

A nitrate reductase activity in *Rhodopseudomonas capsulata* linked to electron transfer and generation of a membrane potential

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We have isolated from a laboratory strain of *Rhodopseudomonas capsulata* a spontaneous mutant possessing a dissimilatory NO_3^- reductase activity. Reduction of NO_3^- under dark and anaerobic conditions generated a membrane potential, and was inhibited by rotenone, oxygen and illumination.

Nitrate dissimilation

Photosynthetic bacteria

Electron transport

1. INTRODUCTION

Many species of the purple non-sulphur photosynthetic bacteria are capable of NO_3^- assimilation, but only a few, including a strain of *Rhodopseudomonas sphaeroides* isolated by enrichment on NO_3^- media [1], and some strains of *Rps. palustris* [2], are capable of growth by dissimilation of NO_3^- . Of 9 strains of *Rps. capsulata* examined, 7 were capable of NO_3^- assimilation but none could grow using NO_3^- as an electron acceptor under anaerobic-dark conditions [2,3]. Here, we show that repeated phototrophic sub-culturing of a laboratory strain of *Rps. capsulata* on a medium containing NO_3^- results in the appearance, presumably by mutation, of a hitherto unrecognised dissimilatory NO_3^- reductase activity. This observation enlarges the known repertoire of this 'most versatile prokaryotic organism' [4] and may help to rationalise other observations on NO_3^- reductases of *Rhodospirillaceae* in the literature.

2. MATERIALS AND METHODS

Rps. capsulata strain St Louis and a green mutant, N22, isolated from it, were obtained originally from O.T.G. Jones (Department of Biochemistry, University of Bristol). Single colony

isolates from our stock cultures of these strains and of the NO_3^- -reducing mutant were grown phototrophically at 30°C in the medium of [5], except that $(\text{NH}_4)_2\text{SO}_4$ was replaced by 14 mM NaNO_3 . Cells for experiments were grown by inoculating ~15 ml of a 24 h culture into a completely filled 300 ml flat-sided bottle that was positioned at 30 cm from each of two banks of 100 W tungsten bulbs. Cells were harvested after 24–28 h, when they were at a late stage of logarithmic growth as judged by A_{650} , and then washed and finally resuspended in the same medium as used for growth except that NO_3^- was omitted. The cells were stored in the dark as a thick suspension on ice and the bacteriochlorophyll content was determined as in [6]. NO_2^- was determined as in [7].

3. RESULTS AND DISCUSSION

We described the kinetics and inhibitor sensitivities of the assimilatory, light-dependent uptake of NO_3^- by cells of *Rps. capsulata* N22 [8]. These experiments were performed on cells which had been adapted to growth on NO_3^- as nitrogen source after transfer from medium containing NH_4^+ . Routinely the cells were harvested after 2–5 transfers (10–20% inoculum at 24 h intervals) following the switch of nitrogen source.

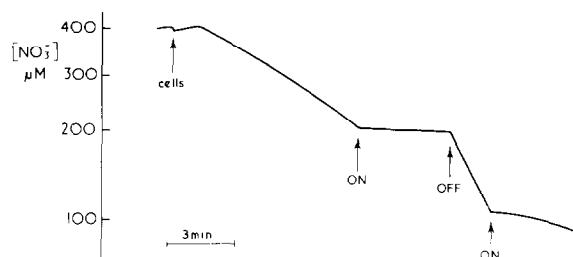


Fig. 1. Reduction of NO_3^- by *Rps. capsulata* in the dark and inhibition by light after repeated serial phototrophic subculture of cells on media containing NO_3^- . Growth medium (14 ml) deficient in both NO_3^- and NH_4^+ were pre-incubated in a vessel fitted with a NO_3^- electrode [8] at 30°C under an atmosphere of argon. The electrode response was calibrated by the addition of aliquots of NaNO_3 to $400\text{ }\mu\text{M}$ final conc. Cells were added to give final bacteriochlorophyll conc. $12.5\text{ }\mu\text{M}$. Photosynthetic exciting light from a 150 W quartz halogen lamp, filtered through 3 cm water and one layer of Wratten 88A gelatin filter was switched on and off where shown.

We have now discovered that after repeated, daily transfer in NO_3^- medium for ~ 4 weeks, the character of NO_3^- uptake in harvested cells changed dramatically (fig. 1). In marked contrast to the original N22 cells, the NO_3^- reductase was active in the dark, produced NO_2^- stoichiometrically with NO_3^- disappearance, was strongly inhibited by light (fig. 1) and was no longer inhibited by NH_4^+ (cf. [8]). We have observed identical changes over a similar period by cells of the parent strain *Rps. capsulata* St Louis.

Cells derived from *Rps. capsulata* N22 that had dark NO_3^- reductase activity produced round, green colonies, characteristic of the original bacteria, on agar plates. Isolates from single colonies were still capable of dark NO_3^- reduction. One such isolate, designated *Rps. capsulata* N22DNAR⁺, was used to provide cells for subsequent experiments. Phase contrast micrographs of the N22DNAR⁺ and N22 cells were indistinguishable and resembled the micrographs in [5], except that the zigzag cell arrangement was less pronounced. Additional evidence that we had isolated N22DNAR⁺ from N22 was that the visible absorbance spectrum of membranes prepared from cells by sonication was indistinguishable from a

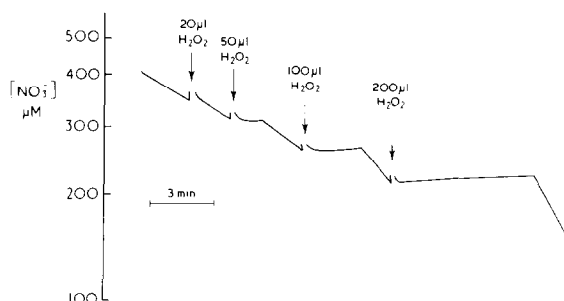


Fig. 2. Inhibition of NO_3^- reduction by O_2 in *Rps. capsulata* N22DNAR⁺. Conditions as in fig. 1 except that bacteriochlorophyll was $22.5\text{ }\mu\text{M}$ and the initial NO_3^- was $600\text{ }\mu\text{M}$ (the initial part of the trace is not shown). The arrows show the addition of aliquots of 1 vol. H_2O_2 which were rapidly converted to O_2 by endogenous catalase activity.

spectrum of membranes from N22 cells. This spectrum is highly characteristic of mutant N22 because of the partial deficiency in carotenoids.

Fig. 2 shows that O_2 strongly inhibited NO_3^- reduction in N22DNAR⁺ cells; no inhibition by O_2 of the light-dependent NO_3^- reduction in N22 cells can be observed. The effect of O_2 means that the slight delay in fig. 1 before the onset of NO_3^- reduction can be attributed to the time required for reduction or residual inhibitory O_2 . NO_3^- reduction by N22DNAR⁺ cells was inhibited strongly by concentrations of rotenone that had a similar effect on the rate of electron flow to O_2 , but sufficient antimycin to block cyclic electron flow through the ubiquinol-cytochrome *c* oxidoreductase had no effect on NO_3^- reduction. Thus electrons for reduction of NO_3^- originated from the rotenone-sensitive NADH dehydrogenase but did not pass through the antimycin-sensitive segment of the electron-transfer chain.

Fig. 3 shows the uptake of the butyltriphenylphosphonium (BTPP⁺) cation in response to the generation of a membrane potential ($\Delta\psi$) by either light-dependent cyclic electron flow or respiratory electron flow to O_2 or electron flow to added NO_3^- (cf. [9]). A second method for detecting $\Delta\psi$ in *Rps. capsulata* is to measure the electrochromic shift in the carotenoid spectrum [9]. Fig. 4 shows that addition of NO_3^- to a dark, anaerobic suspension of N22DNAR⁺ cells resulted in a carotenoid band shift. Both methods for detecting $\Delta\psi$ show that

electron flow to NO_3^- generated a lower $\Delta\psi$ than either cyclic or aerobic dark electron flow, but quantitative comparison between the two methods is not justified because cells with differing specific NO_3^- reductase activities were used for the two types of experiment. As carotenoid band shifts are only seen in certain species of photosynthetic bacteria, their observation adds to the evidence

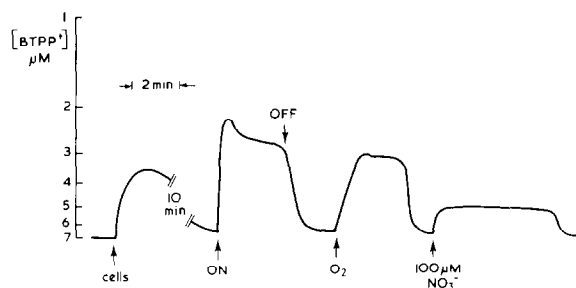


Fig. 3. Uptake of BTPP^+ by N22DNAR^+ cells in response to light-driven cyclic electron flow and respiratory electron flow to either O_2 or NO_3^- . Conditions as in fig. 1 except that the BTPP^+ electrode [9,12] was calibrated by additions of BTPP^+ in steps of $1\text{--}7\text{ }\mu\text{M}$ final conc. in the absence of NO_3^- . Cells were added to give final bacteriochlorophyll conc. $26\text{ }\mu\text{M}$ and incubated until essentially complete anaerobiosis was achieved as judged by the cessation of efflux of BTPP^+ that was initially taken up by the cells. Illumination and oxygenation were as in fig. 1 and 2.

that N22DNAR^+ is derived from N22 and is not a contaminant.

Neither uptake of BTPP^+ nor a carotenoid band shift was observed upon addition of NO_3^- to N22 cells. Both the NO_3^- -dependent BTPP^+ uptake and NO_3^- -dependent carotenoid band shift in N22DNAR^+ cells were reversed by low concentrations of the uncoupler FCCP. $\Delta\psi$ observed after addition of NO_3^- was solely dependent on electron flow to NO_3^- because no $\Delta\psi$ was detected by either method upon addition of NO_2^- . Thus the measurements of $\Delta\psi$ in N22DNAR^+ cells clearly establish that the NO_3^- reductase is linked to a proton translocating electron transfer chain. As NO_2^- is the product of NO_3^- reduction this activity is classified as dissimilatory, hence the designation DNAR^+ .

NO_3^- reductases can sometimes use ClO_3^- as an alternative substrate [10,11]. Addition of ClO_3^- to N22DNAR^+ cells led to a carotenoid band shift (fig. 4), although the initial rate of ClO_3^- reduction was $\sim 10\%$ that of NO_3^- . The band shift and the rate of ClO_3^- reduction declined to zero during the following 10–20 min. This may be due to inactivation of NO_3^- reductase by a product of ClO_3^- reduction (e.g., chlorite) because subsequent addition of NO_3^- did not regenerate a $\Delta\psi$, whereas addition of O_2 or illumination produced $\Delta\psi$ -values of equivalent size to those observed before exposure of cells to ClO_3^- (fig. 4).

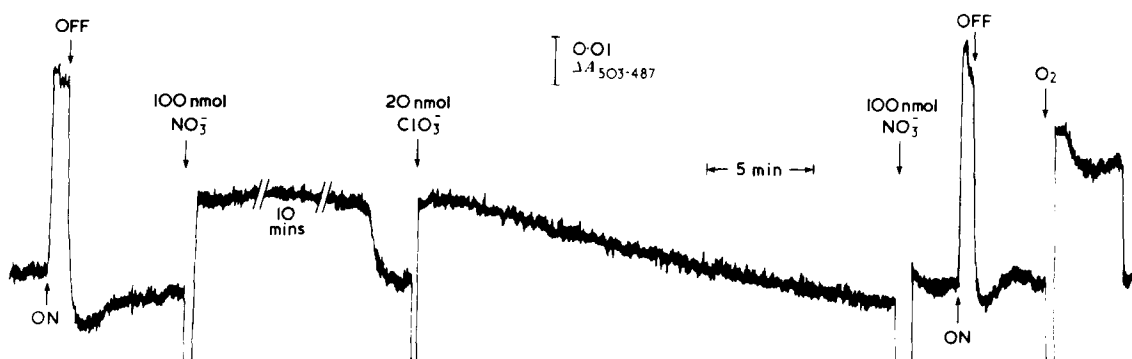


Fig. 4. Carotenoid band shifts in N22DNAR^+ cells in response to light-dependent cyclic electron flow and respiratory electron flow to NO_3^- , ClO_3^- and O_2 . These were measured using a dual wavelength spectrophotometer (perkin-Elmer model 356) [9]. The spectrophotometer cuvette contained 3 ml growth medium from which both NH_4^+ and NO_3^- had been omitted and through which argon had been passed for 30 min at 30°C . Cells were added to give a final bacteriochlorophyll conc. $26\text{ }\mu\text{M}$, and the suspension was incubated for 15 min under argon and with stirring to allow the suspension to become as anaerobic as possible [9]. Illumination and oxygenation were achieved as in fig. 1, 2.

The existence of a ClO_3^- reductase activity, capable of generating $\Delta\psi$, contrasts with the situation in the denitrifier *Paracoccus denitrificans*. Intact cells of this organism have a very low ClO_3^- reductase activity [10,11] and do not take up BTPP^+ in response to ClO_3^- addition, whereas NO_3^- supports uptake [12]. The ability of N22DNAR^+ cells to reduce ClO_3^- suggests either that the permeability barrier that prevents ClO_3^- reduction by intact cells of other bacteria [10,11] is less selective in *Rps. capsulata*, or that the active site of NO_3^- reductase in N22DNAR^+ is at the periplasmic surface of the cell membrane. It has been proposed that the NO_3^- reductase of a denitrifying strain of *Rps. sphaeroides* is a periplasmic and water soluble enzyme [13], in contrast to the membrane-bound NO_3^- reductases of denitrifiers.

We have not yet been able to grow N22DNAR^+ anaerobically in the dark with NO_3^- as electron acceptor. Our working hypothesis is that the $\Delta\psi$ generated by electron flow to NO_3^- is insufficient to provide the energy necessary for growth. It is possible that dissimilatory NO_3^- reduction serves as a supplementary generator of $\Delta\psi$ during photosynthetic growth, particularly in relatively dense, and therefore light-shaded cultures. This is consistent with our findings that:

- (i) The NO_3^- concentration in the N22DNAR^+ growth medium increases dramatically to 7 mM as the cells approach stationary phase;
- (ii) In NO_3^- containing medium N22DNAR^+ grows to slightly higher density than N22.

This hypothesis also accounts for the selective advantage of spontaneous mutants such as N22DNAR^+ in our cultures which are grown repeatedly to early stationary phase. Presumably *Rps. capsulata* N22 and St Louis have lost by mutation the capacity for dissimilatory NO_3^- reduction during prolonged periods of culture on medium lacking NO_3^- . By analogy with the slow 'adaptations' of *Rps. capsulata* to growth on glycerol and acetate [14–16], N22DNAR^+ may be provisionally described as a 'gain of function' mutation.

This is the first demonstration of dissimilatory NO_3^- reduction in *Rps. capsulata*, but we believe that the phenomenon may be more widespread

than is generally appreciated. It is interesting that a range of properties for NO_3^- reductases from a number of strains of *Rps. capsulata* have been reported [17]. Our results with N22DNAR^+ suggest that lack of dark anaerobic growth on NO_3^- is not a sufficient criterion for concluding that a dissimilatory NO_3^- reductase is absent and we suspect that some of these strains may possess dissimilatory NO_3^- reductases.

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