Evidence for Carbon Monoxide Insensitive Respiration in the Aerobic Nitrogen Fixing Bacteria *Azotobacter vinelandii* OP and *Xanthobacter autotrophicus* GZ 29

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In intact cells of the non-gummy *Azotobacter vinelandii* OP CO-insensitivity of the respiratory activity increased with decreasing dissolved oxygen tension in the bacterial suspension. Upon changing from low to high aeration conditions the CO-sensitive respiration was restored. Measurement of the oxidative activity of small particles of *A. vinelandii* OP with NADH, ascorbate-DCPIP and ascorbate-TMPD as substrate in the presence and absence of CO indicated that the CO-insensitive site is probably identical with cytochrome a_1, which preferably is reduced by electrons from ascorbate-DCPIP. Small particles of the gum producing *A. vinelandii* NCIB 8660 appeared to be more sensitive towards CO with ascorbate DCPIP than the non-gummy *A. vinelandii* OP. In small particles the CO-insensitive DCPIP oxidase was present irrespective of the dissolved oxygen tension during cell growth. In intact cells, however, CO-insensitivity was only expressed at low dissolved oxygen tension when electrons are directed to cytochrome a_1/o in the branched respiratory chain. Intact cells of *Xanthobacter autotrophicus* GZ 29 exhibited a similar CO-insensitive respiration as *A. vinelandii* OP.

Introduction

In respiratory chains terminal oxidases are classically defined as heme proteins which are readily complexed in the presence of carbon monoxide (CO). The CO-enzyme complex of terminal oxidases exhibits distinct spectroscopic properties, is enzymically inactive and appears to be photodissociable [1, 2]. Originally, the CO-binding property was almost exclusively associated with terminal oxidase cytochromes. However, CO-binding properties of b-type and c-type cytochromes have been discovered in various organisms [3, 4]. On the other hand, some investigators reported CO-insensitive terminal oxidase activity. Kikuchi and Motokawa [5] found that preparations of terminal oxidases from light-grown *Rhodopseudomonas sphaeroides* and dark-grown *Rhodospirillum rubrum* cells, were not significantly inhibited by CO. Zavarzin and Nozhevnikova [6] and Meyer and Schlegel [7] reported CO-tolerant respiratory systems in aerobic CO-oxidizing bacteria. Appleby [18] found a CO-insensitive respiration in *Rhizobium japonicum* bacteroids at air oxygen tension whereas free living cells of the same specimen responded to the presence of CO with a pronounced decrease of respiratory activity. Both types of cells differed with respect to their respiratory chain components [18]. Sargent and Tayler [20] measured CO-insensitive respiration in the alga *Chlorella pyrenoidosa* and Meyer and Jones [21] reported on a CO-insensitive oxidase in *Kurthia zopfii*; the latter terminal oxidase was used only under low aeration conditions. In this paper we give evidence for a CO-tolerant respiratory activity at 0.20 bar O_2 in the aerobic nitrogen-fixing bacteria *Azotobacter vinelandii* OP and *Xanthobacter autotrophicus* GZ 29.

Abbreviations: TMPD, N,N,N′,N′,-tetramethyl-*p*-phenylenediamine dihydrochloride; DCPIP, 2,6-dichlorophenol indophenol.

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Materials and Methods

Azotobacter vinelandii OP (ATCC 13 705) and NCIB-strain 8660 were grown batchwise in a 11 Quickfit fermentor at 30 °C in modified Burk's medium [8] with NH_4Cl or under nitrogen fixing conditions. *Xanthobacter autotrophicus* GZ 29 (formerly *Corynebacterium autotrophicum,* see [9]) was grown autotrophically or heterotrophically with NH_4Cl or N_2 in batch culture as described previously [10] or autotrophically in continuous culture under N_2-fixation conditions [11]. Dissolved oxygen tensions were measured polarographically. The oxygen concentration in N_2-fixing cultures of *X. autotrophicus* was

kept constant at about 0.03 bar O_2 by means of an automatic oxygen supply control system [11].

Respiratory rates were measured polarographically (Yellow Springs Instruments, model 53); cells were diluted to an appropriate optical density with 50 mM K-PO_4 buffer, pH 7.0, containing 0.5% sucrose. Respiration rates of autotrophically grown *X. autotrophicus* cells were estimated polarographically, too, with 0.20 bar H_2, 0.05 bar O_2 and 0.75 bar argon. Carbon monoxide (CO) inhibition of heterotrophically grown intact cells was measured under an atmosphere of 0.50 bar air and 0.50 bar CO at 30 °C. With autotrophically grown cells CO inhibition was estimated under an atmosphere of 0.20 bar H_2, 0.05 bar O_2, 0.25 bar argon and 0.50 bar CO.

A. vinelandii small particles were prepared as described by Jones and Redfearn [12]. Oxidase activities were measured polarographically (YSI, model 53) with NADH, ascorbate-TMPD and ascorbate-DCPIP as substrates; the tests were carried out according to Jones and Redfearn [12, 13] except that oxidase reactions were measured at optimal pH values (Fig. 2). For CO inhibition experiments, the reaction mixture was sparged 1 min with 0.50 bar CO, 0.20 bar O_2 and 0.30 bar argon (NADH oxidase) or 0.80 bar CO and 0.20 bar O_2 (TMPD and DCPIP oxidase). The reaction was started by addition of substrate.

Absorption spectra were recorded with a Zeiss DMR 10 spectrophotometer at room temperature using small particles prepared from *A. vinelandii*. The difference spectra were measured with $Na_2S_2O_4$-reduced minus oxidized, ascorbate-DCPIP-reduced minus oxidized and ascorbate-TMPD-reduced minus oxidized particles. Besides, CO reduced difference spectra were measured at room temperature with the three above mentioned reductants.

Results

N_2 and NH_4^+ grown intact cells of *A. vinelandii* OP, a non-gummy chromogenic strain of *A. vinelandii* (Bush and Wilson, 1959), exhibited a CO-insensitive respiration when kept at low dissolved oxygen tension. Fig. 1 shows a batch growth experiment with *A. vinelandii* cells under air (N_2 fixation conditions) without pO_2 regulation. With increasing cell density a dramatic decrease of CO sensitivity of the respiratory system occurred. At high optical density respiration appeared to be completely insensitive to the inhibitory effect of CO. Since the polarographically measurable dissolved oxygen tension (Fig. 1, dashed line) decreased to zero and since the growing culture was increasingly subjected to oxygen-limited conditions we assumed a correlation between low oxygen tension and CO-insensitive respiration in *A. vinelandii* cells.

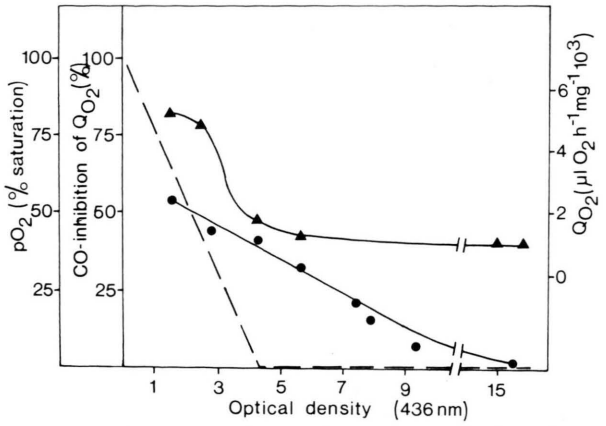

Fig. 1. Batch culture of *Azotobacter vinelandii*. Development of carbon monoxide insensitive respiration. Cells were grown in a 11 Quickfit fermentor with sucrose under N_2-fixation conditions. The dissolved oxygen tension was followed polarographically. Respiration rates were estimated polarographically with air (▲) or 50% air plus 50% carbon monoxide expressed as % inhibition (●); (– – –) dissolved oxygen tension.

In fact, as shown by high aeration to low aeration (and vice versa) changeover experiments with polarographically controlled dissolved oxygen tension (Table I) the CO-tolerant respiration was operative at low oxygen concentration. On the other hand, CO sensitivity was fully restored at high dissolved oxygen tension. The increase or decrease of CO sensitivity required a 2 – 3 h time period to be fully manifested. Besides, upon changing from high to low aeration conditions, the respiration rate decreased about 5-fold from approximately 5000 µl O_2 per h and mg protein to about 1000 µl O_2 per h and mg protein (Fig. 1). The reverse effect was measured upon change from low to high aeration conditions.

In order to elucidate the operative regulatory mechanism of alteration of CO sensitivity in *A. vinelandii* OP we measured respiratory activities with and without CO using small particles of aerobically (CO-sensitive) and microaerobically (CO-insensitive) grown cells with NADH, ascorbate-TMPD and as-

Table I. CO inhibition of respiration in *Azotobacter vinelandii* OP cells at different dissolved oxygen tensions.

Optical density of cell suspensions [436 nm]	pO_2 [% saturation]	Incubation before measurement [h]	CO inhibition of Q_{O_2} [%]
0.30	1	–	2 – 5
0.77	50	3.3	59
1.95	1	6.3	2 – 5
2.40	50	3.5	69

corbate-DCPIP as substrates. Activities were measured at the appropriate pH-optima (Fig. 2). While oxidation of NADH (pH 7.6) and DCPIP (pH 7.25) were maximal at alkaline pH values, TMPD was oxidized with maximal rate at pH 6.5. The pH curve with TMPD as substrate exhibiting two maxima was measured consistently using different preparations of small particles.

Oxidative activities of *A. vinelandii* OP small particles with and without inhibitor are given in Table II. With both types of particles NADH oxidase was largely inhibited in the presence of 0.50 bar CO or 50 μM KCN; maximal NADH oxidase activity was measured with particles from CO-sensitive cells. However, with ascorbate-DCPIP and ascorbate-TMPD as substrate maximal activity was estimated with particles from CO-insensitive cells. Upon sparging the reaction mixture with 0.80 bar CO and 0.20 bar O_2, a 50% inhibition (particles from CO-sensitive cells) and 30% inhibition (particles from CO-insensitive cells) of TMPD oxidase was measured whereas, surprisingly, DCPIP oxidase of particles from both types of cells was only inhibited by 0 – 5%. The oxidation of both substrates TMPD and DCPIP was nearly completely abolished by 50 μM KCN; ATP (1 mM) inhibited both reactions to about 50%. NADH oxidase was inhibited by about 20% in the presence of 50 μM KCN.

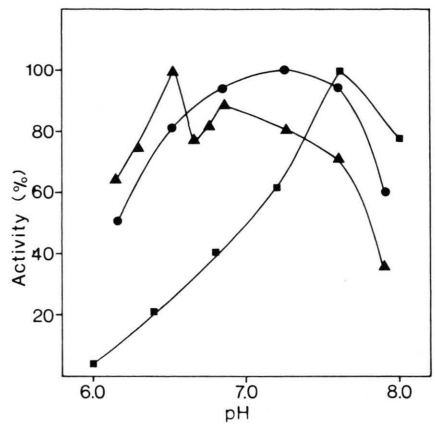

Fig. 2. pH optima of different oxidases of *Azotobacter vinelandii* OP small particles. Small particles were prepared from CO-insensitive cells according to Jones and Redfearn [12]. Oxidase activities were measured polarographically as described under materials and methods. (▲) ascorbate-TMPD oxidase; (●) ascorbate-DCPIP oxidase; (■) NADH oxidase.

Since Jones and Redfearn [13] reported on a CO-sensitive respiration (56% inhibition at a pO_2 of

Table II. Effect of terminal oxidase inhibitors on substrate oxidation by "small particles" of *Azotobacter vinelandii* OP.

Substrate	Additions	"CO-sensitive" particles		"CO-insensitive" particles	
		μmol substrate $mg^{-1}\ h^{-1}$	% activity	μmol substrate $mg^{-1}\ h^{-1}$	% activity
NADH	–	187	100	118	100
	50% CO	21	11	21	18
	50 μM KCN	48	26	21	18
Ascorbate-DCPIP	–	6	100	76	100
	80% CO	6	100	75	99
	50 μM KCN	0	0	3	4
Ascorbate-TMPD	–	14	100	127	100
	80% CO	7	50	88	69
	50 μM KCN	0.5	4	2	2

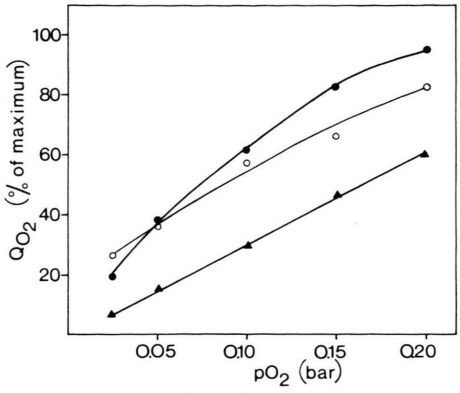

Fig. 3. CO inhibition of small particles from *Azotobacter vinelandii* strain OP and NCIB 8660 at various oxygen partial pressures. Small particles of both strains of *A. vinelandii* were prepared from microaerobically grown cells according to Jones and Redfearn [12]. Oxidase activities were measured polarographically as described under materials and methods. (●) particles (0.33 mg protein) from *A. vinelandii* OP with ascorbate-DCPIP as substrate, (○) particles (0.34 mg protein) from *A. vinelandii* NCIB 8660 with ascorbate-DCPIP as substrate, (▲) particles from *A. vinelandii* OP with ascorbate-TMPD as substrate. Particles from *A. vinelandii* NCIB 8660 exhibited a similar response to CO with TMPD (not shown) as particles from strain OP.

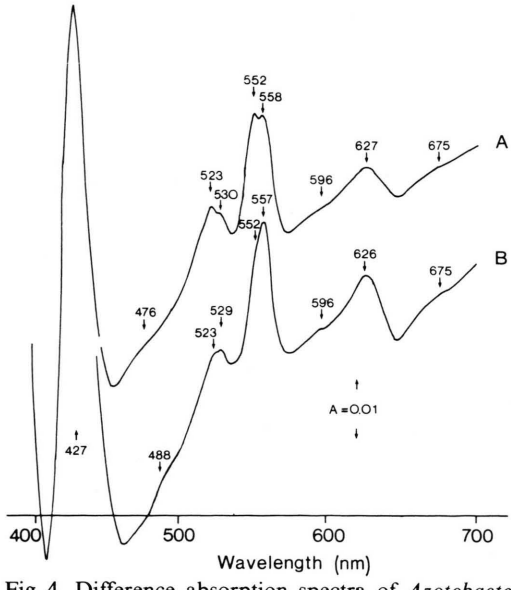

Fig. 4. Difference absorption spectra of *Azotobacter vinelandii* OP small particles. Small particles were prepared according to Jones and Redfearn [12] from aerobically grown cells (CO-sensitive, 1.3 mg protein/ml, curve B) and from microaerobically grown cells (CO-insensitive, 1.1 mg protein/ml, curve A). The spectra were recorded at room temperature with a Zeiss DMR 10, dithionite reduced minus oxidized.

0.16 bar) of small particles from *A. vinelandii* strain NCIB 8660 we estimated, for proper comparison, the respiratory activity of both strains of *A. vinelandii* at various dissolved oxygen tensions in the presence of 0.80 bar CO. As indicated in Fig. 3, small particles of *A. vinelandii* NCIB 8660 exhibited an inhibition of around 20% at 0.20 bar O_2, whereas those of *A. vinelandii* OP were inhibited only to a maximum of 5% with ascorbate-DCPIP as the electron donor system. Using ascorbate-TMPD as substrate particles of both strains showed nearly similar sensitivity towards CO. On the other hand, intact cells of *A. vinelandii* NCIB 8660, when grown under the same conditions as given in Fig. 1 for *A. vinelandii* OP, showed a comparable shift to a relatively CO-insensitive respiration as intact cells of *A. vinelandii* OP (Fig. 1).

From the results given in Fig. 3, it appears that the CO inhibition of the terminal oxidases follows the well known competitive pattern with respect to O_2 [17], however, at 0.20 bar O_2, a nearly completely CO-insensitive respiration occurs with particles from *A. vinelandii* OP.

The results given in Table II show largely increased TMPD oxidase and DCPIP oxidase activity at low dissolved oxygen tension. This feature is in agreement with a branched respiratory chain in *A. vinelandii* [13 – 15]. As will be discussed in the discussion section, it seems likely that the CO-insensitive site is probably identical with the cytochrome a_1-site. To get additional information on this site, we subjected small particles of *A. vinelandii* OP to spectral analysis. In Fig. 4 dithionite reduced minus air oxidized optical spectra of both kinds of particles are shown. The spectra exhibited quite normal features (for comparison see ref. [13]) with increased cytochrome a_2 content in highly aerated cells and larger amounts of c-type cytochromes in microaerobically grown cells. CO difference spectra (not shown) were recorded with both kinds of small particles and different reductants. With dithionite, ascorbate-TMPD and ascorbate-DCPIP as reductants, cytochromes o, a_1 and a_2 (as well as cytochrome b) complexed readily with CO revealing characteristic absorbance at the appropriate wavelengths. However, following the CO complexation process of small particles in a cuvette over a time period of 10 min (in 2 min intervals) the CO difference spectra of DCPIP reduced particles exhibited a pronounced decrease of absorbance in the 431 nm to 456 nm region. Additional changes occurred in the 564 nm region. With dithio-

nite and TMPD as reductants no comparable changes were observed.

Intact cells of *Xanthobacter autotrophicus* GZ 29 exhibited a similar CO-insensitive respiration as *A. vinelandii* OP cells. The CO-insensitive system was found in heterotrophically or autotrophically grown cells with NH_4Cl or N_2 as nitrogen source at low oxygen concentration. Both *A. vinelandii* and *X. autotrophicus* were able to grow in the presence of 50% CO under microaerobic conditions.

Discussion

The aeration changeover experiments with intact cells of *Azotobacter vinelandii* OP showed that the respiration rate decreased about 5-fold upon change from high to low aeration conditions. The reverse effect was measured upon change from low to high aeration conditions. A similar effect on the Q_{O_2} of intact cells of *A. vinelandii* NCIB 8660, a gum producing strain of *A. vinelandii,* was observed by Ackrell and Jones [14]. The shift was reported to occur even in the presence of 200 μg chloramphenicol per ml indicating that the observed alteration of the respiration rate was not linked to protein synthesis. However, at the same time Ackrell and Jones [14] measured a pronounced increase of concentrations of c-type, b-type and o-type cytochromes under oxygen limited conditions.

Concerning the CO sensitivity of the respiratory activity in both strains of *A. vinelandii* pronounced differences were found between the two strains (Fig. 3). It seems likely that the ascorbate-DCPIP oxidase of *A. vinelandii* strain NCIB 8660 and strain OP differ in the affinity towards oxygen, such that DCPIP oxidase of strain OP binds oxygen more readily than that of strain NCIB 8660 thus accounting probably for a higher oxidized and less CO binding state of the ascorbate-DCPIP oxidase in *A. vinelandii* OP.

The presented results (Table II) are in agreement with a branched respiratory chain in *A. vinelandii* [13 – 15] in which, at low dissolved oxygen tension, electrons are directed to the cytochrome b → cytochrome c_4/c_5 → cytochrome a_1, o pathway. On the other hand, under high aeration conditions, the cytochrome b → cytochrome a_2 pathway is used accompanied by low TMPD oxidase and low DCPIP oxidase activity. In any case, CO insensitivity in *A. vinelandii* OP was associated with DCPIP oxidase. Recently, Young and Jurtshuk [16] showed that TMPD oxidase of *A. vinelandii* OP consists of c-type cytochrome(s) and cytochrome o. This site was found to be considerably inhibited by CO (Table II). Thus, it can be inferred that DCPIP directs its electrons preferentially to cytochrome a_1 which then represents the CO-insensitive site. For cytochrome a_1 Meyer and Jones [19] established a considerably lower K_M value for oxygen compared to the other cytochrome oxidases like a_3, o, aa_3. This lower K_M may account for the diminished CO-sensitivity of cytochrome a_1 which may be kept in a higher oxidized and less CO accessible state.

A CO-insensitive DCPIP oxidase (at 0.20 bar O_2) was not only present in small particles of *A. vinelandii* OP prepared from microaerobically grown cells but appeared also in particles from cells wich were grown under high dissolved oxygen tension. Intact cells, however, exhibited a CO-sensitive respiration under high aeration conditions because such cells use preferentially the CO-sensitive cytochrome a_2-pathway. Since the CO-insensitive respiratory system is present in any kind of intact *A. vinelandii* cells but is only expressed under microaerobic conditions, it can be concluded that the response of respiration of intact *A. vinelandii* OP cells to the presence of CO is solely dependent on the direction of the electron flux in the branched respiratory chain (compare ref. [21]).

With small particles of *A. vinelandii* OP, CO difference spectra were recorded using ascorbate-DCPIP as reductant. The spectra showed shifts in absorbance in the 431 nm region as well as at 546 nm within a 10 min incubation time. Although it was not possible to attribute the spectral changes with DCPIP unequivocally to the 432 nm peak of the CO-cytochrome a_1 complex it seems likely that binding of CO to the terminal oxidase is a variable process which is known to depend on the redox state of the cytochrome (see also ref. [17]). In *A. vinelandii* OP the redox state of cytochrome a_1 may be altered under turnover conditions in such a way that the electrons from DCPIP keep the terminal oxidase cytochrome a_1 in a slightly reduced state in which CO has apparently no access to the heme group of the cytochrome.

Acknowledgements

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