in the presence of fumarate or nitrate; however, the red shift was not always observed, and
the reason for the variable results was not apparent. A similar shift in absorbance  
peak (λ_max = 590 nm) attributed to cytochrome a_1 was observed in  S. typhimurium  
from the stationary phase, or after anaerobic growth, but it was absent during aerobic  
exponential growth. A marked increase in absorbance at 556  
nm in  S. typhimurium  ST1 grown anaerobically in medium  
with glycerol and nitrate was presumably due to cytochrome  
b_56, NO_3^-, which is synthesized in  E. coli  under similar  
growth conditions (25). Cytochrome b_56 was prominent in the  
spectra of cells grown anaerobically with fumarate. Only  
minor differences in the cytochrome content of  S. typhimurium  
and  E. coli  were detected, and it is likely that the  
sequence of carriers in the electron transport system is  
similar in these two species.

### DISCUSSION

Cytochrome oxidases can be identified by the ability to  
react with CO when they are in the ferrous state. Cytoc-  
chrome o was the only oxidase that reacted with CO in  
S. typhimurium  ST1 from the early exponential phase of growth  
(Fig. 3) and was the only oxidase detected in these cells by  
the light of CO-inhibited respiration (Fig. 4). This  
study established that although cytochrome o has not  
been reported previously in  S. typhimurium, it is the principal  
nitrate oxide for the respiratory chain in cells grown aerobically.  
Aerobic cells from the stationary phase, where oxygen is  
limiting, and cells grown anaerobically contained an  
additional oxidase, cytochrome d, and cytochrome a_1, which  
may be a component of the cytochrome d complex (20).  
Cytochrome d has a high affinity (K_m = 0.024 μM) for  
oxogen in  E. coli  and apparently scavenges oxygen under  
conditions of limited availability (12, 24). Cytochrome a_1  
binds CO but has not been established as an independent  
oxidase in  E. coli  (10, 22). The synthesis of cytochromes a_1  
and d are regulated coordinately.

We repeated the low-temperature spectra of  E. coli  under  
conditions similar to those used in obtaining the spectra for  
S. typhimurium  and found no major difference between the  
spectra (unpublished data). The similarity of respiratory-  
chain components in  S. typhimurium  and  E. coli  is not  
surprising in view of the close genetic relationship between the  
two species.

The K_0.5 for aerotaxis (0.7 μM) indicates that the aerotaxis  
receptor is a cytochrome. No other class of proteins is  
known to have a K_0.5 for oxygen in the range of 10^{-6} to 10^{-8}  
M. The K_0.5 for aerotaxis and the K_m for respiration were  
similar, and aerotaxis and respiration were both inhibited  
by KCN. These results, together with the evidence for a single  
oxidase in cells from the early exponential phase of growth,  
support the hypothesis that the aerotaxis receptor is cyto-  
chrome o, the terminal oxidase of the respiratory chain.  
Cytochrome o has as its prosthetic group protoporphyrin IX  
instead of the “green heme” of cytochrome a_1. It also differs  
from cytochrome a_1 in its reaction mechanism. Cytochrome o  
from  Viteoscella  spp. can react with oxygen or H_2O_2  
and form a stable intermediate with H_2O_2 at room  
temperature (18). Other evidence that supports the assign-  
ment of cytochrome o as the aerotaxis receptor includes the  
following. Nitrate and fumarate are competitive with oxygen  
in aerotaxis in  S. typhimurium  (16). 2-Heptyl-4-hydroxyqui-  
noline-N-oxide is a noncompetitive inhibitor of both respi- 
ration and aerotaxis in  Bacillus cereus, in which cytochrome  
a-a_1 is the oxidase and receptor for aerotaxis (D. J. Laszlo,  
press). Preliminary experiments to determine whether cyto-  
chrome d is also a receptor for aerotaxis were inconclusive  
and definitive experiments will require adaptation of the  
methodology for aerotaxis to accommodate studies at very  
low oxygen concentrations.

Receptors for chemotaxis to sugars and amino acids are  
either transducing proteins (methyl-accepting chemotaxis  
proteins) or proteins that can interact directly with a trans- 
ducing protein (15, 28). Cytochrome o appears not to be  
a receptor for aerotaxis in the usual meaning of a receptor.  
Recent studies that will be reported elsewhere (J. Shioi  
and B. L. Taylor, J. Biol. Chem., in press) indicate that the  
flow of electrons through the electron transport system and  
the consequent change in proton motive force are the signal  
for aerotaxis. In this respect, and in the mechanism of adapta- 
(21), the pathways for aerotaxis and chemotaxis are  
diverse.

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**TABLE 1.** Cytochrome content of  S. typhimurium  ST1 grown in the  
presence of various electron acceptors

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Cytochrome (nmol/mg of protein)</th>
<th>K_0.5 (μM)</th>
<th>K_m (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential phase</td>
<td>0.10</td>
<td>Not detected</td>
<td>0.07</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>0.18</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.62</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.12</td>
<td>0.07</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Cells were grown and harvested as described in the text. Reduced-minus-oxidized difference spectra were recorded at 25°C, and cytochromes were quantified by using the extinction coefficients of Jones and Redfearn (13) and Daniel (7).


