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Involvement of cytochrome bc_1 complex in the electron transfer pathway for N_2O reduction in a photodenitrifier, *Rhodobacter sphaeroides* f. s. *denitrificans*

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The electron transfer pathway for N_2O reduction in *Rhodobacter sphaeroides* f. s. *denitrificans* was studied. Myxothiazol and antimycin showed inhibiting effects on the consumption of N_2O in whole cells grown under photo-denitrifying conditions. This indicated the involvement of the cytochrome bc_1 complex in the process. Reduced redox dyes that donate electrons directly to cytochrome c_2 overcame this inhibition. Similar results were obtained with *Rhodobacter capsulatus*, in contrast to earlier studies by McEwan et al. [(1985) J. Bacteriol. 164, 823–830] that suggested the lack of involvement of the cytochrome bc_1 complex in this process. Effects of myxothiazol and antimycin were also observed on the N_2O-induced oxidation of cytochromes c_1 and c_2 and on the simultaneous generation of membrane potential measured by the carotenoid band shift. Thus, reducing equivalents for N_2O reduction from substrates pass through the cytochrome bc_1 complex coupling to the generation of an electrochemical gradient of protons.

Cytochrome bc_1 complex; Cytochrome c_2; N_2O reduction; Nitrous oxide; Denitrification; *(Rhodobacter sphaeroides)*

1. INTRODUCTION

Denitrifying bacteria which release gaseous dinitrogen (N_2) are able to use N_2O as a terminal electron acceptor through the action of a reductase that converts N_2O to N_2 [1,2]. It has been generally accepted that N_2O is generated by the action of NO reductase which is another terminal reductase in the pathway of dissimilatory nitrate reduction to N_2 [3,4].

The cytochrome bc_1 complex (ubiquinol:cytochrome c_2 oxidoreductase) was reported to be involved in the electron transport pathway for N_2O reduction in *Paracoccus denitrificans* [5]. On the other hand, in a photosynthetic bacterium *Rhodobacter capsulatus*, the electron flow to N_2O was suggested to be independent of the action of the cytochrome bc_1 complex and to be branched at the level of the ubiquinone pool from other electron pathways [6,7].

Rhodobacter sphaeroides f.s. *denitrificans* is a facultative photosynthetic bacterium which can grow depending on denitrification as an energy-generating system other than O_2 respiration and photosynthesis [8]. We have shown that the electron transfer pathways for NO_2^- [9,10] and NO [11,12] reductions in this photodenitrifier involve the cytochrome bc_1 complex and cytochrome c_2, and that those for NO_3^- reduction [13,14] do not involve them. N_2O reductase of this bacterium was shown to be located in the periplasmic space [15] and, according to Michalski et al. [16], the purified enzyme is water-soluble and contains 4 Cu/mol like that from *P. denitrificans* [17]. In this paper,

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Pipes, piperazine-*N,N'*-bis-(2-ethanesulfonic acid); BChl, bacteriochlorophyll

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we show that the cytochrome bc_1 complex is also involved in the electron transfer pathway for N_2O reduction in *R. sphaeroides* f.s. *denitrificans* and *R. capsulatus*.

2. MATERIALS AND METHODS

A green mutant strain of *R. sphaeroides* f.s. *denitrificans* IL106 was grown under denitrifying conditions in light and harvested by centrifugation as in [9]. *R. capsulatus* G-3, a new isolate of our laboratory, was also cultured in light in the presence of nitrate. Cells were washed with 50 mM Pipes-KOH buffer (pH 7.0) and suspended in the same buffer.

Absorption spectra of pigments in cell suspensions were measured with a Shimadzu UV-160 spectrophotometer equipped with opal glasses behind the cuvettes [18] and bacteriochlorophyll contents were determined as described in [19]. The ratio of bacteriochlorophyll to protein in typical cells of *R. sphaeroides* was 8 nmol/mg. N_2O reduction was measured polarographically following the method described in [5] with a Clark-type electrode with a silver cathode which had a diameter of 1.0 mm (Iijima Seimitsu). 1 M KOH plus 100 mM KCl was used as the electrolyte. The polarizing voltage used was −1.3 V. The N_2O-induced absorption changes of cytochromes and carotenoids were measured with a Shimadzu UV-3000 dual wavelength spectrophotometer. All measurements were taken in 4.4 ml anaerobic suspensions of whole cells in 50 mM Pipes-KOH buffer (pH 7.0) stirred at 30°C. N_2O was added with a syringe as an anaerobic 50 mM Pipes-KOH (pH 7.0) solution (25 mM N_2O) made by equilibrating it with N_2O gas of 1 atm at 25°C.

3. RESULTS

The consumption of N_2O in whole cells of *R. sphaeroides* grown under photo-denitrifying conditions recorded with a Clark-type electrode is shown in fig.1A. Under the dark, anaerobic conditions, N_2O was consumed at a rate of 7.7 nmol N_2O per min per nmol BChl after the first addition of an aliquot of N_2O solution. Illumination for a short period strongly inhibited N_2O consumption (fig.1A), and addition of a protonophore, CCCP (20 μM), prevented the inhibition of N_2O consumption by light (data not shown). When myxothiazol and antimycin (10 μM each), inhibitors of the electron transfer in the cytochrome bc_1 complex, were added and incubated for 5 min, N_2O consumption by whole cells was strongly inhibited. Myxothiazol alone (23 μM) showed a similar effect and antimycin alone (23 μM) was less effective (about 50% of the effect of myxothiazol; data not shown). Incubation of a few minutes was required to observe full inhibitory effects. Ascorbate plus

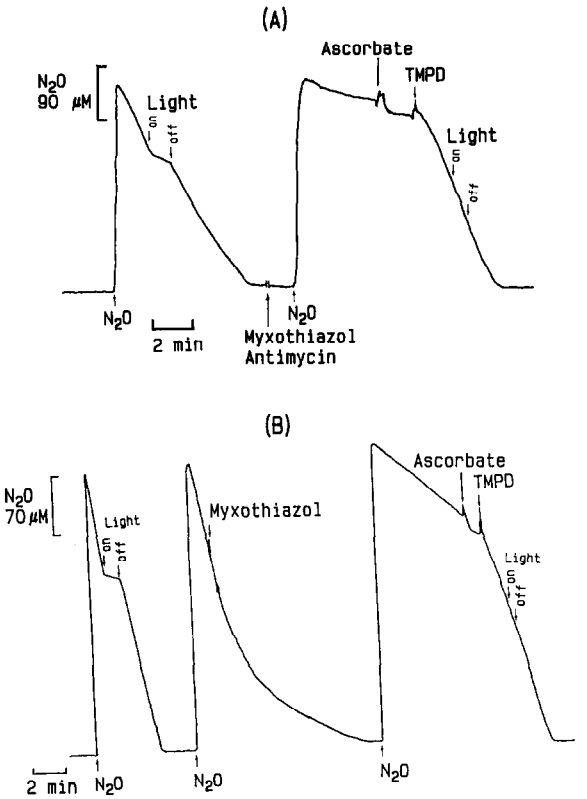

Fig.1. N_2O consumption measured polarographically in whole cells. (A) In cells of *R. sphaeroides* f.s. *denitrificans* grown under photo-denitrifying conditions. Cells (final BChl concentration, 7.6 μM) were suspended in anaerobic 50 mM Pipes-KOH (pH 7.0) buffer. The N_2O reduction was measured at 30°C under dark conditions except when illumination was provided from a tungsten lamp at an intensity of 450 $W \cdot m^{-2}$ (LI-200SA pyranometer sensor, LI-COR, Inc.). After addition of antimycin and myxothiazol (10 μM each), the cell suspension was incubated for 5 min, and then N_2O (350 μM), ascorbate (1.25 mM) and TMPD (100 μM) were added as indicated in the figure. (B) In cells of *R. capsulatus* G-3. Cells containing 4.8 μM BChl (1.8 mg protein per ml) were suspended in anaerobic 50 mM Pipes-KOH (pH 7.0) buffer. N_2O (0.9 mM), myxothiazol (23 μM), ascorbate (1.25 mM) and TMPD (100 μM) were added as indicated in the figure. The other conditions were the same as in A.

TMPD overcame inhibition by myxothiazol and antimycin. Since ascorbate plus TMPD is a potent electron donor to cytochrome c_2, the relief of the inhibition indicates that TMPD donates electrons directly to cytochrome c_2 from which the electrons flow to N_2O reductase. The consumption of N_2O after the addition of ascorbate plus TMPD was not affected by illumination. When cells grown

photosynthetically in the absence of nitrate were used, no significant reduction of N_2O was observed in an experiment similar to that in fig.1A (data not shown) probably because N_2O reductase was not induced in these cells. This is different from the case of *R. capsulatus* in which N_2O reductase is constitutive for anaerobic growth conditions [6].

Fig.1B shows a similar measurement of N_2O consumption in *R. capsulatus* G-3, isolated in our laboratory, which has NO_3^- reductase and constitutive N_2O reductase activities and lacks the ability of NO_2^- reduction. The effects of illumination, myxothiazol, and ascorbate plus TMPD on N_2O consumption were quite similar to those in *R. sphaeroides* (fig.1A), suggesting the involvement of the cytochrome bc_1 complex for N_2O reduction in *R. capsulatus*, too.

When N_2O was added to an anaerobic suspension of *R. sphaeroides* cells grown under photo-denitrifying conditions, the oxidation of *c*-type cytochrome was observed. Fig.2 shows the kinetics of *c*-type cytochrome oxidation (a & c) and the spectrum changes (b & d) in the presence or absence of myxothiazol and antimycin (10 μM each). When N_2O was added to the cell suspension in the absence of inhibitors (fig.2a,b), immediate oxidation of *c*-type cytochrome occurred. Within a minute, the cytochrome was partially re-reduced to a steady level, which lasted for about 2 min probably owing to a steady state of electron flow from endogenous substrates to N_2O. The oxidation level then returned to what it was before the addition of N_2O, probably because of the exhaustion of N_2O. The duration of oxidation was consistent with the results of the direct measurement of N_2O consumption by the polarographic method (fig.1). In the presence of myxothiazol and antimycin (fig.2c,d), the immediate oxidation induced by N_2O addition was larger than that in the absence of the inhibitors, and the high oxidation level continued for more than 30 min. This indicates that the oxidized cytochrome was located after the quinone sites of the cytochrome bc_1 complex in the electron transfer pathway to N_2O. The cytochrome oxidized in the presence of inhibitors was about 60% of the total *c*-type cytochromes which was determined by the dithionite-reduced minus ferricyanide-oxidized difference spectrum of cells. It has been reported that about 55% of the total *c*-type cytochrome is cytochrome c_2 and about 30%

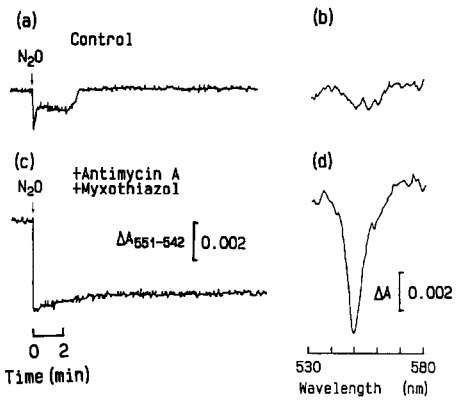

Fig.2. Cytochrome oxidation induced by N_2O addition in *R. sphaeroides* f.s. *denitrificans*. Cells (BChl concentration, 6.4 μM) were suspended in anaerobic 50 mM Pipes-KOH (pH 7.0) buffer. (a and c) Kinetics of the oxidation of *c*-type cytochrome. N_2O (150 μM) was added as indicated in the figure. (b and d) Spectra of 1 min after N_2O addition with a reference of those before the addition. (a and b) Absence of inhibitors. (c and d) After the addition of myxothiazol and antimycin (10 μM each), the cell suspension was incubated for 5 min before the N_2O addition.

of that is cytochrome c_1 in the photo-denitrifying cells [20]. These facts in conjunction with the absorption spectra shown in fig.2b and d suggested that cytochromes c_1 and c_2 were involved in the electron transfer pathway to N_2O. We cannot,

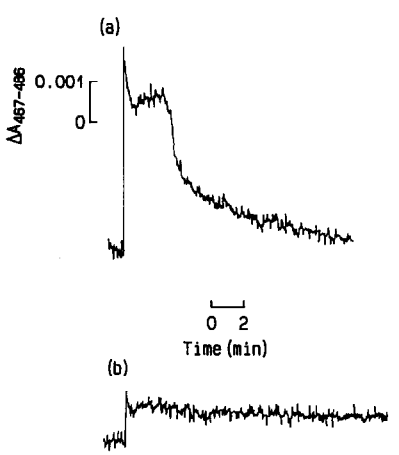

Fig.3. Generation of a membrane potential as detected by the carotenoid band shift after addition of N_2O in *R. sphaeroides* f.s. *denitrificans*. The experimental conditions were the same as those described in fig.2 except the wavelength used. (a) Absence of inhibitors; (b) presence of antimycin and myxothiazol (10 μM each).

however, rule out the possibility that N_2O reductase interacted directly with the cytochrome c_1.

In both intact cells and chromatophores of photosynthetic bacteria, the extent of the red shift in the absorption of the endogenous carotenoids has been used as an indicator of the cytoplasmic membrane potentials [21,22]. In an anaerobic cell suspension, the addition of N_2O induced the carotenoid absorption changes which indicated the development of membrane potential (fig.3a) (see [6,7] in the case of *R. capsulatus*). When cells were incubated for 5 min with 10 μM myxothiazol and 10 μM antimycin, the N_2O-induced membrane potential was about 20% of that in the absence of the inhibitors (fig.3b). This is another piece of evidence for the electron transfer pathway to N_2O through the cytochrome bc_1 complex coupled to the generation of the protonmotive force.

4. DISCUSSION

From the results presented above, we have concluded that the cytochrome bc_1 complex is involved in the electron flow from endogenous substrates to N_2O reductase in *R. sphaeroides* f.s. *denitrificans* and *R. capsulatus* G-3. The participation of cytochrome c_2 in the N_2O reduction was also suggested from the oxidation of both cytochromes c_1 and c_2. In this photo-denitrifier, cytochrome c_2 has been shown to mediate electrons from the cytochrome bc_1 complex to the photosynthetic reaction center complex [20], cytochrome-c_2 oxidase [20], NO_2^- reductase [9,10] and NO reductase [11,12]. N_2O reductase of *R. sphaeroides* f.s. *denitrificans* is water-soluble [16] and is located in periplasmic space [15] as is the case of cytochrome c_2 [23]. It is likely that cytochrome c_2 mediates electrons directly from the cytochrome bc_1 complex to N_2O reductase.

The conclusion that the cytochrome bc_1 complex and probably cytochrome c_2 take part in dissimilatory N_2O reduction is the same as that for *P. denitrificans* [5]. In *R. capsulatus* N22DNAR$^+$ [6], however, McEwan et al. showed that the electron transfer pathway to N_2O was independent of the action of the cytochrome bc_1 complex. According to them, antimycin was not inhibitory for the N_2O reduction. Furthermore, the addition of ascorbate plus TMPD in the dark did not affect the N_2O reduction which was inhibited by rotenone, but in the light ascorbate plus TMPD increased the reduction of N_2O. Our results in *R. sphaeroides* and *R. capsulatus* were different from theirs. We have observed that antimycin alone was less effective than myxothiazol plus antimycin or myxothiazol alone. This observation may be related to the failure of the inhibitory effect of antimycin in their systems.

The physiological consequences of the involvement of the cytochrome bc_1 complex in the electron pathway from substrates to N_2O is that redox energy from quinol to N_2O is coupled to the generation of an electrochemical gradient of protons across the membrane; also that N_2O reduction is under the control of the high-energy state of the membrane. These effects were directly shown in this study as the N_2O-induced carotenoid band shift (fig.3) and the CCCP-sensitive inhibitory effect of illumination on the N_2O reduction (fig.1). Since the reduction of N_2O to N_2 is energetically very favorable (E_{m7} = 1.35 V), the use of the cytochrome bc_1 complex coupling site for N_2O reduction is quite natural, although still a lot of energy must be wasted in the electron transfer from cytochromes c_2 to N_2O.

Since the isolation of the first photo-denitrifying bacterium [8], we have reported the electron transport pathways for denitrification, i.e., for NO_3^- [13,14], NO_2^- [9,10] and NO [11,12] reductions. The present results, together with the previous ones, reveal the outline of the whole electron transfer system for denitrification in *R. sphaeroides* f.s. *denitrificans* as shown in fig.4. The main characteristics of the system are as

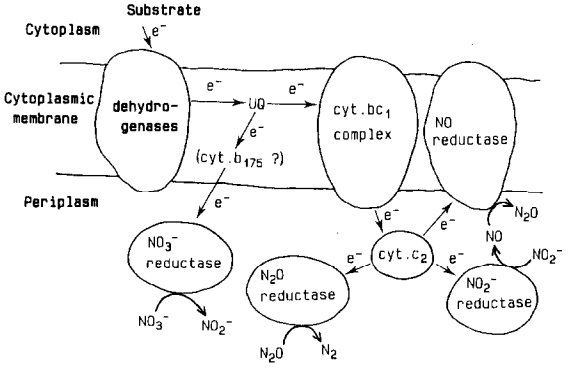

Fig.4. Electron transfer pathways of denitrification in *R. sphaeroides* f.s. *denitrificans*. UQ, ubiquinone; cyt., cytochrome.

follows: (i) ubiquinone, the cytochrome bc_1 complex and cytochrome c_2 are involved in the electron transfer pathways for denitrification as common components with those for O_2 respiration and photosynthesis; (ii) three of the terminal enzymes for denitrification, NO_3^-, NO_2^- and N_2O reductases, are periplasmic proteins and all four terminal reduction reactions take place in periplasmic space; (iii) the electron transfer pathways for the three terminal enzymes, NO_2^-, NO and N_2O reductases, branch at the level of cytochrome c_2.

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