

Purification, Characterization, and Genetic Analysis of Cu-Containing Dissimilatory Nitrite Reductase from a Denitrifying Halophilic Archaeon, *Haloarcula marismortui*

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Cu-containing dissimilatory nitrite reductase (CuNiR) was purified from denitrifying cells of a halophilic archaeon, *Haloarcula marismortui*. The purified CuNiR appeared blue in the oxidized state, possessing absorption peaks at 600 and 465 nm in the visible region. Electron paramagnetic resonance spectroscopy suggested the presence of type 1 Cu ($g_{II} = 2.232$; $A_{II} = 4.4$ mT) and type 2 Cu centers ($g_{II} = 2.304$; $A_{II} = 13.3$ mT) in the enzyme. The enzyme contained two subunits, whose apparent molecular masses were 46 and 42 kDa, according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. N-terminal amino acid sequence analysis indicated that the two subunits were identical, except that the 46-kDa subunit was 16 amino acid residues longer than the 42-kDa subunit in the N-terminal region. A *nirK* gene encoding the CuNiR was cloned and sequenced, and the deduced amino acid sequence with a residual length of 361 amino acids was homologous (30 to 41%) with bacterial counterparts. Cu-liganding residues His-133, Cys-174, His-182, and Met-187 (for type 1 Cu) and His-138, His-173, and His-332 (for type 2 Cu) were conserved in the enzyme. As generally observed in the halobacterial enzymes, the enzymatic activity of the purified CuNiR was enhanced during increasing salt concentration and reached its maximum in the presence of 2 M NaCl with the value of $960 \mu\text{M NO}_2^- \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Denitrification, a biological reduction of nitrate that generates N_2 or N_2O gases, is an important process that contributes to the global nitrogen cycle of the Earth (41). Microorganisms capable of denitrification are distributed not only among bacteria but also among eukaryotic fungi and archaea (41). Denitrifying archaea have been reported to be present in some extreme halophiles (36, 39) and in a hyperthermophile, *Pyrobaculum aerophilum* (2, 38). Because the archaea are predominant microbial populations in extreme environments like saltwater lakes and hot springs, denitrifying archaea sustain the nitrogen cycle under such hypersaline or hot conditions.

The biochemistry of denitrification has been well investigated in some bacterial denitrifiers (4, 41); nitrate is converted to N_2 through nitrite, NO, and N_2O by successive reductions catalyzed by dissimilatory nitrate reductase (NaR), nitrite reductase (NiR), NO reductase, and N_2O reductase, respectively. Reduction of the nitrogen compounds in the denitrifiers has been confirmed to couple with ATP synthesis, which was inhibited by uncoupling reagents (24), indicating that denitrification is an anaerobic respiratory system in which nitrate is used as a terminal electron acceptor instead of O_2 . Additionally, because bacterial denitrification has been considered the ancestor of aerobic respiration (32, 37), biochemical investiga-

tion of denitrification in archaea is, therefore, interesting from the evolutionary aspect.

NiR, which catalyzes the reduction of nitrite and produces NO, is a key enzyme in denitrification. Two types of NiRs with distinct molecular structures, Cu-containing dissimilatory NiR (CuNiR) and “cytochrome *cd*” containing hemes *c* and *d*₁ as the prosthetic cofactors, have been reported in the denitrifying bacteria (41). CuNiR is a homotrimer with a characteristic triangular structure that has been resolved by X-ray diffraction analysis of the crystal (11, 16). The enzyme contains two Cu atoms, which are distinguished from each other by their optical and electron paramagnetic resonance (EPR) spectroscopic properties (blue or green type 1 Cu center and colorless type 2 Cu center), per subunit molecule. In the eukaryotic microorganisms with denitrifying capability, CuNiR has been reported from the fungus *Fusarium oxysporum* (23). CuNiR has also been reported from the denitrifying halophilic archaeon *Haloferax denitrificans* (18). However, the gene structures of the fungal and the archaeal CuNiR have not been reported yet.

In the present study, we purified CuNiR from denitrifying cells of an extreme halophilic archaeon, *Haloarcula marismortui*, and characterized its molecular and enzymatic properties. Further, the *nirK* gene encoding the CuNiR was sequenced and phylogenetic analysis was performed. This is the first report of the genetic analysis on the archaeal denitrifying enzymes.

MATERIALS AND METHODS

Cultivation. Anaerobic cultivation of *H. marismortui*. ATCC 43049 was used in the presence of nitrate as described previously (40). The growth medium contained 1.0 g of peptone (Difco, Detroit, Mich.), 1.0 g of yeast extract (Difco), 5.0 g of K_2SO_4 , 5.1 g of NaNO_3 , 125 g of NaCl, 160 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.13 g

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TABLE 1. Purification procedure of *H. marismortui* NiR

Purification step	Vol (ml)	Total protein (mg)	Total activity (mmol of NO ₂ ⁻ · min ⁻¹)	Sp act (μmol of NO ₂ ⁻ · min ⁻¹ · mg ⁻¹)	Yield (%)
Soluble fraction	238	6,710	7.74	1.14	100
1st hydrophobic (Butyl-Toyopearl)	340	2,700	5.77	2.16	74.6
2nd hydrophobic (Butyl-Toyopearl)	71.0	292	2.74	9.36	35.3
Hydrogen-bound (Sephacryl S-300)	41.0	86.9	2.05	23.6	26.5
Gel filtration (Sephacryl S-300)	42.0	52.9	5.29	100	68.4
3rd hydrophobic (Octyl-Sepharose)	24.0	19.9	2.77	139	35.7
Hydroxyapatite	15.9	1.43	1.37	960	17.8

of CaCl₂, 1.0 mg of MnSO₄, 0.83 mg of Fe₂(SO₄)₃, 10 μg of CuSO₄ · 5H₂O, and 10 μg of (NH₄)₆Mo₇O₂₄ · 4H₂O per liter. The pH of the medium was adjusted to 7.0. After it was autoclaved, Na ascorbate was added to the medium to remove dissolved O₂. Cultivation was performed at 40°C for about 3 days. Cells at the late exponential growth stage were harvested by centrifugation and stored at -20°C until experimental use.

Physical measurements. Protein concentration was determined by a modified Lowry method (12) using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Schägger and von Jagow (34). Spectroscopic measurements in the UV-visible regions were performed using a 220A Spectrophotometer (Hitachi Ltd., Tokyo, Japan) with a 1-cm-long lightpath cuvette. The EPR spectrum was measured with a JEOL RE-1X X-band spectrometer (JEOL Ltd., Tokyo, Japan) at 77 K. The magnetic field was calibrated with diphenylpicrylhydrazine and MnO. The molecular weight of the purified enzyme was measured by size exclusion chromatography using a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column (2.0 by 100 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 2 M NaCl. Hemoglobin (molecular mass, 67 kDa), yeast alcohol hydrogenase (150 kDa), aldolase (156 kDa), and apoferritin (450 kDa) were used as molecular weight standards.

Activity assay. Nitrite-reducing activity was measured as follows: enzyme preparations were mixed with 1 ml of 10 mM Na phosphate buffer (pH 7.0) containing 2 M NaCl and 1 mM NaNO₂, and then the reaction was started by adding 10 μl of 1 M Na₂S₂O₄. After an appropriate reaction time, the concentration of nitrite that remained in the solution was assayed by a diazocoupling method (28).

Purification. Cultured archaeal cells (30 g [wet weight]) were suspended in 10 mM Na phosphate buffer (pH 7.0) containing 2.0 M NaCl, and then the cells were disrupted by treatment with an ultrasonic oscillating device. The resulting solution was centrifuged at 10,000 × g for 30 min to precipitate intact cells. The cell-free supernatant thus obtained was further centrifuged at 70,000 × g for 60 min. The resulting supernatant was used as the starting material for subsequent purification of NiR. The supernatant was dialyzed for 12 h against a 20-fold volume of 50%-saturated (NH₄)₂SO₄ that had been neutralized by NH₃. The resulting solution was centrifuged at 10,000 × g for 15 min, and then the precipitate was removed. The supernatant was combined with (NH₄)₂SO₄ granules to produce a 60%-saturated solution, which was mixed with about 100 ml (bed volume) of Butyl-Toyopearl 650M (Tosoh Co., Tokyo, Japan) that had been equilibrated with neutralized 60%-saturated (NH₄)₂SO₄. The suspension was loaded onto the empty column; then the resulting column was washed with the equilibrating buffer until the eluted solution became clear. The NiR that was adsorbed hydrophobically onto the column was eluted by loading 10 mM Tris-HCl buffer (pH 8.0) containing 2 M NaCl, and then the eluate was pooled for further purification. The preparation thus obtained was mixed with a 1.5-fold volume of saturated (NH₄)₂SO₄ and was loaded onto the Butyl-Toyopearl column (2.5 by 20 cm) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 60%-saturated (NH₄)₂SO₄. The enzyme adsorbed was eluted by the linear gradient generated from 10 mM Tris-HCl buffer (pH 8.0) containing 60%-saturated (NH₄)₂SO₄ and from the buffer containing 2 M NaCl. The eluate showing nitrite-reducing activity was collected, mixed with the same volume of saturated (NH₄)₂SO₄, and then loaded onto the Sepharose CL-6B (Bio-Rad, Richmond, Calif.) column (2.5 by 20 cm) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 50%-saturated (NH₄)₂SO₄. The enzyme adsorbed onto the column was eluted by the linear gradient generated from 10 mM Tris-HCl buffer (pH 8.0) containing 50%-saturated (NH₄)₂SO₄ and from the buffer containing 2 M NaCl. The preparation involving nitrite-reducing activity was mixed with the same volume of a saturated (NH₄)₂SO₄ solution and was charged onto a small Butyl-Toyopearl column (0.5 by 2.0 cm) to concentrate the preparation. The sample that was eluted by 10 mM Tris-HCl buffer (pH 8.0)

containing 2 M NaCl was then subjected to a Sephacryl S-300 (Pharmacia) gel filtration column (2.0 by 100 cm) equilibrated with the same solution. The enzyme thus eluted was mixed with the same volume of a saturated (NH₄)₂SO₄ solution and was then subjected to hydrophobic chromatography on an Octyl-Sepharose (Pharmacia) column (2.0 by 10 cm) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 50%-saturated (NH₄)₂SO₄. The enzyme adsorbed was eluted with a linear gradient generated from 100 ml each of 10 mM Tris-HCl buffer (pH 8.0) containing 50%-saturated (NH₄)₂SO₄ and of the buffer containing 2 M NaCl. The eluate with nitrite-reducing activity was collected and dialyzed against 10 mM morpholineethanesulfonic acid (MES)-NaOH buffer (pH 6.5) containing 2 M NaCl for 12 h. The resulting sample was subjected to a hydroxyapatite (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) column (1.0 by 10 cm) that had been equilibrated with the same buffer that was used for the dialysis. After washing of the column with the same buffer, the NiR adsorbed was eluted with a linear gradient generated from 50 ml each of the same buffer and 10 mM Na phosphate buffer (pH 6.5) containing 2 M NaCl. The eluate that appeared blue was collected, concentrated using a Centricon 25 (Amicon Inc., Beverly, Mass.), and then used as the purified enzyme for the experiments. All the purification procedures were performed at 4°C except the hydroxyapatite chromatography, which was done at room temperature.

Protein sequencing. The purified NiR was subjected to SDS-PAGE, and the protein bands were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, Mass.) using an electroblotting apparatus (Atto Co., Tokyo, Japan). The Coomassie brilliant blue-stained protein bands on

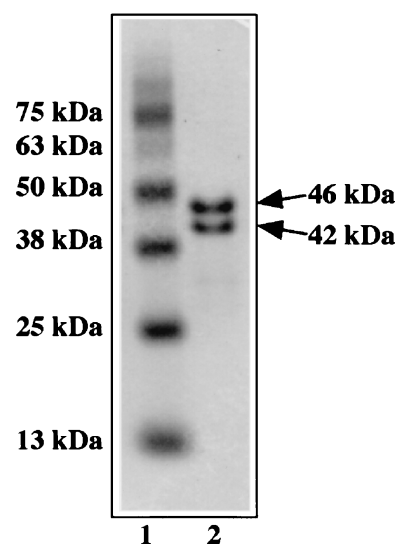


FIG. 1. Subunit composition of the purified *H. marismortui* NiR. SDS-PAGE analysis of the purified NiR was performed, and then the gel was stained with Coomassie brilliant blue. The purified NiR (lane 2) and standard proteins (lane 1) were contained with their molecular masses shown beside the gel.

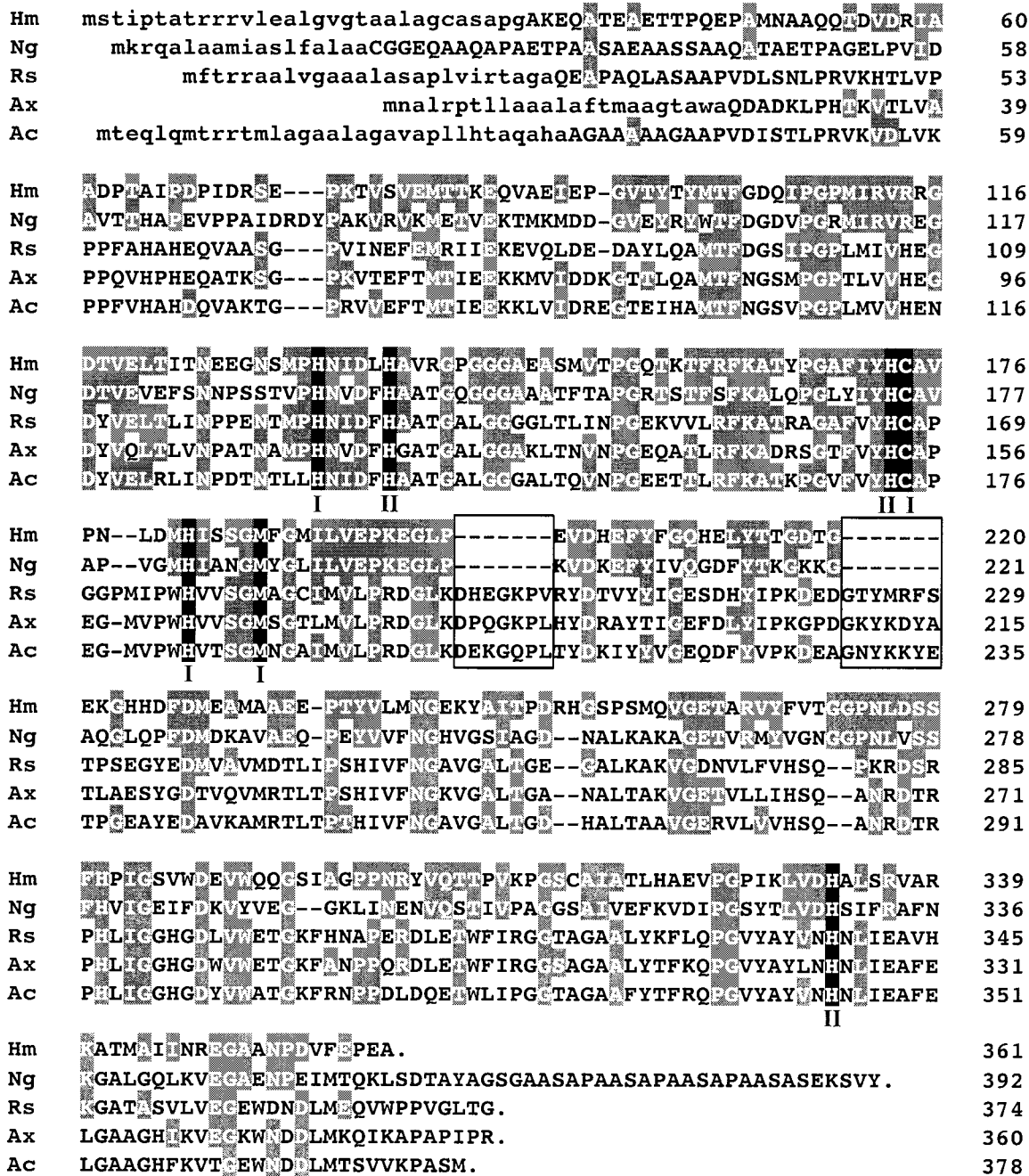


FIG. 2. Amino acid sequence alignment of the *H. marismortui* NiR with bacterial CuNiRs. Shading highlights amino acid residues of bacterial CuNiRs that are identical with those in the *H. marismortui* enzyme. Amino acids are numbered at the right margin. Signal sequences are shown by lowercase letters. Putative Cu-binding residues for type 1 and type 2 Cu are indicated by I and II, respectively. Accession numbers of the sequences shown here are as follows: *N. gonorrhoeae* (Ng) (GenBank M97926), *R. sphaeroides* (Rs) (GenBank U62291), *A. xylooxidans* (Ax) (EMBL AF051831), and *A. cycloclastes* (Ac) (GenBank Z48635).

the membrane were cut out separately and placed on the glass block of a protein sequencer (model PPSQ-21) (Shimadzu Co., Kyoto, Japan) to analyze their N-terminal sequences.

DNA manipulation and sequencing. Extraction of genomic DNA from cultivated *H. marismortui* cells was performed as previously described (7). Plasmid isolation was performed by the alkaline lysis method (6). Standard methods were used for restriction digestions, agarose gel electrophoresis, and ligations. *Taq* polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) was used for PCR, and then the products were cloned into a pT7 Blue T vector (Novagen Inc., Madison,

Wis.). DNA labeling with alkaline phosphatase was done with the materials and protocols of a chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, England) for Southern hybridization. Digested DNA fragments that were cloned into the pUC118 cloning vector were sequenced by the dideoxy chain termination method (8) using a model 4200 DNA sequencer (LI-COR Co., Lincoln, Nebr.). Two degenerate oligonucleotides (5'-GCNATYGARCAGGC NACCVGARGCNGARAC-3' and 5'-GCBGTBGGRTNGCNGCRATNCG RTC-3'; R, Y, V, B, and N represent A+G, C+T, A+C+G, C+G+T, and A+C+G+T, respectively) were synthesized based on the N-terminal amino acid

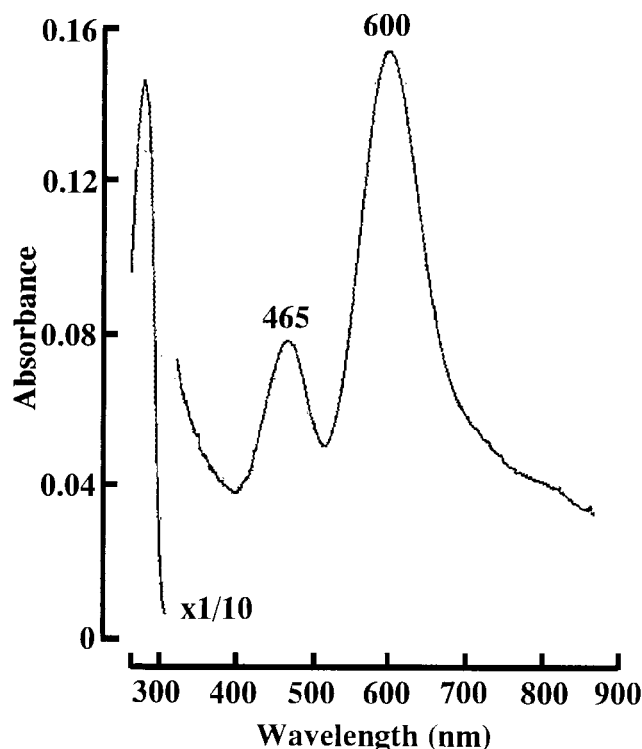


FIG. 3. Absorption spectrum of the *H. marismortui* NiR. Purified NiR (1.91 mg/ml) was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 2 M NaCl. The absorbance of the enzyme at around 280 nm was reduced to a scale of 1/10 the natural intensity.

sequences of the subunits of the purified NiR and were used as primers for the amplification with the archaeal genomic DNA as the template. After cloning and sequencing to confirm its identity, the amplified 93-bp fragment was labeled with alkaline phosphatase and was then used as the DNA probe for Southern hybridization.

Nucleotide sequence accession number. The *nirK* gene from *H. marismortui* has been assigned accession number AJ278286 in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database.

RESULTS

Purification of NiR. When the NiR preparation was transferred to an asaline condition, its activity gradually decreased to about 70% after 1 h. Purification of the enzyme was, therefore, usually performed in coexistence with NaCl or $(\text{NH}_4)_2\text{SO}_4$, the concentrations of which were at least 2 M. Most (96%) of the NiR activity in the cell extract was recovered in the soluble fraction. The blue enzyme was purified 833-fold to gain the final recovery of 17.8% through six preparative steps, including hydrophobic, hydrogen-bound, gel filtration, and hydroxyapatite chromatographies as summarized in Table 1. Enhancement of the nitrite-reducing activity of the enzyme during the gel filtration step was usually observed in the three individual purification procedures, raising the possibility of the presence of an inhibitor against the enzyme in the soluble fraction, as formerly suggested in the purification of the CuNiRs from the denitrifying bacteria *Achromobacter cycloclastes* (26) and *Rhodobacter sphaeroides* (33).

Subunit structure. SDS-PAGE of the purified NiR gave two protein bands whose molecular masses appeared to be

46 and 42 kDa with an approximately 1:1 stoichiometry, as shown in Fig. 1. N-terminal amino acid sequences of the 46- and 42-kDa subunits were determined to be A(I/K)EQATEAETTPQE(P)A MNAAQOTDV and MNAAQOTDVEDRIAADPTAIDD, respectively. The sequence of the 46-kDa subunit after the 17th amino acid was identical to the N-terminal sequence of the 42-kDa subunit, indicating that the NiR was composed of identical subunits, except for the 16-amino-acid difference. The 42-kDa subunit of the enzyme is probably generated by a secondary proteolytic reaction of the 46-kDa subunit. The molecular mass of the enzyme in a mature state was estimated to be 167 kDa by gel filtration, suggesting that the purified enzyme is a multicomplex of an identical subunit.

Structure of gene encoding NiR. Based on the N-terminal sequences of the 46- and 42-kDa subunits of the purified enzyme, the DNA fragment encoding the N-terminal region of the enzyme was amplified and used as a probe for Southern hybridization. Hybridization upon *Hind*III-, *Kpn*I-, *Pst*I-, and *Sma*I-digested gene fragments showed a single positive band corresponding to 5.2, 2.5, 2.8, and 2.4 kbp, respectively, suggesting that only one copy of the gene would be present in the archaeal genomic DNA (data not shown). The nucleotide sequence of the *Kpn*I-digested fragment was determined using standard DNA manipulation methods. The 2.5-kbp DNA fragment of the *H. marismortui* genome included two complete open reading frames, *nirK* and *pcn*, which encoded the present NiR with 361 amino acids and a proliferating cell-nuclear antigen with 256 amino acids, respectively. The N-terminal amino acid sequences of the 46- and 42-kDa subunits of the purified enzyme matched completely with parts of the sequence of the *nirK* product. Molecular weights of the 46- and 42-kDa subunits were calculated to be 35,821 and 34,138, respectively, from the sequence. The calculated molecular weights were quite small compared with those estimated from the SDS-PAGE analysis. Nucleotide and amino acid sequence data are available under accession number AJ278286 in the EMBL database, the number given at the end of Materials and Methods.

Sequence alignment. In Fig. 2, the deduced amino acid sequence of the *H. marismortui* NiR is shown and aligned with those of CuNiRs from four denitrifying bacteria, *Neisseria gonorrhoeae*, *R. sphaeroides*, *Alcaligenes xylosoxidans*, and *A. cycloclastes*. Putative Cu-liganding residues His-133, Cys-174, His-182, and Met-187 for type 1 Cu and His-138, His-173, and His-332 for type 2 Cu were completely retained in the *H. marismortui* enzyme. Sequential coincidence of the archaeal NiR with the bacterial enzymes was observed in the overall region, especially with the gonorrhoeal pathogen *N. gonorrhoeae*, having in common the presence of two short deletions (boxed regions of the sequence in Fig. 2).

Optical absorption and EPR spectra. The purified enzyme appeared blue in an oxidized state, and the color disappeared on reduction with $\text{Na}_2\text{S}_2\text{O}_4$. The absorption spectrum of the enzyme exhibited absorption maxima at 465 and 600 nm with a small shoulder around 820 nm in the visible region (Fig. 3). The extinction coefficient at 600 nm of NiR was estimated to be $2.88 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ when the molecular weight of the subunit was considered to be 35,000, as an average of those of the 46- and 42-kDa subunits.

EPR measurement also revealed the presence of Cu in the

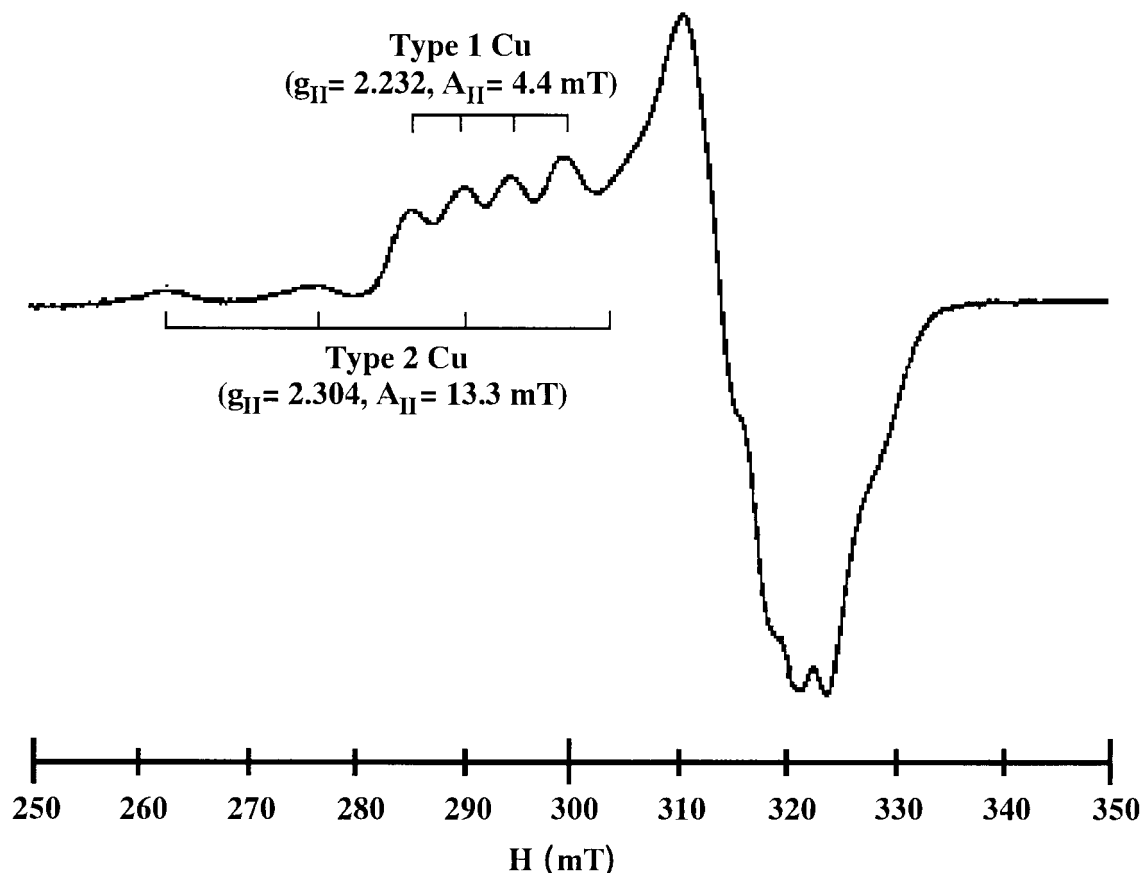


FIG. 4. EPR spectrum of *H. marismortui* NiR at 77 K. The purified NiR (19.1 mg/ml) was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 2 M NaCl. Conditions of the EPR run were microwave frequency, 9.21 GHz; microwave power, 3.13 mW; modulation amplitude, 1.0 mT; sweep time, 8 min; time constant, 0.1 s.

purified enzyme. The spectrum of the oxidized enzyme showed signals characteristic of the type 1 Cu center with narrow, sharp, hyperfine splitting ($g_{II} = 2.232$; $A_{II} = 4.4$ mT) and characteristic of the type 2 Cu center with a broader split ($g_{II} = 2.304$; $A_{II} = 13.3$ mT), as shown in Fig. 4. Overall, spectroscopic features of the purified enzyme resembled those of CuNiRs isolated from denitrifying bacteria. Composition of Cu atoms in the purified enzyme was estimated to be $1.5 \text{ mol} \cdot \text{mol}^{-1}$ of the subunit by double integration of the EPR spectrum, suggesting that two prosthetic Cu atoms are present in a subunit molecule. These EPR spectroscopic analyses provided evidence that one molecule each of type 1 and type 2 Cu centers is present in a subunit of the archaeal NiR.

Catalytic properties. As generally observed in the halobacterial enzymes, the purified CuNiR was activated in the presence of high salt concentrations. As shown in Fig. 5, nitrite-reducing activity of the enzyme reached its maximum at concentrations of NaCl higher than 2 M. Using $\text{Na}_2\text{S}_2\text{O}_4$ as the electron donor, enzymatic activity at 2 M NaCl was estimated to be $960 \mu\text{M NO}_2^- \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Relative activities of the enzyme measured in the presence of 2 M KCl, LiCl, NH_4Cl , and NaNO_3 against the activity in 2 M NaCl were 87, 69, 84, and 65%, respectively, suggesting that the activity of CuNiR is

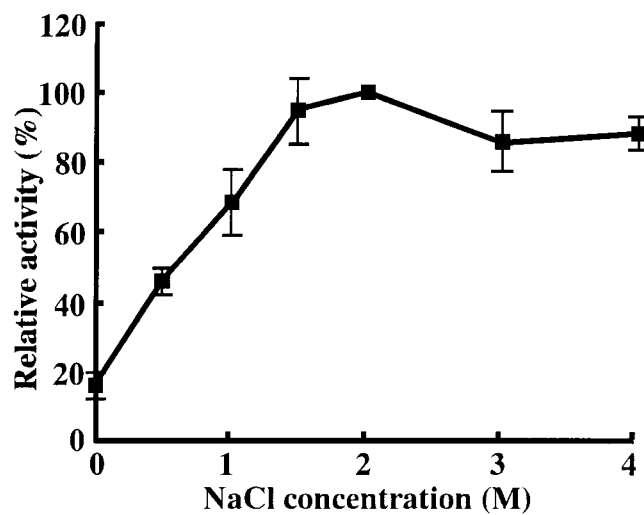


FIG. 5. Effect of NaCl concentration on the enzymatic activity of *H. marismortui* CuNiR. Nitrite-reducing activity was measured in 10 mM Tris-HCl buffer (pH 8.0) containing each concentration of NaCl. Maximum activity (100%), which was measured at 2 M NaCl, was $960 \mu\text{M NO}_2^- \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Each point is the mean value of three individual measurements.

TABLE 2. Properties of CuNiRs

Species (enzyme)	Molecular mass(es) (kDa)	Subunit mass (kDa)	Composition	Absorption peaks ^f (nm)	ϵ_{mM} at maximum peak ($\text{mM}^{-1} \cdot \text{cm}^{-1}$ of subunit)	Cu atom composition ($\text{mol} \cdot \text{mol}^{-1}$ of subunit)
<i>H. marismortui</i> (blue)	167 ^a	46 ^c (35.8) ^d + 42 ^c (34.1) ^d	$(\alpha/\alpha')_3 \times 2^a$	465, 600 , 820	2.88	1.5
<i>H. denitrificans</i> (green)	127 ^a	64 ^c	α_2^a	462 , 594, 682	1.8	ND ^h
<i>A. xylosoxidans</i> (blue)	70 ^a , 103 ^b	36.5 ^d	α_3^e	460, 593 , 770	3.7	1.8
<i>A. cycloclastes</i> (green)	69 ^a , 105 ^b	37 ^d	α_3^e	400, 464 , 590, 700	2.1	1.6
<i>F. oxysporum</i> (blue)	83.3 ^a	41.8 ^c	α_2^a	470, 595 , 782	5.4	1.9

^a Determined by gel filtration.

^b Reflects sedimentation equilibrium.

^c Determined by SDS-PAGE.

^d Calculated from sequence.

^e Crystal structure.

^f Maximum peaks are given in boldface.

^g Blue Cu protein and cytochrome *c* are not present (–) (see text).

^h ND, not determined.

ⁱ DDC, diethyldithiocarbamate.

not affected by specific ion species but requires only high ionic strength.

The purified enzyme was found to accept electrons only from $\text{Na}_2\text{S}_2\text{O}_4$. The physiological electron donor has remained unclear; potent physiological electron donors of the bacterial enzyme, low-molecular-weight blue Cu protein and cytochrome *c*, were not present in the denitrifying cells of *H. marismortui* according to optical spectroscopic observation. Artificial reductants, such as methyl viologen, phenazine methosulfate, and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, showed no electron-donating ability to the purified enzyme. Treatment with 1 mM diethyldithiocarbamate or cyanide completely inhibited the activity of the purified enzyme, as is also true in the bacterial CuNiRs. Incubation with 1 mM EDTA for 1 h decreased the nitrite-reducing activity to below 30%, while the reduced activity was not restored by treatment with 50 μM CuSO_4 .

DISCUSSION

In the bacterial and fungal CuNiRs, blue and green enzymes have been reported (41). The blue CuNiR purified from *A. xylosoxidans* (1, 26), *Pseudomonas aureofaciens* (42) and the fungus *F. oxysporum* (23) showed a maximum peak at a wavelength of 580 to 600 nm that was accompanied by a small shoulder around 460 nm, while the green enzyme from *A. cycloclastes* (20), *Alcaligenes faecalis* (22), and *R. sphaeroides* (33) possessed dual peaks at around 590 and 460 nm with comparable absorptional intensities. The green enzyme has also been reported in another halophilic archaeon, *H. denitrificans* (18). Molecular and enzymatic properties of the CuNiRs from archaea (*H. marismortui* and *H. denitrificans*), bacteria (*A. xylosoxidans* and *A. cycloclastes*), and fungi (*F. oxysporum*) are summarized in Table 2.

In spite of the clear difference in the optical absorption spectroscopic features, EPR signal parameters of the blue CuNiRs, including the present archaeal enzyme, are quite similar and are hardly distinguishable from that of the green enzyme in Table 2. Crystal structures of the blue CuNiR from *A. xylosoxidans* (11) and of the green enzymes from *A. cycloclastes* (16) and *A. faecalis* (27) have already been determined with high resolution, and comparison of the Cu-liganding structures

in the enzymes demonstrates that a small difference in the orientation of the S_8 atom of Met in the tetrahedral geometry around the type 1 Cu center causes variation in optical spectroscopic properties (19). CuNiR purified from *H. marismortui* in this study appeared blue and possessed a maximum absorption peak at 600 nm in the visible region, while it was accompanied by a peak at 465 nm whose intensity was about half that at 600 nm. The spectrum looks like a combination of the blue and green enzymes. Spectroscopic properties of the *H. marismortui* enzyme will be elucidated by future investigation of the crystal structure of the enzyme.

The amino acid sequence of the archaeal CuNiR showed homology with those of enzymes from four denitrifying bacteria, *N. gonorrhoeae*, *R. sphaeroides*, *A. xylosoxidans*, and *A. cycloclastes*, in the overall region of the sequence (Fig. 2). X-ray crystal structure analyses of CuNiRs from *A. xylosoxidans* (11) and *A. cycloclastes* (16) have demonstrated the characteristic triangular structure composed of three identical subunits. The similarity of the amino acid sequences suggests that the minimum functional unit of the archaeal enzyme is a trimeric complex of the identical subunits containing one molecule each of type 1 and type 2 coppers in a subunit molecule. Further, preliminary X-ray analysis of the archaeal CuNiR crystal also suggests that the enzyme is a hexamer, probably a dimer of a trimeric complex, of the subunit molecule in the mature state (H. Ichiki et al., unpublished results).

Interestingly, phylogenetic analysis of the amino acid sequences indicated that the archaeal CuNiR is in a quite close relationship with the enzyme from the gonorrhoeal pathogen *N. gonorrhoeae*, as revealed in the unrooted phylogenetic tree shown in Fig. 6. The ratio of amino acid residues, identical to that for the *N. gonorrhoeae* CuNiR (41%), is remarkably high compared with those for the other enzymes (30 to 32%). Moreover, short deletions in the same two positions (boxed regions of the sequences in Fig. 2) were commonly present in the two enzymes. The structural similarity suggests the lateral transfer of the *nirK* gene between the halophilic archaea and the pathogenic proteobacteria. Lateral gene transfer between the halophilic archaea and the cyanobacteria has been proposed in the [2Fe-2S] type ferredoxin of *Halobacterium salinarum* (30).

In the N terminus of the CuNiR precursor, the positively

TABLE 2—Continued.

EPR parameters for:				Electron donor				Activity ($\mu\text{M NO}_2^- \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Inhibitor(s)	Source or reference(s)
Type 1 Cu		Type 2 Cu		Cu protein	Cytochrome <i>c</i>	Methyl viologen- $\text{Na}_2\text{S}_2\text{O}_4$	Phenazine methosulfate- ascorbate			
g_{II}	A_{II} (mT)	g_{II}	A_{II} (mT)							
2.232	4.4	2.304	13.3	— ^g	— ^g	+ ($\text{Na}_2\text{S}_2\text{O}_4$)	—	960	DDC ⁱ , CN^- , EDTA	This study
ND	ND	ND	ND	ND	ND	ND	+	25	DDC	18
2.208	6.3	2.298	14.2	+	+	+	+	240	ND	1, 10, 11, 26
				(azurin)	(cytochrome c_{553})					
2.195	7.3	2.262	17.5	+		ND	+	260	DDC, CN^- , CO	16, 20, 21, 25
				(pseudoazurin)						
2.22	6.82	2.32	ND	+	+	+	+	447	DDC, CN^- , CO	23
				(azurin)	(cytochrome c_{549})					

charged (-RRR-) and subsequent hydrophobic (-ALGVGTA ALAG-) regions, which are characteristic to the bacterial signal peptide (29), were present (Fig. 2). By using the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>), a possible cleavage site of the archaeal CuNiR precursor by the signal peptidase was predicted to be between the 32nd and 33rd (-PGA/KEQ-, gram-positive mode) positions or between the 29th and 30th (-ASA/PGA-, gram-negative mode) positions, which are close enough to the cleavage site (-APG/AKE-) to give the 46-kDa subunit of CuNiR. The results suggest that the archaeal CuNiR is a periplasmic protein, as was previously revealed of the bacterial NiRs (9). However, a spheroplasting experiment for resolving the intracellular localization of the enzyme has not succeeded until now, because the denitrifying cells of *H. marismortui* are easily disrupted only by the precipitation of the cells by centrifugation.

The physiological electron donor to the CuNiR remains unclear. Azurin- or pseudoazurin-like blue Cu proteins (10, 23, 25) and soluble *c*-type cytochromes (23, 26) have been reported as the direct electron donors to the CuNiRs in some

denitrifying bacteria. Although a pseudoazurin-like blue Cu protein named “halocyanin” has been reported from a haloalkaliphilic archaeon (35), no blue protein except the present enzyme was observed in *H. marismortui* during the fractionation of the proteins. Further, cytochromes *c* with low-spin hemes are detected in neither soluble nor membrane fractions of the archaeon from their difference spectra ([reduced] – [oxidized]), as formerly reported of the other halophilic archaeon, *H. salinarum* (14).

Enzymes from halophilic archaea generally require extreme salinity for their stabilization and/or enzymatic activity. It is known that the halophilic proteins contain an excess of negatively charged residues compared to their nonhalophilic homologs (15). Recent X-ray crystallographic analysis of some halobacterial enzymes showed the presence of characteristic salt bridge structures composed of acidic residues and solvent cations and the presence of an excess of bound water molecules on the surface of the protein molecule (13, 31). Denaturation of the halobacterial enzymes in the nonhalophilic condition is explained by the presence of clusters of the negatively charged residues that lead to instability of the protein molecule from the electrostatic repulsion among the clusters in a condition of low ionic strength. The *H. marismortui* CuNiR purified in this study is a typical halophilic enzyme, showing maximum activity at above 2 M NaCl while being denatured in the absence of salinity. However, the ratio of negatively charged residues in the archaeal enzyme (13.3%) was only a little higher than those in the nine bacterial enzymes (9.4 to 11.8%) that are shown in Fig. 6. Understanding the structural implication to a “haloadaptation” of CuNiR requires X-ray crystal structure analysis in the future.

Recent investigations of dissimilatory nitrate reduction in the archaea have indicated that the archaeal NaR resembles the bacterial membrane-bound NaR in terms of enzymatic properties (2, 3, 5, 17, 40), subunit composition (5, 17, 40), and DNA sequence (EMBL database accession no. AJ277440). In this study, we purified CuNiR from the denitrifying archaeon *H. marismortui* and cloned the *nirK* gene encoding the enzyme. In spite of the similarity of the sequence, the purified enzyme showed some peculiarities in the structural, spectroscopic, and catalytic properties compared with those of the bacterial CuNiRs. The biochemistry of the NO reducing system in archaeal denitrification, therefore, will be the focus of our next study.

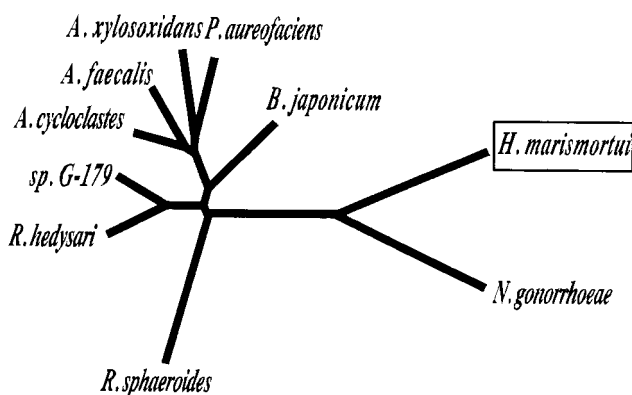


FIG. 6. Phylogenetic relationship of the *H. marismortui* CuNiR with bacterial CuNiRs based on the sequence alignment. Construction of an unrooted phylogenetic tree is inferred from the alignment of the amino acid sequences based on the ClustalW program (<http://clustalw.genome.ad.jp/>). Accession numbers for the sequences shown here (see Fig. 2 legend for other accession numbers) are *A. faecalis* (GenBank D13155), *Bradyrhizobium japonicum* (EMBL AJ002516), *P. aureofaciens* (GenBank Z21945), *Pseudomonas* sp. strain G-179 (GenBank M97294), and *Rhizobium hedysari* (GenBank U65658).

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