INDUCTION BY CYANIDE OF CYTOCHROME *d* IN THE PLASMA MEMBRANE OF *PARACOCCUS DENITRIFICANS*

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1. Introduction

Paracoccus denitrificans can synthesize various electron carriers depending on the conditions of growth. When grown aerobically, *P. denitrificans* possesses a mitochondrion-like electron transport chain [1,2]. *P. denitrificans* can also develop an anaerobic electron transport chain when growing in the presence of either nitrate or nitrite as terminal electron acceptors [1,3]. The purpose of this paper is to show that when grown aerobically in the presence of cyanide, *P. denitrificans* incorporates an additional redox carrier, namely cytochrome *d*, into the cytoplasmic membrane. This carrier partially replaces cytochrome oxidase aa_3, and provides an alternative route of electron transport. Such changes in the redox carrier pattern reflect attempts by the bacterium to overcome significant decreases in respiration rates, since the synthesis of cytochrome *d* is also accompanied by an increased resistance to respiratory inhibitors and a greater affinity for O_2.

Promotion of the synthesis of cytochrome *d* has already been observed in *Achromobacter* or *Escherichia coli* cells grown in the presence of cyanide [4,5]. A close relationship was observed [4] between the amount of cytochrome *d* and the resistance of respiration of *Achromobacter* cells to cyanide. In these cells, cytochrome *d* would be the major terminal oxidase.

Abbreviations: HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; DBMIB, 2,5-dibromo-6-isopropyl-3-methylbenzoquinone; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethane sulfonic acid

2. Materials and methods

2.1. *Growth conditions and membrane preparation*

P. denitrificans (strain 381 DSM 65) was grown in the medium [6], at 27°C, in a 15 l fermenter, with succinate as carbon source and either O_2 or nitrate as terminal electron acceptor. Potassium cyanide (300 μM), when present, was added to the culture medium prior to inoculation, as an aqueous, unbuffered solution. The cultures were maintained at pH 7.5–8.5 by additions of NaOH, and the cyanide concentration checked and maintained at 250–300 μM during the growth period (6 days). The cells were collected by centrifugation (1000 × *g*, 45 min) washed twice with distilled water and the pellet diluted 4-fold in a 10 mM Hepes, 2 mM $MgCl_2$ buffer (pH 7). The cells were disrupted by sonication, 30 s × 4, at 0°C, with a Branson sonifier at maximum output. The suspension was then centrifuged at 10 000 × *g*, 20 min to remove intact cells and cell debris. The pellet was treated again by sonication and centrifuged at low speed. The resulting pellet was discarded. The pooled supernatant fluids were centrifuged at 105 000 × *g*, 3 h. After removal of the fluffy layer, the reddish pellet of membranes was resuspended in 10 mM Hepes (pH 7). The membranes were stored at −20°C as 1.5 ml aliquots. Protein was determined as in [7] with bovine serum albumin (Sigma, fraction V) as a standard.

2.2. *Respiration measurements*

The oxygen uptake was measured amperometrically at 25°C with a Clark-type oxygen electrode (Yellow Springs Instruments, OH) equipped with a standard

Teflon membrane as in [8]. The incubation mixture contained 1 mg protein with 10 mM Hepes buffer (pH 6.5) and 2 mM $MgCl_2$ in total vol. 1 ml. The reaction was initiated by the addition of 30 μl NADH to give 2.5 mM NADH final conc.

The app. K_m for oxygen of isolated membrane vesicles was determined by measuring oxygen uptake at a low initial O_2 concentration, which was obtained by bubbling N_2 into the 1 ml respiration medium. When O_2 was reduced to $\leqslant 30$ μM, the sensitivity of the recorder was increased so that a full scale deflection corresponded to $\leqslant 30$ μM O_2. The anaerobic suspension of membrane particles was allowed to equilibrate with the respiration medium; the reaction started by addition of the substrate and followed until O_2 was exhausted.

The chart speed, initially at 25 mm/min, was raised to 250 mm/min when the kinetics of oxygen uptake changed from zero order to first order. The rates of oxygen uptake were calculated from the tangents on the traces and were plotted as a function of O_2 concentration in the medium, taking 240 μM for the air-saturated medium, at 25°C.

2.3. *Cyanide*

Cyanide was determined colorimetrically by the picric acid method [9] as in [10].

2.4. *Spectrophotometric determinations*

Low temperature absorption difference spectra were recorded with a Cary model 15 spectrophotometer equipped with sample holders containing liquid N_2 and cuvettes of 2 mm lightpath.

3. Results and discussion

3.1. *Effect of cyanide on growth of* P. denitrificans

Cyanide, at concentrations increasing to 300 μM KCN, was added to an aerobic culture of *P. denitrificans* prior to inoculation. Cyanide at 10 μM or 30 μM had little effect on the growth pattern, the mean generation time and the final cell yield. After 40 h growth, an A_{550} of 5.9 was observed in the culture containing 30 μM KCN, compared to 6.5 for the cells grown in the absence of cyanide. On the other hand, 100 μM KCN caused a lag in the onset of growth and increased the mean generation time 2–3-fold. The latter culture reached a final A_{550} of 4.0. In the presence of 300 μM KCN, no growth was observed during a period of 40 h. However, when the culture was kept at 27°C and the cyanide maintained at 250–300 μM by repeated additions of KCN growth eventually resumed after a lag of several days and reached A_{550} 1.0 after 6–7 days.

Low temperature absorption difference spectra of whole cells of *P. denitrificans* indicated that increasing cyanide concentrations in the culture medium resulted in a progressive decline of the absorption band at 604 nm (the α band of cytochromes $a+a_3$) and a parallel increase of a broad band at 628–632 nm, which is assigned in [11] to cytochrome d (a_2).

3.2. *Inhibitor studies*

The particulate fractions obtained from cells grown in the absence (S–O membranes) or in the presence of 300 μM KCN (S–O–CN membranes) actively oxidized NADH but differed in their sensitivity towards the classical inhibitors of the mitochondrial respiratory chain. With S–O membranes titration of NADH oxidation with rotenone, antimycin A, HOQNO and cyanide proved monophasic, indicating only one inhibition constant for each inhibitor (fig.1). The following K_i values were determined: rotenone (10^{-6} M or 0.4 μg/mg protein), antimycin A (2×10^{-7} M or 0.1 μg/ml protein), HOQNO (10^{-4} M) and KCN (10^{-5} M). These values are comparable to those obtained with mitochondria.

Figure 2 shows that, contrary to S–O membranes,

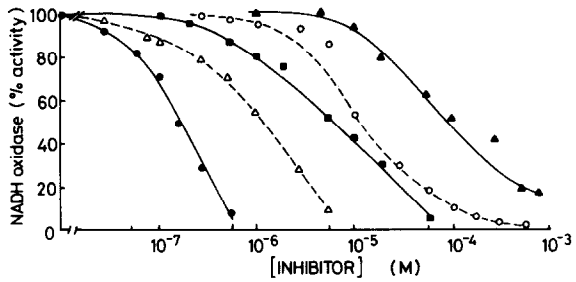

Fig.1. Titration with inhibitors of NADH oxidase activity in plasma membrane vesicles of *P. denitrificans*. (A) Membranes were isolated from cells grown on succinate–O_2 (S–O membranes) as in section 2. The S–O membranes (1.2 mg/ml) were preincubated for 5 min with the various inhibitors before starting the reaction by addition of 3 mM NADH. Inhibitors: (●—●) antimycin A; (△—△) rotenone; (■—■) DBMIB; (○- -○) KCN; (▲—▲) HOQNO.

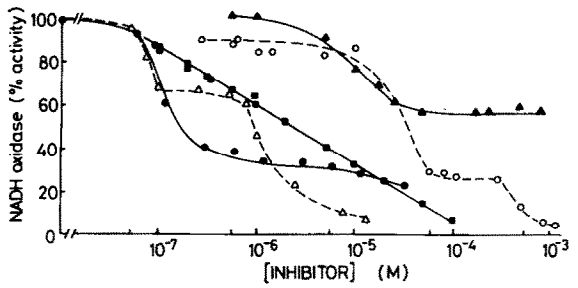

Fig.2. Titration with inhibitors of NADH oxidase activity in plasma membrane vesicles of *P. denitrificans*. Membrane vesicles were isolated from cells grown aerobically for 1 week on succinate in the presence of 300 μM KCN. The membranes (1 mg protein/ml) were preincubated for 5 min with the various inhibitors before starting the reaction by addition of 2.5 mM NADH. Inhibitors: (●—●) antimycin A; (△—△) rotenone; (■—■) DBMIB; (○- -○) KCN; (▲—▲) HOQNO.

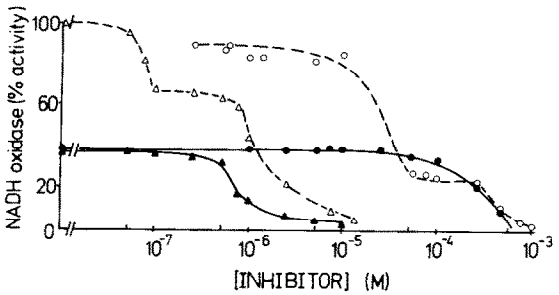

Fig.3. Sensitivity of the second respiratory pathway to rotenone and cyanide. The main respiratory chain was blocked by either 10^{-4} M KCN (for titration with rotenone (▲—▲)) or 2.5×10^{-6} M antimycin A (for titration with KCN (●· · ·●)). S–O–CN membranes (1 mg/ml) were preincubated for 5 min with: (△- -△) rotenone; (▲—▲) 10^{-4} M KCN + rotenone; (○- -○) KCN; (●—●) 2.5×10^{-6} M antimycin A + KCN. Thereafter 2.5 mM NADH was added to start the reaction.

S–O–CN membranes exhibited biphasic titration curves for rotenone and KCN, when NADH was being oxidized. Antimycin A and HOQNO caused only partial inhibition of respiration. In the case of antimycin A, a 'plateau' region is reached at 70% inhibition of NADH oxidase activity. Concentrations in antimycin A as high as 2.5×10^{-5} M did not cause any further inhibition. It can be seen that the inhibition by both antimycin A (up to 5×10^{-5} M) and by KCN (5×10^{-5} M) reached the same level. The second site titrated by KCN indicated K_i 5×10^{-4} M. About 1 mM cyanide was required to block respiration by 95%.

These results suggest that two electron transport chains are operative in S–O–CN membranes, one sensitive to low cyanide concentrations, and the second one, alternative to cytochrome aa_3, very resistant to high concentrations of all inhibitors.

To ascertain that in S–O–CN membranes the higher K_i for cyanide refers to the inhibition of an alternative respiratory pathway, NADH oxidation was titrated with cyanide in the presence of concentrations of antimycin A which completely blocked the main respiratory chain, but still allowed the electron flux to proceed through the second respiratory pathway. Figure 3 shows that in this case a monophasic titration curve was obtained with a K_i value for cyanide, 5×10^{-4} M, similar to that determined in fig.2, and corresponding to the inhibition of the second oxidase.

It is well known [12] that the cyanide-insensitive, alternative respiratory pathway of eukaryotic microorganisms and of higher plant mitochondria is specifically inhibited by hydroxamic acid derivatives [13]. The alternative respiratory pathway of *P. denitrificans* was not affected by up to 5 mM salicylhydroxamic acid, when present alone or together with cyanide or antimycin A. Metal chelators, such as 8-hydroxyquinoline, or KSCN had negligible effect on the oxygen uptake.

The biphasic nature of the titration curve with rotenone (fig.2), which had been repeatedly observed with different bacterial preparations, might reflect a non-homogeneous batch of membranes, derived from cells having either cytochrome *d* or cytochrome aa_3 as terminal oxidase rather than two sites of inhibition for rotenone, which was to our knowledge, never observed. Such a possibility could occur with cultures growing for long periods in the presence of KCN if the concentration of KCN in the culture is not maintained at its initial level. We have indeed observed that under strong aeration the concentration of KCN may rapidly decrease; under these conditions the newly synthesized membranes incorporating cytochrome aa_3, are lighter and can be separated by differential centrifugation. We have therefore checked that the same spectral pattern was obtained with cells collected at $20\,000 \times g$. min and at $100\,000 \times g$. min and have considered this fact as indicative of a fairly homogeneous population of cells.

When *P. denitrificans* is grown anaerobically in the presence of nitrate as terminal acceptor, a nitrite reductase is induced. This nitrite reductase is a soluble enzyme which holds hemes *c* and *d* on the same protein [14]. Membranes have been isolated from bacteria grown anaerobically on succinate + nitrate (S–N membranes) and compared to S–O membranes and S–O–CN membranes for their cytochrome content and their sensitivity to respiratory inhibitors (results not shown). S–N membranes were able to use O_2 as terminal electron acceptor to oxidize NADH. The inhibition of NADH oxidation by antimycin and cyanide was biphasic showing the presence of two pathways to oxygen but rotenone displayed a monophasic titration curve. These results suggest that the second pathway of electron transfer from NADH to O_2 branches after the site of action of rotenone, and that each branch shows a different sensitivity to cyanide and antimycin A as has also been observed with the photosynthetic bacterium *Rhodopseudomonas capsulata* [15,16].

3.3. *Difference in the cytochrome components of cells grown under different conditions*

To interpret the biphasic inhibitory effect of antimycin A on the oxidation of NADH in S–O–CN membranes the effect of antimycin was examined on the redox state of the cytochromes with particular attention to the behaviour of the *b*-type cytochromes. Figures 4 and 5 show low temperature difference spectra of membrane vesicles obtained from S–O and S–O–CN cells, respectively. From the relative peak heights it can be seen that membranes prepared from cells grown in the presence of cyanide (or grown anaerobically with nitrate, spectrum not shown) contain higher amounts of *b*- and *c*-type cytochromes. On the other hand, the membranes obtained in the presence of cyanide contained much lower amounts of cytochromes $a+a_3$ than S–O membranes.

With S–O membranes (fig.4) the degree of reduction of cytochrome *b* in the aerobic steady state (NADH reduced) (fig.4b) was increased, as expected, in the presence of antimycin A (fig.4c). On the contrary, in S–O–CN membranes, while cytochrome b_{562} became more reduced, when antimycin A was added (fig.5c), cytochrome b_{555} remained more oxidized in the presence of antimycin (fig.5c), than in its absence

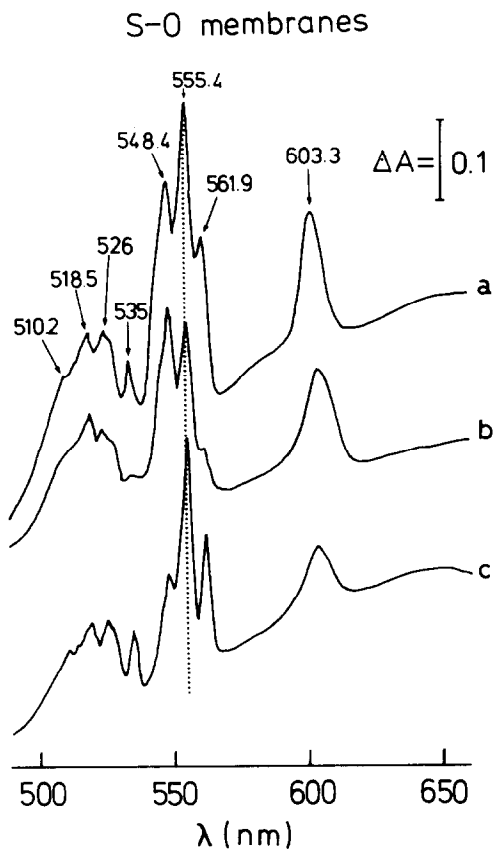

Fig.4. Low temperature difference spectra of membrane vesicles from *P. denitrificans*. Difference spectra were recorded at 77 K (a) Dithionite reduced minus H_2O_2 (+ catalase) oxidized; (b) NADH (5 mM) reduced minus H_2O_2 (+ catalase) oxidized; (c) NADH (5 mM) + antimycin A (2.5×10^{-6} M) reduced minus H_2O_2 (+ catalase) oxidized. S–O membranes 12.6 mg protein/ml.

(fig.5b). This observation is consistent with the occurrence of an alternative respiratory pathway, branching off from cytochrome b_{555} or at a point connected to it.

3.4. *Affinity for O_2*

Assuming the existence of a branched respiratory pathway with 2 terminal oxidases, the kinetics of O_2 uptake were determined at various O_2 concentrations with the 3 types of membranes (S–O, S–O–CN, S–N). The rate of oxygen uptake remained constant, down to very low levels of O_2 ($\simeq 10\ \mu M\ O_2$). The O_2

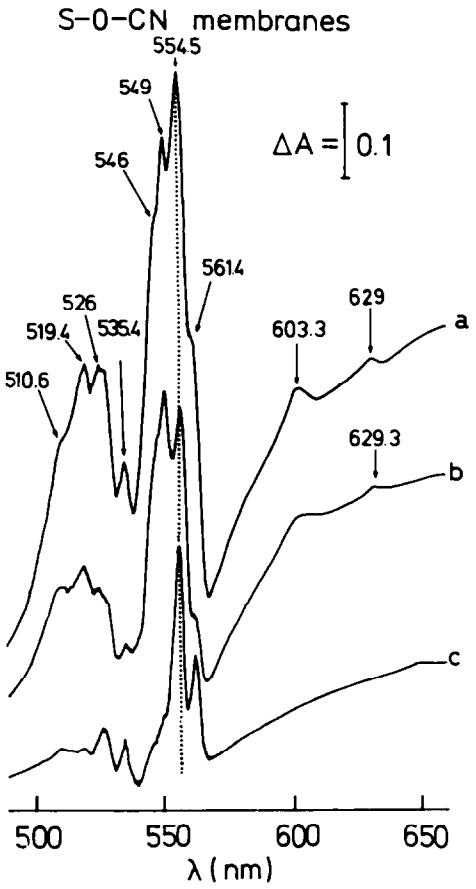

Fig.5. Same conditions as in fig.4. S–O–CN membranes 12 mg protein/ml.

consumption by isolated membranes followed a hyperbolic saturation curve which yielded the K_m values shown in table 1.

A K_m value for oxygen of ~4.5 μM was found for the two cytochrome aa_3-containing membranes, namely S–O and S–O–CN membranes. When electron transport was blocked by either antimycin A or KCN, a second oxidase having about 10-times more affinity for O_2 could be detected (table 1). The lowest K_m for oxygen (0.35 μM) was obtained with S–O–CN membranes inhibited by antimycin A. It is tentatively assigned to cytochrome *d* acting as terminal oxidase. An even lower K_m for oxygen (0.024 μM) had been determined for cytochrome *d* found in *E. coli* grown under O_2 limitation [17].

As mentioned earlier, *P. denitrificans* may synthesize a soluble cytochrome *d*-containing protein having nitrite reductase activity. Nitrite reductase has been reported [18] to also exhibit a cytochrome oxidase activity but the K_m for oxygen (27 μM) of nitrite reductase was much higher than that found with cytochrome *d*-containing membranes (0.35 μM) (table 1).

No cytochrome aa_3 was detectable by low temperature spectrophotometry in cells grown anaerobically with nitrate. However, these cells could respire on O_2 and exhibited a biphasic titration curve for antimycin A and cyanide indicative of branched terminal respiratory pathways. Cytochrome *o*, reported to occur in high concentrations in anaerobic cells of *P. denitrificans* [19], could function as terminal oxidase. The presence and functioning of cytochrome *o* could indeed explain the occurrence of a terminal oxidase having an app. K_m for oxygen of 0.85–1.0 μM as was detected with S–N membranes or with S–O–CN membranes inhibited by KCN.

From the above-mentioned observations, we conclude that a terminally branched respiratory system

Table 1
Affinity for O_2 of terminal branches of the respiratory system

Membrane preparation	Inhibitor present	$K_m^{O_2}$ (μM)	V_{max} (relative rates)
S–O membrane	–	4.33	100[a]
S–O–CN membrane	–	4.5	52
S–O–CN membrane	Antimycin A (3.6 μM)	0.35	5
S–O–CN membrane	KCN (200 μM)	0.75	3
S–N membrane	Antimycin A (3.6 μM) or KCN (200 μM)	0.85–1.0	14

[a] Corresponding to 26 nmol O_2 .min^{-1}.mg^{-1} protein

exists in *P. denitrificans.* The alternate oxidase could be cytochrome *o* (as possibly in S–N membranes) or cytochrome *d* or both cytochrome *o* and *d* (in S–O–CN membranes). In the latter case we could expect that, in the absence of inhibitors when all the oxidases are operative, the app K_m value for O_2 would be that of the pathway with the greater affinity for O_2 (cyanide-resistant pathway). This was not what was found experimentally (table 1). The reason may be that, while the affinity for O_2 of the alternate cyanide-resistant pathway was greatly increased, the rate of O_2 consumption was at the same time very significantly decreased (10-times lower) so that it becomes technically difficult to estimate K_m values at very low O_2 tension and very low respiratory rates.

In S–O–CN membranes, a lower value of the app. K_m for O_2 of the alternative cyanide-resistant oxidase was indeed anticipated since this second respiratory chain is induced when the respiratory rate is markedly diminished under O_2-limitation (as in *E. coli* [17]) or when cytochrome oxidase is blocked by cyanide.

3.5. *Hypothesis of a branched respiratory system in* P. denitrificans

Resistance to cyanide inhibition has revealed terminal branching of bacterial respiratory systems in, e.g., *Azotobacter vinelandii* [20], *Rhodopseudomonas capsulata* [15,16], *Beneckea natriegens* [21].

The occurrence of a branched respiratory system in S–O–CN membranes from *P. denitrificans* is supported by the data reported here:

(1) The biphasic nature of the titration curves with inhibitors;
(2) The presence of two very different K_m values for O_2.

The pathway is branched since two oxidases may be distinguished by KCN sensitivity. The branch point must be located before the site of action of antimycin A since the oxidase activity mediated by the alternative path is insensitive to that antibiotic and displays a sensitivity to cyanide different from that of the main pathway.

It is proposed that the alternative respiratory pathway contains cytochrome *d* as terminal oxidase.

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