ELECTRON TRANSPORT TO NITROUS OXIDE IN *PARACOCCUS DENITRIFICANS*

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1. Introduction

Many bacteria can use nitrogenous oxides, instead of oxygen, as terminal electron acceptor during respiration [1]. *Paracoccus denitrificans* is capable of growing anaerobically in the presence of nitrate, nitrite or nitrous oxide. When nitrate is the electron acceptor growth is accompanied by the evolution of nitrogen and nitrous oxide [2]. Cells grown anaerobically with nitrate as electron acceptor contain flavin, UQ-10, *b*-, *c*-, and *o*-type cytochromes, a membrane-bound nitrate-reductase and a soluble two-haem (*c*- and *d*-type) nitrite-reductase [3,4]. There are strong indications that *b*-type cytochromes are involved in electron transport to nitrate and *c*-type cytochromes in electron transport to nitrite [5,6]. The occurrence of obligate intermediates, like nitrous oxide, in the reduction of nitrite to nitrogen gas is a point of controversy [7–10]. Therefore we started a more profound study of the role of nitrous oxide in denitrification by *Pa. denitrificans*. The results indicate that nitrous oxide could be an obligate intermediate in the reduction of nitrite to nitrogen gas and that *c*-type cytochrome(s) are involved in electron transport to nitrous oxide.

2. Materials and methods

Pa. denitrificans NCIB 8944, formerly *Micrococcus denitrificans* [11], was the experimental organism.

Bacteria were grown anaerobically at 37°C in the liquid medium described in [12] with succinate as carbon and energy source, NH_4Cl as nitrogen source and nitrate as electron acceptor. Anaerobic chemostat experiments were performed as described in [13] in a Bioflo C30 chemostat (New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA). The culture volume was ~300 ml. The pH was controlled at a value of 7.5, unless otherwise stated. In succinate-limited continuous cultures the concentrations of succinate and nitrate were 25 mM and 100 mM, respectively. With electron acceptor limitation the concentration of nitrate was 40 mM, while succinate was added in a concentration of 50 mM. Succinate- and nitrate-limited chemostat cultures were grown at dilution rates of 0.13 and 0.18/h, respectively. After at least six generation times, the chemostat effluent was collected overnight in an ice-cooled vessel and cells were harvested by centrifugation. After washing twice with phosphate-buffer (10 mM K-phosphate, pH 7.0) cells were resuspended in the same buffer.

Bacteria were grown in batch culture on methanol (100 mM) and nitrate (100 mM) in a 1-l vessel at 35°C under stagnant conditions. The medium was supplemented with 0.01% yeast-extract (Difco) and 0.05% $NaHCO_3$. The starting pH was 7.5. Cells were harvested when the culture had reached an absorbance of 0.25–0.35, measured at 660 nm.

Cells were grown in batch culture at 35°C on succinate (50 mM) in an anaerobic jar, only filled with N_2O. After 4 to 5 days the cells were harvested.

All cytochrome studies were performed using an Aminco DW-2 UV/Vis spectrophotometer. Cytochrome oxidation-reduction kinetics were studied in dual wavelength experiments under anaerobic conditions in a Thunberg cuvette at 25°C. Nitrous oxide was added by means of injection of a known volume of N_2O-saturated water at 25°C (nitrous oxide concentration: 24.5 nmol/μl). Known quantities of nitrite were introduced by injecting an anaerobic $NaNO_2$ solution. NaN_3 and KCN were dissolved in 10 mM K-phosphate buffer (pH 7.0). Rotenone and antimycin A were added as ethanolic solutions. In

experiments with *Pa. denitrificans* cells, grown on methanol and NO_3^-, rotenone and antimycin A were solubilized in *N,N*-dimethyl formamide, because ethanol is an electron donor for methanol dehydrogenase [14,15].

3. Results

A number of steady-state 'dithionite-reduced minus nitrous oxide-oxidized' difference spectra of cells of *Pa. denitrificans* are shown in fig.1. From the peaks at 522–523 nm and 550–552 nm and the shoulders at 528–529 nm and 558–559 nm in the α- and β-region of the spectra, it is clear that nitrous oxide oxidizes *b*- and *c*-type cytochromes in *Pa. denitrificans* cells, grown under succinate- (a) or NO_3^--limitation (c) in the chemostat, with N_2O and succinate in batch culture (b) and with NO_3^- and methanol in batch culture (d). There are no spectral indications for the oxidation of *a*-type cytochromes. To investigate the involvement of cytochromes in the reduction of N_2O, kinetic experiments with cytochrome *c* were performed. The absorption of cytochrome *c* was measured at 551–552 nm minus 577 nm. The effects of several electron transport inhibitors (KCN, NaN_3 and antimycin A) on the nitrous oxide reduction rate and the oxidation level of cytochrome *c* were studied. It appeared that N_2O-reduction by cells of succinate-limited chemostat cultures (pH 6.9) was very sensitive to cyanide. A concentration as low as 2 μM KCN in the cuvette, containing about 2 mg dry weight cells/ml, resulted in a 50% inhibition of the rate of reduction of nitrous oxide by endogenous substrates. 90% inhibition was achieved at a KCN-concentration of 10 μM. The steady-state oxidation level of cytochrome *c* gradually decreased by increasing the KCN-concentration from 0 to 33 μM. We found essentially the same results with cells from a batch culture of *Pa. denitrificans*, grown on succinate and N_2O as electron acceptor.

The reduction of nitrous oxide by cells from a

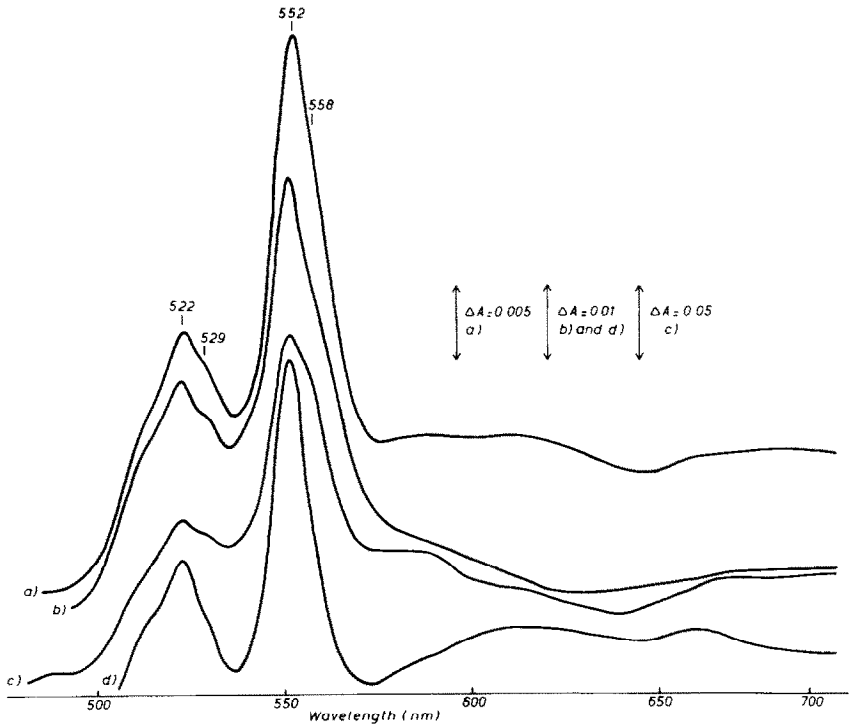

Fig.1. Dithionite-reduced minus N_2O-oxidized difference spectra (490–700 nm) of *Pa. denitrificans* cells, grown (a) under succinate-limitation (pH of the chemostat culture was 6.9; 1.4 mg dry weight cells/ml); (b) with N_2O and succinate in batch culture (2.4 mg dry weight cells/ml); (c) under NO_3^- limitation (2 mg dry weight cells/ml); and (d) with NO_3^- and methanol in batch culture (2 mg dry weight cells/ml).

succinate-limited continuous culture with nitrate as electron acceptor was also rather sensitive to azide. The N_2O-reduction velocity was 50% inhibited by 15 μM NaN_3, while 90% inhibition was achieved at a concentration of 65 μM (about 2 mg dry weight cells/ml). As in the case of KCN, addition of NaN_3 in increasing concentrations (0–200 μM) resulted in a gradual decrease of the steady-state N_2O-induced oxidation level of cytochrome *c*. The same picture was obtained for the inhibition of N_2O reduction by azide with bacteria from a nitrate-limited chemostat culture and a batch culture, grown on succinate and N_2O.

Although these effects of KCN and NaN_3 are compatible with an interaction of N_2O with the respiratory chain after cytochrome *c*, of course it is no conclusive evidence. Therefore we also used antimycin A as electron transport inhibitor, which is known to inhibit electron transfer between cytochromes *b* and *c* [15,16]. After addition of 230 nmol N_2O to a 3-ml cell suspension of *Pa. denitrificans*, grown under nitrate limitation, a drop in the absorbance precedes the N_2O-steady-state (fig.2a,b). A possible explanation for this phenomenon will be given in section 4. Following the addition of 10 μg antimycin A the oxidation level of cytochrome *c* due to N_2O is higher than the one before addition of this inhibitor and the consumption of N_2O takes more time (fig.2b). Obviously, the reduction of N_2O by endogenous substrates is inhibited by antimycin A. This experiment suggests that cytochrome *c* indeed is involved in electron transport to nitrous oxide.

To obtain more evidence in favour of this view we decided to grow *Pa. denitrificans* on methanol as carbon and energy source and nitrate as electron acceptor. It has been shown that *Pa. denitrificans* is able to grow under these conditions [14]. Reduction equivalents of methanol enter the respiratory chain at the level of cytochrome *c* in aerobic [15] and anaerobic cells [14] of *Pa. denitrificans*. Consequently, when cytochrome *c* is involved in N_2O reduction, it must be possible to couple oxidation of methanol to formaldehyde with the reduction of N_2O. A way to demonstrate this is to block electron transport between endogenous substrates and cytochrome *c* and observe whether subsequent addition of methanol to the cell suspension results in a stimulation of N_2O reduction. A typical experiment is shown in fig.3. Endogenous substrates reduced the injected N_2O within 1 min (fig.3a). After addition of 10 μg AA and subsequent addition of N_2O to the suspension, the oxidation level of cytochrome *c* is increased and much more time is needed for reduction of the added N_2O (fig.3b). When thereafter methanol (1.8 mmol) is added to the cuvette the N_2O-induced oxidation level of cytochrome *c* is lowered and equal to that before the addition of antimycin A and the time required for N_2O consumption is strikingly reduced (fig.3c). Because oxidation of methanol to formaldehyde can be followed by oxidation of formaldehyde to formate by the NAD-linked formaldehyde-dehydrogenase and of formate to CO_2 by the NAD-linked formate-dehydrogenase, it is possible, although not

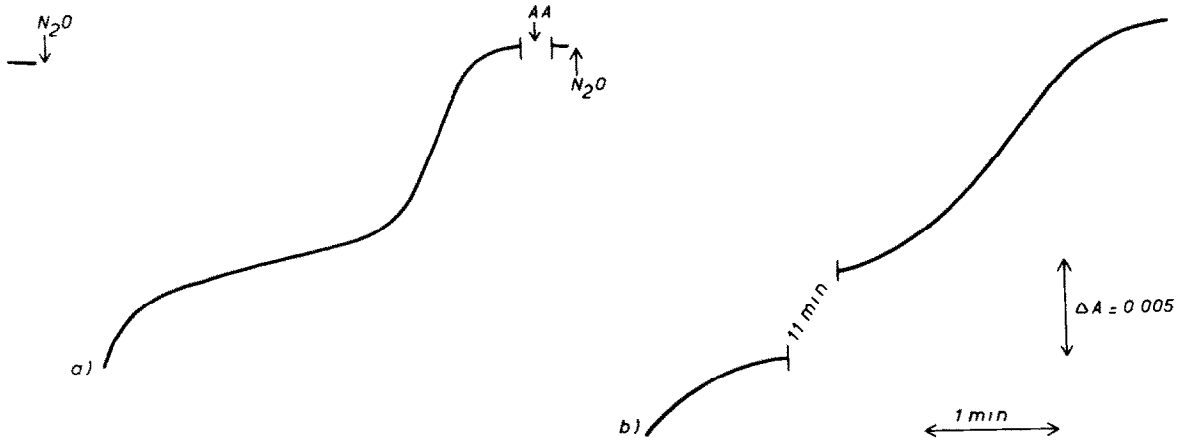

Fig.2. Absorbance at 551 nm minus 576 nm (cyt. *c*) of cells of *Pa. denitrificans* grown under succinate-limitation (2 mg dry weight cells/ml) after successive addition of (a) nitrous oxide (230 nmol), antimycin A (AA; 3.3 μg/ml); and (b) nitrous oxide.

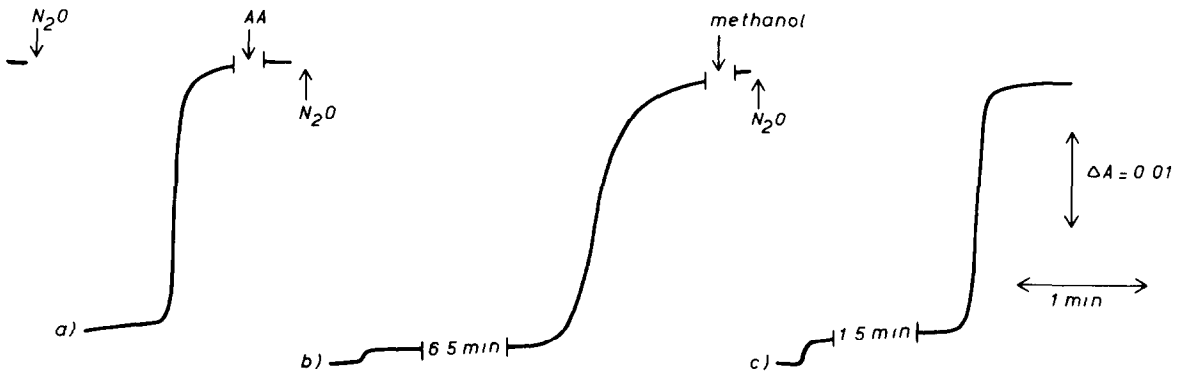

Fig.3. Absorbance at 549 nm minus 576 nm (cyt. *c*) of *Pa. denitrificans* cells grown in batch culture with methanol and NO_3^- (1.5 mg dry weight cells/ml) after successive addition of (a) nitrous oxide (230 nmol), antimycin A (3.3 μg/ml); (b) N_2O, methanol (0.6 M); and (c) N_2O.

probable, that the antimycin A blockade is overrun by the extra NADH formed in this way. To exclude this possibility two experiments were done. Firstly, the tricarboxylic acid cycle intermediate L-malate was used as a potential source of NADH. Addition of excess L-malate before and after addition of antimycin A did not result in a stimulation of the nitrous oxide reduction, on the other hand, methanol stimulated the consumption of N_2O in the presence of antimycin A and L-malate (results not shown). Secondly rotenone, an electron transport inhibitor acting on or in a very close proximity of iron-sulfur centre 2 of the NADH-ubiquinone segment of the respiratory chain [17], was used next to antimycin A. 300 μM rotenone alone inhibited the N_2O-reduction by about 60%. Addition of 1.8 mmol methanol resulted again in an increased nitrous oxide consumption rate, approximately equal to the one before addition of rotenone. Moreover methanol also increased the N_2O-reduction which was inhibited by the simultaneous presence of 6.5 μg antimycin A/ml and 300 μM rotenone, while L-malate did not. The results of these experiments strengthen the view that cytochrome *c* is involved in electron transport to nitrous oxide.

The reduction of nitrite by *Pa. denitrificans* has also been studied with the aid of dual wavelength spectroscopy. The oxidation level of cytochrome *c* (552-557 nm) is followed with time. As can be seen in fig.4 the back-reduction of cytochrome *c* after a NO_2^--pulse is biphasic (fig.4b). The first steady-state must result from reduction of nitrite, while the second one is most probably caused by reduction of nitrous oxide, because a N_2O-pulse results in an almost identical steady-state oxidation level of cytochrome *c* (fig.4a). The addition of azide up to a concentration of 155 μM has no significant influence on the rate of NO_2^--reduction and on the NO_2^--induced oxidation level of cytochrome *c*. However, azide has a pronounced effect on the second steady-state (fig.4c, d and e). Increasing concentrations of NaN_3 result in a decreasing second oxidation level of cytochrome *c* and increasing length of the second steady-state. Addition of N_2O in the presence of 155 μM NaN_3 brings about inhibition of the reduction of nitrous oxide and the same oxidation level as from the N_2O produced from NO_2^- (fig.4f). Consequently the second steady-state, which appears after addition of NO_2^-, most probably can be attributed to the production of N_2O from NO_2^-.

4. Discussion

The inhibition of the nitrous oxide reduction by antimycin A and the stimulation of the N_2O-reduction by methanol in the presence of rotenone and antimycin A clearly indicate the involvement of *c*-type cytochrome(s) in electron transport to nitrous oxide. Because we have no evidence for the presence of *a*-type cytochromes in *Pa. denitrificans* cells grown under anaerobic conditions, it is very likely that the N_2O-reductase is attached to the respiratory chain in the region of the *c*-type cytochromes.

The observation that KCN and NaN_3, in concentrations which inhibit the N_2O-reduction by endogenous substrates, have no significant effect on the

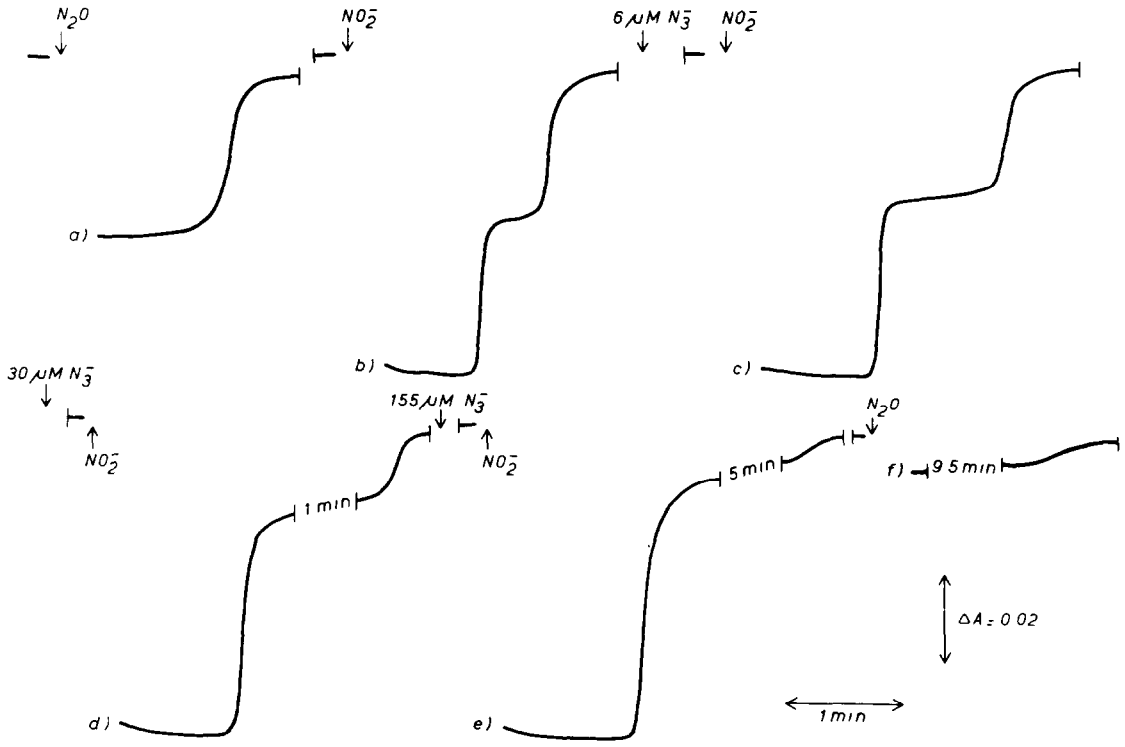

Fig.4. Absorbance at 552 nm minus 577 nm (cyt. *c*) of *Pa. denitrificans* cells grown under succinate-limitation (2 mg dry weight cells/ml) after successive addition of (a) N_2O (230 nmol); (b) NO_2^- (200 nmol), azide (6 μM); (c) NO_2^-, azide (30 μM); (d) NO_2^-, azide (155 μM); (e) NO_2^-; and (f) N_2O.

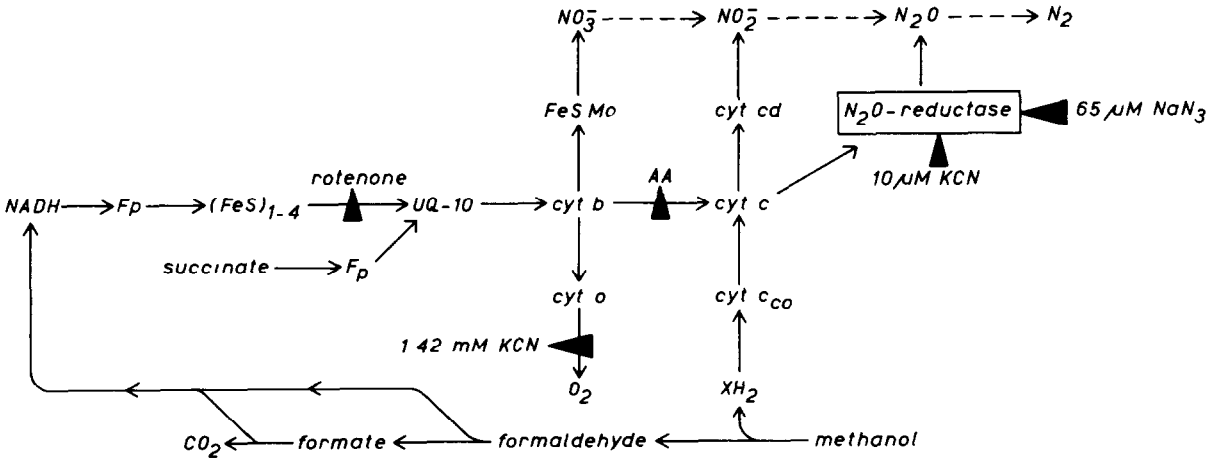

Fig.5. The proposed scheme of the anaerobic respiratory chain of *Pa. denitrificans*. The concentrations of KCN and NaN_3 at which 90% inhibition of respiration occurs are given. *Abbreviations:* Fp, flavoprotein; FeS, iron-sulfur center; UQ-10, ubiquinone-10; cyt, cytochrome; AA, antimycin A; X, methoxatin [19]; Mo, molybdenum.

NO_2^--reduction probably means that these inhibitors exert their inhibiting effect after cytochrome *c*, where the N_2O-reductase itself is the most likely candidate. Therefore we propose that the anaerobic electron transport chain of *Pa. denitrificans* has the composition described in fig.5 (an extended version of the one presented in [18]).

We believe that the often observed change in absorbance immediately after injection of N_2O is caused by a relative slow establishment of the N_2O-steady-state (fig.2). Besides this, sometimes a short O_2-steady-state, preceding the N_2O-steady-state, could be observed (for example in fig.3). This contamination with oxygen did not significantly influence the results reported here.

It has been reported that azide inhibits nitrogen production from nitrous oxide in *Pa. denitrificans* at low concentrations, bus has little effect on the production of nitrogen from nitrite [8]. Sidransky et al. [20] have suggested that an explanation of this phenomenon might be that nitrite can counteract the inhibition by azide of nitrous oxide reductase activity. However in fig.4 we have shown that azide has little or no influence on the NO_2^- reduction but a clearly inhibiting effect on the reduction of N_2O produced from NO_2^- in the same order as in the case of the reduction of N_2O itself. This result is opposite to the findings of Sidransky et al. [20] with *Pseudomonas aeruginosa* but in agreement with those of Matsubara and Mori [21], who claimed that only N_2O was produced from nitrite in *Pseudomonas denitrificans* in the presence of an adequate concentration of azide.

Concerning the role of N_2O in the denitrification of NO_2^- the results presented in fig.4 do suggest that N_2O is an obligate intermediate in the conversion of NO_2^- to N_2. A more penetrating study is necessary for a clear-cut conclusion about the obligate occurrence of N_2O.

Acknowledgements

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