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Effect of O_2 Limitation on Growth and Respiration of the Wild Type and an Ascorbate-tetramethyl-*p*-phenylenediamine-oxidase-negative Mutant Strain of *Azotobacter vinelandii*

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Abstract

Azotobacter vinelandii strain AVOP (wild type) and an ascorbate-*N,N,N',N'*-tetramethylene-*p*-phenylenediamine oxidase-negative mutant (AV11) were each grown in O_2-limited chemostat cultures. The results showed that the mutant strain grew and used O_2 less efficiently than the wild-type strain. Respiration rates of membrane particles with NADH or malate as the substrate were similar for each strain. Succinate oxidase activity was about fourfold lower in membrane particles prepared from mutant than from wild-type strain. Cyanide at a concentration that completely inhibited ascorbate-TMPD oxidase activity resulted in a 50% inhibition of NADH oxidase activity in membrane particles of AVOP. These data suggest that the cytochrome *o*, a_1, oxidase branch of the respiratory chain may be important in the physiology of *A. vinelandii* under O_2-limiting growth conditions.

Key Words: *Azotobacter;* respiration; nitrogen fixation.

Introduction

Azotobacter vinelandii is a Gram-negative, obligately aerobic bacterium that fixes atmospheric nitrogen. The reduction of N_2 is catalyzed by an oxygen-sensitive enzyme system, nitrogenase (Yates and Planque, 1975). During growth, the bacterium must maintain its intracellular oxygen concentration low enough to protect nitrogenase but high enough to provide for adequate

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ATP synthesis. Thus, the respiratory chain of *A. vinelandii* has two functions: (1) to conserve energy by oxidative phosphorylation (Ackrell and Jones, 1971a; Downs and Jones, 1975; Eilerman *et al.*, 1970; Laane *et al.*, 1979) and (2) to protect nitrogenase by removal of excess oxygen (Dalton and Postgate, 1969b; Drozd and Postgate, 1970; Hill *et al.*, 1972; Jones *et al.*, 1973). The respiratory chain contains hydrogenase, flavin-dependent NADH and malate dehydrogenases, ubiquinone-8, and various cytochromes, (a_1, b_1, c_4 and c_5, *d*, and *o*), (Ackrell and Jones, 1971a; Jones and Redfern, 1966, 1967). These components are arranged in a branched electron transport system (Fig. 1) with cytochrome *d* and cytochromes *o*, a_1 as the terminal oxidases (Ackrell and Jones, 1971a; Downs and Jones, 1975; Laane *et al.*, 1979; Sagi-Eisenberg and Gutman, 1979). The oxidation of physiological substrates (NADH or malate) proceeds mainly via the cytochrome *d* oxidase branch (Ackrell and Jones, 1971a; Hoffman *et al.*, 1979, 1980a, b; Jones and Redfern, 1966; Sagi-Eisenberg and Gutman, 1979) which is relatively insensitive to inhibition with cyanide (K_i > 115 μM) (Ackrell and Jones, 1971a; Jones, 1973; Jones and Redfern, 1966). The oxidation of artificial electron donors (ascorbate-tetramethyl-*p*-phenylenediamine) (TMPD) proceeds via the cytochrome *o*, a_1, oxidase branch (Hoffman *et al.*, 1979, 1980a, b) and is readily inhibited by cyanide (K_i 0.5 μM) (Ackrell and Jones, 1971a; Jones, 1973; Jones and Redfern, 1966).

Mutants of *A. vinelandii* that lack detectable ascorbate-TMPD oxidase activity have recently been isolated (Hoffman *et al.*, 1979, 1980a, b). These mutants have similar growth rates under nitrogen-fixing conditions with normal oxygen tensions as well as similar respiration rates with NADH or malate and comparable energy conservation efficiencies as determined by the amount of phosphate esterified (P/O ratio) or protons extruded per oxygen atom consumed (H/O ratio) (Hoffman *et al.*, 1979, 1980a, b). It was concluded that the cytochrome *o*, a_1 oxidase branch does not appreciably contribute to respiration or energy conservation (Hoffman *et al.*, 1979, 1980a,

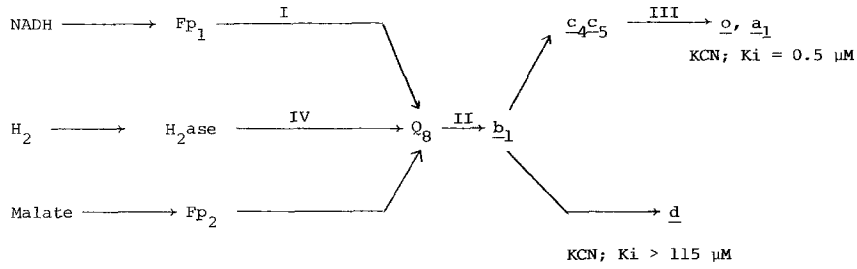

Fig. 1. Schematic diagram of the respiratory chain of *Azotobacter vinelandii* adapted from Laane *et al.* (1979). Symbols: Fp, flavoprotein; H_2ase, hydrogenase; Q_8, ubiquinone; *a, b, c, d, o,* cytochromes; roman numerals, proton-translocating sites.

b). However, other workers have shown that the contribution of the cytochrome *o*, a_1 branch to respiration and energy conservation increases when oxygen becomes limiting (Ackrell and Jones, 1971b; Dalton and Postgate, 1969b; Haaker and Veeger, 1976; Lisenkova and Khnel, 1967). We decided to study the effect of oxygen limitation on growth and respiration of the wild-type strain and an ascorbate-TMPD oxidase-negative mutant of *A. vinelandii*.

Methods

Strains AVOP (wild-type) and AV11 (ascorbate-TMPD oxidase-negative mutant) (Hoffman *et al.*, 1979) of *A. vinelandii* were each grown in an oxygen-limited chemostat (New Brunswick Scientific Co., Model C30, Edison, New Jersey) using a modified Burk's minimal medium (Strandberg and Wilson, 1968) with sucrose as the energy source and atmospheric nitrogen as the nitrogen source. Each culture was grown at a dilution rate of 0.1 per hour using a Buchler peristaltic pump (Fort Lee, New Jersey) to control the flow rate. Air was supplied to the culture to provide an oxygen influent rate of 32 nmol $\cdot$ l^{-1} $\cdot$ hr^{-1}. Air influent rate was regulated using a calibrated Gilmont Instruments flowmeter (Great Neck, New York). Medium in the reservoir was bubbled with oxygen-free argon to reduce its dissolved oxygen concentration. Oxygen limitation was verified by showing that the steady-state cell concentration was not affected by doubling the concentration of the medium components but was decreased when oxygen influent rate was decreased (Dalton and Postgate, 1969a). Total gas influent rate was maintained at a constant value by using an auxilary supply of nitrogen gas. After four turnovers of the culture volume, steady state was verified when the pH, cell concentration, and dissolved oxygen concentration remained constant for two additional turnovers of the culture volume.

The cell density of the culture was determined from a standard curve relating optical density to the viable number of cells. Dissolved oxygen concentration was determined polarigraphically (Dalton and Postgate, 1969a, b). The electrode was sterilized by 30-min incubation in each of the following solutions: 4% (v/v) H_2O_2 and 70% (v/v) isopropanol. The pH was determined with an autoclavable electrode (New Brunswick Scientific Co., Inc.). Culture purity was confirmed by microscopic analysis and plating aliquots onto nutrient agar medium (Difco).

Membrane particles were prepared from effluent samples collected on ice under an argon atmosphere. Bacterial cells were collected by centrifugation (7000 $\times$ *g*, 15 min, 4°C), resuspended in an equal volume of 20 mM phosphate buffer (pH 7.4), and stored at -20°C. Membrane particles were prepared as described by Ackrell and Jones (1971a) except that the

membranes were pelleted by centrifugation at 100,000 × *g* for 90 min. Respiration rates and cytochrome levels of membrane particles prepared from fresh or frozen cells as well as from effluent cells or cells collected from the vessel were identical. Oxygen uptake by membrane particles was measured polarigraphically (Hoffman *et al.*, 1979). When KCN was present, it was allowed to react 1 min before addition of the substrate. Types and amounts of cytochromes were determined by difference spectroscopy (Hoffman *et al.*, 1979).

Results and Discussion

The steady-state cell density of the mutant (0.8×10^8 cells/ml) was about one-half that obtained by the wild-type strain (1.5×10^8 cells/ml). The dissolved oxygen concentration of the medium was undetectable (< 1 μM) in cultures of AVOP but was 27 μM for cultures of AVII.

The respiration rate of membrane particles of AVOP or AVII were similar when NADH or malate served as the electron donor (Table I). The rate for succinate was about four times higher in membrane particles from AVOP than from AVII. Oxygen uptake was not observed with membrane particles of AVII when ascorbate-TMPD was the substrate, showing that a revertant was not selected. The addition of KCN at a level that completely inhibited O_2 uptake with ascorbate-TMPD resulted in a 50% inhibition in the respiration rate with NADH using membrane particles prepared from AVOP (data not shown). This verifies that the cytochrome *o*, a_1 branch was participating in the oxidation of NADH (Ackrell and Jones, 1971a; Jones, 1973; Jones and Redfern, 1966). The levels of *c* and *o*-type cytochromes were slightly higher and levels of *b*-type cytochromes were lower in membrane particles of AVII than those of AVOP, while those of the *d*-type cytochromes

Table I. Respiration Rates of Membrane Particles of AVOP and AV11 Grown Under O_2 Limitation[a]

Substrate	μmol of O_2 consumed/mg protein/min	
	AVOP	AV11
NADH	1.33	1.15
Malate	0.40	0.52
Succinate	0.20	0.05
Ascorbate + TMPD	0.15	<0.01

[a]Each strain was grown in continuous culture at a dilution rate of 0.1/hr and O_2 influent rate of 4.4 ml/min.

Table II. Levels of Cytochromes in Membrane Particles Prepared from AVOP and AV11 Grown Under O_2 Limitation[a]

Cytochrome type[b]	nmol/mg of protein in strain	
	AVOP	AV11
c	0.56	0.73
b	0.54	0.42
d	0.28	0.24
o	0.07	0.10
a	*t*[c]	*t*

[a]See the footnote of Table I for growth conditions.
[b]See Hoffman *et al.* (1979) for wavelength pairs, extinction coefficients, and correction for overlapping peaks.
[c]t = detectable as a shoulder but not quantifiable.

were similar in both strains (Table II). Other workers (Haaker and Veeger, 1976), using a different strain of *A. vinelandii,* reported that *c* and *o* type cytochromes are present in greater amounts when grown under oxygen-limiting conditions.

It was previously reported that the cytochrome *o,* a_1 oxidase branch did not appreciably contribute to energy conservation or respiration since the loss of ascorbate-TMPD oxidase activity did not affect growth rate, respiration rates, and H/O or P/O ratios (Hoffman *et al.,* 1979, 1980a, b). However, these studies were conducted under conditions where the participation of the cytochrome *o,* a_1 oxidase branch would not be expected, i.e., at non-growth-limiting concentrations of O_2 (Ackrell and Jones, 1971b; Dalton and Postgate, 1969b; Haaker and Veeger, 1976). Our results show that the ascorbate-TMPD oxidase-negative mutant grew and used oxygen less effectively than the wild-type strain when the O_2 concentration was growth-limiting. These data support the contention that the cytochrome oxidase *o,* a_1 branch is important in respiration under O_2-limiting condition. (Ackrell and Jones, 1971b; Haaker and Veeger, 1976). This may be important in understanding the role *A. vinelandii* plays in N_2 fixation in soil environments where adaption to changing oxygen concentrations would be essential to the survival of the organism.

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