*Biochimica et Biophysica Acta*, 1056 (1991) 225–232

*ADONIS* 000527289100063I

BBABIO 43329



# The denitrifying nitrite reductase of *Bacillus halodenitrificans*

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(Received 17 May 1990)
(Revised manuscript received 25 September 1990)

Key words: Halophile; Copper protein nitrite reductase; Nitrate reductase; EPR

**Denitrifying nitrite reductase was purified from *Bacillus halodenitrificans*, a newly isolated moderate halophile, and identified as a dimeric nonheme copper protein with a molecular mass of 82 kDa comprising two identical 40-kDa subunits. Unlike its counterparts in other denitrifiers, this enzyme is firmly bound in the cytoplasmic membrane, a characteristic that may be related to the Gram-positive nature of this bacillus (and thus lack of a periplasmic space). The enzyme functioned most effectively at pH 6, and was activated by elevated salt concentrations, a property shared by the copper nitrite reductase from another, but nonhalophilic, denitrifier, *Achromobacter cycloclastes*. In contrast, high salt activation was not exhibited by cytochrome $cd_1$ nitrite reductases from either nonhalophilic *Thiobacillus denitrificans* or halophilic *Paracoccus halodenitrificans*. The enzyme was rich in aspartate/asparagine and methionine residues. Electron paramagnetic resonance spectroscopy revealed both type 1 and type 2 copper in the protein. The physiological electron donor that transfers electrons to the copper nitrite reductase from *A. cycloclastes*, a small blue copper protein, did not transfer electrons to the copper nitrite reductase of *B. halodenitrificans*.**

## Introduction

The moderate halophile, *Bacillus halodenitrificans*, tolerates high concentrations of nitrate and nitrite and may thus be useful for nitrogen-abatement in high-nitrate waste water [1,2].

Nitrite reductase activity in *B. halodenitrificans* appeared somewhat fragile in stationary phase cells grown on complex medium, owing perhaps to weak binding of components of the electron transfer chain leading to nitrite. Study of the nitrite reductase (nitric-oxide (acceptor) oxidoreductase, EC 1.7.99.3) from the bacillus was chosen as the first step toward understanding this behavior.

Previous experiments [2] showed that *B. halodenitrificans* produced a copper-containing nitrite reductase, characteristic of a type considered to differ from the better known cytochrome $cd_1$ but perhaps more prevalent than previously assumed. Bacteria synthesizing copper nitrite reductase include *Achromobacter xylosoxidans* (formerly called *Alcaligenes* sp. NCIB 11015 and *Pseudomonas denitrificans*) [3], *Alcaligenes faecalis* strain S-6 [4], *Achromobacter cycloclastes* [5,6], and *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides* forma sp. *denitrificans* [7], to which *Pseudomonas aureofaciens* [8] and *Haloferax* (formerly *Halobacterium*) *denitrificans*; [9] have recently been added.

The genus *Bacillus* contains representative denitrifiers [10,11]. Yet, nitrite reductase(s) of denitrifying bacilli have not been characterized or even identified to date. As Gram-positive organisms, bacilli possess no periplasmic compartment. If nitrite reductase is located on the outer side of the cytoplasmic membrane in bacilli, as in other denitrifiers [12–14], special structures must hold them to the membrane to prevent loss into the surrounding medium. Moreover, external location on the cells would require tolerance to varying environmental effects such as pH and ionic strength. This notion seems especially appropriate for *B. halodenitrificans*, a moderate halophile. Studying the enzyme from *B. halodenitrificans* should therefore provide useful insights into comparative enzymology.

## Materials and Methods

### *Preparation of cellular fractions*

*B. halodenitrificans* cells were grown anaerobically to early stationary phase at 37°C on DNYA medium, which contained: nutrient broth powder (Difco), 8 g;

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yeast extract (Difco), 5 g; $MgSO_4 \cdot 7H_2O$, 1 g; sodium acetate $\cdot 3H_2O$, 0.5 g; Tris base, 1.21 g; NaCl, 30 g; $NaNO_3$, 5 g, and enough water to provide 1 liter of medium. Each liter also contained 10 ml of a metal salt solution containing $CaCl_2 \cdot 2H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 1 g; $MnCl_2 \cdot 4H_2O$, 0.5 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; and $CuSO_4 \cdot 5H_2O$, 0.1 g, in 1 liter of 0.1 M HCl. The bacteria were harvested with a Sharples centrifuge at 3000 rpm for 4 h and suspended in an equal volume of 100 mM Tris-HCl buffer (pH 7.6) supplemented with NaCl, 1.2 M, and $\alpha_2$-macroglobulin (a universal proteinase inhibitor), 1 mg $\cdot l^{-1}$. Cells were disrupted by passage through a Manton Gaulin homogenizer at a pressure of 633 kg/cm$^2$. A few milligrams of DNase I and II were added to lessen the viscosity of the crude extract. The preparation was treated with neutralized streptomycin sulfate (0.5 mg/ml protein), stirred for 30 min at 4° C, and centrifuged at 13200 × *g* in a Sorvall RC-5B refrigerated centrifuge for 30 min. The pellet was discarded and the supernatant was centrifuged at 144000 × *g* for 90 min at 4° C. The membrane pellet was washed once with 10 mM Tris-HCl buffer (pH 7.6) (final protein concentration = 10 mg $\cdot$ ml$^{-1}$). The dark-red pellet obtained after ultracentrifugation is referred to as the membrane fraction. The supernatant resulting from the low ionic strength wash was pooled with the soluble extract and dialyzed for 24 h against 10 mM Tris-HCl (pH 7.6)/NaCl 150 mM, with two changes of dialysis buffer. This preparation is referred to as the soluble fraction.

*Enzymatic assays*

Nitrite reductase activity was assayed using ascorbate-phenazine methosulfate (PMS) as electron donating system in 15-ml anaerobic vials flushed and left filled with argon. The reaction mixture consisted of Tris-HCl (pH 7.6), 100 μmol; ascorbate, 5 μmol; PMS, 10 nmol; nitrite, 500 nmol, in a total volume of 1 ml. The vials were preincubated at 30° C for 2 min and the reaction was started by injection of the cell extract into the vial. Samples containing 40 μl were withdrawn and mixed with 960 μl of distilled water. Nitrite was then assayed using a modification of the method described by Nicholas and Nason [15]. 100 μl of a 4% sulfanilamide solution in 25% HCl were added to the samples. The tubes were vortexed and rapidly supplemented with 100 μl of a 0.1% *N*-naphthylethylenediamine dihydrochloride solution. The intensity of the pink color that developed within 10 min was measured at 543 nm. A unit of activity is defined as the reduction of 1 μmol nitrite/min at 30° C.

The enzymatic assay utilizing hydroxylamine instead of ascorbate-PMS as electron donor was carried out as described by Iwasaki and Matsubara [5]. The product of the reaction was determined by gas chromatography using an electron capture detector [2].

Oxidase activity as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation in 100 mM phosphate buffer (pH 7.6) was followed spectrophotometrically at 520 nm [16], or by following oxygen consumption with a Clark-type electrode.

*Activity staining*

Non-denaturing gel electrophoresis was performed as described by Jovin et al. [17]. A modification of the nitrite reductase activity assay method described by Sawada et al. [7] served when the purpose was to locate the enzyme for electroelution. The method of Mancinelli and co-workers [18] was used for long-term staining.

*Analytical methods*

Protein concentrations were determined by the method of Bradford as described by Read and Northcote [19] with bovine serum albumin as standard. Molecular mass was determined by gel filtration on Ultrogel AcA 44 (Pharmacia) in 10 mM Tris-HCl buffer (pH 7.6) containing 200 mM NaCl, and by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) [20]. Calibration standards for gel filtration were α-chymotrypsinogen (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and aldolase (157 kDa). Standards for SDS-PAGE were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin, bovine serum albumin and phosphorylase *b* (92 kDa).

Optical spectra were recorded at room temperature with a Shimadzu UV-265 double-beam spectrophotometer (slit width = 1.0 nm). EPR spectra were recorded as previously described [21]. The data were recorded in a Bruker Aspect 2000 computer and transferred to an IBM AT computer for analysis. Metal content was determined by plasma emission spectroscopy, using a Jarrel-Ash model 750 Atomcomp.

*Amino acid analysis*

Cysteine/cystine residues in the enzyme were derivatized to pyridylethylcysteine (PITC) prior to hydrolysis in ultrapure 6 M HCl at 165° C for 1 h under an atmosphere of argon [22]. Following evaporation, the amino acids were treated with PITC and analyzed with an Applied Biosystems 120 A PTH analyzer.

*Materials*

DE-52 was purchased from Whatman. Bio-Gel HTP was a product of Bio-Rad Laboratories. Sephacryl S-200 and S-300 were obtained from Pharmacia-LKB corporation.

The blue copper protein and copper nitrite reductase from *A. cycloclastes* and cytochrome $cd_1$ from *Thiobacillus denitrificans*, generous gifts from M.-Y. Liu, were used for comparative studies. Crude extract of *Paracoccus halodenitrificans* was also prepared for comparative

studies from cells grown on complex medium [23]. Purification steps were carried out at 4 °C.

## Results

*Isolation of nitrite reductase*

The enzyme was partitioned between the soluble and the membrane fraction of cell extracts. Between 70 and 80% of the activity sedimented along with the membranes, whether low (10 mM Tris-HCl) or high (600 mM NaCl) ionic strength washes were applied.

*Purification of the soluble enzyme*

During the first attempts to isolate the enzyme, up to 90% of the activity was lost after the first chromatographic step (anion exchange), perhaps by removal of a protecting agent that results in oxygen sensitivity [23] and/or cofactor depletion. Subsequent purification steps were therefore carried out under anaerobic conditions, using an anaerobic chamber filled with 5% hydrogen in nitrogen whenever possible. Buffers were saturated with argon gas in other cases. All chromatography and dialysis buffers were supplemented with 1 $\mu$M $Cu^{2+}$.

The soluble fraction was loaded onto a DE-52 column (25 × 6 cm) equilibrated with Tris-HCl buffer (pH 7.6) containing 150 mM NaCl. After washing with 1 liter of identical buffer, the proteins were eluted by a NaCl gradient (150 to 400 mM; 3 liters total) in 10 mM Tris-HCl buffer. The nitrite reductase activity emerged near 290 mM NaCl, following, with some overlapping, cytochrome *c*-550. Nitrite reductase fractions were pooled and directly loaded onto Bio-Gel HTP column (22 × 4.5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.6)/200 mM NaCl. The column was washed with 200 ml of 2 mM Tris-HCl (pH 7.6)/200 mM NaCl, and a gradient of phosphate buffer (5 to 200 mM; 2 liters total) was applied. This step separated cytochrome *c*-550, which eluted between 15 and 20 mM phosphate, and nitrite reductase, which emerged near 90 mM phosphate. Nitrite reductase fractions were pooled, concentrated by ultrafiltration on an Amicon YM 30 membrane, and loaded on a Sephacryl S-200 column (60 × 2.5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.6)/200 mM NaCl. Proteins were chromatographed at a flow rate of 3 ml · $h^{-1}$. The enzyme appeared on the column as a light blue band. Fractions with high activity were pooled and further purified by anion-exchange-HPLC chromatography. A TSK-DEAE-5PW preparative column (15 × 2.15 cm) was used for this purpose. The chromatography proceeded at 4 ml · $h^{-1}$, with the following elution program: wash with 10 mM phosphate buffer (pH 7.0)/160 mM NaCl (equilibration buffer), for 10 min; 160 to 250 mM NaCl in 5 min; 250 to 300 mM NaCl in 60 min; 300 to 510 mM NaCl in 5 min. Nitrite reductase eluted at 285 mM NaCl just ahead of a major protein contaminant. The DEAE-HPLC chromatography was repeated once, after which the enzyme was about 95% pure as judged by non-denaturing gel electrophoresis.

Nitrite reductase was purified 343 fold, with a recovery yield of 17% by this procedure (Table I). The yield after the DE-52 chromatography was high, suggesting that the use of anaerobic conditions may be critical for the purification of nitrite reductase. Inhibition or inactivation of nitrite reductase by oxygen or $H_2O_2$ [24], a consequence of removal of superoxide dismutase and catalase, might be implicated in the loss of activity in air after the DE-52 step. The enzyme purified under anaerobic conditions was stable at −80 °C in 50% glycerol for at least 10 months.

Further purification of the enzyme by HPLC-gel filtration on a TSK-3000 SW column (60 × 2.15 cm) did not remove the protein contaminants. For analyses requiring samples with high purity (e.g., amino acid composition), the enzyme was further purified by disc gel electrophoresis and electroelution.

*Solubilization and isolation of the membrane-bound enzyme*

The enzyme in the membrane fraction was solubilized by detergents. At a concentration of 2% (v/v; protein concentration = 10 mg · $ml^{-1}$), Triton-X-100 solubilized the proteins in the membranes. Sequential release of membrane proteins by increasing detergent concentrations (0.8, 1 and 2%) proved ineffective for nitrite reductase extraction, as only a portion of the enzyme was solubilized at the lower concentrations.

The solubilized and soluble enzymes behaved similarly on chromatography media and were therefore purified in a similar manner. The major modification employed in work with the detergent-solubilized enzyme was that inclusion of low concentrations of deter-

TABLE I

*Purification of soluble nitrite reductase*

| Purification step | Protein (mg) | Total activity | Specific activity | Yield % | Purification |
|---|---|---|---|---|---|
| Soluble extract | 10780 | 650 | 0.06 | 100 | – |
| DE-52 | 767 | 620 | 0.81 | 95.4 | 13.5 |
| HTP | 306 | 300 | 0.98 | 46 | 16.33 |
| S-200 | 73.65 | 200 | 2.71 | 30.7 | 45 |
| DEAE-HPLC (×2) | 5.3 | 110 | 20.6 | 17 | 343 |

gents in the chromatography buffers was required. Otherwise the membrane-bound enzyme attached irreversibly to the chromatographic supports. Chromatography on DE-52 and DEAE-HPLC was carried out in the presence of 0.05% Triton X-100. Other chromatographic procedures made use of buffers supplemented with 0.6% cholate, an ionic detergent producing smaller micelles than Triton X-100 but not usable with anion-exchange supports. As a second modification, the gel-filtration chromatography utilized Sephacryl S-300 instead of Sephacryl S-200 because of the larger size of the nitrite reductase micelles in cholate. Anaerobic purification of the membrane-bound enzyme has not been attempted yet. Yields were consequently very low.

The enzyme obtained after the DEAE-HPLC step, specific activity 4.5 units $\cdot$ mg$^{-1}$, was subjected to non-denaturing preparative gel electrophoresis in the presence of 0.1% Triton X-100. Activity staining of the gel gave two bands with relative mobilities of 0.2 (NIR-2) and 0.75 (NIR-1) (polyacrylamide concentration = 7.5%). The second value indicates the greater mobility of the soluble enzyme. The two bands were cut from the gel, electroeluted, and analyzed by SDS-PAGE. NIR-1 moved as one band at 40 kDa and may therefore be considered identical to the soluble enzyme. NIR-2, on the other hand, yielded two protein bands of equal intensity at 42 and 40 kDa. Such a pattern suggests that NIR-1 was complexed with another polypeptide and appeared only partially resolved by non-denaturing gel electrophoresis. Solubilization of membrane proteins by Triton X-100 reportedly results in isolation of protein complexes in other cases [25,26]. We noted that non-denaturing gel electrophoresis eliminated a major protein contaminant of about 76 kDa.

The nature of the 42 kDa polypeptide is unknown. The existence of a physiologically relevant complex with nitrite reductase is possible. The fraction obtained after DEAE-HPLC was unable to reduce nitrite in the absence of the electron transfer mediator, PMS, and the products of the reaction with ascorbate-PMS was preponderantly NO. This suggests that the 42 kDa polypeptide complex was neither the electron donor to nitrite reductase nor a NO reductase.

As the enzyme isolated from the membranes appeared identical to the soluble enzyme, characterization studies were conducted on the more easily isolated soluble nitrite reductase.

*Spectroscopic properties*

In the spectrum of the oxidized enzyme, two maxima appeared in the visible region, 595 nm ($\epsilon = 4.94$ mM$^{-1} \cdot$ cm$^{-1}$) and 454 nm ($\epsilon = 4.1$ mM$^{-1} \cdot$ cm$^{-1}$), with weak shoulders around 710 nm and 800 nm (Fig. 1). The ultraviolet absorption maximized at 277 nm with a marked shoulder at about 282 nm. Partly defined features were displayed at 258, 268 and 290 nm. Addition of dithionite eliminated absorption in the visible region.

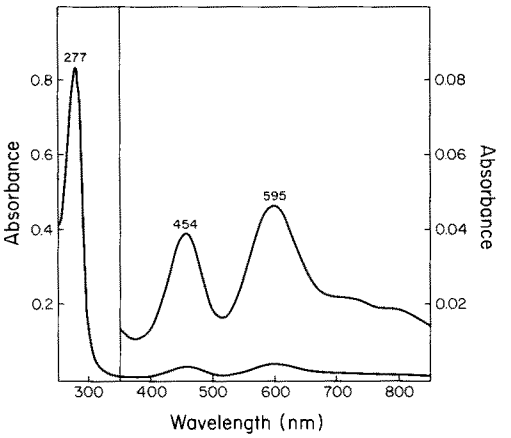


Fig. 1. Electronic absorption spectrum of soluble nitrite reductase. Protein concentration, 0.82 mg$\cdot$ml$^{-1}$ in 10 mM potassium phosphate buffer (pH 7.0)/300 mM NaCl.

In the EPR spectrum of native nitrite reductase (Fig. 2), both type 1 and 2 copper sites appeared with $|A_{\parallel}| =$ 49 G and $g_{\parallel} = 2.23$ (type 1) and $|A_{\parallel}| = 129$ G and $g_{\parallel} = 2.32$ (type 2); $g_{\perp} = 2.10$.

*Physical properties*

The molecular mass of the enzyme, determined under non-denaturing conditions (gel filtration), was $82 \pm 3$ kDa. SDS gel electrophoresis gave a single protein band at $40 \pm 2$ kDa, indicating a dimeric structure with two subunits of identical molecular mass. Plasma emission spectroscopy indicated a copper content of 1.56 gatom/82 000 $M_r$ molecule. The N-terminal residue appeared to be blocked, and therefore the peptide was not amenable to amino acid sequencing by Edman degrada-

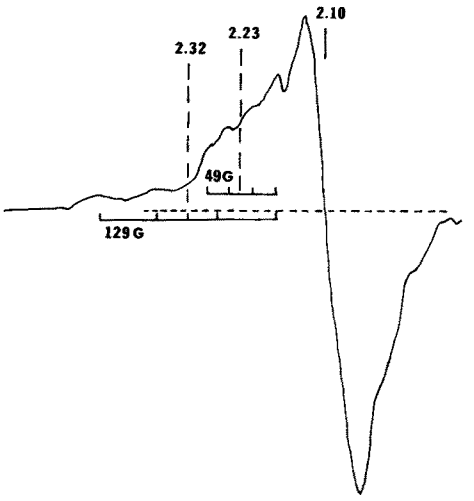


Fig. 2. EPR spectrum of nitrite reductase. Conditions: temperature, 10 K; protein concentration, 72.7 $\mu$M of $M_r$ 82000; microwave frequency 9.4308 GHz; modulation amplitude, 5 G; attenuation, 20 dB; gain $3.2 \cdot 10^5$.

TABLE II

*Amino acid composition of B. halodenitrificans nitrite reductase*

| No. of residues/ 40-kDA molecule | | No. of residues/ 40-kDa molecule | |
|---|---|---|---|
| Asx | 60 | Tyr | 13 |
| Glx | 33 | Val | 42 |
| Ser | 12 | Met | 8 |
| Gly | 30 | Ile | 18 |
| His | 13 | Leu | 14 |
| Arg | 3 | Phe | 17 |
| Thr | 28 | Lys | 20 |
| Ala | 28 | Cys | 1 |
| Pro | 28 | Trp | n.d. |

n.d., not determined.

tion. Amino acid composition analysis (Table II) revealed an abundance of aspartate/asparagine and methionine residues.

*Enzymatic properties*

Optimal activity appeared at approx. pH 6 (not easily tested at pH < 6 as ascorbate-PMS non-enzymatically reduced nitrite), with specific activity of 90 μmol nitrite reduced/min per mg protein, which is 4.5-times the value obtained at pH 7.6. The enzyme was activated by high NaCl concentrations; the activity in buffers containing 1 to 3 M NaCl was twice that obtained for reaction mixtures lacking added salt (Fig. 3). Unexpectedly, the copper enzyme from the non-halophilic *A. cycloclastes* reacted identically. In contrast, activation by NaCl appeared minimal with cytochrome $cd_1$ nitrite reductases. The cytochrome $cd_1$ nitrite reductase from *Thiobacillus denitrificans* was unresponsive to, or even inhibited at, NaCl concentrations > 2 M. The cytochrome $cd_1$ nitrite reductase activity in crude extracts from the halophilic denitrifier, *P. halodenitrificans*, was also not adversely affected by high salt concentration and was only slightly activated by

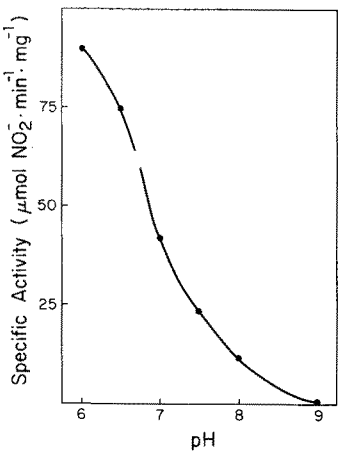


Fig. 3. pH profile of nitrite reductase activity. pH 6–7.5; 0.1 M phosphate buffers. pH 7.5–9; 0.1 M Tris-HCl buffers.

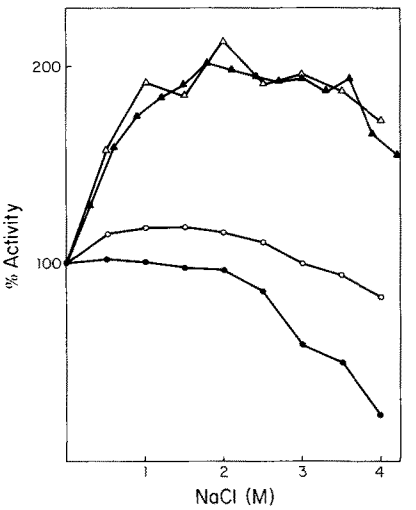


Fig. 4. Effect of NaCl on the activity of cytochrome $cd_1$ and copper-containing nitrite reductases. ▲, *B. halodenitrificans* CuNIR; △, *A. cycloclastes* CuNIR; ●, *T. denitrificans* cytochrome $cd_1$; ○, *P. halodenitrificans* soluble extract (containing cytochrome $cd_1$).

NaCl (Fig. 4). Grant and Hochstein [27] reported a slight activation of purified cytochrome $cd_1$ from this bacterium at NaCl concentrations around 200 mM.

Incubation at 25 °C in air for 24 h failed to diminish the activity of the *B. halodenitrificans* nitrite reductase. The enzyme also remained stable in NaCl concentrations as great as 2 and 4 M for at least 15 h. As the purified enzyme remained active under aerobic conditions, the apparently damaging effects of oxygen observed during the first chromatographic purification step appear to have been eliminated. Metal chelators (ethylene diamine tetraacetic acid, EDTA, 1 mM; KCN, 1 mM; 8-hydroxyquinoline, 0.1 mM; and diethyldithiocarbamate, DDC, 1 mM) and sulfhydryl group inhibitors (thioglycollate, 0.1 mM; and *p*-chloromercuribenzene sulfonate, 1 mM) inhibited nitrite reduction. Azide (1 mM) failed to affect activity (Table III).

The product of nitrite reduction by the purified enzyme was predominantly NO, but a small amount of $N_2O$ was also produced, accounting for about 5% of the nitrite reduced. Non-enzymatic NO reduction to the

TABLE III

*Inhibitors of nitrite reductase activity*

pCMBS, *p*-chloromercuribenzenesulfonate.

| Inhibitor | Concentration (mM) | % Inhibition |
|---|---|---|
| Azide | 1 | 0 |
| Cyanide | 1 | 100 |
| EDTA | 1 | 70 |
| DDC | 1 | 100 |
| OH-quinoline | 0.1 | 100 |
| Thioglycolate | 0.1 | 50 |
| pCMBS | 0.1 | 20 |
| | 1 | 36 |

TABLE IV

*Properties of copper-containing nitrite reductase from denitrifiers*

n.d., no data. pCMB, *p*-chloromercuribenzoate. BCP, blue copper protein.

| Property | *A. cycloclastes* IAM 1013 [a,b] | *R. sphaeroides* forma sp. *denitrificans* [c] | *A. faecalis* strain S-6 [d] | *A. xylosoxidans* NCIB 11015 [e] | *P. aureofaciens* [f] | *B. halodenitrificans* [g] |
|---|---|---|---|---|---|---|
| Molecular mass (kDa) | 69 (2×36.8) | 80 (2×39) | 110 (4×30) | 70 (2×37) | 85 (2×40 | 82 (2×40) |
| Cu atom/mol. | 2 [a],3 [b] | 2 | 4 | 2 | 2 | 2 |
| Cu(II) type | 1 and 2 | 1 and 2 | 1 and 2 | 1 only | 1 only | 1 and 2 |
| Electronic absorption spectrum maxima (nm) | 283 | 280 | 280 (116.8) | 282 | 280 | 277 (88.8) |
| | 400 (1.6) [a] | 450 | 400 (4.53) | 470 (0.64) | 474 | 454 (4.1) |
| | 464 (2.6) [a] | 590 | 457 (6.98) | 594 (3.7) | 595 (7) | 595 (4.94) |
| | 590 (2.0 [a] | | 587 (5.42) | 780 (1.6) | 780 | |
| $\epsilon(mM^{-1}\cdot cm^{-1})$ | 700 (1.7) [a] | | 700 (4.65) | | | |
| Purity index ($A_{-590}/A_{-280}$) | 0.045 [b] | 0.1 | 0.046 | 0.068 | 0.074–0.077 | 0.0556 |
| | 0.034 [a] | | | | | |
| Physiological electron donor | *c*-551, *c*-553 or *c*-555 [a] BCP [b] | $c_2$, horse heart cyt. *c* | BCP | *c*-553 | BCP | ? |
| Product of nitrite reduction | | | | | | |
| + ascorbate/PMS | NO | NO | NO | NO | NO | NO |
| + hydroxylamine | $N_2O$ | n.d. | n.d. | $N_2O$ | $N_2O$ | $N_2O$ |
| Optimum pH | 6.2 | 7.0 | 5.9 | n.d. | ≈ 6 | ≈ 6 |
| Specific activity | 150 [a] | 26.2 [c] | 380 | 25 (110 after freeze-thaw) | 3 | 90 |
| | 280 [b] | 40 [d] | | | | |
| Inhibitors | KCN, DDC, CO *p*CmB | KCN, DDC, CO | KCN, DDC | n.d. | KCN, DDC, *p*CMB *o*-phenanthroline | KCN, DDC EDTA, *p*CMBS OH-quinoline |

[a] [5], [b] [6], [c] [7],
[d] [4], [e] [3], [f] [8],
[g] This work.

small amount of $N_2O$ by $Fe^{2+}$-ascorbate complexes [28] appeared unlikely, as the iron chelator EDTA also inhibited nitrite reductase activity. Like other copper nitrite reductases [8], the enzyme produced $N_2O$ as the only product of nitrite reduction when hydroxylamine served as electron donor. The reaction rate with hydroxylamine was only 1.4 μmol $NO_2^-$ reduced to $N_2O$/min per mg protein compared to 78 μmol $NO_2^-$ reduced/min per mg protein with ascorbate-PMS as donor (pH 6.5 for both assays). Iwasaki and Matsubara [5] reported similar results with the counterpart enzyme of *A. cycloclastes*. Both nitrite and hydroxylamine reportedly contribute one nitrogen atom of the emergent $N_2O$ in this reaction [8].

Neither the soluble cytochrome *c*-550 isolated from *B. halodenitrificans* nor the blue copper protein from *A. cycloclastes*, the apparent physiological electron donor for *A. cycloclastes* copper-containing nitrite reductase [6], transferred electrons to *B. halodenitrificans* nitrite reductase. The purified copper nitrite reductase exhibited no oxidase activity.

## Discussion

*B. halodenitrificans* is to our knowledge the first denitrifying *Bacillus* species whose nitrite reductase has been purified, although Coyne et al. [29] found that DDC inhibited nitrite reductase activity in crude extracts of a *Bacillus* species in which the enzyme did not react with antibody against *A. cycloclastes* nitrite reductase. The presence of copper-containing enzyme in a *Bacillus* species may indicate a broader distribution of this type of nitrite reductase among denitrifiers than previously assumed. The presence of a copper-containing nitrite reductase in the archaebacterium, *H. denitrificans* [9], may further indicate that this type predates the more frequently seen cytochrome $cd_1$ in an evolutionary sense [29].

Nitrite reductase isolated from the membrane fraction of *B. halodenitrificans* by detergent solubilization appeared identical to that found in the soluble fraction. Thus the binding to the cytoplasmic membrane appears an intrinsic property of the enzyme not attributable to

the existence of two different enzymes. A 42-kDa protein that copurified with the membrane-associated enzyme in a presumably natural complex was neither the electron donor for nitrite reductase nor for the NO reductase; it may serve, however, to anchor nitrite reductase to the membrane.

The N-terminal amino acid seems to be blocked in nitrite reductase, perhaps by involvement in membrane attachment. The recently reported fatty acid chain covalently attached by a thioester bond to the N-terminus of *Rhodobacter viridis* cytochrome *c* appeared to bind that cytochrome to the cytoplasmic membrane [30]. Covalent lipid attachment to membrane-associated proteins is now a well established phenomenon [31,32]. Adequate modification of nitrite reductase in *B. halodenitrificans*, a Gram-positive organism, appears logical if, as suggested by the general trend in Gram-negative bacteria, the protein is located on the outer side of the cytoplasmic membrane.

Aside from this possible modification, *B. halodenitrificans* nitrite reductase resembles other copper-containing nitrite reductases (Table IV). Even so, it did not accept electrons from *A. cycloclastes* blue copper protein, the electron donor to nitrite reductase in that organism, revealing differences between the two. The small copper proteins that donate electrons to the copper-containing nitrite reductase in several denitrifiers were identified and purified using their intense blue color as a guide [4,6,8], but no such proteins were observed in extracts of *B. halodenitrificans* during the various chromatographic steps in the present study. Specific activity varies among the various copper-containing nitrite reductases (Table IV). Assay procedures have not been identical from one study to another, but such differences hardly explain the variations observed. Whether these differences arise from damage done during isolation of nitrite reductases or from intrinsic properties of the enzymes remains unclear.

Activation of the halophilic *B. halodenitrificans*' nitrite reductase by high salt concentrations was expected, but a similar response by the enzyme from the non-halophilic *A. cycloclastes* was not. As high salt concentrations did not influence the activity of cytochrome $cd_1$ from either the non-halophilic *T. denitrificans* or the halophilic *P. halodenitrificans*, activation by salt seems therefore an intrinsic characteristic of only the copper-containing nitrite reductases. Moreover, the salt effect may be related to the enzyme's mechanism of action. Induction of critical inter- or intramolecular conformational changes by high salt concentrations might result in activation. Activation of *A. xylosoxidans* nitrite reductase by freeze-thawing, which may also induce conformational changes, was reported by Masuko and co-workers [3]. The physiological significance of either type of activation remains open to question.

*B. halodenitrificans* nitrite reductase was stable at high salt concentrations. This property would be consistent with the location of the enzyme on the outer side of the cytoplasmic membrane. The general stability of the purified enzyme contrasts with the observed lability of the activity in whole cells. Other cell components or functions may thus bring about the rapid loss of nitrite reductase activity we have observed in *B. halodenitrificans*.

## Acknowledgement

Supported by grants number DMB-8718646 from the National Science Foundation and C/VD 4036400 from the Commissariat à l'Energie Atomique, France. We thank J.J.G. Moura for carrying out the EPR studies.

## References

1 Denariaz, G., Payne, W.J. and LeGall, J. (1989) Int. J. Syst. Bacteriol. 39, 145–151.
2 Denariaz, G., Payne, W.J. and LeGall, J. (1989) in Microbiology of Extreme Environments and its Potential for Biotechnology (DaCosta, M.S., Duarte, J.C. and Williams, R.A.D., eds.), pp. 328–345, Elsevier, New York.
3 Masuko, M., Iwasaki, H., Sakurai, T., Susuki, S. and Nakahara, A. (1984) J. Biochem. 96, 447–454.
4 Kakutani, T., Watanabe, H., Arima, K. and Beppu, T. (1981) J. Biochem. 89, 453–461.
5 Iwasaki, H. and Matsubara, T. (1972) J. Biochem. 71, 645–652.
6 Liu, M.-Y., Liu, M.C., Payne, W.J. and LeGall, J. (1986) J. Bacteriol. 166, 604–608.
7 Sawada, E., Satoh, T. and Kitamura, H. (1978) Plant Cell Physiol. 19, 1339–1351.
8 Zumft, W.G., Gotzmann, D.J. and Kroneck, P.M.H. (1987) Eur. J. Biochem. 168, 301–307.
9 Tomlinson, G.A. and Hochstein, L.I. (1988) p. 209, Abst. Ann. Meet. Am. Soc. Microbiol., Miami.
10 Payne, W.J. (1981) Denitrification, p. 40, John Wiley & Sons, New York.
11 Knowles, R. (1982) Microbiol. Rev. 46, 43–70.
12 Meijer, E.M., Van der Zwaan, J.W. and Stouthamer, A.H. (1979) FEMS Microbiol. Lett. 5, 369–372.
13 Wood, P.M. (1978) FEBS Lett. 92, 214–218.
14 Ferguson, S.J. (1988) in Bacterial Energy Transduction. (Anthony, C., ed.), pp. 151–182, Academic Press, London.
15 Nicholas, D.J.D. and Nason, A. (1957) Methods Enzymol. 3, 981–984.
16 Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1982) FEBS Lett. 139, 255–258.
17 Jovin, T., Chrambach, A. and Naughton, M.A. (1964) Anal. Biochem. 9, 351–369.
18 Mancinelli, R.L., Cronin, S. and Hochstein, L.I. (1986) Arch. Microbiol. 145, 202–208.
19 Read, S.M. and Northcote, D.H. (1981) Anal. Biochem. 116, 53–64.
20 Laemmli, U.K. (1970) Nature 227, 680–685.
21 Villalain, J., Moura, I., Liu, M.-C., Payne, W.J., LeGall, J., Xavier, A.V. and Moura, J.J.G. (1984) Eur. J. Biochem. 141, 305–312.
22 Fullmer, C.S. (1984) Anal. Biochem. 142, 336–339.
23 Sadler, M., McAninch, M., Alico, R. and Hochstein, L.I. (1980) Can. J. Microbiol. 26, 496–502.

24 Kakutani, T., Beppu, T. and ARima, K. (1981) Agric. Biol. Chem. 45, 23–28.
25 Hederstedt, L. (1980) J. Bacteriol. 144, 933–940.
26 Lichtenberg, D., Robson, R.J. and Dennis, E.A. (1983) Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim. Biophys. Acta 737, 285–304.
27 Grant, M.A. and Hochstein, L.I. (1984) Arch. Microbiol. 137, 79–84.
28 Zumft, W.G. and Frunzke, K. (1982) Biochim. Biophys. Acta 681, 459–468.
29 Coyne, M.S., Arunakumari, A., Averill, B.A. and Tiedje, J.M. (1989) Appl. Environ. Microbiol. 55, 2924–2931.
30 Weyer, K.A., Schäfer, W., Lottspeich, F. and Michel, H. (1987) Biochemistry 26, 2909–2914.
31 Towler, D.A., Gordon, J.I., Adams, S.P. and Glaser, L. (1988) Annu. Rev. Biochem. 57, 69–99.
32 Ferguson, M.A.J. and Williams, A.T. (1988) Annu. Rev. Biochem. 57, 285–320.