Pseudomonas stutzeri soluble nitrate reductase αβ-subunit is a soluble enzyme with a similar electronic structure at the active site as the inner membrane-bound αβγ holoenzyme

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Abstract **A two-subunit (αβ) form of dissimilatory nitrate reductase from *Pseudomonas stutzeri* strain ZoBell was separated from the membrane-residing γ-subunit by a heat solubilization step. Here we present an optimized purification protocol leading to a soluble αβ form with high specific activity (70 U/mg). The soluble form has the stoichiometry $\alpha_1\beta_1$ consisting of the 130 kDa α-subunit and the 58 kDa β-subunit. We did not observe any proteolytic cleavage in the course of the heat solubilization. The enzyme is competively inhibited by azide, but not by chlorate. It exhibits a K_M value of 3.2 mM for nitrate. We compare the enzymatic and electron paramagnetic resonance (EPR) spectroscopic properties of the αβ form with the αβγ holoenzyme which resides in the membrane and can be prepared by detergent extraction. The nearly identical EPR spectra for the Mo(V) signal of both enzyme preparations show that the active site is unaffected by the heat step. The factors influencing the binding of the α- and β-subunit to the γ-subunit are discussed.**
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Key words: Nitrate reductase; Molybdenum; Electron paramagnetic resonance; Oxidoreductase; Enzymology; *Pseudomonas stutzeri*

1. Introduction

Oxidoreductases are the largest class of known enzymes. Since the action of these enzymes can be directly linked with electron transfer processes and hence with electronics, they are of particular interest for technical applications. We are interested in deciphering the properties of these enzymes. Nitrate reductases (NaR) are widespread in both eukaryotes and prokaryotes and play an important role in the assimilation [1,2] and dissimilation of nitrogen [3]. These enzymes catalyze the following reaction [4]:

$$NO_3^- + 2e^- + 2H^+ \leftrightarrow NO_2^- + H_2O \ (E'_o = +420 \text{ mV})$$

Nitrate reductases are diverse enzymes when considering their active site constitutions, subunit structures and cell localization (reviewed e.g. in [5,6]).

All nitrate reductases, however, contain molybdenum in the form of a molybdopterin as has been shown by extensive studies on various enzymes from differing sources. In the past three years, the crystal structures of six molybdopterin-containing enzymes have been reported (for reviews see [7–9]). Dias et al. [10] purified, characterized and solved the crystal structure of the periplasmic nitrate reductase from *Desulfovibrio desulfuricans* (NAP). We analyzed the membrane-bound nitrate reductase (EC 1.7.99.4) of *Pseudomonas stutzeri* strain ZoBell (ATCC 14405) which contains three subunits (αβγ). The α-subunit (NarG) harbors the catalytic center consisting of a molybdenum and two pterin cofactors. The iron–sulfur complexes of the β-subunit (NarH) are supposed to be involved in the electron transport from the quinol pool of the membrane [11]. The small γ-subunit (NarI) is a cytochrome *b* with two b-type heme groups and spans the membrane.

Wentworth [12] has proposed that the minimal chemical reaction (oxidation of the molybdenum under oxygen atom transfer)

$$Mo^{IV}O + NO_3^- \leftrightarrow Mo^{VI}O_2 + NO_2^-$$

may occur in these enzymes. At some point in this redox cycle, one or two pterins must be involved. That some reductases contain a single pterin may be an indication that only one is required. In all cases, the cofactor plays a well-defined structural role by anchoring the molybdenum core in the center of the protein matrix [10]. In addition, the pterin unit mediates the electron flow to and from the molybdenum (depending on the enzyme) by linking it to the iron–sulfur center.

The two αβ-subunits can be separated from the membrane-spanning γ-subunit by using a heat step. This has been described before for the nitrate reductases of different organisms [11,13,14]. Here we present an optimized purification protocol leading to an αβ form with higher specific activity. We compare the enzymatic and electron paramagnetic resonance (EPR) spectroscopic properties of the αβ form with the αβγ holoenzyme which resides in the membrane and can be prepared by detergent extraction.

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2. Materials and methods

2.1. Fermentation

P. stutzeri strain ZoBell (ATCC 14405) was a kind gift from Prof. Dr. W.G. Zumft (University of Karlsruhe, Germany). The strain was stored at −80°C in Luria–Bertani broth (LB) with 20% (v/v) glycerol. Media were sterilized by autoclaving (20 min, 120°C) or sterilized by filtration through 0.2 μm filters (Pall-Gelman). ACN medium with 15 g l^{-1} agar agar (Difco) was used for agar plates. LB agar plates were used for routine strain control. Cell growth was monitored offline by following the optical density of a properly diluted culture at 600 nm. Centrifuged culture supernatant was used as blank.

The medium composition described by Matsubara et al. [15] was modified. Sodium chloride was substituted by the sulfate and borate was added to the trace element solution. The synthetic ACN medium contained (in g l^{-1}): L-asparagine 2.0, trisodium citrate×$2H_2O$ 7.0, KH_2PO_4 2.0, $MgSO_4\times7H_2O$ 2.0, Na_2SO_4 1.22, $CaCl_2$ 0.075, $FeCl_3\times6H_2O$ 0.02. Control experiments revealed that stock solutions with only asparagine and citrate in 10-fold concentration were suitable for the feeding of medium. No growth limitation could be observed when all further components were kept at their original one-fold concentrations. A stock solution of trace elements was prepared with molybdate, borate and with chlorides from Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} at 10 mM concentrations and filter-sterilized. Trace element solution was added to give final concentrations of 3 μM for each component.

Bacteria were grown anaerobically in the presence of nitrate in a 1000 l fermenter (GBF, Braunschweig, Germany). To achieve higher yields of cell mass, we used an optimized fed-batch protocol [16]. The pH of the culture was checked in regular time intervals and sulfuric acid was added when alkalization of the medium occurred. Cells were centrifuged in a continuous flow milk centrifuge. The soft pellet (about 40 l) was filled in 1 l plastic bags and frozen on dry ice.

2.2. Protein purification

2.2.1. Preparation of membranes. The purification of membrane-bound nitrate reductase from *P. stutzeri* follows [11,17]. Bacterial cells were disintegrated by several passages through a Manton-Gaulin press (MAV, Germany). Cytosolic proteins were separated from the membrane fraction by several filtrations through filters with 0.16 μm pores (300 kDa cut-off; Omega Filtration Membranes, Pall-Filtron, Germany) in 50 mM potassium phosphate buffer, pH 7.5. These purification steps were performed at 4°C. Membrane fractions were stored in 50 ml tubes (Falcon) at −70°C. For further use, the tubes were melted slowly either at 4°C or room temperature.

2.2.2. αβγ holoenzyme. The membrane fraction was further concentrated by ultracentrifugation (at 180 000×*g*). Then, the membranes were treated by ultrasonic for 30 s and were again ultracentrifuged. 12 ml 10% (w/w) dodecyl-β-D-maltoside (Biomol, Calbiochem) in buffer 1 (20 mM HEPES–KOH pH 8, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.25 mM dithiothreitol (DTT), 10% (w/v) Glycerol) was added slowly to 120 ml of concentrated membranes (about 20–25 mg/ml protein concentration). This solution was stirred for 1 h at room temperature to solubilize membrane proteins. The solution was centrifuged for 40 min at 50 000 rpm at 4°C in a Ti 70 rotor, Beckman L-80. The gray pellet did not contain nitrate reductase activity and was discarded. The dark red-brown supernatant (12 mg/ml protein, 19 U/ml NaR activity) was loaded on an anion exchange column (POROS D50, 250×27 mm). The flow-through did not contain nitrate reductase. The column was washed with a linear gradient of 0–0.15 M NaCl in buffer 2 (=0.05% (w/w) dodecyl-β-D-maltoside in buffer 1) and nitrate reductase was eluted in two further gradients of 0.15–0.25 M NaCl and 0.25–0.6 M NaCl in elution buffer. Nitrate reductase eluted from the column was collected in four large fractions with fraction 2 containing the majority of nitrate reductase activity.

Fraction 2 from the D50 column was concentrated and 1 ml of the concentrate (9.6 mg/ml protein, 24 U/ml NaR activity) was applied to a gel filtration column (Sephacryl S 300, 170 ml bed volume). The column was run at 0.5 ml/min flow rate at 4°C. Elution buffer was 110 mM K_2SO_4 in buffer 2. Fractions containing nitrate reductase activity were pooled.

2.2.3. αβ-subunit preparation. At the beginning of our studies we used a purification protocol based on the procedure of Blümle and Zumft [11] with some modifications, like substituting centrifugation steps by cross-flow filtration steps: 'method I'. To obtain higher yield of nitrate reductase activity in less steps we optimized the heat step of the purification protocol and switched to 'method II'.

2.2.3.1. Method I. Membranes (see Section 2.1) were diluted to a protein concentration of 2 mg/ml with 50 mM potassium phosphate buffer, pH 7.5 to yield a final volume of 400 ml. The solution was stirred for 20 min at 60°C, cooled on ice and filtered through a 300 kDa Omega-Filter in cross-flow filtration technique. The retentate was again adjusted to 400 ml with 50 mM potassium phosphate buffer, pH 7.5 and the heat step and the filtration step were repeated. The filtrate from both filtrations was combined and concentrated by cross-filtration through a 10 kDa filter. By repeated dilution/filtration steps through the 10 kDa filter, the buffer in the retentate was exchanged to buffer 3 (25 mM Tris/bis-Tris–propane/HCl, pH 8.0).

This solution was applied to an anion exchange column (POROS D50, 100×10 mm). The flow-through did not contain nitrate reductase. Nitrate reductase was eluted in a gradient of 0–0.6 M NaCl in buffer 3. Fractions containing nitrate reductase activity were pooled. A desalting and buffer exchange step was performed on a gel filtration column (Sephadex G-25) HiPrep 26/10 (Pharmacia, Sweden), with 50 mM potassium phosphate buffer, pH 7.5 as elution buffer.

The αβ-NaR was further purified by hydrophobic interaction chromatography. One volume of a saturated solution of ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5 (saturated at 4°C) was added to the desalted nitrate reductase solution obtained after anion exchange. This solution was applied to a phenyl-ether column (POROS PE, 100×10 mm). A linear gradient of ammonium sulfate at 50% saturation in potassium phosphate buffer to 0% ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5 was applied. Fractions containing nitrate reductase activity were combined, concentrated and desalted. They contained 30% of the NaR activity compared to the holoenzyme (100%).

2.2.3.2. Method II. The membrane fraction (at a protein concentration of 3 mg/ml) was heated in 140 mM guanidinium hydrochloride (G·HCl; Fluka), 3 mM EDTA (Merck), 25 mM Tris–HCl (Roth) to 40°C for 3 h. This process results in a loss of enzyme activity of about 3%.

The dissociation of the αβ-subunit from the holoenzyme was optimal at pH 7.2. Values between pH 6.0 and 8.5 were analyzed. The specific αβ-subunit activity and yield show a weak pH dependence with a maximum at pH 7.0 and 7.5, respectively.

Membrane fraction together with dissociated αβ-NaR was cooled down to room temperature and loaded on an Expanded Bed DEAE chromatography column (Streamline DEAE, Pharmacia). Cellular parts, contaminating proteins and membrane particles were washed off the DEAE column with first 0 mM NaCl followed by 200 mM NaCl in 25 mM Tris–HCl, pH 8.0, until absorption at 280 nm reached a minimum. αβ-Subunit was eluted from the DEAE column in 400 mM NaCl, 25 mM Tris–HCl, pH 8.0. The activity yield of αβ-subunit from holoenzyme (100%) was 70%.

2.3. Protein and enzymatic assays

Protein amount was determined according to Bradford [18]. As protein standard for quantification bovine serum albumin (BSA) was used at concentrations from 6 to 30 μg/ml in distilled water. Protein-bound dyes were detected at 595 nm using the microtiter plate reader Spectra Max 250 (Molecular Devices, USA). Protein concentrations were determined three-fold.

Protein amount in samples with low protein concentration was determined spectroscopically using a colorimetric assay with bicinchoninic acid (BCA Protein Assay, Pierce, USA) following the protocol supplied by the manufacturer. BSA was used as standard protein. The optical density of the colored protein–BCA complex was determined using a Shimadzu UV1202 photometer.

The amount of nitrate in samples was determined with the assay described by Borcherding et al. [19]. Enzyme activity was measured at room temperature and at pH 7.5 with an assay based on the nitrite-dependent diazotation of *N*-(1-naphthyl)-ethylenediamine (Merck) according to Werner [20] as described in [19]. To determine the pH optimum for the nitrate reductase the reaction buffer (50 mM potassium phosphate, pH 7.5) in this assay was modified as described in [19].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Denaturing polyacrylamide gel electrophoresis was performed ac-

cording to Laemmli [21]. The acrylamide and bisacrylamide concentration in the separating gel was adjusted to 13% total concentration and 1% cross-linkage. Protein samples were diluted with the same volume gel loading buffer containing 2% (v/v) β-mercaptoethanol and 2% (w/v) SDS. Samples were heated for 5 min to 95°C prior to loading the gel. Gels were stained with Coomassie brilliant blue R-250. SDS–PAGE standard 'broad range' (Bio-Rad, Germany) was used as molecular weight marker.

2.5. Analytical gel filtration of native αβ-NaR

We determined the molecular mass and stoichiometry of native αβ-NaR by gel filtration using a fast performance liquid chromatography (FPLC) system with pump P-500, fraction collector FRAC 100, detector UV-1 (used at 280 nm) and recorder REC 102 (Pharmacia, Sweden). We loaded 1 mg protein per application in 250 μl volume (0.2% of the column bed volume) onto the gel filtration column Hi-Load 16/60 Superdex 200 prep grade (separation range 10–600 kDa; Pharmacia, Sweden). The protein loading buffer (250 mM NaCl, 17 mM EDTA, 220 mM guanidinium·HCl, 50 mM potassium phosphate buffer, pH 7.5) was identical to the column equilibration buffer. Exchanging NaCl with KSCN or 250 mM NaCl, 50 mM potassium phosphate buffer with 25 mM Tris–HCl, 350 mM NaCl did not change the elution behavior of αβ-NaR.

2.6. EPR experiments

The EPR spectra were taken using an X-band Bruker ER 200 D-SRC spectrometer (Bruker). Temperature was controlled by a helium flow-through cryostat ESR 910 (Oxford Instruments). The magnetic field was regulated using a Bruker Field Controller ER 031-M (Bruker) calibrated by a Bruker NMR Gaußmeter B-NM 12 (Bruker). Microwave frequency was determined using a frequency counter 6054 D (Cystron-Donner). Spectra were taken at a temperature of 120 K with 20.0 mW/10 dB microwave power at a frequency of 9.6459 GHz and 2.0 G modulation amplitude. The enzyme was reduced by 0.35 mM NADH for 4 min at room temperature. Subsequently, the sample was poured in an EPR tube and frozen in liquid nitrogen. To obtain the EPR spectra in the presence of nitrate, the above prepared sample was thawed and then incubated with 0.5 M nitrate for 5 min at room temperature. Subsequently, the sample was frozen again in liquid nitrogen, as described above.

3. Results

3.1. Enzyme properties of αβ-NaR

A denaturing polyacrylamide gel with samples of the αβ form at different steps of the purification protocol (method I) is displayed in Fig. 1. The apparent molecular weights of the two subunits have been determined as 130 and 58 kDa.

The enzyme purified by this method showed a specific activity of 71 U/mg with one unit of nitrate reductase activity being defined as the production of 1 μmol nitrite per min. To our knowledge this is the highest value reported for any nitrate reductase. Previous preparations of this enzyme showed only 26.97 U/mg at slightly different assay parameters (pH 7.2, 30°C [11] instead of pH 7.5, 25°C (this study)). Preparations of the dissimilatory nitrate reductase from *Escherichia coli* exhibited a specific activity of about 60 U/mg [22], and the enzyme from *Paracoccus denitrificans* showed a specific activity of 50 U/mg [23].

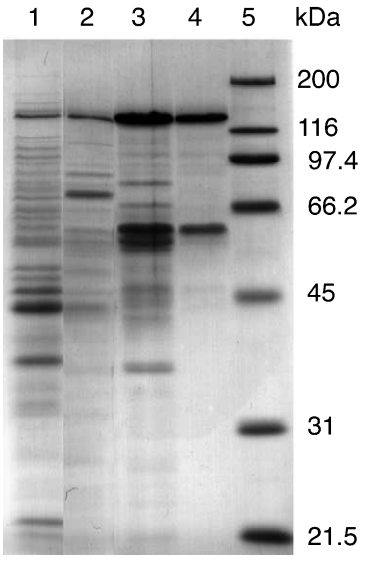

Fig. 1. PAGE analysis of αβ-NaR at different stages of the purification protocol (see Section 2, method I). From left to right: 34 μg of of membrane fraction (lane 1), 19 μg of retentate after heat solubilization and cross-flow filtration (lane 2), 27 μg of combined fractions after ion exchange chromatography (lane 3), 10 μg of combined fractions after hydrophobic interchange chromatography (lane 4). Lane 5 was loaded with a mixture of standard proteins (about 2 μg of each protein) with their molecular weights shown on the right of the gel. The gel was stained with Coomassie brilliant blue R-250.

The enzyme activity was measured in the range from pH 6 to 9 (see Section 2). The enzyme shows a broad pH optimum between 7.5 and 8.0. The K_M value for nitrate was determined in a Lineweaver–Burk plot to be 3.2 mM (data not shown).

The activity is not inhibited by up to 100 mM sodium chlorate but it is inhibited by sodium azide. This inhibition is of the competitive type and can be overcome by higher nitrate concentrations. The presence of up to 1 M sodium chloride did not influence the activity of the enzyme (data not shown).

3.2. The dissociated subunit has the stoichiometry $\alpha_1\beta_1$

The dissociated subunit might have various compositions like single subunits (α), dimers (α_2), mixed complexes ($\alpha_1\beta_1$)

Table 1
Amino-terminal amino acid sequences of α- and β-subunits of nitrate reductases from different *Pseudomonas* species

Subunit	Organism	Prepared by	Amino acid sequence (position)												
			1	2	3	4	5	6	7	8	9	10	11	12	13
α	*P. stutzeri*	heat solubilization		S/G	A	L	L	D	Q	L	R	F	F		
		detergent solubilization		S	H	L	L	D	Q/I	L	R	F	F	N	R
	P. aeruginosa	N/A	M	S	H	L	L	D	R	L	Q	F	F	K	K
	P. fluorescens	N/A	M	S	H	L	L	D	Q	L	R	F	F	N	R
β	*P. stutzeri*	heat solubilization	M	K	I	R	S	Q	V	G	M	V			
		detergent solubilization	M	K	I	R	S	Q	V	G	M	V	L	D	K
	P. aeruginosa	N/A	M	K	I	R	S	Q	V	G	M	V	L	N	L
	P. fluorescens	N/A	M	K	I	R	S	Q	I	G	M	V	L	N	L

The amino acid sequences from *P. stutzeri* have been determined by amino acid sequencing (see Section 3); the sequences from *P. aeruginosa* and *P. fluorescens* were taken from published amino acid sequences based on the gene sequences [27,28].

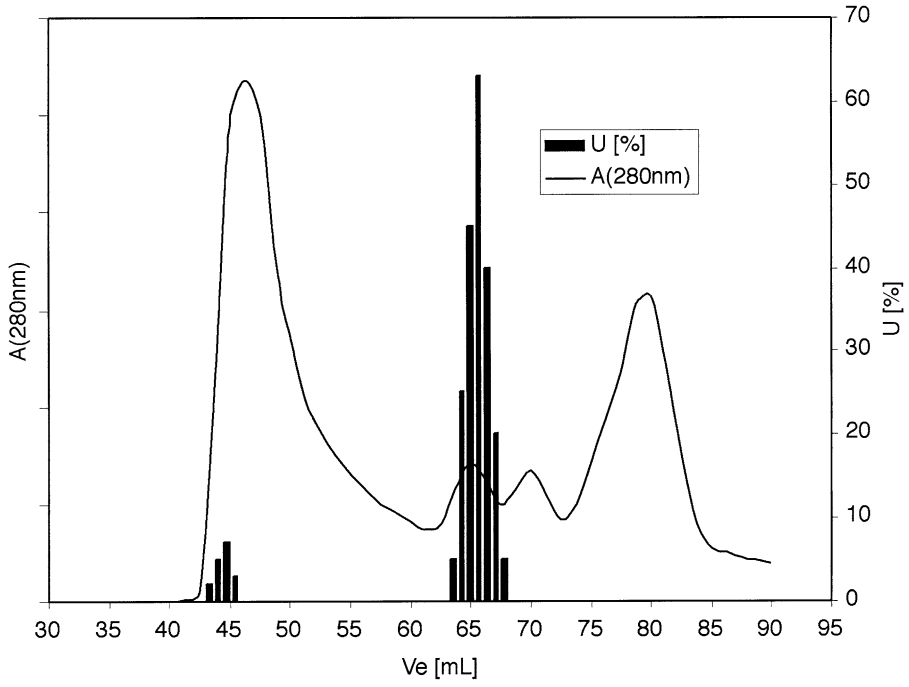

Fig. 2. Estimation of the molecular weight of *P. stutzeri* αβ-NaR by size exclusion FPLC. The sample loaded on the column was αβ-NaR extracted from membranes by incubation at 40°C (method II, see Section 2). The absorption of the eluted proteins was monitored at 280 nm and is given in arbitrary units. The fractions of the eluate were tested for nitrate reductase activity as described in Section 2. Activity is given in % with 3 U/ml set to 100%. The elution volume V_E is given in ml.

and multimers of the units (e.g. $\alpha_2\beta_2$). β monomer and β multimers are excluded since β was shown not to contain the active molybdenum center [11]. Nitrate reductases of other organisms vary in composition and molecular weight from monomers with 90 kDa (*Clostridium perfringens*; [24]) to a multimer of 1060 kDa ($\alpha_4\beta_4\gamma_8$; *Klebsiella aerogenes*; [25]). We therefore determined the molecular mass and stoichiometry of the dissociated subunit by gel filtration (see Section 2). The HiLoad gel filtration column (bed volume 120 ml) was calibrated with the proteins ferritin (440 kDa molecular weight), aldolase (156 kDa) and BSA (monomer: 66 kDa, dimer: 132 kDa). The void volume of the column was determined using Dextran blue (2 kDa molecular weight) as 42 ml. The elution volumes were determined as follows: ferritin (56 ml), aldolase (66 ml) and BSA (monomer: 74 ml, dimer: 64 ml).

The absorbance of the eluate was measured at 280 nm and plotted versus elution volume (see Fig. 2). Fractions of the eluate were collected in 2 ml steps and analyzed for enzyme activity (see Fig. 2). 90% of enzyme activity were found at 66 ± 2 ml elution volume corresponding to a molecular weight of 150–200 kDa. This molecular weight is not consistent with a subunit composition of α monomers (130 kDa), dimer of the α monomer (α_2 260 kDa) or higher multimers but agrees well with the composition $\alpha_1\beta_1$ (188 kDa).

10% enzyme activity elutes in the void volume (see Fig. 2) thus having a molecular weight of over 600 kDa. We interpret this as nitrate reductase αβγ holoenzyme still bound to membrane fractions.

3.3. N-terminal amino acid sequence

The N-terminal amino acid sequences of the α- and β-subunit were determined for both NaR preparations: for the αβ-NaR obtained by heat solubilization and for the αβγ-NaR obtained by detergent solubilization.

The subunits were separated by SDS–PAGE, blotted on polyvinylidene difluoride membranes, and stained with Coomassie brilliant blue R-250. Bands were cut out, and the amino acid sequence for the α- and the β-subunit was determined by automated microscale Edman degradation on an Applied Biosystems protein sequencer model 494A (Applied Biosystems, Foster City, CA, USA).

The two nitrate reductase forms, obtained by these two preparation methods, show identical amino N-termini for the β-subunits (see Table 1). The two sequences for the α-subunit differ slightly: in position 3 an alanine was detected for the heat solubilized protein and a histidine for the detergent solubilized protein. This is probably a reading error as histidine usually shows only weak signals in amino acid sequencing. By comparing the two sequences with the known sequences from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* a histidine in this position seems to be more probable. In position 2 the sequence for the heat solubilized protein was not clear and gave signals for serine and glycine while the detergent solubilized protein showed a clear serine signal. In position 7 the sequence for the detergent solubilized protein was a mixture between glutamine and isoleucine while the sequence of the heat solubilized protein unambiguously showed a glutamine. Both sequences lack the initial methionine (position 1) which is probably cleaved during the maturation of the active α-subunit.

The amino acid sequence of the *P. stutzeri* enzyme is only partially known [26]. The sequences we obtained, however, align very well with the known amino-termini from the membrane-bound nitrate reductases of *P. aeruginosa* and *P. fluorescens* [27–29] as they had been deduced from the DNA se-

quence of these proteins. The intact N-termini of the α- and β-subunit show that no N-terminal degradation occurred (except for the aforementioned cleavage of methionine in the α-subunit).

3.4. αβ-NaR does not bind to phospholipids

To support the hypothesis that the αβ-subunit is not, also not partly, membrane associated we measured the binding of αβ-NaR to phospholipids. Phospholipids were immobilized onto polystyrol beads (Nimbus). Beads were either loaded with the neutral phospholipid palmitoyl-oleoyl-phosphatidyl-choline (POPC) or, alternatively, with a mixture of 20% negatively charged palmitoyl-oleoyl-phosphatidyl-choline (POPG) and 80% POPC. αβ-NaR was incubated with phospholipids carrying beads at a lipid concentration of 0–0.9 mM at either 25 or 60°C for 30 min potassium phosphate buffer, pH 7.5 following the instruction of the manufacturer. The lipid loaded beads were centrifuged down; they would carry with them any αβ-NaR binding to the lipids. αβ-NaR activity was determined in the supernatant. Within experimental error (about 10%) all αβ-NaR activity was found in the supernatant independent of lipid concentration (up to 10^6-fold excess over αβ-NaR concentration) and temperature (data not shown). Thus, αβ-NaR is not centrifuged down with the lipid loaded beads indicating that it does not bind to these phospholipids.

3.5. EPR

The molybdenum center of *P. stutzeri* nitrate reductase was studied in both enzyme forms by EPR spectroscopy of molybdenum (V) in partially reduced forms of the enzyme. The resting, as prepared, enzyme in the αβ form displayed already small amplitudes of resonances attributable to Mo(V). On reduction with NADH, the Mo(V) resonances increased with time but decreased later after prolonged exposure to the excess substrate as the enzyme became more reduced. The *g* values of the NADH-reduced enzyme at pH 8.5 could be determined as follows: $g_1 = 1.986$, $g_2 = 1.976$ and $g_3 = 1.960$ yielding an averaged *g* value of 1.974 (Fig. 3a, upper trace). The same *g* values could also be observed in the NADH-reduced detergent solubilized NaR (Fig. 3a, lower trace). EPR spectra of samples clamped at different reduction potentials indicated a three-fold decrease of the signal amplitude of the Mo(V) resonances by lowering the reduction potential from +24 mV to −40 mV vs. NHE. After addition of nitrate (0.5 M) to the NADH-reduced high pH form of the enzyme in the αβ form, new resonances due to hyperfine coupling of Mo(V) to solvent exchangeable protons could be observed. The experimental spectrum could be simulated with the following parameters: $g_{1,2,3}$: 1.9992, 1.9858, 1.9659; A $(^1H)_{1,2,3}$: 1.46, 1.11, 1.09 mT; half linewidths: 0.81, 0.81, 0.81 mT (Fig. 3b, upper trace). The same *g* values were also obtained for the NADH-reduced detergent solubilized NaR in the presence of nitrate (Fig. 3b, lower trace).

4. Discussion

The dissimilatory nitrate reductase of *P. stutzeri* was either solubilized by detergents or eluted from the membranes by heat extraction. While the detergent solubilized form is the αβγ holoenzyme (with not exactly known stoichiometry), the heat extracted form is a heterodimer of the $\alpha_1\beta_1$ stoichiometry.

4.1. Properties of the αβγ and $\alpha_1\beta_1$ enzyme

In denaturing polyacrylamide gels we found an apparent molecular weight of 130 kDa for the larger α-subunit and 58 kDa for the smaller β-subunit. While the latter is in good accordance with the value of 60 kDa determined by Blümle and Zumft [11], the former value differs significantly from the 112 kDa determined in the same study. This difference might be explained by differences in the method applied (denaturing gradient gel in contrast to a discontinuous denaturing gel according to Laemmli [21]) leading to different separation behavior. Alternatively, the preparation of Blümle and Zumft might contain a protease while our preparation does not. Blümle and Zumft postulate the action of a protease during the heat solubilization procedure as they find two forms of the β-subunit: one with 60 kDa, the other one with 46 kDa apparent molecular mass.

Recently we determined enzymatic properties of the αβγ nitrate reductase (NaR; EC 1.7.99.4) of *P. stutzeri* using exogenous redox mediators [19]. The Michaelis–Menten constant of substrate (nitrate) affinity K_m was determined to 0.49 ± 0.07 mM at saturating methyl viologen concentrations. The NaR activity shows a broad pH optimum between pH 7.5 and 8.5 and is independent of NaCl concentration (1 mM to 1 M) present in the reaction buffer. The NaR activity is strongly temperature dependent with an activity maximum at 76°C. In contrast, for the αβ-NaR we measured a K_M value for nitrate of 3.2 mM which is in accordance with the literature value of 3.8 mM [11]. We explain this difference between the αβ and the αβγ form of about one order of magnitude by an easier access of the electron donors to the iron–sulfur centers, a prerequisite for reducing the active center. In the holoform the electron donors have to reduce the γ-subunit first (which might well be a slow process) while in the αβ form they have direct access to the iron–sulfur centers. Thus, the αβγ form reaches substrate saturation already at a lower nitrate concentration.

The activity for the αβ-NaR also exhibits a broad pH optimum between pH 7.5 and 8.0. It is not influenced by NaCl of up to 1 M, too. In addition, we examined the influence of sodium chlorate and found no inhibition with concentrations of up to 100 mM $NaClO_3$.

The inhibitory effect of azide and cyanide has been shown previously by Blümle and Zumft [11], but the independence of the activity on chloride and chlorate of the *P. stutzeri* enzyme as well as the pH and temperature optima have not been reported before.

4.2. Binding of αβ- to γ-subunit

The amino-termini of both preparations, the detergent solubilized αβγ form as well as the heat extracted αβ form, were determined by sequencing and were found to be identical for the β-subunits. The slight differences in the sequences of the α-subunits can be explained by interpretation errors in the amino acid sequencing.

The amino-terminal sequences of both subunits show high homology with the respective sequences of the membrane-bound nitrate reductases of *P. fluorescens* and *P. aeruginosa*. In addition, the SDS–PAGE analysis of the purified αβ-NaR showed only one band for the α-subunit as well as the β-subunit unaltered from the bands of the αβγ protein. These findings do not agree with previous publications on the nitrate reductase from *P. stutzeri* [11] and *E. coli* [14]. These articles

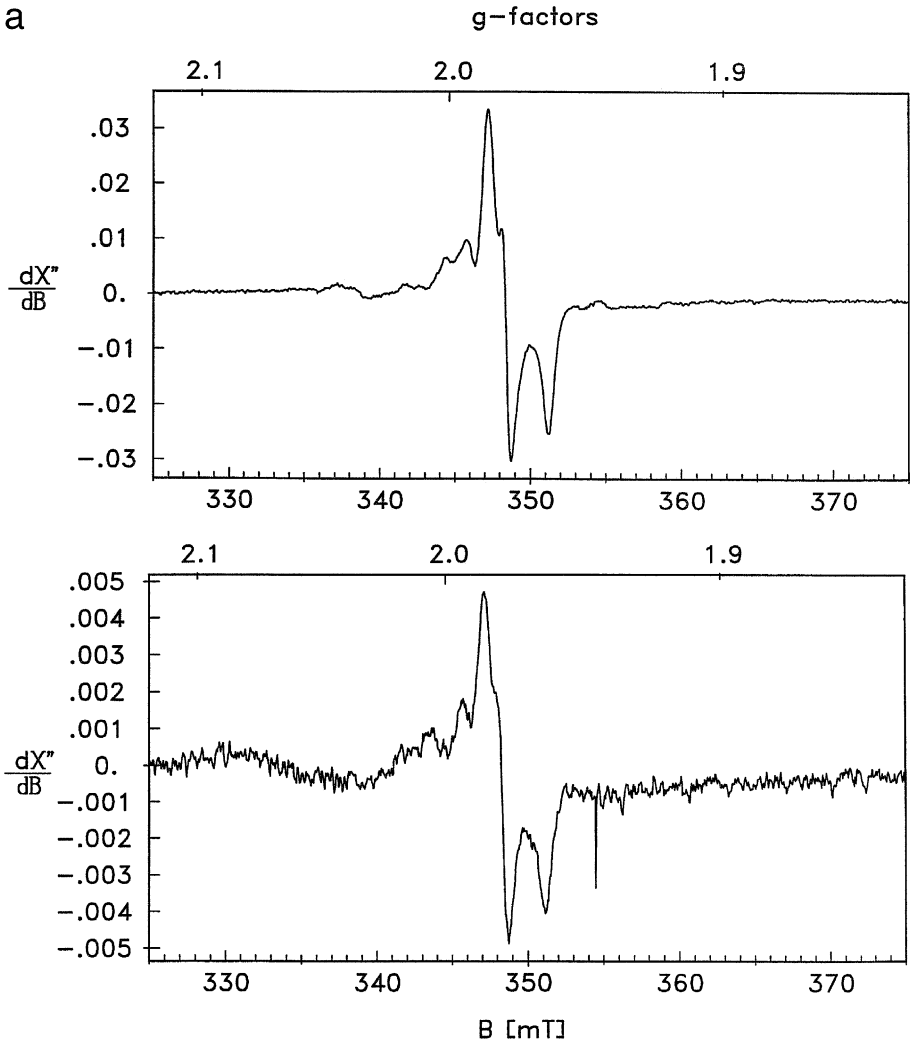

Fig. 3. a: EPR spectra from heat solubilized (upper trace) and detergent solubilized (lower trace) *P. stutzeri* nitrate reductases. The heat solubilized enzyme was prepared according to method I (see Section 2). The detergent solubilized form of the enzyme was a sample taken after the ion exchange chromatography step of the purification protocol. See Sections 2 and 3 for experimental details. b: EPR spectra from heat solubilized (upper trace) and detergent solubilized (lower trace) *P. stutzeri* nitrate reductase in the presence of 0.5 M nitrate. The two samples of the enzyme are identical with those in a. See Sections 2 and 3 for experimental details.

postulate the action of an endogenous protease during the heat solubilization step. In contrast, Ballard and Ferguson [13] examined the soluble form of nitrate reductase from *P. denitrificans* and found no relation between proteolytic cleavage of α- or β-subunit and their release from the membrane.

The participation of a proteolytic activity in the dissociation process would result in different amino-terminal sequences and in the appearance of altered subunits (α and/or β) with lower molecular weight with – probably – α- and β-subunit gel bands at altered positions. Instead, our data suggest that electrostatic factors rule the dissociation of the αβ-NaR from the membrane-residing γ-subunit. Our experiments show that αβ-NaR does not bind to hydrophobic surfaces. Instead, we observed that the dissociation of the αβ-NaR from the γ-subunit can either be inhibited by the addition of 50 mM Mg^{2+} [16] or can be enhanced by the addition of EDTA [30]; see also Section 2. Therefore we infer that the binding between the αβ-subunits and the γ-subunit in *P. stutzeri* probably occurs by electrostatic interaction supposedly supported by divalent cations.

The very same observation on the influence of Mg^{2+} has been made by Ballard and Ferguson [13] for the nitrate reductase from *P. denitrificans*. However, in contrast to our EDTA results, they found in their system that EDTA neither enhances nor impairs the dissociation of αβ-NaR from the membrane but instead promotes the dissociation of other proteins from the membrane, thus lessening the specificity of the dissociation process.

4.3. Holo αβγ and αβ alone have the same active center

Heat extracted as well as detergent solubilized nitrate reductase was analyzed by EPR spectroscopy. The *g* values of both types of NaR in the NADH-reduced state coincided nicely and were nearly identical to those determined for the high pH species of the dissimilatory nitrate reductase of *P. aeruginosa* [31]. Also in the latter enzyme the EPR spectrum of the heat extracted protein was reported to be nearly identical to the detergent solubilized species. Therefore, the ligand environment of the molybdenum in the active center seems to be unaffected by the heat treatment in both cases. The heat

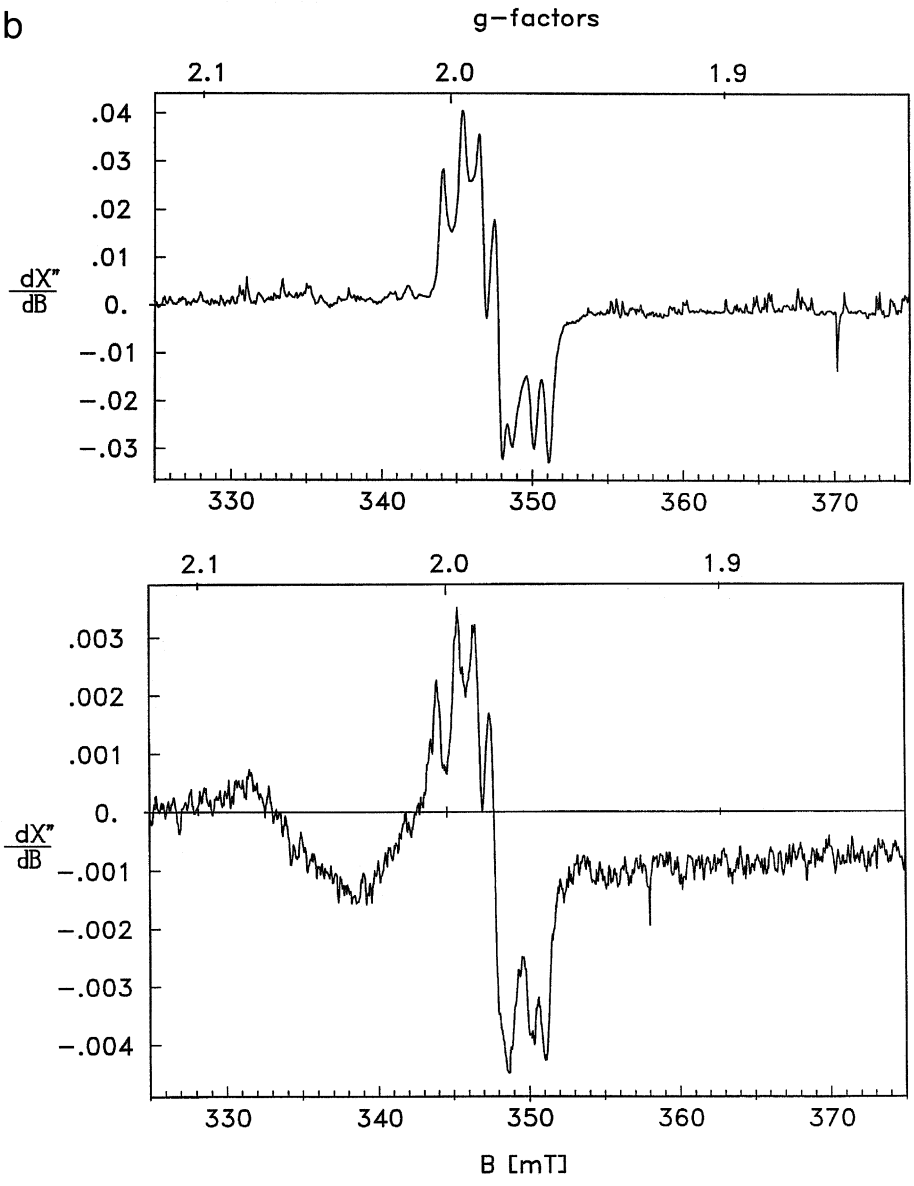

Fig. 3 (Continued).

stability of the molybdenum complex might be explained through the crystal structures of several molybdoenzymes, which seem to indicate the molybdenum cofactor to be positioned well within the mature protein, at the end of a funnel shaped passage making the access of the substrate possible [32–34].

Furthermore, also in the NADH-reduced state in the presence of nitrate, both forms of the NaR of *P. stutzeri* exhibited the same *g* values. The average hyperfine splitting value of 1.22 mT used in the simulation of the spectrum indicates coupling to a single proton. The same *g* values and hyperfine couplings had also been observed for the enzymes from *P. denitrificans* [35] and *P. aeruginosa* [31] and *E. coli* [36]. Therefore, the environment of the molybdenum seems to be nearly identical in all four different enzymes.

The dissimilatory nitrate reductase (NarGHI) of *E. coli* has extensively been studied by the group of Blasco (as reviewed in [37] and references cited therein). Their work examines the nitrate reductase mainly in the native, membrane-bound state. The Mo(V) signals of the wild-type enzyme as well as the signals of selected mutants have been studied by EPR spectroscopy [38]. These studies of the Mo(V) signal showed a transition between a low pH and a high pH form, both of which are involved during enzyme turnover. Not only the molybdenum center but also the iron–sulfur centers in the β-subunit and the heme centers in the γ-subunit have been examined by this approach [22,39–46]. Amino acids ligating these metal centers have been exchanged and these mutants of the enzyme in the membrane-bound form have been characterized by EPR and optical spectroscopy.

In contrast, we compared the membrane-bound form of the dissimilatory nitrate reductase with the soluble two-subunit form of the enzyme. Our data suggest that our purification protocol for the soluble two-subunit form yields a form of the enzyme which closely resembles the native state in the detergent solubilized αβγ-NaR holoenzyme, as these two enzyme preparations exhibit nearly identical EPR spectra for the catalytically active molybdenum center.

This soluble two-subunit form might serve as a more suitable starting point for crystallization experiments than the membrane-bound three-subunit form.

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