Purification and Characterization of the MQH$_2$NO Oxidoreductase from the Hyperthermophilic Archaeon Pyrobaculum aerophilum*

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The membrane-bound NO reductase from the hyperthermophilic denitrifying archaeon Pyrobaculum aerophilum was purified to homogeneity. The enzyme displays MQH$_2$NO oxidoreductase (qNOR) activity, consists of a single subunit, and contains heme and nonheme iron in a 2:1 ratio. The combined results of EPR, resonance Raman, and UV-visible spectroscopy show that one of the hemes is bis-His-coordinated low spin ($g_s = 3.015$; $g_L = 2.226$; $g_{ax} = 1.45$), whereas the other heme adopts a high spin configuration. The enzyme also contains one nonheme iron center, which in the oxidized enzyme is antiferromagnetically coupled to the high spin heme. This binuclear high spin heme/nonheme iron center is EPR-silent and the site of NO reduction. The reduced high spin heme is bound to a neutral histidine and can bind CO to form of a low spin complex. The oxidized high spin heme binds NO, yielding a ferric nitrosoyl complex, the intermediate causing the commonly found substrate inhibition in NO reductases ($K_i(NO) = 7 \mu M$). The qNOR as present in the membrane is, in contrast to the purified enzyme, quite thermostable, incubation at 100 °C for 86 min leading to 50% inhibition. The purified enzyme lacks heme b and instead contains stoechiometric amounts of hemes O$_{P1}$ and O$_{P2}$ ethynylgeranylated and hydroxyethylgeranylated derivatives of heme b, respectively. The archael qNOR is the first example of a NO reductase, which contains modified hemes reminiscent of cytochrome $b_o$ and $a_a$ oxidases. This report is the first to describe the purification and structural and spectroscopic properties of a thermostable NO reductase.

Denitrification serves as an alternative to aerobic respiration to generate a proton motive force and is found in many prokaryotic microbes that thrive under anaerobic conditions. In denitrification, nitrate is reduced via nitrite, nitric oxide, and nitrous oxide to dinitrogen (1–3). The denitrification pathway and the properties of the four specific enzymes have been extensively studied in Gram-negative proteobacteria such as Paracoccus denitrificans, Paracoccus halodenitrificans, Ralstonia eutropha, Pseudomonas aeruginosa, and Pseudomonas stutzeri (4–9). Studies on denitrification in other branches of the bacteria are scarce; the properties of the NO reductase from the Gram-positive Bacillus azotoformans have been reported recently (10).

Several archaea are capable of denitrification (e.g. the halophiles Halofex denitrificans and Haloarcula marismortui and the hyperthermophiles Ferroglobus placidus and Pyrobaculum aerophilum) (11–14). These organisms were shown to reduce nitrate as in bacteria via nitrite, NO, and N$_2$O to N$_2$, P. aerophilum is a hyperthermophilic archaeon growing optimally at temperatures of 100 °C and can utilize nitrate or oxygen as terminal electron acceptors (11, 12). In P. aerophilum, both the nitrite reductase and nitrous oxide reductase are bound to the membrane, in contrast to Gram-negative bacteria (11, 15). Furthermore, P. aerophilum is also the only denitrifier identified so far in which menaquinol is the electron donor to all four denitrification reductases (15).

In this paper, the purification of the NO reductase from P. aerophilum is described. NO reductases are integral membrane proteins and ancient members of the superfamily of heme-copper oxidases (16–18). The crystal structures of several oxidases are available (19–24), but crystallization of NO reductases has so far been unsuccessful. The large subunit of NO reductases or oxidases contains 12–14 transmembrane $\alpha$-helices and six conserved histidine residues and harbors the active binuclear site, where reduction of two molecules of NO to N$_2$O or O$_2$ to H$_2$O occurs (6, 8, 18, 25–29). Two conserved histidines are ligands to a high spin heme center, which serves as the site where electrons enter; one histidine residue acts as a ligand to a high spin heme center, and the remaining three histidine residues coordinate to Fe$_b$ in the NO reductases or to Cu$_{a2}$ in the oxidase branch. The oxidized NO reductases contain a binuclear center consisting of a nonheme iron center, Fe$_b$, and a high spin heme group, $\sim 3.5$ Å apart, connected through an oxo-bridge conveying a strong antiferromagnetic coupling (3, 6, 28, 29). In contrast to the activity of oxidases, which is associated with the transmembrane movement of “chemical” and pumped protons from the cytoplasm to the periplasm (5, 6, 8, 30–32), the NO reductases do not pump protons, rendering the overall reaction nonelectrogenic (33). Although NO reductases can reduce oxygen and oxidases can slowly reduce NO, both enzymes are optimized for their reaction with their natural substrates (5, 27, 34).

At present, three different bacterial NO reductases have been characterized. Whereas the properties of the binuclear active site are very similar for all three, the site of electron entry is quite different. Gram-negative bacteria contain the...
two-subunit cytochrome bc complex type of enzyme (cNOR)\(^1\) (5, 6, 8, 9). Another type of NOR, the qNOR, consists of one subunit and uses menahydroquinone as electron donor (4). Genes encoding the qNOR have been identified in denitrifying soil (R. eutropha) and marine bacteria (Synecocystis), in the genome of the denitrifying marine archaeon P. aerophilum and in non-denitrifying pathogenic micro-organisms such as Neisse- ria meningitides, Neisseria gonorrhoeae, and Corynebacterium diphtheriae (4, 35–37). A third type of NOR is present in the Gram-positive bacterium B. azotoformans (10). This NOR uses menahydroquinone as electron donor, consists of two subunits (the smaller one containing CuA), and is called qCuANOR (3, 10).

A qNOR has been purified for the first time as a histidine-tagged enzyme from R. eutropha (4). The purified enzyme consists of a single subunit (84,000 kDa). The enzyme lacks heme c and contains heme b and nonheme iron in an approximately 2:1 ratio, respectively. The enzyme is inactive with cytochrome c and contains heme b and nonheme iron in an approximately 8:0), 0.5 mM PMSF, and centrifuged for 30 min at 20,000 g.

The genome of P. aerophilum contains a gene (QSZSS8) putatively encoding a qNOR (38). Membranes of P. aerophilum contain a NO reductase oxidizing MQH\(_2\), thus classifying it as a functional qNOR. This paper describes its purification and presents a structural and spectroscopic characterization. Whereas the purified enzyme is similar in several respects to the qNOR from R. eutropha, which was purified as the Histagged mutant, the Pyrobaculum enzyme is the first NO reductase shown to contain modified hemes rather than b hemes (39). Furthermore, the qNOR as present in the membrane is very thermostable. The modified hemes might be one of the factors contributing to thermostability of the qNOR.

EXPERIMENTAL PROCEDURES

Cell Growth and Enzyme Purification—Cells from P. aerophilum were grown, harvested, and stored essentially as described previously (11, 40). Membranes were prepared as follows. Frozen cells (30–60 g) were thawed and diluted five times with 20 mM Tris-HCl buffer (pH 8.0), 10 mM MgSO\(_4\), 2–4 mg of each DNase and RNase, and 0.5 mM PMSF. The suspension was homogenized with a Potter-Elvehjem homogenizer. The P. aerophilum cells were completely broken as a result of this hypotonic treatment. The broken cells were centrifuged for 5 min at 1000 \(\times g\). The pellet, mainly cell debris, was discarded, and 0.5 mM PMSF was added to the supernatant. The supernatant was subsequently centrifuged for 30 min at 20,000 \(\times g\). The pellet representing the membrane fraction was washed with 20 mM Tris-HCl buffer (pH 8.0), 0.5 mM PMSF, and centrifuged for 30 min at 20,000 \(\times g\). The resulting pellet was suspended in a small volume of the same buffer and stored at \(-40^\circ C\) until use.

After thawing, the membranes were brought to a protein concentration of 10 mg/ml with 20 mM Tris-HCl buffer (pH 8.0). Immediately after the addition of 0.5 mM PMSF, the membranes were extracted with 1% dodecyl maltoside, incubated at room temperature for 15 min, and centrifuged for 30 min at 45,000 \(\times g\). The supernatant was applied to the first CM-Sepharose column (\(V = 60 ml\) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) plus 0.5% dodecyl maltoside; the flow-through was directly applied to a POROS-HQ column (\(V = 60 ml\) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) plus 1% dodecyl maltoside). NO reductase activity was exclusively found in the flow-through, which was applied to a second CM-Sepharose column identical to the first one. After washing with 5 column volumes, a linear NaCl gradient (0–0.5 M) was applied over 5 column volumes. Fractions with more than 45% of NO reductase activity bound to the second CM-Sepharose column. The flow-through of the first CM-Sepharose column was subsequently applied to an anion exchanger, POROS-HQ. As expected on the basis of its calculated pi (9.4), NO reductase eluted again in the flow-through, but other denitrification enzymes did bind; their purification will be described elsewhere.

RESULTS

Purification—Membranes from P. aerophilum contain a NO reductase, which uses MQH\(_2\) as substrate and thus belongs to the subclass of qNORs (15, 36). This qNOR was purified from P. aerophilum membranes after solubilization with 1% dodecyl maltoside, extracting \(-85\%\) of the NOR activity (Table 1). The solubilized enzyme did bind to CM-Sepharose, however, only after application to a second CM-Sepharose column. We suggest that because the genome of P. aerophilum encodes many (putative) genes with high pi values (average pi of 8.4–8.5) (38), the qNOR is excluded from the first CM-Sepharose column. The flow-through of the first CM-Sepharose column was subsequently applied to an anion exchanger, POROS-HQ. As expected on the basis of its calculated pi (9.4), NO reductase eluted again in the flow-through, but other denitrification enzymes did bind; their purification will be described elsewhere.

The abbreviation used are: cNOR, bc-type NO reductase; qNOR, (mena)quinol: NO oxidoreductase; NOR, NO reductase; HS, high spin; LS, low spin; MQH\(_2\), menaquinol; RR, resonance Raman; PMSF, phenylmethylsulfonyl fluoride.

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eluted between 0.9 and 1.0 M phosphate. After these two chromatographic steps, the enzyme was purified ~54-fold with respect to the membranes and was found to be essentially pure on the basis of its SDS-PAGE profile (Fig. 1 and Table I), prosthetic group content (see below), and its chromatographic behavior on gel filtration.

The SDS-PAGE profile of the urea-denatured purified (Fig. 1, lane 2) protein shows a main band around 75–80 kDa, in agreement with the molecular mass of 78.8 kDa calculated from the DNA sequence (38). The relatively faint bands at ~41–43 kDa are not impurities but represent remaining partially folded states of the enzyme. The bulk of the NO reductase migrates at this position when urea is omitted during denaturation (Fig. 1, lane 3). The N termini of the bands at 41–43 and 75 kDa were all blocked as determined after blotting to polyvinylidene difluoride paper. Direct sequencing of the nonblotted enzyme preparation did yield a sequence, corresponding to ~1% of the total amount of protein. The 30 N-terminal residues obtained, APAAGVA...QLFP, correspond precisely to residues 122–151 of the published sequence of gene Q8ZSS8 (38). It is suggested that the peptide starting at residue 122 represents a minor (e.g. proteolytic) fragment and that the majority of the protein is blocked at the N terminus.

Chromatography on Superdex 200 yielded a single peak with an apparent molecular mass of 127 ± 7 kDa (not shown). Assuming that the qNOR like other membrane proteins binds one dodecyl maltoside micelle of about 40–50 kDa (42), the apparent molecular mass of 78.8 kDa calculated on the basis of the DNA-derived protein sequence (25.4 and 12.7 nmol/mg, respectively, yielding a stoichiometry of enzyme/heme/nonheme iron of 1:2.13:1.27. The total heme and nonheme iron contents of the pure protein were 26.6 and 16.1 nmol/mg, respectively, yielding a stoichiometry of enzyme/heme/nonheme iron of 1:2.13:1.27. The prosthetic group content corresponds to a protein of ~75 kDa based on heme content and is close to that calculated on the basis of the DNA-derived protein sequence (25.4 and 12.7 nmol/mg, assuming two hemes and one nonheme iron, respectively, and a mass of 78.8 kDa) confirming its high purity (95% on the basis of heme). A final indication of purity and homogeneity of the purified enzyme.

The total heme and nonheme iron contents of the pure protein were 26.6 and 16.1 nmol/mg, respectively, yielding a stoichiometry of enzyme/heme/nonheme iron of 1:2.13:1.27. The prosthetic group content corresponds to a protein of ~75 kDa based on heme content and is close to that calculated on the basis of the DNA-derived protein sequence (25.4 and 12.7 nmol/mg, assuming two hemes and one nonheme iron, respectively, and a mass of 78.8 kDa) confirming its high purity (95% on the basis of heme). A final indication of purity and homogeneity of the qNOR was the observation that the heme/protein ratio measured as the absorbance ratio at 410 nm/280 nm was constant over the peak eluting from the hydroxyapatite column; neither this ratio nor the specific activity increased after subsequent chromatography with CM-Sepharose or Superdex 200 (data not shown). We conclude that the qNOR is essentially pure and contains one nonheme iron center and two heme centers.

Identity of the Hemes—The identity of the heme groups of purified qNOR was determined with the pyridine hemochrome method. Heme c was absent. The pyridine hemochrome absorbance maximum was at 555 nm, which corresponds to a shift of ~1 nm to the blue with respect to pyridine hemochrome obtained from myoglobin. Reversed phase chromatography of the heme extracts of the pure enzyme identified only very small amounts of heme b and the presence of two hydrophobic modified forms of heme b (Fig. 2) in accordance with the heme composition of complete membranes (39). Following the identification and nomenclature in Ref. 39, the more hydrophobic and slower migrating heme corresponds to heme O₉₁, an ethenylgeranylgeranyl derivative of heme b; the other corresponds to O₂₂, containing the hydroxyethylgeranylgeranyl modification. The ratio of the areas of heme b, heme O₉₁, and O₂₂ are 0.035, 0.51, and 0.45, respectively. Assuming similar extinction coefficients for the three hemes at 400 nm, we conclude that the major components, hemes O₉₁ and O₂₂, are present in a 1:1 stoichiometric ratio.

Although the pyridine hemochrome spectral maximum at 555 nm of the mixture of heme O₉₁ and O₂₂ is close to that of heme b, heme O₉₁ had a pyridine hemochrome α-band maximum at 558 nm and heme O₂₂ at 553 nm. Their γ-band maxima in the oxidized as isolated state are at 399 and 395 nm, respectively.

UV-visible Spectroscopy—The UV-visible spectrum (Fig. 3A) in oxidized and MQH₂-reduced enzyme is typical of heme. Menaquinol elicits complete reduction of the hemes, since reduction with sodium dithionite alone or subsequent to menaquinol yielded essentially the same optical spectrum (data not shown). The oxidized spectrum (Fig. 3, A and B) shows a γ-band maximum at 412 nm (ε = 210 nm⁻¹ cm⁻¹) and a broad absorbance around 585–590 nm, partially overlapping with the α-band at 557 nm. The β-band maximum is at 527 nm. In the spectrum of the reduced enzyme the γ-band is shifted to 421 nm (ε = 217 nm⁻¹ cm⁻¹), the α- and β-band maxima are at 560 nm (ε = 31.0 nm⁻¹ cm⁻¹) and 524 nm, respectively. The positions of the various maxima in the oxidized and reduced qNOR are very similar to those of the cNOR from P. denitrificans (15, 36).

The oxidized enzyme binds NO. In the γ-band region, a small shift is seen as well as a small reduction in intensity (Fig. 3A). Shifts of a few nm are observed in the region between 530 and 570 nm as well as a sharpening of the two absorbances in that spectral region, which is particularly clear from the shape of the difference spectrum showing peaks at 534 and 570 nm (Fig. 3B). Furthermore, the broad absorbance around 585–590 nm disappears upon the addition of NO, resulting in a minimum at 590 nm in the difference spectrum (Fig. 3B).

The reduced enzyme binds CO. The γ-band maximum shifts to 419.5 nm, sharpens, and slightly intensifies (CO diff traces in Fig. 3, A and B). Between 500 and 630 nm, shifts of peaks of relatively low intensity are observed, yielding maxima at 538 and

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**Table I**

Summary of the purification of the P. aerophilum NO reductase

| Preparation       | Total protein (mg) | Specific activity at 64.5 °C (μmol NO/mg min) | Yield (%) | Purification |
|-------------------|--------------------|-----------------------------------------------|-----------|-------------|
| Membranes         | 4375               | 0.06                                          | 100       | 1.00        |
| Extract*          | 3420               | 0.07                                          | 91.2      | 1.1         |
| CM-Sepharose      | 140.9              | 0.46                                          | 24.7      | 7.7         |
| Hydroxyapatite    | 6.3                | 3.28                                          | 7.8       | 54.6        |

* High speed supernatant after the dodecyl maltoside extraction of membranes. Data are an average of three independent purifications. The spread in values for specific activity, yield, and purification factor is about ±20%. Note that: activities in the various fractions employing MQH₂ are listed in the table. Activities obtained with Ase/phenazine ethosulfate were essentially the same.
568 nm and a minimum at 600 nm in the difference spectrum (Fig. 3B). Collectively, the spectral shifts obtained upon the addition of CO are consistent with an HS to LS spin state change of the reduced heme iron (see also “Resonance Raman Spectroscopy”); a similar spin state change occurs in, for example, myoglobin and in cNOR from P. denitrificans (15, 36).

**EPR Spectroscopy**—Fig. 4 shows the EPR spectrum of the purified NO reductase. The major features are the resonances at \( g_z = 3.015 \), \( g_y = 2.226 \), and \( g_x = 1.45 \) derived from a highly anisotropic low spin heme center. Quantitation and comparison with the optically determined concentration indicates a stoichiometry of 0.3–1.0 spins/enzyme. Two minor low spin heme resonances are observed at \( g \approx 2.80 \) and 2.70, representing together about 5% of the spins of the major peak. The other resonances in the spectrum at \( g \approx 6 \), \( g = 4.3 \), and around \( g = 2 \) collectively represent a fraction of <2% of the enzyme concentration and are in part due to adventitious heme and non-heme iron. The majority of the nonheme iron present in the enzyme and the high spin center are EPR-silent, a feature common to all NO reductases characterized so far (4, 6, 8, 10, 43). The reduced enzyme was EPR-silent at the temperatures and microwave powers investigated, 5–120 K and 200 micro-watts to 200 milliwatts, in the perpendicular mode. Parallel mode EPR experiments have not yet been performed.

**Resonance Raman Spectroscopy**—The high frequency region of the Resonance Raman spectrum of oxidized qNOR from P. aerophilum is shown in Fig. 5A. The spectrum is dominated by vibrational frequencies of a hexacoordinated low spin heme with a \( \nu_2 \) spin state marker band detected at 1503 cm\(^{-1} \), a \( \nu_3 \) at 1585 cm\(^{-1} \), and a \( \nu_{10} \) at 1638 cm\(^{-1} \) (Fig. 5A, trace A). Some five-coordinated high spin heme species is also detected via its \( \nu_2 \) band, which appears as a shoulder at 1491 cm\(^{-1} \). Attempts to identify a \( \nu_{10}(Fe^{II}−O−Fe^{III}) \) using a 442-nm excitation and \( ^{18}O/^{16}O \) labeling with bulk \(^{18}O\)-water, as previously performed
with P. denitrificans cNOR (29), were inconclusive. In the fully reduced enzyme, the porphyrin modes indicate the presence of HS and LS heme configurations with common \( v_{\text{g}} \) at 1358 cm\(^{-1}\) and distinct \( v_{\text{g}} \) at 1491 and 1470 cm\(^{-1}\), respectively (Fig. 5A, trace B). Binding of CO to the ferrous HS heme is confirmed by a loss of the HS \( v_{\text{g}} \) at 1491 cm\(^{-1}\) and upshift of its \( v_{\text{g}} \) from 1358 to 1370 cm\(^{-1}\) (Fig. 5A, trace C).

As carried out with P. denitrificans cNOR, the conversion of the ferrous HS heme to LS upon binding of CO was utilized to identify the axial ligand of the reduced HS heme (28). Using a 442-nm excitation, a band at 213 cm\(^{-1}\) in the RR spectrum of the reduced enzyme disappears upon binding of CO (Fig. 5B). The 213-cm\(^{-1}\) band is assigned to the \( v_{\text{g}} \) of the reduced HS heme species with near Soret excitations (44). The same vibration was observed at 218 cm\(^{-1}\) in P. denitrificans NOR (28). In both the qNOR and the cNOR, the observed frequency is at the lower end that such vibrations can display, indicating that only a weak hydrogen bond may be engaged by the proximal histidine. The 5-cm\(^{-1}\) difference in \( v_{\text{g}} \) near Soret is likely to relate to a small change in orientation of the proximal histidine with respect to the (Npyrrole-Fe-Npyrrole) axis of the porphyrin plane (45, 46).

The ferrous-CO complex was characterized using \(^{12}\text{CO}/^{13}\text{CO}\)-isotope labeling. In the low frequency region, the \( v_{\text{g}} \) of CO is observed at 476 cm\(^{-1}\) and downshifts by 6 cm\(^{-1}\) with \(^{13}\text{CO}\), whereas the \( \delta_{\text{g}} \) of CO is at 570 cm\(^{-1}\) and downshifts by 15 cm\(^{-1}\) (Fig. 6). A high background in the high frequency region precluded the identification of the \( v_{\text{g}} \) (C-O). However, because the \( v_{\text{g}} \) of CO and \( \delta_{\text{g}} \) of CO are very similar in the qNOR and the cNOR from P. denitrificans, we can conclude that the catalytic high spin hemes in these enzymes have similar distal pocket environments.

**Steady-state Kinetic Properties and Temperature Stability—**

Steady-state kinetic traces of NO reductases from P. denitrificans or B. azotoformans are curved, indicating substrate inhibition at relatively high concentrations of NO (6, 10). The enzyme activity is highest at [NO] \(<2-4\ \mu\text{M}\), which is below the lowest [NO] that can be measured reliably with the Clark electrode. High sensitivity electrodes could not be used at temperatures above 23 °C because of an extremely high background current, and as a consequence we could not estimate a \( K_{m} \) (NO) for the qNOR. Activity traces with membranes of P. aerophilum using MQH\(_2\) are curved as well (15). The apparent \( K_{i} \) for NO for qNOR is 7 \( \pm 3\ \mu\text{M}\); apparent \( K_{i} \) for NO for purified qNOR is 15 \( \pm 3\ \mu\text{M}\) (not shown); a \( K_{i} \) of 13.5 \( \mu\text{M}\) was determined for the cNOR from P. denitrificans (6).

The enzyme activity in pure enzyme or as present in the membrane was found to be completely inhibited by 100 \( \mu\text{M}\) CO or 80 \( \mu\text{M}\) potassium cyanide, whereas azide (1 \text{mM}) or 2-heptyl-4-hydroxyquinolone-N-oxide (1 \text{mM}) did not inhibit the enzyme activity (data not shown).

The purified detergent-solubilized qNOR was inactivated by 75% after incubation for 5 min at 100 °C and by more than 95% after 1 h (Fig. 7). When membranes from the mesophiles P. denitrificans or B. azotoformans were incubated at 100 °C, all NO reductase activity had disappeared completely within 5 min (data not shown). In contrast, qNOR from P. aerophilum as present in membranes is much more stable than the detergent-solubilized enzyme. At room temperature, qNOR in membranes does not lose activity after 24 h (cf. Fig. 7). When incubated at 100 °C, half of the activity was lost in 86 min, and progressively longer incubations times were required to obtain half-inactivation at the lower temperatures (see legend to Fig. 7).

The purified qNOR is active over a broad pH range of pH 4-10; a broad pH optimum is seen between pH 7 and 9 (Fig. 8A). In view of the limited stability of the purified enzyme at high temperature, only the relation between temperature and activity of the qNOR as present in the membranes was determined (Fig. 8B). The enzyme activity could be measured up to 93 °C. Below 25 °C, the activity was too low to be detected. The relation between rate and temperature indicates an Arrhenius activation energy of 8.3 kJ/mol for the qNOR in membranes.

**DISCUSSION**

This paper describes the first purification of a thermophilic NO reductase. The enzyme from P. aerophilum is a MQH\(_2\)/NO oxidoreductase or qNOR consisting of a single subunit (Fig. 1). Its somewhat heterogeneous migration on SDS-PAGE is due to incomplete denaturation. The molecular mass of the purified enzyme determined by gel filtration corresponds to that of a single subunit monomeric enzyme, assuming the binding of a single lauryl maltoside micelle to the purified enzyme (42).

The qNOR contains, in addition to nonheme iron, two modified heme groups that are specific for P. aerophilum membranes (39). Whereas heme Op1 is present in both aerobically and anaerobically grown P. aerophilum, heme Op2 is found only under anaerobic conditions and in an amount about half of that of heme Op1. In the isolated qNOR, both heme groups are present in an approximate 1:1 stoichiometry. The purified qNOR or the membranes contain only a very small amount of heme b, the likely precursor of both hemes Op1 and Op2. We do not know whether incorporation of heme b in qNOR leads to the formation of an active or inactive enzyme. Whether heme Op2 is specific for the qNOR or also present in other membrane-bound oxidoreductases like nitrate reductase or formate reductase remains to be established.

The hemes of the NO reductase are fully reducible by MQH\(_2\) in accordance with its identity as a qNOR. The UV-visible absorbance around 585–590 nm similar to that observed for the qNOR from R. eutropha (4). This absorbance may be the equivalent of the 595–600-nm absorbance observed in the cNOR from P. denitrificans and represent a ligand to ferric iron charge transfer band from high spin heme Op1 or Op2 (28). The disappearance of this band upon the addition of NO (Fig. 3B) is consistent with a spin state change from HS to LS in the nitrosyl adduct as has
been observed in the cNOR from \textit{P. denitrificans} (47). The formation of a stable ferric-nitrosyl species in the qNOR from \textit{P. aerophilum}, in the cNOR from \textit{Pa. denitrificans} (47), and in the qCu\textsubscript{3}NOR from \textit{B. azotoformans} (47) during steady-state turnover may provide an explanation for the phenomenon of substrate inhibition by micromolar concentrations of NO observed in all NO reductases characterized so far (6, 8, 10).

The presence of a high spin heme center in the oxidized heme is confirmed by resonance Raman spectroscopy. However, it is also evident from the UV-visible and resonance Raman data that this configuration may represent less than one heme equivalent and that the catalytic heme may be in a mixture of five-coordinated high spin and hexacoordinated low spin state. The shifts observed upon binding of CO to reduced enzyme also suggest the presence of a high spin center becoming low spin after binding of the ligand.

EPR spectroscopy shows the presence of a low spin heme center. Its g values are consistent with a parallel over meso configuration of the two histidine residues liganding the low-spin heme.
The minor low spin heme signals with $g_\text{z}$ of $P. \text{aerophilum}$ turnover of the purified absorbance at 600 nm not observed in the archaeal qNOR. The copper. Further, the reduced enzyme. We presume that these are due to contaminants (e.g., copper). However, the EPR spectrum of the enzyme contains some signals not present in the archaeal enzyme (36, 43). The various structural determinants contributing to protein thermostability have been reviewed extensively (48–51). Clearly, factors like hydrogen bonding, the presence of salt bridges, ion binding, or reduction of solvent-accessible area cannot be evaluated at present. Nevertheless, one might draw a few conclusions from a comparison of the primary sequences of the approximately 10 qNOR sequences from mesophilic organisms and the two thermophilic organisms $P. \text{aerophilum}$ and Sulfolobus solfataricus P2 (cf. Ref. 52). The S. solfataricus qNOR consists of two subunits, NorB1 (194 residues) and NorB2 (534 residues). The first 172 residues of NorB1 are homologous to the N-terminal part of the qNOR from $P. \text{aerophilum}$, whereas NorB2 is homologous to the remaining C-terminal residues. The primary sequences of all qNORs in the public data base were compared and aligned with the ClustalW program, yielding the following conclusions. The two sequences from $P. \text{aerophilum}$ and S. solfataricus have 43% identical residues, whereas the comparison between the two thermostable enzymes and the nine mesophilic qNORs indicate 21–25% identical residues. The number of cysteine residues is low in all qNORs, but it is even lower in the thermophilic enzymes (one residue per sequence compared with two or three). Glu is lower in the thermophilic enzymes (2.5% versus 4%). The percentage of hydrophobic residues with branched side chains (Ile, Leu, and Val) is significantly larger in the thermophilic enzymes (31% versus 24%), mainly due to the increase of about 5–10% in Ile. The two thermophilic polypeptides are shorter by some 5–7% or 35–50 residues; they seem to lack specifically two hydrophilic stretches (potential surface loops) of 10–16 residues and 7 residues in length situated in the hydrophilic C-terminal part, which is presumed to harbor the quinone-binding site. Collectively, these differences between the thermophilic qNOR and mesophilic qNORs are in line with the analyses described in the literature (48–51). Other predicted properties like a larger total charge or increased number of Arg or Tyr in the thermophilic proteins could not be confirmed. Another specific factor contributing to the thermostability of the $P. \text{aerophilum}$ qNOR might be the, for NO reductases, so far unique presence of the modified hemes $O_{26}$ and $O_{27}$, containing the ethenylgeranylgeranyl and hydroxyethylgeranylgeranyl modification, respectively. These hydrophobic side chains might engage in extra hydrophobic contacts leading to the compaction and stabilization of the protein structure. The ethenylgeranylgeranyl modification is not only present in $P. \text{aerophilum}$ but also in the heme $\Delta_6$ of the $P. \text{aerophilum}$ and $R. \text{eutropha}$ enzyme is the presence of modified hemes in the thermostable enzyme.

In comparison with other purified thermophilic enzymes, the thermostability of the isolated qNOR from $P. \text{aerophilum}$ is very low, whereas that of the qNOR as present in the membrane is average. It seems plausible that removal of the membrane lipids upon solubilization by detergent is the main cause of the lower thermostability, but it is unclear how this would occur. Like cytochrome oxidases, the enzyme might bind specifically one or more lipids (19–24), the removal of which would lead to lower stability but not to lower activity (see Table I). An analysis of other potential factors contributing to the thermostability is necessarily speculative and incomplete in the absence of a high resolution three-dimensional structure. However, general insight into the secondary structure of NO reductases has been obtained from a comparison of the primary structures of cytochrome oxidases and NO reductases (18, 36). Five different cytochrome oxidase structures have been determined (19–24), and this knowledge together with the hydropathy analysis was used to determine the location and number of the 13–14 transmembrane $\alpha$-helices and interhelical loops. The various structural determinants contributing to protein thermostability have been reviewed extensively (48–51). Clearly, factors like hydrogen bonding, the presence of salt bridges, ion binding, or reduction of solvent-accessible area cannot be evaluated at present. Nevertheless, one might draw a few conclusions from a comparison of the primary sequences of the approximately 10 qNOR sequences from mesophilic organisms and the two thermophilic organisms $P. \text{aerophilum}$ and Sulfolobus solfataricus P2 (cf. Ref. 52). The S. solfataricus qNOR consists of two subunits, NorB1 (194 residues) and NorB2 (534 residues). The first 172 residues of NorB1 are homologous to the N-terminal part of the qNOR from $P. \text{aerophilum}$, whereas NorB2 is homologous to the remaining C-terminal residues. The primary sequences of all qNORs in the public data base were compared and aligned with the ClustalW program, yielding the following conclusions. The two sequences from $P. \text{aerophilum}$ and S. solfataricus have 43% identical residues, whereas the comparison between the two thermostable enzymes and the nine mesophilic qNORs indicate 21–25% identical residues. The number of cysteine residues is low in all qNORs, but it is even lower in the thermophilic enzymes (one residue per sequence compared with two or three). Glu is lower in the thermophilic enzymes (2.5% versus 4%). The percentage of hydrophobic residues with branched side chains (Ile, Leu, and Val) is significantly larger in the thermophilic enzymes (31% versus 24%), mainly due to the increase of about 5–10% in Ile. The two thermophilic polypeptides are shorter by some 5–7% or 35–50 residues; they seem to lack specifically two hydrophilic stretches (potential surface loops) of 10–16 residues and 7 residues in length situated in the hydrophilic C-terminal part, which is presumed to harbor the quinone-binding site. Collectively, these differences between the thermophilic qNOR and mesophilic qNORs are in line with the analyses described in the literature (48–51). Other predicted properties like a larger total charge or increased number of Arg or Tyr in the thermophilic proteins could not be confirmed. Another specific factor contributing to the thermostability of the $P. \text{aerophilum}$ qNOR might be the, for NO reductases, so far unique presence of the modified hemes $O_{26}$ and $O_{27}$, containing the ethenylgeranylgeranyl and hydroxyethylgeranylgeranyl modification, respectively. These hydrophobic side chains might engage in extra hydrophobic contacts leading to the compaction and stabilization of the protein structure. The ethenylgeranylgeranyl modification is not only present in $P. \text{aerophilum}$ but also in the heme $\Delta_6$ of the

![Graph A](image1.png)

**Graph A:** Dependence of the MQH$_2$:NO oxidoreductase of purified qNOR on pH (A) and on temperature of qNOR as present in membranes (B). The line through the data points (B) is a simulation according to the Arrhenius equation with activation energy of 8.3 kJ/mol.

![Graph B](image2.png)

**Graph B:** Specific activity (nmol/min/mg) versus Temperature (°C) for the MQH$_2$:NO oxidoreductase of purified qNOR.
cytochrome oxidase from *S. solfataricus*, perhaps a factor involved in increased thermostability of that enzyme. Crystallization of the *P. aerophilum* qNOR and of mesophilic NO reductases is currently being pursued to obtain further insight into the relevant factors contributing to enzyme structure stabilization.

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Purification and Characterization of the MQH$_2$:NO Oxidoreductase from the Hyperthermophilic Archaeon *Pyrobaculum aerophilum*

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