

# Purification and Characterization of a Dissimilatory Nitrite Reductase from the Phototrophic Bacterium *Rhodopseudomonas palustris*

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A dissimilatory nitrite reductase from the facultatively phototrophic bacterium, *Rhodopseudomonas palustris* strain 1a1 was studied. A basic level of the enzyme (10–50 mU/mg protein) was measured in dark, aerated and anaerobic, photosynthetic cultures. A marked derepression of enzyme synthesis occurred under conditions of oxygen limitation (200–300 mU/mg protein). The addition of nitrite (or nitrate) to the culture medium had only a slight effect on the maximal nitrite reductase titer of cells. The enzyme was purified from photosynthetically grown cells by precipitation with ammonium sulfate, gel filtration through Sepharose 6B and repeated chromatography on DE 52-cellulose. As estimated by gel filtration, the nitrite reductase had a molecular weight of about  $120\,000 \pm 12\,000$  and yielded only one band (mol. wt. of about  $68\,000 \pm 7000$ ) in SDS-gel electrophoresis. The isoelectric point of the enzyme was at pH 5.1. Nitric oxide (NO) was identified as the reaction product of nitrite reduction. The enzyme also exhibited cytochrome *c*-oxidase activity and was active with chemically reduced viologen dyes, FMN and cytochrome *c* as electron donors. Highly purified nitrite reductase preparations contained 10 mol% of a *c*-type cytochrome. Trace metal analyses indicated the presence of Cu in the enzyme. Consistent with the detection of Cu was the finding that the Cu-chelator, diethyl-dithiocarbamate, strongly inhibited the nitrite reductase.

## Introduction

In denitrifying prokaryotes, at least two types of dissimilatory nitrite reductases, *i.e.* enzymes catalyzing the reduction of nitrite to gaseous NO or N<sub>2</sub>O, can be distinguished. A cytochrome *cd* containing enzyme with a four-heme moiety (EC 1.9.3.2) has been isolated from various *Pseudomonas* species [1, 2], *Paracoccus denitrificans* [3], *Thiobacillus denitrificans* [4], *a.o.*, whereas heme-free Cu-containing enzymes (EC 1.7.99.3) have been reported to occur in *Achromobacter cycloclastes* [5], *Alcaligenes faecalis* [6] and a denitrifying strain of the phototrophic bacterium, *Rhodopseudomonas (Rps.) sphaeroides* [7]. In a previous paper [8], we have shown that certain strains of *Rps. palustris*, another member of the facultatively phototrophic bacteria, contain a nitrite reductase of the dissimilatory type. In this communication we describe the purification of the enzyme from *Rps. palustris* strain 1a1 and present data on its catalytic, structural and physiological properties.

## Material and Methods

*Rps. palustris* strain 1a1 (originally obtained from Prof. Dr. G. Drews, Universität Freiburg, as strain 1e1) was maintained photosynthetically (30 °C, 2500 lux) on a culture medium containing 22 mM Na-D,L-malate, 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05% (w/v) yeast extract and mineral salts as specified in [9]. For photosynthetic growth (30 °C, 2500 lux) of larger cell batches in 100, 500 or 1000 ml-screw cap bottles, the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> of the latter medium was replaced by 25 mM NH<sub>4</sub>NO<sub>3</sub>. The bacteria were also grown aerobically in Erlenmeyer flasks (malate-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-medium) agitated vigorously at 30 °C in the dark by the aid of a magnetic stirrer, or semianaerobically (30 °C) in the dark in Erlenmeyer flasks filled to 4/5 of their volume (malate-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-medium slowly agitated by magnetic stirrer).

Growth was followed by measuring the OD<sub>660</sub> in a "Spectronic 20" photometer (Bausch & Lomb, München) of appropriately diluted samples taken aseptically from the culture medium.

Cell free extracts were prepared by treatment of cells with ultrasonic oscillation (Ultrasonic disintegrator of Schoeller & Co., Frankfurt) followed

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by removal of cell fragments and intact cells by centrifugation (4 °C) at  $10\,000\times g$  for 15 min. Protein concentrations in extracts and purified enzyme preparations were determined by the Lowry method [10].

Nitrite reductase activity in cell free extracts or purified enzyme preparations was determined at 30 °C by measuring the disappearance of nitrite in reaction mixtures (pH 6.5) containing dithionite-reduced benzylviologen as electron donor [8]. In experiments aimed at measuring the formation of the gaseous reaction products of nitrite reduction, the reaction was performed in  $N_2$ -flushed Warburg vessels (30 °C) in the presence and absence of alkaline  $KMnO_4$  [3]. Cytochrome oxidase activity of purified nitrite reductase with  $O_2$  as electron acceptor was measured manometrically at 30 °C in reaction mixtures containing 20 mM Na-ascorbate as electron donor and 0.25 mM cytochrome *c* (horse heart) in 50 mM K-phosphate, pH 7.5.

One unit (U) is the activity catalyzing the conversion of 1  $\mu$ mol substrate per min at 30 °C. Ammonia was analyzed colorimetrically by using Nessler's reagent [11].

For purification of nitrite reductase, the cells harvested from about 20 liters of an exponential phase photosynthetic culture were suspended in 200 ml K-phosphate (100 mM, pH 6.5) supplemented with 1 mM EDTA and 0.1 mM  $NaN_3$  and ruptured by ultrasonic treatment (30 s per ml cell suspension at maximal output). The resulting homogenate was centrifuged for 15 min (4 °C) at  $10\,000\times g$  to remove unbroken cells and large cell debris. The dark-red colored supernatant (= crude extract) was then subjected to a fractionation by ammonium sulfate. The protein fraction precipitating between 35 and 75% saturation was collected by centrifugation and dissolved in about 10 ml of the buffer mixture quoted before.

This enzyme solution was filtered through a column of Sepharose 6B ( $2.6\times 95$  cm) equilibrated with the same buffer mixture. The nitrite reductase peak fractions were pooled and saturated with ammonium sulfate up to 70%. The precipitated protein was centrifuged down, dissolved in buffer and filtered through a second column of Sepharose 6B ( $1.5\times 90$  cm).

Nitrite reductase in the pooled peak fractions was again precipitated with ammonium sulfate, collected by centrifugation and dissolved in a small

volume of 10 mM Tris-HCl, pH 7.8. This enzyme solution was dialyzed overnight (4 °C) against 2 liters of the latter buffer and was then applied to a column of DE 52-cellulose ( $1.5\times 30$  cm) equilibrated with 10 mM Tris-HCl, pH 7.8. The DE 52-column was washed with 100 ml of the latter buffer and then with 100 ml of a mixture of 10 mM Tris-HCl, pH 7.8, and 10 mM NaCl, to remove the unbound proteins. Nitrite reductase was finally eluted with a NaCl-gradient (50–75 mM NaCl) in 10 mM Tris-HCl, pH 7.8 (Fig. 1). Enzyme in the peak fractions was concentrated by precipitation with ammonium sulfate at 70% saturation, dissolved in a small volume of 10 mM Tris-HCl, pH 7.8, dialyzed against this buffer and rechromatographed on a smaller DE 52-column ( $1.6\times 10$  cm) with elution conditions described for the first DE 52-column. The enzyme in the peak fractions was concentrated by using CX-10 filter units of Millipore GmbH, Neu-Isenburg. The purification procedure outlined before is summarized in Table I.

Absorption spectra were recorded with the "Acta M VI" spectrophotometer (Beckman Instruments GmbH, München). Electrophoretic separation of native proteins were run in polyacrylamide gels (gel system No. 1a) according to [12]. SDS gel electrophoresis for subunit analysis was performed according to [13] using bovine serum albumin, trypsin inhibitor and the subunits of RNA polymerase as molecular weight markers. Determination of the isoelectric point (IEP) of nitrite reductase

Table I. Purification of *Rps. palustris* 1a1 nitrite reductase.

Fraction	Vol. [ml]	Protein [mg]	Nitrite reductase activity	
			Total units	U/mg protein
Crude extract	170	1615	129	0.08
35–70% $(NH_4)_2SO_4$ fraction	17	1105	122	0.11
1 <sup>st</sup> Sepharose 6B-filtrate	57	319	72	0.23
2 <sup>nd</sup> Sepharose 6B-filtrate	29	206	71	0.34
1 <sup>st</sup> DE 52-eluate	2	1.1	11	10
2 <sup>nd</sup> DE 52-eluate	3	0.15	1.1	6.6

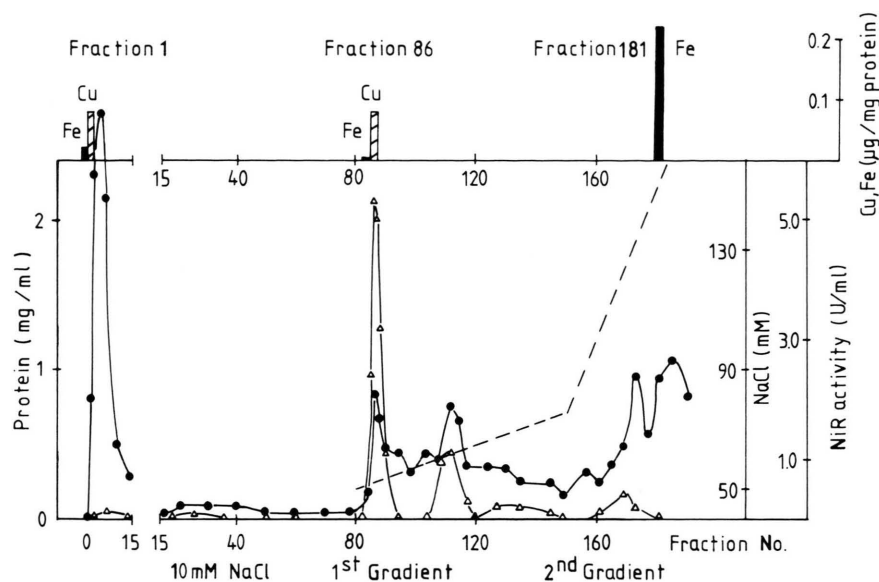


Fig. 1. Fractionation of *Rps. palustris* 1a1 soluble proteins by DE 52-chromatography, distribution of dissimilatory nitrite reductase, and trace metal (Cu, Fe) content of various fractions. Eight ml of a Sepharose 6B-filtrate (see section Materials and Methods) containing 192 mg protein were dialyzed against 10 mM Tris-HCl, pH 7.8, and then applied to a column (1.5 × 30 cm) of DE 52-cellulose equilibrated with the latter buffer. The first protein fraction was eluted by washing the column with 10 mM Tris-HCl, pH 7.8. Nitrite reductase activity and other proteins were eluted with two successive NaCl-gradients (50 mM to 75 mM; and 75 mM to 250 mM) in 10 mM Tris-HCl, pH 7.8. Fractions of 2 ml were collected and assayed for protein (●), nitrite reductase activity (△) and – in some cases – Fe and Cu content.

was carried out by isoelectric focusing with the Multiphor 2117 (LKB) using Ultrodex gel (LKB) containing ampholine (LKB) with a pH range of 3 to 11 [14].

Trace metal concentrations of purified enzyme preparations were analyzed by atomic absorption spectroscopy (Mikroanalytisches Labor E. Pascher, 5300 Bonn) using a Perkin Elmer model 300 AA spectrophotometer equipped with a HGA graphite furnace cuvette. NADH, cytochrome c and marker proteins for molecular weight determination were obtained from Boehringer, Mannheim; Sephadex G-25, Sepharose 6B and Sepharyl S-300 from Deutsche Pharmacia, Freiburg; DE 52-cellulose from Whatman, Maidstone, Kent; riboflavin, viologen dyes and chemicals for analytical polyacrylamide gel electrophoresis from Serva, Heidelberg; ampholines for IEP determinations from LKB Instrument GmbH, Gräfelfing; and all other chemicals from Merck, Darmstadt.

## Results and Discussion

In our previous paper [8], we have reported the presence of both, nitrate and nitrite reductase (the

latter of the dissimilatory type) in a strain (1a1) of the phototrophic bacterium, *Rps. palustris*. For an unknown reason, strain 1a1 has lost the ability to synthesize active nitrate reductase during a two year period of maintenance under photosynthetic conditions in malate- $\text{NH}_4\text{NO}_3$ -medium. However, formation of nitrite reductase activity was a stable property of the strain. As shown in Table II, the

Table II. Nitrite reductase activities in *Rps. palustris* 1a1 cells grown under different culture conditions.

Culture condition (all at 30 °C)	Doubling time [h]	Maximal nitrite reductase activity [mU/mg protein]
Dark, aerated (see text)	6.9	10–40
Dark, oxygen limited (see text)	27.7	200–300
Light, anaerobic		
no addition	6.4	40–60
plus 10 mM $\text{KNO}_3$	6.6	50–90
plus 2 mM $\text{KNO}_2$	7.8	48–75

The basal culture medium (pH 6.8) contained 22 mM Na-D,L-malate, 7.5 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.05% (w/v) yeast extract and mineral salts as specified in [9].

specific activities of nitrite reductase in *Rps. palustris* 1a1 ranged from 0.01 to at maximum 0.30 U/mg protein in cell populations grown under various culture conditions. In accordance with results obtained with other denitrifying bacteria [15], exposure of *Rps. palustris* 1a1 cells to conditions of high aeration repressed the formation of nitrite reductase, whereas nitrite and nitrate had only a minor effect on nitrite reductase expression. We conclude that biosynthesis of dissimilatory nitrite reductase in *Rps. palustris* 1a1 is mainly controlled by the oxygen supply to the growing culture. Under all culture conditions that allowed expression of nitrite reductase activity (dark-anaerobic; dark-aerobic and semianaerobic; light-anaerobic), the specific activity of the enzyme was found to increase markedly during exponential cell proliferation and to decline sharply at the end of the logarithmic growth phase. This general pattern is shown for a photosynthetic, anaerobic culture in Fig. 2. Note, that a drastic increase of culture medium-pH did not occur during the phase of nitrite reductase decay.

Cultivation of *Rps. palustris* 1a1 in media with increased or decreased trace metal concentrations (Cu, Fe) had no marked effect on the nitrite reductase titer of cells.

Because of the rather low cell yields in semi-anaerobic (or strictly anaerobic cultures supplemented with 25 mM Na-pyruvate and 1 mM KNO<sub>2</sub>), the nitrite reductase was purified from photosyn-

thetically grown cells. The purification procedure outlined in the foregoing section increased the specific activity of the enzyme to about 10 U/mg protein. With respect to the presence of heme or Cu as functional groups in nitrite reductases of different origin, the absorption spectra and trace metal contents of some protein fractions eluted from the first DE 52-column (Fig. 1) were measured. The nitrite reductase containing peak fraction No. 86 was practically free of heme but contained a significant amount of Cu and only traces of Fe. In contrast, the reddish-brown protein fraction No. 181 was almost free of Cu, but contained a significant amount of Fe, due to the presence of a cytochrome of the *c*-type as revealed by the absorption spectrum of that fraction. It should be remembered in this connection that soluble cytochromes of the *c*-type (cytochrome *c*<sub>2</sub>) with absorption bands at 417–418, 522 and 551.5 nm (reduced state) have been purified from various *Rps. palustris* strains [16].

Nitrite reductase preparations purified up to the stage of the second DE 52-column were nearly homogeneous when subjected to analytical gel electrophoresis (Fig. 3) and revealed an isoelectric point at pH 5.1. The molecular weight of the native enzyme, as determined by gel filtration through Sephacryl S-300, was found to be 120 000 ± 12 000. After SDS-gel electrophoresis, only one band at a position of a molecular weight of 68 000 ± 7000 dalton was found, regardless of whether gels with 5 or 7.5% acrylamide were used. This suggests that the

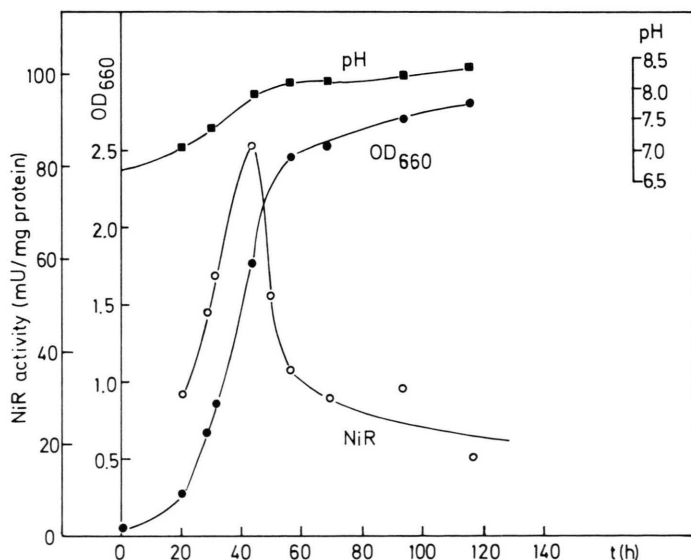


Fig. 2. Specific nitrite reductase activity of *Rps. palustris* 1a1 and pH of culture medium during anaerobic, photosynthetic growth (2500 lux, 30 °C) in a medium containing 22 mM Na-D,L-malate, 25 mM NH<sub>4</sub>NO<sub>3</sub>, 0.05% (w/v) yeast extract and mineral salts as specified in [9].  
○ nitrite reductase (NiR) activity;  
● turbidity of culture (OD<sub>660</sub>);  
■ pH of culture medium.

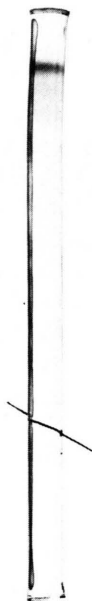


Fig. 3. Polyacrylamide gel electrophoresis of highly purified nitrite reductase of *Rps. palustris* 1a1. 32  $\mu$ g enzyme was applied to the gel (7.5% acrylamide). The dye front was marked by a plastic thread. Protein was stained with Coomassie blue.

nitrite reductase was composed of two identical subunits.

Measurement of the absorption spectrum of a nearly homogeneous enzyme preparation (about 90% pure; 0.9 mg/ml) in the dithionite-reduced state revealed absorption bands at 418, 522, and 552 nm, with an absorbance  $A_{552} = 0.02$  (Fig. 4). Based on a molar extinction coefficient of 26.7 liter/mmol for the 552-band (reduced state) of the *Rps. palustris* cytochrome  $c_2$  [17], the nitrite reductase preparation had a cytochrome  $c$ -content of 0.82  $\mu$ mol/g enzyme protein, corresponding to 9.8 mol%. Note in this connection that the double-heme nitrite reductase of *Thiobacillus denitrificans*, at the same concentration as that used for the *Rps. palustris* 1a1 nitrite reductase (0.9 mg per ml), would exhibit an  $A_{552} = 0.40$  in the reduced state [18], compared to the value of  $A_{552} = 0.02$  obtained with the *Rps. palustris* 1a1 enzyme. Thus, we cannot exclude the possibility that the cytochrome-specific absorption bands detected in the nitrite reductase preparation of *Rps. palustris* 1a1 are due to a contamination of the enzyme by soluble cytochrome  $c_2$ .

$\text{NH}_3$  was not detectable as reaction product of nitrite reduction catalyzed by the *Rps. palustris* 1a1 nitrite reductase. Manometric experiments with ascorbate-reduced cytochrome  $c$  as electron donor revealed the formation of a gaseous reaction product which was absorbed by alkaline permanganate (see [3]). The ratio between  $\mu$ mol gas produced and  $\mu$ mol nitrite consumed approached values of close to 1, suggesting that the gaseous reaction product of nitrite reduction was nitric oxide (NO). The enzyme was active with dithionite reduced benzylviologen, methylviologen, FMN and cytochrome  $c$ , the relative activities being 100, 61, 54 and 25, respectively. The  $K_m$  for nitrite (estimated in the standard reaction mixture with dithionite-benzylviologen) was 27–30  $\mu$ M.

Like the nitrite reductase of other denitrifiers, the *Rps. palustris* 1a1 enzyme also exhibited cyto-

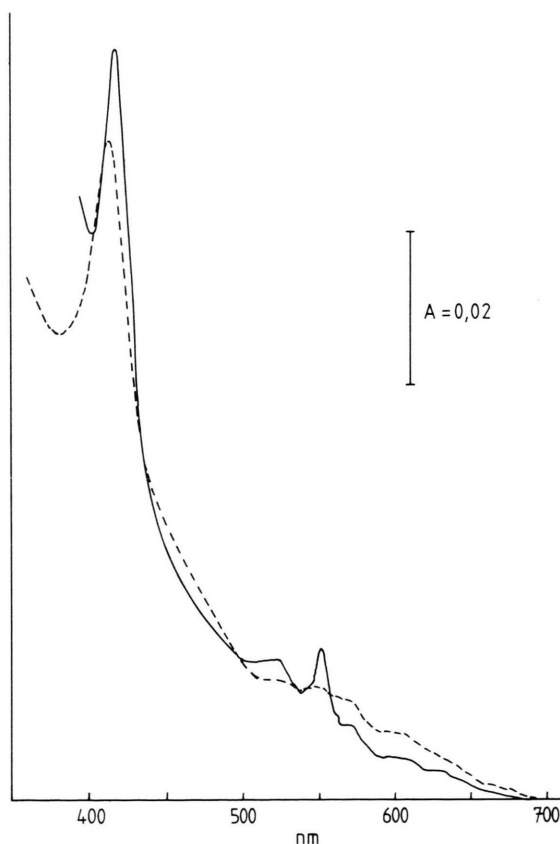


Fig. 4. Absorption spectra of highly purified nitrite reductase of *Rps. palustris* 1a1 (0.9 mg protein/ml in 10 mM Tris-HCl, pH 7.8). Oxidized preparation (enzyme as obtained from the second DE 52-column without addition of oxidant): ----; reduced preparation ( $\text{Na}_2\text{S}_2\text{O}_4$ -reduced): —.



chrome *c*-oxidase activity. A preparation with a specific nitrite reductase activity (standard test system) of 2.6 U/mg protein catalyzed O<sub>2</sub>-dependent cytochrome *c*-oxidation with Na-ascorbate as electron donor at a rate of 0.21 U/mg protein.

Trace metal analyses by atomic absorption spectroscopy of highly purified nitrite reductase preparations repeatedly yielded Cu-contents of the enzyme in the range of 1.7–2.4 µg/mg protein, corresponding to 3.2–4.5 g-atoms Cu per mol enzyme. In some preparations, also iron was found (0.5–0.6 µg/mg protein). Mn was always below the detection limit of atomic absorption spectroscopy. Consistent with the detection of Cu in highly purified enzyme preparations was the finding that the metal chelator, diethyldithiocarbamate, strongly inhibited the nitrite reductase (more than 90% inhibition at 0.5 mM of chelator). This compound has been shown to be a potent inhibitor of nitrite reductases of the Cu-type (enzymes of *Rps. sphaeroides* [7] and *Alcaligenes* [6]) but to be practically without effect on nitrite reductases of the

double-heme-type (like that of *Paracoccus denitrificans* [3]).

Our experimental results show that the phototrophic bacterium, *Rps. palustris* 1a1 contains a nitrite reductase, most probably of the "Cu-type" whose catalytic and regulatory properties are consistent with a respiratory function. In a denitrifying strain of the phototrophic bacterium, *Rps. sphaeroides*, both, nitrate reductase and dissimilatory nitrite reductase of the Cu-type are released from the cells together with other periplasmic proteins upon preparation of sphaeroplasts by enzymatic lysis of the cell wall, suggesting an association of both enzymes with the outer face of the cytoplasmic membrane [19].

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