Assimilatory Nitrate Reductase of *Rhodopseudomonas capsulata* AD2: A Molybdo-Hemeprotein

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The assimilatory nitrate reductase of the phototrophic bacterium *Rhodopseudomonas capsulata* strain AD2 was purified to homogeneity by a combination of ammonium sulfate fractionation, chromatography on DEAE-cellulose and isoelectric focusing (isoelectric point of 4.8). The purified enzyme was active only with reduced viologen dyes or reduced flavin as electron donors. Contrary to other bacterial assimilatory nitrate reductases, the enzyme was not inhibited by chlorate, but rather accepted this substance as an alternate substrate. The molecular weight of the enzyme was 185 000 dalton as determined by gelfiltration. Subunit analysis by sodium dodecyl sulfate (SDS) gel electrophoresis yielded a single protein band with a molecular weight of 85 000 dalton, suggesting that the enzyme was composed of two identical subunits. The nitrate reductase contained 0.8 g-atoms molybdenum per 1.85×10^5 g protein and exhibited absorption maxima at 418, 523 and 552 nm in the reduced state (dithionite as reductant). The nitrate reductase of *Rps. capsulata* AD2 is the first prokaryotic enzyme of the assimilatory type that has been shown to contain heme.

Introduction

In a previous paper [1], we have described some properties of an assimilatory nitrate reductase prepared from nitrate-grown cells of the phototrophic bacterium, *Rhodopseudomonas (Rps.) capsulata* strain AD2. The enzyme was located in the soluble fraction, had a molecular weight of about 180 000 dalton, and was active only with reduced viologen dyes or reduced flavins as electron donors. Since partially purified enzyme preparations regularly contained cytochrome, we decided to purify the enzyme further in order to draw definite conclusions regarding the heme content. The present communication describes the method developed for obtaining electrophoretically homogeneous enzyme preparations and shows that the nitrate reductase of *Rps. capsulata* AD2 is a molybdo-hemeprotein containing heme of the c-type.

Materials and Methods

Organisms and culture conditions. The present study was conducted with the newly isolated *Rps. capsulata* strain AD2. The strain was identified as a representative of the species *Rps. capsulata* on the basis of its morphological and cultural properties, and its sensitivity to penicillin G [2]. The strain grew well in a malate-medium with nitrate as the sole N-source, but was unable to grow under dark-anaerobic conditions with NH_4^+ as N-source and nitrate as a possible electron acceptor for anaerobic respiration. Stock cultures of *Rps. capsulata* AD2 were grown photosynthetically (malate-ammonium sulfate-medium according to ref. [2]) in 50 ml-screw cap-bottles and transferred every month. For enzymatic studies, the bacteria were grown under photosynthetic conditions (2500 lux, 30 °C) in the malate-nitrate-medium described in ref. [1]. Cultures were harvested when the turbidity had attained an OD_{660} of about 1. If necessary, cell pastes were stored at −15 °C until use.

Preparation of cell free extracts. The cells harvested from a 10 liter-culture were washed twice with 0.1 M K-phosphate, pH 7, and finally resuspended in the same buffer (2–4 g wet cells per 10 ml). The cells were ruptured at 5 °C by ultrasonic treatment (60 sec per ml cell suspension) using the KLK 250 system of Schoeller & Co., Frankfurt, at maximal output (250 w). The resulting homogenates were centrifuged at 140 000 g for 2.5 h (4 °C) to separate cell fragments and chromatophores from the soluble fraction containing the nitrate reductase.

Nitrate reductase assay. The activity of the enzyme was assayed in a reaction mixture with dithionite as electron donor [1]. If not otherwise stated, 0.1 mM benzylviologen (BV) was added as additional electron carrier. When using chlorate as

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an alternate substrate, the activity test was conducted at 30 °C in Warburg vessels containing a reaction mixture (2.5 ml) with 40 mM K-phosphate, pH 7; 3 mM methylviologen; 0.6 units of *Desulfovibrio gigas*-hydrogenase (prepared according to ref. [3]); 16 mM $KClO_3$ and 0.05 – 0.1 units of purified *Rps. capsulata* AD2-nitrate reductase. The vessels were gassed for 10 min with O_2-free hydrogen, and the reaction was started by tipping in the chlorate from the side arm into the main compartment. One unit is the enzyme activity catalyzing the conversion of 1 μmol substrate per min at 30 °C.

Analytical methods. Protein concentrations of crude extracts were determined by the method of Lowry *et al.* [4] using bovine serum albumin as standard. In purified enzyme preparations, protein was analyzed by measuring the absorbancies at 280 and 260 nm. Absorption spectra were recorded with the "Acta MVI" spectrophotometer (Beckman Instruments GmbH, München). The molybdenum content of the enzyme was analyzed by atomic absorption spectrophotometry carried out by the Mikroanalytisches Laboratorium Dr. Franz Pascher, 5300 Bonn 1. Dialyzed enzyme (0.6 mg) was used for the analysis. Electrophoretic separation of proteins was performed with the analytical disc-electrophoresis system of Desaga GmbH, Heidelberg, using 7.5% acrylamide gels according to ref. [5]. The gels were loaded with 30 μg protein and then subjected to electrophoresis in Tris-glycine buffer, pH 8.3, at 6 – 8 mA per tube. Proteins were stained with 0.12% Coomassie blue R250 in 22% methanol-4.6% glacial acetic acid. The gels were destained in a solution of 5% methanol-7.5% glacial acetic acid. SDS-gel electrophoresis for subunit analysis was performed according to the method of Weber and Osborn [6]. Protein samples with 50 – 60 μg *Rps. capsulata* AD2-nitrate reductase or 100 μg marker protein (bovine serum albumin, α-, β- and β′-subunits of *Escherichia coli*-RNA polymerase) in 10 mM Na-phosphate, pH 7, containing 1% SDS and 1% β-mercaptoethanol were denatured by heating at 100 °C for 5 min. 10 – 50 μg of protein was applied on 5% polyacrylamide gels containing 0.1% SDS. Electrophoresis was carried out at a constant current of 6 – 8 mA per gel. Staining and destaining procedures were as described above.

Isoelectric focusing. Preparative isoelectric focusing was performed in the LKB "Multiphor" apparatus supplemented with the equipment for isoelectric fucosing (LKB Instruments, Gräfelfing). Six electrofocusing paper strips (LKB, type 2117 – 106) were wetted with 10 ml of a 5% solution of carrier ampholytes (LKB "Ampholine" pH 4 – 6) and placed in the electrofocusing chamber according to the instructions given by the manufacturer. The paper strips were overlaid by a mixture of 6 g "Ultrodex" gel (LKB, type 211 – 510) and 150 ml of 5% carrier ampholytes (LKB "Ampholine" pH 4 – 6). The gel mixture was dried under a gentle stream of air until about 35% of the original weight had disappeared. The electrofocusing chamber was then placed on the top plate (covered by a thin film of 0.1% Triton X-100) of the "Multiphor" apparatus. A paper strip was wetted with 1 M phosphoric acid and connected with the anode, a second strip was wetted with 1 N NaOH and connected with the cathode. 4.5 ml of the enzyme solution (dialyzed for 15 h against 50 mM glycine, pH 6.5) was added to the sample applicator and applied to the gel at a position corresponding to about pH 5.5. Isoelectric focusing was performed at 4 °C for 24 h at a constant output of 8 w. After the end of electrofocusing, a piece of filter paper was slightly pressed on the surface of the gel. The paper was dried for 15 min at 110 °C and then washed three times with 10% trichloroacetic acid. Proteins were stained with 0.2% Coomassie blue R250 in a mixture of methanol, water and glacial acetic acid (50:50:10). The paper was destained in a mixture of 500 ml methanol, 500 ml water and 100 ml glacial acetic acid. The gel portions containing protein bands were scraped off the gel plate and eluted with 100 mM K-phosphate, pH 7. BV-linked nitrate reductase was then assayed in the eluates.

The isoelectric point of the enzyme was determined by analytical isoelectric focusing on commercially available Ampholine PAG-plates (pH 3.5 – 9.5) (LKB, type 1804 – 401). The plates were loaded with 10 μg nitrate reductase by the aid of a small piece of paper (5 × 5 mm). Electrofocusing was carried out at 4 °C for 18 h at a constant output of 8 w. After electrofocusing, the gel plates were placed for 1 h in a solution of 17.2 g sulfosalicylic acid and 57.5 g trichloroacetic acid in 500 ml of 30% methanol. Staining and destaining was performed as described above. The isoelectric point of the enzyme was located by use of the calibration curve indicating the pH-gradient between the two electrodes.

Chemicals. Bovine serum albumin, benzylviologen, methylviologen, FAD, FMN and Triton X-100 were obtained from Serva Feinbiochemica GmbH & Co., Heidelberg; NADH, lysozyme and the marker proteins for molecular weight estimations by gelfiltration and SDS-gelelectrophoresis from Boehringer Mannheim GmbH, Mannheim; Sepharose 6B from Deutsche Pharmacia GmbH, Freiburg; DEAE-cellulose type DE 52 from Whatman Ltd., Maidstone, Kent, England; gels and ampholytes for isoelectric focusing from LKB Instruments, Gräfelfing; and all other chemicals from E. Merck, Darmstadt.

Results

The nitrate reductase of *Rps. capsulata* AD2 was fully induced (specific activities of crude extracts in the range of 0.02 – 0.03 units/mg protein) only in cells growing exponentially under photosynthetic conditions in a medium with nitrate as N-source. Under the conditions specified under Materials and Methods, the enzyme was released from photosynthetically grown cells in a soluble form. It should be noted, however, that enzymatic lysis of the organism (treatment of cells with lysozyme in the presence of 1 mM EDTA) or ultrasonic rupture of cells at energy levels less than 250 w released the nitrate reductase in a particle-bound state.

In the following, a purification procedure will be described yielding an electrophoretically homogeneous enzyme preparation. Soluble extract from cells of a 25 liter-culture was subjected to fractionation by ammonium sulfate. The protein fraction precipitating between 35 and 50% saturation was dissolved in 10 ml K-phosphate buffer (20 mM, pH 6.5). The resulting protein solution was dialyzed for 18 h against 5 liters of the same buffer. The dialyzed solution was then applied to the top of a DEAE-cellulose (DE 52, Whatman) column (2 × 15 cm) previously equilibrated with 20 mM K-phosphate, pH 6.5. After washing of the column with 50 ml of the same buffer, the proteins were eluted with a linear K-phosphate gradient (20 to 300 mM, pH 6.5). Samples of about 5 ml were collected and assayed for protein (absorbancy at 280 nm) and BV-linked nitrate reductase. As shown in Fig. 1, the nitrate reductase was eluted at a phosphate concentration of about 150 mM. The activity peak corresponded to a small protein peak. Fractions 41 through 47, which had a reddish-brown color, were combined

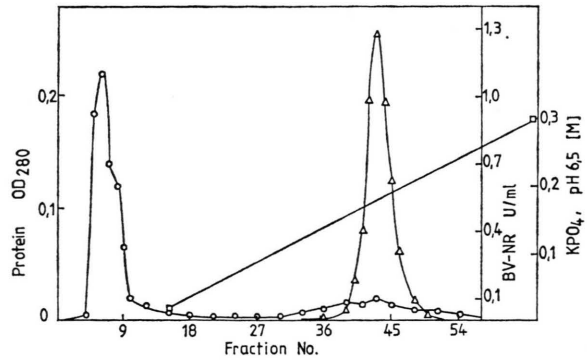

Fig. 1. Chromatography of nitrate reductase from *Rhodopseudomonas capsulata* AD2 on DEAE-cellulose. 17 ml of a 35 – 50% ammonium sulfate fraction (450 mg protein) from nitrate-grown cells were dialyzed against 20 mM K-phosphate, pH 6.5, and then applied on the top of a DEAE-cellulose (DE 52, Whatman) column (2×15 cm) previously equilibrated with the same buffer. After washing of the column with 50 ml of that buffer, adsorbed proteins were eluted with a linear K-phosphate gradient (20 to 300 mM, pH 6.5; □). Fractions of 5 ml were collected and assayed for protein (OD_{280}; ○) and benzylviologen-linked nitrate reductase (BV-NR; △). For OD_{280}-determinations, the fractions were diluted 100-fold.

and saturated with ammonium sulfate up to 50%. The precipitated protein was collected by centrifugation, dissolved in 5 ml of 20 mM K-phosphate, pH 6.5, and dialyzed for 15 h against 5 liters of 50 mM glycine, pH 6.5. Further purification of the enzyme was performed by preparative isoelectric focusing as described before.

A summary of a typical purification procedure is given in Table I. Due to activity losses during dialysis and electrofocusing, the specific activities of the homogeneous enzyme preparations obtained by the method outlined before, were not as high as the value reported for the partially purified enzyme (see ref. [1]). However, the enzyme preparations obtained by electrofocusing contained only one major protein band and a very weak contaminant band when subjected to analytical gel electrophoresis. It was estimated that the enzyme was about 95% pure. The isoelectric point of the enzyme was 4.8, and the molecular weight estimated according to the method of Andrews [7] with aldolase, catalase and ferritin as marker proteins was found to be 185 000 dalton. The absorption spectrum of the nitrate reductase, when recorded in the presence of dithionite, showed the typical absorption maxima of a heme of the c-type at 418, 523 and 552 nm (Fig. 2). By using the extinction coefficient of cytochrome c_2

Table I. Purification of *Rhodopseudomonas capsulata* AD2 nitrate reductase.

Fraction	Volume [ml]	Protein [mg]	Nitrate Reductase Activity Total units [U]	Spec. activity [U/mg protein]
Soluble fraction	126	1846	44	0.024
35 – 50% $(NH_4)_2SO_4$-fraction	17	448	40	0.089
DEAE-cellulose eluate	35	50	30	0.60
Eluate from electrofocusing gels	4	2.5	2	0.80

from *Rhodospirillum (R.) rubrum* ($\varepsilon_{551} = 26.9$ liter/mmol, ref. [8]), a heme content of 1.5 mol per 1.85×10^5 g of pure enzyme protein was calculated. The heme moieties of the enzyme, if previously reduced by dithionite, could be oxidized by an excess of nitrate both under aerobic and anaerobic conditions (Fig. 2).

The pure enzyme was only active with dithionite-reduced viologen dyes or FMN as electron donors, the activity obtained with the latter compound being about 3% of that with reduced benzylviologen. The K_m-value for nitrate (assayed at pH 7) was found to be 0.8 mM. Like other pro- and eukaryotic nitrate reductases [9 – 13], the enzyme of *Rps. capsulata* AD2 contained molybdenum (0.8 g-atoms per 1.85×10^5 g of pure enzyme protein). In agreement with this finding, the biosynthesis of an active nitrate reductase in culture media with 1 μM molybdate was completely inhibited by 1 mM Na-wolframate. However, this inhibition by wolframate was nearly completely overcome in the presence of 1 mM molybdate.

The subunit molecular weight of the nitrate reductase was analyzed by SDS-polyacrylamide gel electrophoresis following the method of Weber and Osborn [6]. In contrast to other bacterial nitrate reductases [11, 12] the enzyme of *Rps. capsulata* AD2 yielded only one protein band when subjected to SDS-electrophoresis. The data given in Fig. 3 suggest that the enzyme is a dimer composed of two identical subunits of 85 000 dalton.

Azide, cyanide, cyanate and thiocyanate are inhibitors of various nitrate reductases [12 – 14]. The enzyme of *Rps. capsulata* AD2 was only slightly inhibited (about 10%) by 10 mM cyanide and completely insensitive against thiocyanate. Interestingly, the activity of the enzyme was significantly enhanced in the presence of 1 mM Na-azide or 1 mM Na-cyanate (about 2-fold activation). According to Pichinoty [15], chlorate is a competitive inhibitor of assimilatory bacterial nitrate reductases. In contrast, chlorate (10 mM) had no effect on the rate of nitrate reduction catalyzed by the purified *Rps. capsulata* AD2-enzyme. When chlorate (16 mM) was used as an alternate substrate, a reaction rate of about 25% of that obtained with nitrate was recorded.

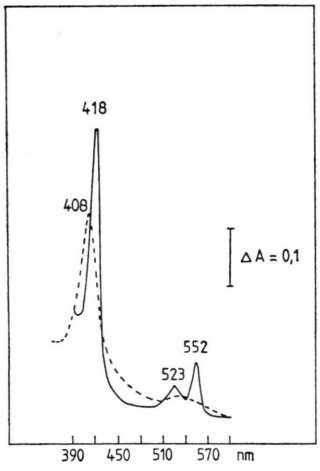

Fig. 2. Absorption spectra of oxidized and reduced nitrate reductase from *Rhodopseudomonas capsulata* AD2. An enzyme preparation of 0.18 mg per ml in 0.1 M K-phosphate, pH 7, was used. Solid line: Sample reduced with dithionite. Dashed line: Dithionite-reduced sample oxidized by an excess of KNO_3 under aerobic or anaerobic conditions.

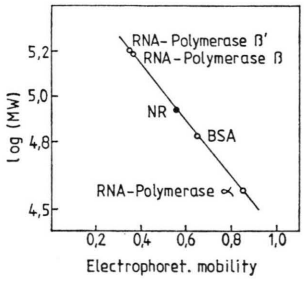

Fig. 3. Subunit molecular weight determination of purified nitrate reductase from *Rhodopseudomonas capsulata* AD2 by SDS polyacrylamide gel electrophoresis. The procedure is described in the section Materials and Methods. ●: nitrate reductase (NR) subunits. Molecular weight markers: α-subunit of *E. coli* RNA-polymerase, 39 000 dalton; bovine serum albumin, 68 000 dalton; β-subunit of *E. coli* RNA-polymerase, 155 000 dalton; β'-subunit of *E. coli* RNA-polymerase, 165 000 dalton.

Discussion

The experimental results reported here are of interest both in enzymological and taxonomical respect. According to Pichinoty's classification of nitrate reductases [15], the assimilatory enzymes (type B) are soluble and competitively inhibited by chlorate, whereas the dissimilatory ones (type A) are membrane-bound and accept chlorate as an alternate substrate. It is obvious that this classification is not very useful in the present case. The assimilatory nitrate reductase of *Rps. capsulata* AD2 appears to be a membrane-bound enzyme that is solubilized by ultrasonic treatment of the cells and is not inhibited by chlorate.

The assimilatory nitrate reductases of the eukaryotes *Chlorella vulgaris* [10], *Neurospora crassa* [13] and *Rhodotorula glutinis* [14] have been reported to contain cytochromes of the b-type. In contrast, the assimilatory enzyme of the N_2-fixing bacterium *Azotobacter chroococcum* did not contain heme [16]. The membrane-bound dissimilatory nitrate reductases of *Escherichia coli* [17] and *Pseudomonas denitrificans* [18] were also shown to be associated with a b-type cytochrome (at least under certain purification conditions). It must be noted also that the nitrate reductase of the phototrophic bacterium *R. rubrum* was claimed to be a hemeprotein (heme of the b-type; see ref. [19]). It seems, however, that the low degree of purification of the *R. rubrum* enzyme (about 6-fold) does not allow to exclude the possibility of a contamination of the nitrate reductase preparation by the soluble cytochrome of the b-type which is regularly found in extracts of this organism. [20]. Thus, the enzyme from *Rps. capsulata* AD2 is the first bacterial assimilatory nitrate reductase that has unequivocally been shown to contain heme.

The subunit structure of the *Rps. capsulata* AD2-enzyme is far from being understood. The analytical data show that the enzyme contains 1.5 mol heme and 0.8 g-atoms molybdenum. The rather low content of molybdenum relative to heme may be due to a loss of this metal in the course of the purification procedure (compare ref. [10]). It is difficult to decide, however, what the actual heme content of the enzyme is. If the heme moieties were attached to polypeptides different from the larger subunits, one should expect the corresponding peptide band in SDS-gels. However, our SDS-gel electrophoreses repeatedly yielded only one type of subunit. We interpret this finding in that both 85 000 dalton-subunits contain a heme group.

Obviously, further work is required to elucidate the actual subunit structure of the enzyme as well as to answer the question whether association of heme with nitrate reductase is typical for all nitrate assimilating Rhodospirillaceae.

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