Nitrate Photo-Assimilation by the Phototrophic Bacterium *Rhodopseudomonas capsulata* E_1F_1

F. Castillo, F. J. Caballero, and J. Cárdenas

Departamento de Bioquímica, Facultad de Biología y C.S.I.C. Universidad de Sevilla, Apartado 1095 Sevilla, Spain

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Rhodopseudomonas capsulata E_1F_1 bacteria photo-assimilated nitrate under anaerobic conditions, but were incapable of reducing it in the dark. The bacteria utilized dissolved N_2 as nitrogen source, but the growth in the presence of nitrate was 5-fold higher. Nitrite was excreted to the medium, but not stoichiometrically with respect to nitrate consumption. Nitrate reductase is particulate and used reduced viologens or flavins as electron donors for nitrate reduction. The enzyme was inhibited *in vitro* by KCN, NaN_3, dithioerythritol and mercurials. Ammonia and other sources of reduced nitrogen repressed nitrate reductase synthesis.

Introduction

The ability of photosynthetic bacteria to use dinitrogen or ammonia and other reduced forms of nitrogen is a fact long ago well documented [1, 2]. However, the data on nitrate assimilation in these organisms are scarce and less clear. After the earlier claims of Katoh [3] and Taniguchi and Kamen [4] about nitrate assimilation by certain phototrophic bacteria, the subject has been recently revised, and only several strains of *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides* have been found to be capable of photo-assimilating nitrate as sole nitrogen source [5]. Nitrate reductases from *R. capsulata* AD2 and BK5 have been isolated [6] and more recently nitrate assimilation has been demonstrated in *R. capsulata* E_1F_1 and its nitrite reductase partially characterized [7]. The present work reports on the partial characterization of the assimilatory nitrate reductase of this bacterium *R. capsulata* E_1F_1.

Materials and Methods

R. capsulata E_1F_1 cells (a gift of Dr. W. G. Zumft, Erlangen, Bundesrepublik Deutschland) were grown phototrophically in the RCV medium containing DL-malate and $(NH_4)_2SO_4$ [8] with continuous illumination (2000 lux, 30 °C), using alternatively as nitrogen source the following compounds per liter: KNO_3, 1 g; NH_4NO_3, 1 g; NH_4Cl, 1 g; yeast extract, 1 g; L-glutamate, 1 g; and KNO_2, 85 mg. The cultures were maintained anaerobic either by filling completely with culture medium 125 ml screw capped bottles or by sparging them with high purity argon. The purity of the cultures was checked routinely on yeast extract agar plates.

Cells were harvested by centrifugation at 20000×*g*, 20 min, in a Sorvall RC-2B refrigerated centrifuge, washed once with 50 mM Tris-HCl buffer (pH 7.5) and lysed by osmotic shock as described by Kerber *et al.* [9]. The lysate was spun down at 10000 × *g*, 15 min, and the resulting membrane-containing supernatant was used as source of enzyme.

Nitrate and nitrite reductases were assayed as described previously [10, 11]. For the photochemical assay of nitrate reductase the conditions of Zumft *et al.* were used [12]. Nitrogenase was determined in whole cells under argon in flasks placed within an illuminated Warburg bath (2000 lux, 25 °C). Acetylene reduction was followed in a Pye Unicam gas chromatograph.

Nitrite was determined according to Snell and Snell [13]. Nitrate was estimated spectrophotometrically as described by Cawse [14], and ammonia by the Conway microdiffusion technique [15]. Protein was determined by the Lowry procedure using bovine serum albumin as standard [16]. When needed, cells were digested by boiling with 2 N NaOH prior to protein determination [17]. Cell growth was determined turbidimetrically by measuring the absorbance of bacterial suspensions at 680 nm. A unit of enzyme is that amount of enzyme

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catalyzing the reduction of 1 μmol of substrate per minute, and specific activity is expressed as units per mg protein.

Results and Discussion

R. capsulata E_1F_1 cells grew phototrophically under argon in media with nitrate as the sole nitrogen source (Fig. 1). When nitrate was omitted from the media no growth took place. In the presence of nitrate and under argon, the cells lacked nitrogenase (results not shown).

Nitrite was excreted to the medium but in much less quantity then the nitrate was taken up (Fig. 1). The cells entered the stationary phase of growth when excreted nitrite reached the maximum level. In this situation nitrate ceased being taken up, and the growth obtained under argon was always half that observed in anaerobic cultures without argon which excreted much less nitrite (Fig. 2A).

In these latter cultures growth was observed in the absence of nitrate, albeit in its presence a higher and more vigorous growth took place (Fig. 2A). In this respect we did not observe any lag phase in the growth with nitrate nor any difference in comparison with cultures utilizing ammonia or glutamate as recently reported for *R. capsulata* B_{10} [18]. The difference may be due to the fact that inocula used in our experiments came from cultures with nitrate. The rates of nitrate uptake and nitrate and nitrite reductases synthesis were similar to those observed in cultures under argon (see also ref. [7]). However, the quantity of nitrate taken up and the growth were higher, and the excretion of nitrite 8-fold lower (Fig. 2A). The growth observed in nitrate-deficient cultures was due to the utilization of the molecular nitrogen dissolved in the medium as deduced from the high cellular levels of nitrogenase measured under these condition (Fig. 2B). A similar growth at the expense of dissolved nitrogen has been reported in *R. sphaeroides*, *R. rubrum* and *R. capsulata* B_{10} [18, 19].

As may be seen by comparing Fig. 2A and Fig. 2B, nitrite present in the medium inhibited

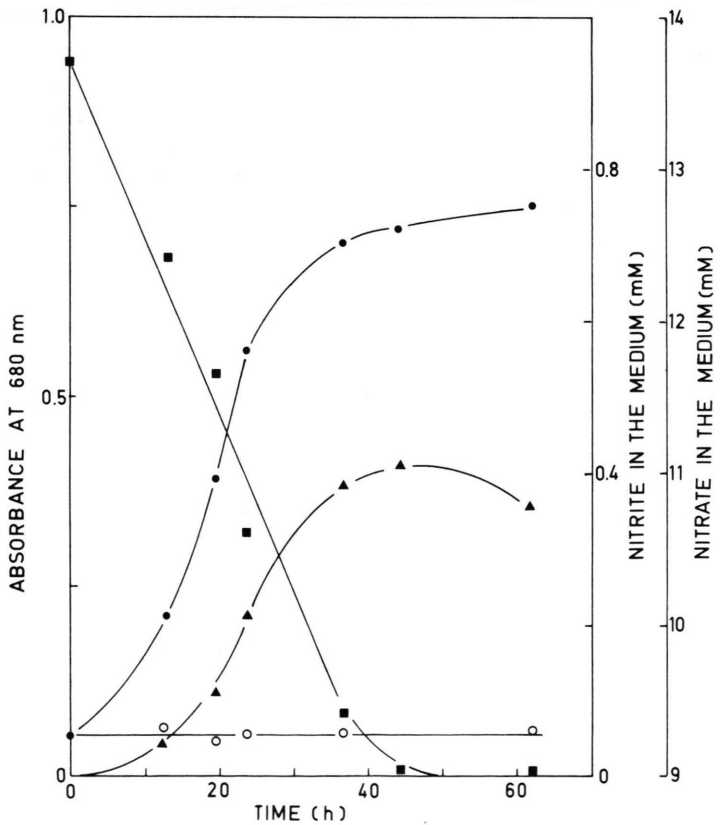

Fig. 1. Growth representation and time courses of nitrate uptake and nitrite excretion by *R. capsulata* E_1F_1 cells growing with or without nitrate under argon. Cells were grown anaerobically in the light as described in Material and Methods. ■—■: nitrate in the medium; ▲—▲: nitrite in the medium; ●—●: absorbance of cultures with nitrate; ○—○: absorbance of cultures without nitrate.

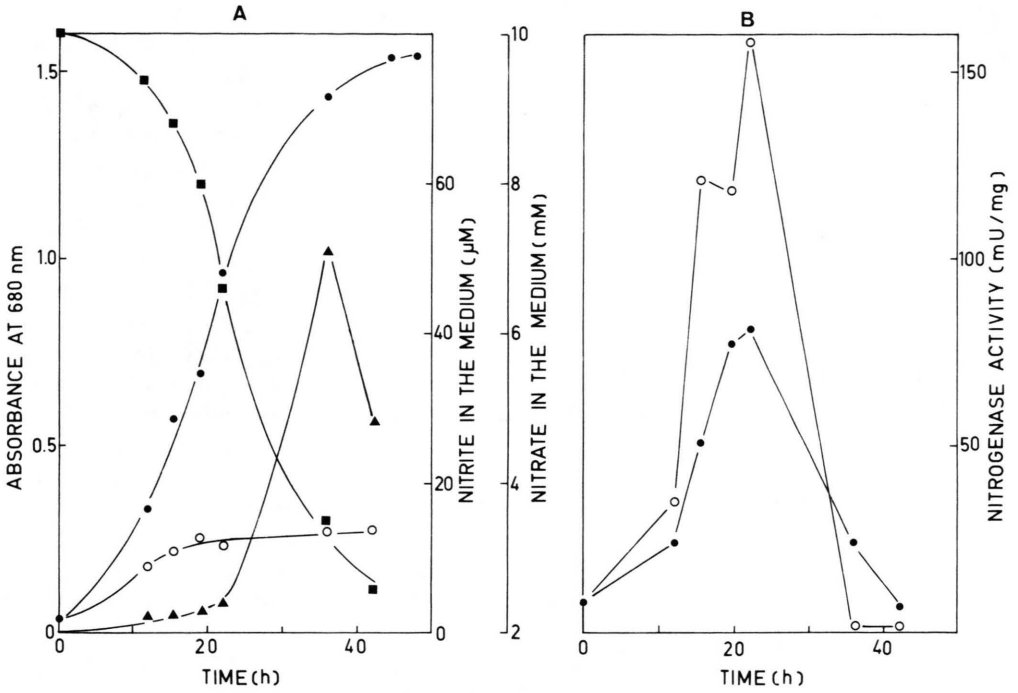

Fig. 2. A) Growth representation and time courses of nitrate uptake and nitrite excretion by *R. capsulata* E_1F_1 cells growing anaerobically in the light with or without nitrate in media not sparged with argon. ■—■, nitrate in the medium; ▲—▲, nitrite in the medium; ●—●, absorbance of cultures with nitrate; ○—○, absorbance of cultures without nitrate. B) Time course of nitrogenase activity in whole cells of *R. capsulata* E_1F_1 cells growing anaerobically in the light in media not sparged with argon. ●—●, cultures with nitrate; ○—○, cultures without nitrate.

nitrogenase activity since the cellular levels of the enzyme in cells grown on nitrate were always lower than those measured in cultures lacking nitrate (Fig. 2B). Inhibition of nitrogenase by nitrite has been reported in *R. capsulata* B_{10} [18], in *R. palustris* [20] and in other non-photosynthetic nitrogen fixers [21]. The sharp decline in nitrogenase of Fig. 2B after 30 h of growth is probably due to the destruction of the enzyme in ageing cells.

Only chemically reduced viologens and photochemically reduced flavins supported nitrate reduction to an appreciable extent in membrane preparations of *R. capsulata* E_1F_1. In contrast, neither chemically reduced flavins nor reduced pyridine nucleotides (with or without added flavins) acted as electron donors in the enzyme reaction (Table I). In this respect, nitrate reductase of *R. capsulata* E_1F_1 is similar to that of other photosynthetic [22, 23] and chemotrophic bacteria [24, 25], and differs from that of fungi and higher plants [26] and some purple bacteria [4] which have NAD(P)H-linked assimila-

Table I. Electron donors and cofactors for nitrate reductase from *R. capsulata* E_1F_1.

Electron donors and cofactors	Concentration [mM]	Nitrate reductase (relative units)
$S_2O_4^{2-}$	5	0
Methyl viologen/$S_2O_4^{2-}$	0.2/5	100
Benzyl viologen/$S_2O_4^{2-}$	0.2/5	35
Riboflavin/EDTA, light	0.5/10	41
FMN/EDTA, light	0.5/10	45
FAD/EDTA, light	0.5/10	36
Riboflavin/$S_2O_4^{2-}$	0.1/5	0
FMN/$S_2O_4^{2-}$	0.1/5	0
FAD/$S_2O_4^{2-}$	0.1/5	0
NAD(P)H	0.3	0
NAD(P)H/FMN or FAD	0.3/0.1	0

Nitrate reductase activity was measured at 30 °C in standard reaction mixtures containing besides the enzyme, in a final volume of 1 ml, the indicated concentrations of donors and cofactors, 10 mM KNO_3 and 0.05 M HCl-Tris buffer (pH 8.0). The photochemical assays were carried out anaerobically under argon at 25 °C. Other experimental conditions were as described in Materials and Methods. 100% activity corresponded to 20 mU/mg protein.

Table II. Effect of different inhibitors on *R. capsulata* E_1F_1 nitrate reductase activity.

Inhibitor	Concentration [M]	Inhibition [%]
None		0
p-hydroxymercuribenzoate [a]	5×10^{-4}	13
	2×10^{-3}	83
dithioerythritol [a]	2×10^{-3}	59
o-phenanthroline [b]	2×10^{-3}	40
KCN	10^{-4}	76
NaN_3	2×10^{-3}	61
KCNO	10^{-3}	35
$KClO_3$	2×10^{-3}	10

Experimental conditions were the same as in the standard assay for reduced methyl viologen nitrate reductase of Table I. The inhibitors were added at the final concentrations indicated.
[a] Enzyme incubated with the inhibitor 5 min before starting the assay.
[b] Enzyme reduced by illumination (2.8 kW/m²) at 25 °C under anaerobic conditions with 0.5 mM FMN and 10 mM EDTA.

tory nitrate reductases. The characterization of the reaction catalyzed by nitrate reductase with reduced methyl viologen indicated that no nitrate reduction took place either in the absence of methyl viologen or dithionite or when the enzyme was omitted or boiled. Besides, the reduction of nitrate was linear and stoichiometric during the first 10 min. The optimum pH for the reaction was 8.0 (results not shown). The characterization of the photochemical assay for the nitrate reductase, indicated that no nitrate reduction occurred in the dark, in the presence of boiled enzyme, or in the absence of EDTA, flavins or enzyme. Similar values of activity have been found for the photochemical assay of assimilatory nitrate reductases from *Neurospora crassa*, *Chlorella fusca* and *Chlamydomonas reinhardii* [12].

Cyanide, azide and *o*-phenanthroline were inhibitors of nitrate reductase whereas chlorate was practically without effect (Table II). This metal chelating effect strongly suggests the involvement of heavy metal(s) in enzyme activity. Similar effects on nitrate reductase have been reported in other photosynthetic bacteria [4]. Actually, the presence of iron and molybdenum has been detected in the nitrate reductases of some strains of non sulfur purple bacteria [23]. The strong inhibition by *p*-hydroxymercuribenzoate points to a possible role of SH-groups in binding or catalysis. Besides, a considerable inhibition by dithioerythritol, a well known agent responsible for the reductive cleavage of disulfide bonds [27], was also observed. Physiological studies with molybdenum and tungsten indicate the possible involvement of molybdenum in the reduction of nitrate by *R. capsulata* E_1F_1 cells (results not shown).

As in certain chemotrophic bacteria, cyanobacteria and eukaryotic algae [26] ammonia and reduced nitrogen compounds repressed the nitrate reductase of *R. capsulata* E_1F_1, even in the presence of nitrate (Table III). When NH_4NO_3 was the nitrogen source, ammonia was utilized first, and only after exhaustion of ammonia, nitrate was used. Whether the effect of these compounds is direct at the transcriptional or post-transcriptional level, or through a product of their incorporation, or otherwise, remains to be clarified. Curiously, nitrite was a better inducer of nitrate reductase than nitrate itself.

Attempts to grow the cells in the dark either with nitrate or nitrite as nitrogen source and under anaerobic conditions were unsuccessful.

Table III. Effect of different nitrogen sources on the nitrate reductase of *R. capsulata* E_1F_1.

Nitrogen source	Nitrate reductase activity (relative units)
NH_4Cl	1
NH_4NO_3	0
KNO_3	86
KNO_2	100
N_2	0
Yeast extract	1
L-glutamate	1

Cells from inocula with NH_4Cl were centrifuged aseptically and then transferred into the media with the above nitrogen sources at the concentrations indicated in Materials and Methods, harvested at the mid-logarithmic phase of growth, and activity was assayed with chemically reduced methyl viologen as described. 100% activity corresponded to 22 mU/mg protein.

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