The assimilatory nitrate reductase from the phototrophic bacterium, *Rhodobacter capsulatus* E1F1, is a flavoprotein

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Abstract The assimilatory nitrate reductase from the phototrophic bacterium *Rhodobacter capsulatus* has been purified to electrophoretic homogeneity and its molecular and kinetic parameters determined. The native nitrate reductase is a dimer of 144 kDa composed of two subunits of 46 and 95 kDa. The purified enzyme catalyzes the electron transfer from NADH, reduced bromophenol blue or reduced viologens to nitrate. The nitrate reductase contains 1 mol FAD per mole of enzyme and also reduces cytochrome *c* or dichlorophenol indophenol with NADH as the electron donor. The diaphorase activity is located in the small subunit.

Key words: Nitrate reductase; Flavoprotein; Phototrophic bacterium

1. Introduction

The assimilation of nitrate is a key process of the nitrogen cycle that is an object of current research in higher plants, fungi, green algae, cyanobacteria and heterotrophic bacteria [1–8]. Nevertheless, the assimilatory nitrate and nitrite reductases have only been scarcely studied in phototrophic bacteria [9,10]. By contrast, the molecular bases of nitrate respiration/denitrification and periplasmic nitrate reduction are well known in both heterotrophic and phototrophic bacteria [11–18].

The electron donor for the assimilatory nitrate reduction is NAD(P)H in eukaryotic organisms [1,2,7] and ferredoxin in cyanobacteria [8,19]. In spite of the assimilatory nitrate reduction having been studied in bacteria, the physiological electron donor has remained unidentified. The purified assimilatory nitrate reductase from *Azotobacter vinelandii* consists of a single subunit of 105 kDa that contains a 4Fe4S center and 1 Mo atom per enzyme molecule [4]. No evidence for the presence of FAD or cytochromes was found in the purified enzyme, which can use the flavodoxin 1 as an electron donor in vitro [20].

In *Klebsiella pneumoniae* the genes involved in nitrate assimilation are clustered in the *nasFEDCBA* operon: *nasC* codifies a 43 kDa NADH–dehydrogenase necessary for nitrate assimilation, *nasB* encodes the nitrite reductase and *nasA* codifies a 92 kDa nitrate reductase [21,22]. The expression of *nas* operon is dependent on a dual control exerted by the *ntr* regulatory system [23,24] and by nitrate/nitrite induction [25]. Anti-termination of transcription has been suggested to be involved in the expression of the *nas* operon in *K. pneumoniae* [3,26]. A positive regulation of nitrate assimilation by nitrite has also been reported in cyanobacteria [27].

In *Bacillus subtilis* the assimilatory nitrate reductase is encoded by *nasBC* genes. NasB contains putative NAD(P)H- and FAD-binding domains and nasC is homologous to the nitrate reductase from *Synechococcus* [28]. In *A. vinelandii* an operon coding the nitrate assimilation system has also been characterized [5]. Both in *Bacillus* and *Azotobacter* the expression of *nas* genes is under global nitrogen control [29,30]. In *A. vinelandii* the *nasST* operon is required for the expression of the *nasAB* operon, which codifies the nitrate and nitrite reductases [30].

Regarding nitrate transport, a nitrate permease multicomponet system has been described in *K. pneumoniae* [22] and *Azotobacter chroococcum* [31]. A 47 kDa periplasmic protein is involved in nitrate uptake and nitrate reductase induction in *Rhodobacter capsulatus* [32]. In *B. subtilis*, the NasA protein has been reported as a nitrate transporter [28].

On the basis of genetic studies it has been suggested that NADH couples to the assimilatory nitrate reductase through the NasC protein in *Klebsiella* [22] and through the NasB protein in *Bacillus* [28]. Nevertheless, this coupling has not been demonstrated in purified nitrate reductase preparations. In this work it is shown for the first time that the assimilatory nitrate reductase from a phototrophic bacterium is a NADH-dependent flavoprotein. The molecular and kinetic properties of the enzyme are also presented.

2. Materials and methods

2.1. Culture conditions

Rhodobacter capsulatus E1F1 was cultured under light-anoxic conditions as previously described [33] with 10 mM KNO_3 as the nitrogen source and 30 mM malate as the carbon source. Under dark-oxic conditions the bacterium was grown in Erlenmeyer flasks filled up to 10% of its volume with culture medium, capped with sterile cotton stoppers and shacken at 140 r.p.m. Under air, the carbon source was 5.5 mM glucose and the nitrate concentration was lowered to 2.5 mM.

2.2. Preparation of cell-free extracts

The cells were harvested at the mid-logarithmic phase by centrifugation, resuspended in 50 mM phosphate buffer (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride and disrupted by cavitation at 75 W for 45 s. The cell debris was removed by centrifugation at 100 000 × *g* for 90 min. The clear supernatant is referred to as the cell-free extract.

2.3. Enzymatic assays

NADH–nitrate reductase and reduced methyl viologen nitrate reductase were measured as previously described [34].

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Abbreviations: p-HMB, *p*-hydroxymercuribenzoate; BPB(H), (reduced) bromophenol blue; DCPIP, dichlorophenol indophenol; MV, methyl viologen; MTA, mixed alkyl trimethylammonium bromide

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NADH–diaphorase was measured in a reaction mixture containing, in a final volume of 1 ml, 50 μmol Tris-HCl buffer (pH 8.0), 0.4 μmol NADH, 50 nmol DCPIP or cytochrome *c* as electron acceptors and an appropriate amount of enzyme. Reduction of cyt *c* or DCPIP were followed at 550 or 600 nm, respectively.

Reduced bromophenol blue–nitrate reductase activity was measured colorimetrically at 540 nm. The reaction mixture contained 100 μmol Bis-Tris propane buffer (pH 9.5), 10 μmol potassium nitrate, 0.2 μmol bromophenol blue, 5 μmol sodium dithionite and an appropriate amount of enzyme in a final volume of 1 ml. The nitrite produced was estimated according to Snell and Snell [35].

In polyacrylamide gels, the nitrate reductase and the diaphorase activities were detected as previously described [7,36].

2.4. Purification procedure

Cell-free extracts were fractionated with solid ammonium sulfate (35–50% saturation). The pellet from the 50% saturation was resuspended in 50 mM phosphate buffer (pH 6.8), treated with 0.075% MTA (w/v) and centrifuged at 30 000×*g* for 10 min. The supernatant was loaded onto a G-75 gel filtration column (5.5×1.5 cm) equilibrated with 10 mM NaCl. All further chromatographic steps were run at 4°C in a Pharmacia FPLC system. The exclusion volume of the G-75 chromatography was loaded onto a hydroxyapatite column (4.0×1.5 cm) equilibrated with 10 mM NaCl. The column was washed with 14 ml of the same buffer and the activity was eluted by using a 25 ml linear gradient of potassium phosphate pH 6.8 (0–25 mM). The active fractions were pooled, glycerol added up to 3% (w/v) and loaded onto a Mono-Q HR 5/5 anion-exchange column equilibrated with 50 mM Bis-Tris propane (pH 9.5) containing 3% glycerol (buffer A). The column was washed twice, first with 20 ml of buffer A and then with 10 ml of buffer A containing 200 mM NaCl. The activity was eluted by using a 16 ml linear gradient from 200 to 300 mM NaCl. The active fractions were pooled and the proteins were precipitated with solid ammonium sulfate at 50% saturation. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0) buffer, containing 0.5 M ammonium sulfate. The suspension was loaded onto a phenyl superose HR 5/5 column which was washed with 5 ml of the same buffer. The enzyme activity was eluted with a 20 ml linear gradient of ammonium sulfate (0.5–0 M).

2.5. Analytical methods

The protein concentration was estimated according to Lowry et al. [37]. The flavins were measured by HPLC as previously described [36]. The molecular parameters of the native nitrate reductase were determined as follows: the Stokes' radius was calculated as described by Siegel and Monty [38]; the sedimentation coefficient was measured according to Martin and Ames [39]; the frictional quotient f/f_0 was calculated as described by Brewer et al. [40]. The protein standards used in these methods were as previously described [41]. The molecular mass of the native nitrate reductase was also estimated by PAGE (5–15% polyacrylamide gradient). The following protein standards were used: bovine liver catalase (240 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and soybean trypsin inhibitor (22 kDa). For the determination of the molecular masses of the nitrate reductase subunits, SDS electrophoresis was performed according to Laemmli [42], using as protein standards: rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and bovine erythrocytes carbonic anhydrase (29 kDa).

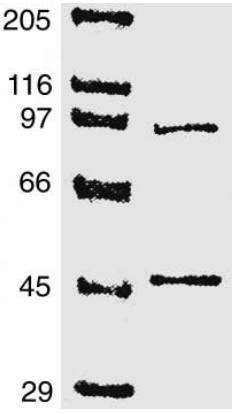

Fig. 1. SDS-PAGE of the purified nitrate reductase from *R. capsulatus* E1F1. The eluate from the Phenyl Superose column (20 μg) and the protein standards were applied to a SDS-polyacrylamide gel as described in Section 2.

3. Results and discussion

The assimilatory nitrate reductase from *Rhodobacter capsulatus* E1F1 has been purified to electrophoretic homogeneity. The purification procedure is shown in Table 1. The purified protein exhibited three enzymatic activities: NADH–nitrate reductase (total activity), BPBH–nitrate reductase (terminal activity) and NADH–cytochrome *c* reductase (diaphorase activity). The preparation was homogeneous according to electrophoretic criteria and showed two protein bands in SDS-PAGE of 46 and 95 kDa (Fig. 1 and Table 2). The molecular mass of the enzyme calculated from its hydrodynamic parameters was 147 kDa (Table 2).

The terminal and the diaphorase activities could also be assayed in gels when the purified enzyme was subjected to non-denaturing pore gradient PAGE. A major protein band of 144 kDa showing both the BPBH–nitrate reductase and the NADH–diaphorase activities was detected (Fig. 2). The diaphorase activity was also associated to a tiny protein band of about 50 kDa (Fig. 2), that probably corresponded to the 46 kDa subunit observed in SDS gels (Fig. 1). These results suggest that the diaphorase activity was located in the small subunit of the nitrate reductase, and that the native protein was partially dissociated upon non-denaturing pore gradient PAGE. Therefore, it can be concluded that the native enzyme is a molecular entity composed of two subunits of 46 and 95 kDa (as deduced from SDS-PAGE) corresponding to the diaphorase and the terminal nitrate reductase activities, respectively. The physiological role of the NADH–dehydrogenase

Table 1
Purification of the nitrate reductase from *Rhodobacter capsulatus* E1F1

	Volume (ml)	Protein (mg/ml)	Total activity (mU)	Specific activity (mU/mg)	Y (%)	*F*
Cell-free extract	215	5.8	92 880	74	100	1
Ammonium sulfate+MTA	17.5	19	81 527	245	87	3.3
Sephadex G-75	38	4.7	64 514	361	69	4.9
Hydroxy apatite	22	2.22	19 198	393	20	5.3
Mono Q	10	0.11	9 100	8 273	10	112
Phenyl Superose	1	0.07	1 980	28 286	2.1	382

MTA treatment was performed by mixing the 35–50% ammonium sulfate supernatant with MTA up to a final concentration of 0.075% at 0°C for 5 min. F, purification factor.

Table 2
Kinetic and molecular parameters of nitrate reductase from *Rhodobacter capsulatus*

Molecular mass (kDa)	
Gel filtrationand ultracentrifugation	147
Native electrophoresis	
NR	144
Diaphorase	50
SDS electrophoresis	
Small subunit	46
Large subunit	95
Sedimentation coefficient ($S_{20,w}$)	9.17
Stokes radius (nm)	3.84
Friction quotient (f/f_0)	1.108
Optimal pH	
NADH–NR	9.0
MV–NR	9.5
NADH–diaphorase (DCPIPR)	8.5
Activation energy (kJ/mol)	57.7
Km NO_3^- (mM)	
NADH–NR	0.096
MV–NR	0.13
BPB–NR	13.0
K_m NADH (μM)	
NR	52
DCPIPR	67
K_m DCPIP (mM)	100
K_m cyt *c* (mM)	80
K_m BPB (mM)	0.54
K_m MV (mM)	0.87

NR, nitrate reductase; DCPIPR, DCPIP reductase.

moiety is probably to transfer electrons from NADH to nitrate in vivo.

The molecular properties of the assimilatory nitrate reductase from *R. capsulatus* differs from those described for the purified enzyme from *Azotobacter*, which seems to be a single subunit of 105 kDa [4]. Nevertheless, the enzyme from *R. capsulatus* E1F1 might be similar to the one codified by the *nasCA* genes in *K. pneumoniae* [21,22], since the predicted molecular masses of the NasC and NasA proteins, as deduced from DNA sequencing, agree with the sizes of the two moieties of the nitrate reductase purified from *R. capsulatus* E1F1. Moreover, the fact that the mutation in *nasC* gene results in the inability of *Klebsiella* to grow with nitrate [22] supports the hypothesis that the role of the diaphorase subunit is the coupling between NADH and the terminal subunit of the nitrate reductase. A similar diaphorase moiety is codified by the *nasB* gene of *B. subtilis* [28].

The purified assimilatory nitrate reductase from *R. capsulatus* E1F1 exhibited the same molecular properties independently of the bacterial growth conditions, either under light-anoxic conditions with nitrate and malate or under dark-oxic conditions with glucose and nitrate (data not shown). This suggests that the strain E1F1 only synthesizes the assimilatory nitrate reductase and not the periplasmic or the membrane-bound enzymes. In the darkness, to avoid inhibition of growth by nitrite [43], nitrate concentration in the medium was lowered to 2 mM.

Table 2 shows the molecular and kinetic parameters of the nitrate reductase from *R. capsulatus* E1F1. The enzyme could be assayed with NADH or reduced viologens and bromophenol blue as electron donors. The ability of bromophenol blue as an electron donor for nitrate reduction has been previously shown in the assimilatory nitrate reductase from eukaryotic organisms [7,44] and seems to be a characteristic of the assimilatory nitrate reductases. Actually, the periplasmic enzyme from *Rhodobacter sphaeroides*, a dimer composed of a cytochrome *c* subunit and a catalytic nitrate reductase moiety of 92 kDa [17], does not use reduced bromophenol blue as an electron donor (unpublished results).

The assimilatory nitrate reductase from *Azotobacter vinelandii* does not possess flavin as prosthetic group [4]. Taking into account that the nitrate reductase from *R. capsulatus* E1F1 used NADH as an electron donor and exhibited NADH–diaphorase activity, we tested the presence of flavin in the purified enzyme. When samples of the purified nitrate reductase were heated and analysed by HPLC coupled to a diode array detector, a chromophore showing the same retention time and spectrum as standard FAD was detected (Fig. 3). The amount of the chromophore corresponded to an stochiometry of 1 mol FAD/1 mol enzyme. This result agrees with the predicted FAD-binding domain previously reported in NasB protein from *B. subtilis* [28]. Therefore, we concluded that the native assimilatory nitrate reductase from *R. capsulatus* E1F1 is a flavoprotein. This is consistent with the inactivation of the nitrate reductase by atebrine (a flavin analogue) and NADH. Like the eukaryotic assimilatory nitrate reductase, the enzyme of *R. capsulatus* was strongly inactivated by NADH under air (data not shown). This inactivation is probably due to the formation of superoxide anion at the flavin center since the activity was protected in the presence of superoxide dismutase (data not shown). A similar process has been shown for the NADH inactivation of the nitrate reductase from *Ankistrodesmus braunii* [45]. Other nucleophiles such as cyanide or acetylene also inactivated nitrate reductase from *R. capsulatus* (data not shown). These results also agree with those previosly reported for the eukaryotic enzyme [46]. The lability of the enzyme towards oxygen and other nucleophiles may account for the poor yield achieved in the purification

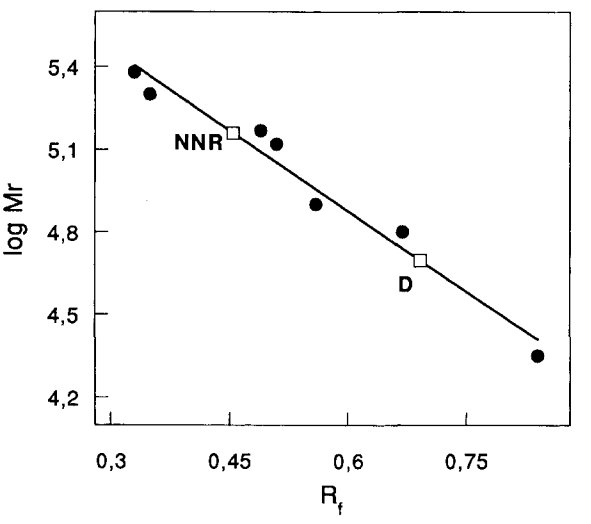

Fig. 2. Gradient pore PAGE of the purified nitrate reductase from *R. capsulatus* E1F1. The protein standards (●) and the purified nitrate reductase were applied to a gradient pore polyacrylamide gel as described in Section 2. BPBH–nitrate reductase and NADH–diaphorase activities (□) were stained as previously described. NNR represents the native 144 kDa enzyme exhibiting both activities; D represents the 50 kDa protein that only exhibitis diaphorase activity.

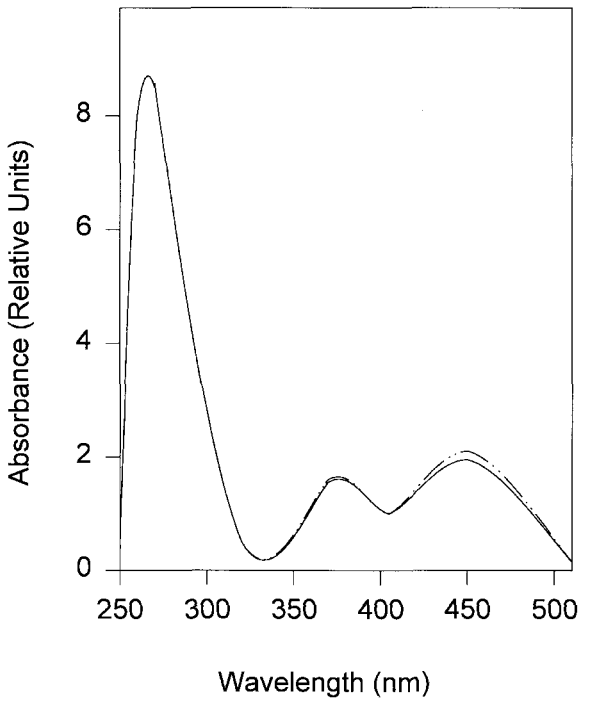

Fig. 3. Comparative spectra of standard FAD (——) and the chromophore extracted from the purified nitrate reductase (—— · · ——). The purified protein was heated and the sample injected in an HPLC column as described in Section 2. The HPLC peak with the same retention time than an FAD standard was analyzed by the diode array system and the spectrum was normalized and compared with the standard.

procedure (2% of the initial activity). In addition, the assimilatory nitrate reductase from *Rhodobacter* seemed to be cleaved into a 66 kDa polypeptide that only retained the terminal activity (not shown), thus lowering the final yield even more.

In conclusion, the assimilatory nitrate reductase from *R. capsulatus* E1F1 seem to be completely different from the enzyme previously described in *Azotobacter* or *Anabaena*. Nevertheless, the molecular structure of the native nitrate reductase from *R capsulatus* E1F1 agrees with that predicted for the *Klebsiella* enzyme, as deduced from DNA sequencing of the *nas* operon [21,22]. In addition, the regulatory pattern of nitrate assimilation in *R. capsulatus* E1F1 [32] could also be similar to that described in *Klebsiella* [3]. Due to the flavin group of the diaphorase moiety, NADH is probably the physiological electron donor for the assimilatory nitrate reductase in *R. capsulatus* E1F1.

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