# Involvement of a Novel Hydroxylamine Oxidoreductase in Anaerobic Ammonium Oxidation†

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ABSTRACT: In this study a novel hydroxylamine oxidoreductase (HAO) was purified and characterized from an anaerobic ammonium-oxidizing (Anammox) enrichment culture. The enzyme, which constituted about 9% of the protein mass in the soluble fraction of the cell extract, was able to oxidize hydroxylamine and hydrazine. When phenazine methosulfate and methylthiazolyltetrazolium bromide were used as electron acceptors, a $V_{max}$ [21 and 1.1 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$] and $K_m$ (26 and 18 $\mu$M) for hydroxylamine and hydrazine were determined, respectively. The hydroxylamine oxidoreductase is a trimer and contains about 26 hemes per 183 kDa. As deduced from UV/vis spectra, hydroxylamine reduced more and different cytochromes than hydrazine. The dithionite-reduced spectrum showed an unusual 468 nm peak. Inhibition experiments with $H_2O_2$ showed that hydroxylamine bound to this P-468 cytochrome, which is assumed to be the putative substrate binding site. Cyanide and hydrazine inhibited the oxidation of hydroxylamine. The amino acid sequences of several peptide fragments of HAO from Anammox showed a clear difference with the deduced amino acid sequence of HAO from the aerobic ammonia-oxidizing bacterium *Nitrosomonas europaea*. In EPR spectra of the Anammox HAO, two *g*-values ($g_z$ = 2.37 and 2.42) were observed, which were not present in HAO of *N. europaea*.

Recently, it was demonstrated that anaerobic ammonium oxidation (Anammox) is a biological process in which nitrite is used as the electron acceptor (*1*, *2*), according to the equation $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$ [$\Delta G^{\circ\prime} = -358$ kJ (mol of $NH_4^+$)$^{-1}$]. The Anammox process is a completely new process, which could be used for the sustainable treatment of wastewater with high nitrogen content (*3*). Using a medium containing $HCO_3^-$, $NH_4^+$, and $NO_2^-$, it was possible to enrich and characterize the organisms responsible for the Anammox process (*4*). Very recently, the bacteria responsible for the Anammox process have been identified as a novel deeply branching Planctomycete (*5*). The process is strictly anaerobic and is inhibited by phosphate, oxygen, and high nitrite concentrations (*6*).

Studies with $^{15}N$-labeled compounds showed that hydroxylamine and hydrazine are intermediates in the process, and a reaction mechanism was postulated (Figure 1) (*7*, *8*). It was proposed that hydroxylamine and ammonium are combined to yield hydrazine. Hydrazine itself is then oxidized to $N_2$, generating four reducing equivalents. These electrons could be used for the reduction of nitrite to hydroxylamine. The reverse of this reaction, the oxidation of ammonium to nitrite via hydroxylamine, is well-known for aerobic nitrification, which occurs in both autotrophic and heterotrophic microorganisms.

In autotrophic bacteria, a membrane-bound, mixed-function (mono)oxygenase (AMO)[1] catalyzes the initial oxidation of ammonium to hydroxylamine. The two reducing equivalents for this reaction are derived from the quinone pool (*9*). The second step is the oxidation of hydroxylamine to nitrite, catalyzed by the periplasmic enzyme hydroxylamine oxidoreductase (HAO) (*10*). In autotrophic bacteria this reaction is the energy-yielding step, generating four electrons (*9*). Two of these are returned to AMO to catalyze the oxygenase reaction, while the other two are used for generating a protonmotive force or for the production of NADH via a reverse electron flow. Heterotrophic aerobic nitrifiers, on the other hand, are incapable of using the electrons derived during the oxidation of hydroxylamine as a source of energy for growth (*11*−*13*).

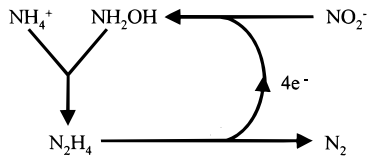


FIGURE 1: Possible reaction mechanism for anaerobic ammonium oxidation postulated after $^{15}N$-labeling studies (*8*).

The biochemistry and genetics of HAO have been studied in detail in the autotrophic bacterium *Nitrosomonas europaea* (*14*, *15*). This enzyme is a complex $\alpha_3$ multimer containing a subunit of around 63 kDa with seven *c* hemes and one P460 heme in each subunit (*16*, *17*). The exact native molecular mass of HAO has always been difficult to

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[1] Abbreviations: HAO, hydroxylamine oxidoreductase; AMO, ammonium monooxygenase; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PMS, phenazine methosulfate; MTT, methylthiazolyltetrazolium bromide; DCPIP, 2,6-dichlorophenolindophenol; BV, benzyl viologen; MV, methyl viologen; NAD, nicotinamide adenine dinucleotide.

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determine, but estimates vary between 125 and 200 kDa (*16*, *18*, *19*). Several HAO enzymes from heterotrophic nitrifiers have also been (partially) purified: *Paracoccus denitrificans* GB17 (*12*), *Pseudomonas* species (*20*, *21*), *Alcaligenes faecalis* species (*13*, *22*), *Methylococcus capsulatus* (*23*), and *Arthrobacter globiformis* (*24*). Comparison of HAO's from heterotrophic nitrifiers with HAO's from autotrophic bacteria showed great differences in molecular mass, heme content, $V_{max}$, and $K_m$. Since nitrite is proposed to be reduced to hydroxylamine in the Anammox process, it is postulated that this reaction might be catalyzed by an HAO type of enzyme. Therefore, this research was focused on the purification and characterization of a putative HAO from the Anammox enrichment culture.

## MATERIALS AND METHODS

*Origin of Biomass and Cultivation*. The Anammox biomass (*1*) used for the purification of a novel hydroxylamine oxidoreductase was cultivated in a 15 L sequencing batch reactor (SBR). The SBR was fed with an anaerobic synthetic medium containing 10 mM $HCO_3^-$, 30 mM ammonium, and 30 mM nitrite, yielding 1.7 g dry weight/mol of $NH_4^+$ at a doubling time of 20 days (*25*). These selective conditions led to an enrichment of about 75% of the Planctomycete-like Anammox bacteria. *N. europaea* ATCC 19178 was grown in 1 L serum bottles under static conditions at 25 °C. The mineral medium was supplemented with 10 mM $NH_4$-Cl. The culture was kept at a constant pH of 7.8 by addition of sterile $Na_2CO_3$. Purity was tested regularly by plating on trypton−yeast extract agar plates.

*Enzyme Purification*. The preparation of the cell-free extract and the purification on Macro Q were performed at 4 °C. UNO Q-6 and Superdex 200 HR chromatography were performed at room temperature. Cells from the enriched Anammox culture (20 g wet weight) were first washed three times with 10 mM Tris-HCl, pH 8.0 (buffer A), and finally resuspended in 40 mL of buffer A with 1 mM DTT and DNase. Subsequently, the cell suspension was passed five times through a French pressure cell (American Instrument Co., Silver Spring, MD) at 110 MPa. Whole cells and cell debris were removed by centrifugation at 48000*g* for 30 min, and the supernatant was used as the cell-free extract. The cell-free extract was loaded on a Macro Q column (2.5 × 17 cm; Bio-Rad, Veenendaal, The Netherlands) equilibrated with buffer A. A linear gradient of 0−0.5 M NaCl in buffer A (750 mL) was applied at 3 mL/min. Active fractions were pooled, diluted 5-fold, and concentrated with a Centriprep 30 (Amicon, Capelle a/d Ijssel, The Netherlands). Further purification was achieved by anion-exchange chromatography on a prepacked UNO Q-6 column (Bio-Rad). Proteins were eluted in a 0−0.5 M NaCl gradient (90 mL) in buffer A at a rate of 2 mL/min. Active fractions were pooled and concentrated with a Microsep 30 K filter (Filtron, Breda, The Netherlands). The concentrated enzyme solution was finally purified by gel filtration on a prepacked Superdex 200 HR 10/30 column (Pharmacia, Roosendaal, The Netherlands) fitted to a HPLC system equipped with a diode array detector (Hewlett-Packard). The gel filtration column was preequilibrated with buffer A containing additional 0.2 M KCl at a rate of 0.5 mL/min. Elution of the HAO enzyme was followed at 280, 416, 470, and 525 nm. Active fractions were stored in liquid nitrogen.

*Enzyme Assays*. The standard assay for HAO was performed under anaerobic conditions at 35 °C by following the reduction of MTT ($\epsilon_{578}$ = 13.0 $mM^{-1}\cdot cm^{-1}$) via PMS using an UV/vis HP 8453 spectrophotometer (Hewlett-Packard, Amsterdam, The Netherlands) equipped with a thermostatic cell holder. One unit of activity is defined as 1 μmol of MTT reduced/min. The reaction mixture contained, in a 1 mL volume, 50 μmol of Tris-HCl, pH 8.0, 20 nmol of PMS, 0.4 μmol of MTT, and 1 μmol of hydroxylamine. The reaction was started by the addition of an appropriate amount of enzyme. The standard assays were performed in 1 mL cuvettes sealed with butyl-rubber stoppers and were made anaerobic with argon. Kinetic parameters were determined at 35 °C (pH 8.0) and analyzed by the nonlinear fitting program GRAFIT from Erithacus software (Staines, U.K.). For determination of the enzyme activity at different pH values, the Tris-HCl buffer was replaced by 50 mM phosphate, Hepes, or bicarbonate buffer which was adjusted by adding 0.1 M NaOH or 0.1 M HCl. The pH was measured before and after determination of the enzyme activity. The temperature dependence of HAO over a range of 20−75 °C was determined. The standard assay, including the enzyme, was first preincubated at the desired temperatures. The enzyme activity was measured 1 min after the addition of an appropriate amount of hydroxylamine.

The assay for NO reduction by HAO was performed using a Clark electrode polarized at 0.85 V as described by Girsch and de Vries (*26*). With this method high NO concentrations can be detected (10−100 μM).

The assay for $NO_2^-$ reduction by HAO was performed in an anaerobic cuvette sealed with a butyl-rubber stopper and was made anaerobic with argon. The reaction mixture contained, in a 1 mL volume, 50 μmol of phosphate buffer, pH 7.0, 1 μmol of MV, and 1 μmol of $NaNO_2$. An appropriate amount of $Na_2S_2O_4$ solution in 100 mM $NaHCO_3$ was added until $A_{600}$ = 2. The reaction was started by the addition of an appropriate amount of enzyme.

*Electron Donors and Acceptors*. Substrate specificity was investigated in the enzyme assay described above. Hydrazine ($N_2H_4\cdot H_2SO_4$) and methanol were tested as substrates (1 mM final concentration). To test alternative electron acceptors, PMS and MTT were replaced in the standard assay (1 mL) by horse heart cyt *c* ($\epsilon_{550}$ = 19.6 $mM^{-1}\cdot cm^{-1}$), 50 nmol, DCPIP ($\epsilon_{600}$ = 16.3 $mM^{-1}\cdot cm^{-1}$), 75 nmol, $K_3Fe(CN)_6$ ($\epsilon_{420}$ = 1 $mM^{-1}\cdot cm^{-1}$), 0.9 μmol, Wurster's blue ($\epsilon_{600}$ = 9.0 $mM^{-1}\cdot cm^{-1}$), 0.5 μmol, BV ($\epsilon_{600}$ = 14.7 $mM^{-1}\cdot cm^{-1}$), 0.8 μmol, and $NAD^+$ ($\epsilon_{340}$ = 6.2 $mM^{-1}\cdot cm^{-1}$), 0.9 μmol.

*Inhibitors*. The effect of potential inhibitors was studied using the standard assay described above. The assay mixture containing 40 μg of HAO was preincubated with the inhibitor for 5 min before the specific activity was determined. The following inhibitors (concentrations) were used: EDTA (1 mM), SDS (0.75−8%), $H_2O_2$ (0.4−40 μM), sodium azide (1 mM), potassium cyanide (0.1−0.35 mM), sodium nitrite (1 mM), methanol (1 mM), and hydrazine (0.01−1 mM).

*Protein Electrophoresis*. After each chromatography step the protein composition was checked, and samples were loaded on SDS−PAGE and nondenaturing gels. SDS−PAGE was performed at 13 °C on 8% and 12% polyacrylamide slab gels according to the method of Laemmli (*27*) using Mini protein gel equipment (Hoefer, Pharmacia). Enzyme samples were denatured by incubation for 5 min in 4% SDS

and 5% $\beta$-mercaptoethanol without boiling. Nondenaturing polyacrylamide slab gels (8%, 10%, and 12%) were run without SDS and $\beta$-mercaptoethanol. Besides HAO from Anammox, the cell-free extract from *N. europaea* also was loaded on a nondenaturing PAGE gel. The cell-free extract from *N. europaea* was obtained using sonification (6 × 30 s) and centrifugation (30 min at 48000*g*). Gels were stained for protein with Coomassie Brilliant Blue G250. A low and high molecular mass calibration kit (Pharmacia) was used to estimate the molecular mass. Heme staining was performed with 3,3′,5,5′-tetramethylbenzidine (*28*). Enzyme activity after native PAGE was performed by preincubation of the gel in anaerobic 50 mM Tris-HCl (pH 8.0) buffer, containing 20 $\mu$M PMS and 0.4 mM MTT. The assay was started by adding an appropriate amount of 20 mM hydroxylamine. To determine the isoelectric point of HAO, a PhastSystem from Pharmacia using PhastGel IEF markers 2.5−6.5 was used.

*Heme Removal*. A chemical method according to Fontana et al. was used to remove the *c*-type hemes from the HAO (*29*). The enzyme was dissolved in 50% acetic acid and incubated with 100 equiv of sulfenyl chloride (2-nitrophenylsulfenyl chloride; Sigma) dissolved in glacial acetic acid up to a final acetic acid concentration of 66%. After 10 min of stirring at room temperature, the reaction was stopped by diluting the mixture three times with water and then extracted four times with equal volume of ethyl acetate. The aqueous layer was applied on a PD-10 column (Pharmacia) and concentrated in a Microsep 10 K filter (Filtron). The protein was applied on an 8%, 12%, and 15% SDS−PAGE gel as described above.

*Molecular Mass Determination*. The apparent molecular mass of the native HAO was determined by gel filtration on a Superdex 200 HR 10/30 column (Pharmacia) and by SDS and nondenaturing PAGE gels (see Protein Electrophoresis). The gel filtration column was calibrated with marker proteins (Pharmacia): thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and horse heart cyt *c* (12.4 kDa). Retention times were plotted as $K_{av}$ against the logarithm of the molecular mass.

*Spectroscopy*. Spectra of HAO in 50 mM Tris-HCl (pH 8.0) at room temperature were recorded on an UV/vis HP 8453 spectrophotometer (Hewlett-Packard, Amsterdam, The Netherlands). The spectral bandwidth was 1 nm. HAO-reduced spectra (40 $\mu$g/mL) were obtained by adding hydroxylamine (0.5 mM final concentration), hydrazine (0.5 mM final concentration), or dithionite. The effect of $H_2O_2$ (0.1 mM) was studied by incubation of HAO (40 $\mu$g/mL) for 30 min before spectra of oxidized and reduced HAO were recorded. The pyridine hemochrome method (*30*) was used to determine the heme *c* content.

EPR spectroscopy was performed on a Varian E-9 spectrometer operating at X-band frequency and equipped with a home-built He flow cryostat.

*Analytical Procedures*. Protein concentrations were determined with the BCA protein assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin was used as standard. Nitrate, ammonium, hydroxylamine, nitrite, and hydrazine were measured colorimetrically (*31*−*35*). The standard addition method was used for all of the nitrogen compounds to correct for the influence of the medium components. Dinitrogen gas and nitrous oxide were analyzed using gas chromatography (*36*). Nitric oxide and nitric dioxide were analyzed using chemiluminescence (*37*).

Table 1: Purification of a Hydroxylamine Oxidoreductase (HAO) from the Anammox Culture

| purification step | total protein (mg) | total activity (units) | recovery (%) | specific activity (units/mg) | purification (*x*-fold) |
|---|---|---|---|---|---|
| cell-free extract | 150 | 288 | 100 | 1.9 | 1 |
| Macro Q | 7.4 | 86 | 30 | 12 | 6 |
| UNO Q-6 | 1.2 | 20 | 7 | 17 | 9 |
| Superdex 200 | 0.5 | 11 | 4 | 21 | 11 |

*Product Analyses.* Anaerobic batch experiments were used to determine the conversion products of HAO when different substrates were used. The experiments with hydroxylamine, nitric oxide, and nitrite were performed in a 50 mL serum bottle. The reaction mixture for the conversion of $NH_2OH$ contained 10 mL of 50 mM phosphate buffer (pH 7.5), 2 mM $NH_2OH$, 0.5 mM PMS, and 4 mM MTT. The reaction mixture for NO conversion contained 10 mL of 50 mM phosphate buffer (pH 6.0), 20 $\mu$M horse heart cyt *c*, 0.1 mM PMS, and 10 mM ascorbate. NO is chemically in equilibrium with nitrite and nitrate; therefore, the reaction mixture was stirred for 10 min before the reaction was started. For the conversion of $NO_2^-$, the mixture contained 3 mL of 100 mM phosphate buffer (pH 7.0), 2.5 mM $NaNO_2$, 1 mM MV, and 5 mM $Na_2S_2O_4$ (in 100 mM $NaHCO_3$). The conversion with hydrazine was performed in a 10 mL serum bottle, and the mixture contained 1 mL of 50 mM phosphate buffer (pH 7.5), 0.5 mM $N_2H_4$, and 2 mM horse heart cyt *c*. All bottles were sealed with butyl-rubber stoppers and were made anaerobic with helium. The experiments were started by addition of 50 $\mu$g of purified HAO. In the experiment for the nitrite conversion, 200 $\mu$g of HAO was used. Control experiments were performed in the absence of HAO. All nitrogen compounds were measured as described above (see Analytical Procedures).

*Amino Acid Sequence Analysis*. Attempts to determine the amino acid sequence of the N-terminus of HAO failed, since it was blocked. Therefore, a trypsin or V8 protease digestion was performed to derive smaller peptides from HAO. Subsequently, the N-terminus of different peptides was determined for amino acid sequences. The analysis was performed with a Procise 494 protein sequencer (Perkin-Elmer/Applied Biosystems).

## RESULTS

*Purification.* A novel hydroxylamine oxidoreductase (HAO) from the Anammox culture was purified to homogeneity from the soluble fraction (Table 1). The preparation contained more than 99% HAO, as indicated by the symmetrical peak on the Superdex 200 at different wavelengths and one intense band at 58 kDa on SDS−PAGE after the removal of the hemes. Sequential anion-exchange chromatography (Macro Q and UNO Q-6) was used for the initial purification. The protein eluted from these columns at 0.2 M NaCl. Finally, the fractions with the highest specific activity were collected, subsequently concentrated, and applied to a Superdex 200 gel filtration column. In total, an 11-fold purification was

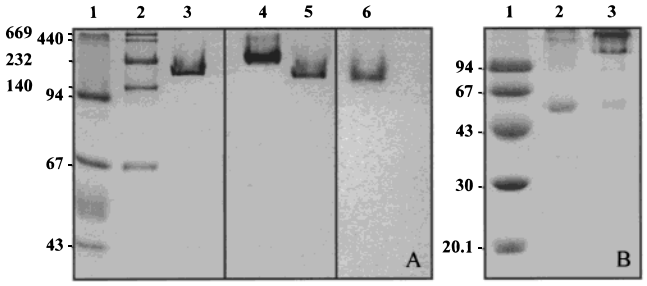


FIGURE 2: (A) 10% nondenaturing PAGE (of purified HAO from Anammox. Coomassie (lanes 1−3), activity (lanes 4 and 5), and heme (lane 6) staining after electrophoresis of HAO from Anammox and the cell-free extract from *N. europaea*. Lanes: 1, low molecular mass marker; 2, high molecular mass marker; 3, HAO from Anammox (5 $\mu$g); 4, cell-free extract of *N. europaea* (25 $\mu$g); 5, HAO from Anammox (2 $\mu$g); 6, HAO from Anammox (1 $\mu$g). Activity staining was performed by preincubating the gel in anaerobic 50 mM Tris-HCl (pH 8.0) buffer containing 20 $\mu$M PMS and 0.4 mM MTT. The staining was started by addition of hydroxylamine at a final concentration of 2 mM. (B) 12% SDS−PAGE of purified HAO from Anammox after removal of the *c*-type hemes with sulfonyl chloride stained with Coomassie Brilliant Blue G250. Lanes: 1, low molecular mass marker; 2, HAO from Anammox after chemical treatment with sulfonyl chloride (4 $\mu$g); 3, HAO without chemical treatment (8 $\mu$g).

obtained, indicating that the HAO content of the total cell-free extract was about 9%.

*Molecular Mass Determination*. To determine the native molecular mass, the HAO was applied to a Superdex 200 gel filtration column. The enzyme eluted at a volume (13 mL) corresponding to a molecular mass of 150 ± 3 kDa when compared to marker proteins. Nondenaturing PAGE and SDS−PAGE showed that the migration pattern of HAO depended on the percentage of acrylamide in the gel. On nondenaturing gels the HAO from Anammox migrated with an apparent molecular mass of about 183 ± 12 kDa (Figure 2A). Activity staining revealed that the HAO band from Anammox migrated faster than the HAO band in the cell extract of *N. europaea*. The migration distances after activity staining on native gels of purified HAO and HAO in the cell-free extract from Anammox were the same. On SDS−PAGE gels most of the enzyme remained on top of the gel, probably due to aggregate formation (Figure 2B). However, after removal of the *c*-type hemes from HAO with sulfonyl chloride, one intense band appeared on all SDS−PAGE gels, with different acrylamide percentages, at an apparent molecular mass of 58 ± 2 kDa.

*Spectroscopy*. The UV/visible absorption spectrum of dithionite-reduced HAO gave maxima at 420 nm (Soret band), 524 nm ($\beta$-band), and 552 nm ($\alpha$-band) and an unusual peak at 468 nm (Figure 3A). This absorption peak at 468 nm seemed similar to that of the P-460 of *N. europaea* (*10*). The addition of hydroxylamine or hydrazine induced a partially reduced spectrum, compared to the spectrum of HAO reduced with dithionite, whereas the absorption at 468 nm did not change. The difference absorption spectra showed that different *c*-type cytochromes are reduced when $NH_2OH$ or $N_2H_4$ is used as a reductant compared to dithionite. UV/vis absorption spectra of HAO incubated with 0.1 mM $H_2O_2$ for 30 min resulted in spectral changes, similar to results obtained with the HAO of *N. europaea* (*38*). Subsequent reduction of the incubated enzyme with dithionite no longer showed the peak at 468 nm (Figure 3B). The other

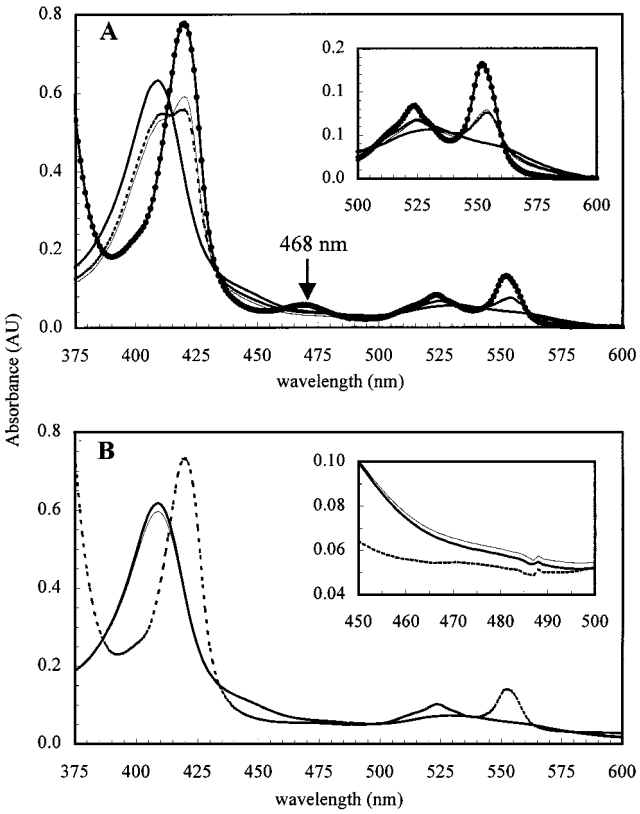


FIGURE 3: UV/visible absorption spectra of purified HAO from Anammox. (A) HAO (40 $\mu$g/mL) reduced with 0.5 mM $NH_2OH$, 0.5 mM $N_2H_4$, and dithionite: (—) air oxidized; (−) $NH_2OH$ reduced; (- - -) $N_2H_4$ reduced; (− ● −) dithionite reduced. The inset shows the spectra of the $\alpha$ and $\beta$ bands. (B) HAO (40 $\mu$g/mL) incubated for 30 min with 0.1 mM $H_2O_2$: (—) oxidized; (−) $NH_2OH$ reduced; (- - -) dithionite reduced. The inset shows the HAO spectra of the unusual 468 nm peak.

*c* hemes, however, could be reduced by dithionite. Hydroxylamine could not reduce HAO after incubation with $H_2O_2$, and the enzyme was no longer active. When, however, $H_2O_2$ and hydroxylamine were added simultaneously to the enzyme, the HAO activity was protected from inactivation by $H_2O_2$. The absorption maximum of the pyridine heme−chrome complex was used to determine the identity and concentration of heme. The maximum at 550 nm is typical for *c*-type cytochromes. Using a molecular mass of HAO of 183 kDa, it was calculated that 1 mol of HAO contained 26 ± 4 mol hemes. The p*I* of the enzyme, estimated on PhastGel IEF, was 5.5.

The electron paramagnetic resonance (EPR) spectrum of oxidized HAO consists of multiple resonances of highly anisotropic low-spin heme centers. The resonances ascribed to various low-spin hemes are as follows: $g_z = 3.38$, 2.99 (shoulder), 2.42, and 2.37; $g_y = 2.20$; $g_x = 1.83$, 1.66, and 1.44 (Figure 4). EPR signals due to high-spin iron, seen around $g = 6$ ($g_{y,x} = 6.2$ and 5.8) are also visible but represent less than 0.2% of the total heme iron. Similarly, the signal at $g = 4.3$ represents a very low amount of high-spin iron and is assigned as adventitious iron. The derivative-like feature at $g = 2.20$ is typical for low-spin heme centers and most likely is an overlap of several $g_y$ resonances. The resonances and peaks seen in the region around $g = 2.00$ including the minimum at $g = 1.83$ are difficult to assign. In part, this spectral region may contain contributions from the most anisotropic heme center ($g = 3.38$), but the sharp

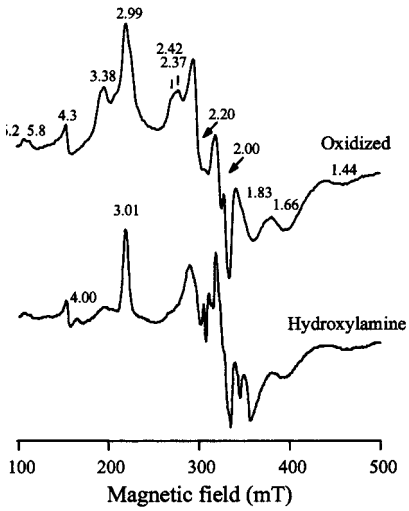


FIGURE 4: X-band EPR spectra of HAO in various redox states. EPR conditions: frequency, 9.232 GHz; modulation amplitude, 1.6 mT; microwave power, 2 mW; temperature, 12 K. The numbers in the figure refer to the *g*-values of the resonances. Hydroxylamine was added to a concentration of 0.5 mM. The spectra are represented at the same gain.

features are more likely to be due to complex magnetic coupling between various heme centers (*39*). Parts of the low-spin heme centers, i.e., those with $g_z = 3.38$, 2.42, and 2.37, are reducible by hydroxylamine (Figure 4). Also, the shoulder accompanying the peak at $g = 2.99$ becomes reduced. The position of the resonance at $g = 2.99$ apparently shifts to lower field, to $g = 3.01$. A small signal around $g = 4.00$ is visible after reduction with hydroxylamine. This signal is due to an $S = {}^3/_2$ system, but its amount is very low (<0.05% of the total heme iron). A weighted subtraction of the EPR spectra, i.e., oxidized minus hydroxylamine-reduced enzyme, to identify *g*-values of individual heme centers revealed in addition to the signals assigned above, several different absorption-like and derivative-like signals which, however, were not "pure EPR spectra". The reason for this is most likely that the total spectrum not only is due to magnetically isolated heme centers but also contains contributions of magnetically interacting hemes (*39*). Magnetic interaction also prevents a detailed quantitation of the low-spin heme centers. Taking into account the fact that the smaller the value of $g_z$, the more isotropic the EPR signal, and the smaller the amount represented by the area under the $g_z$ peak (*40*); The two signals at $g_z = 2.37$ and 2.42 together represent about 1.0−1.3 low-spin hemes and those at $g_z = 2.99$ and 3.38, each about 2.5−3.0 hemes.

*Kinetic Properties.* The kinetic properties of hydroxylamine oxidoreductase were determined at pH 8.0 and 35 °C with PMS and MTT as the electron acceptors. From the Michaelis−Menten plot, an apparent $V_{max}$ and $K_m$ for hydroxylamine of 21 ± 2 units/mg and 26 $\mu$M, respectively, could be calculated. For hydrazine, a $V_{max}$ and $K_m$ of 1.1 ± 0.3 units/mg and 18 ± 2 $\mu$M were obtained, respectively. Addition of higher concentrations of PMS and MTT did not influence the $V_{max}$. Methanol could not be used as a substrate for HAO. The highest activity observed for HAO with hydroxylamine as the substrate was at 65 °C, with a $V_{max}$ of 49 ± 6 units/mg and a $K_m$ of 127 ± 5 $\mu$M. Table 2 shows different electron acceptors that could be used by HAO when hydrazine and hydroxylamine were used as the substrate.

Table 2: Highest Specific Activity of Hydroxylamine Oxidoreductase (HAO) from the Anammox Culture Using Different Electron Acceptors with Hydroxylamine and Hydrazine as the Electron Donors

| | specific activity (units/mg) | |
|---|---|---|
| | $NH_2OH$ | $N_2H_4$ |
| PMS + MTT | 21 | 1.1 |
| horse heart cyt *c* | 18 | 2.1 |
| DCPIP | 20 | 1.2 |
| $K_3Fe(CN)_6$ | 40 | 6.2 |
| $NAD^+$ | *a* | *a* |
| benzyl viologen | *a* | *a* |
| Wurster's blue | *a* | *a* |

[a] No HAO activity found.

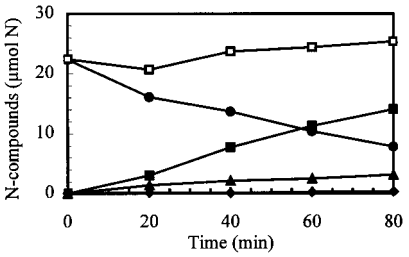


FIGURE 5: Anaerobic batch experiment with purified HAO from Anammox and hydroxylamine as the substrate. The reaction mixture contained 50 mM phosphate buffer, pH 7.5, 2 mM $NH_2OH$, 0.5 mM PMS, 4 mM MTT, and 50 $\mu$g of HAO. Symbols: (●) $NH_2OH$; (■) $N-N_2O$; (▲) NO; (◆) $NO_2^-$; (□) total N.

Ferricyanide as the electron acceptor resulted in the highest specific activity at 30 °C of 40.3 ± 3 and 6.2 ± 2 units/mg for hydroxylamine and hydrazine, respectively. The pH optimum for the enzyme under standard conditions was found to be pH 8.0. The specific activity of HAO did not change significantly when different buffers such as phosphate, Hepes, or carbonate replaced Tris-HCl. HAO from Anammox was also capable of reducing NO to $N_2O$ with a specific activity of 2.9 ± 0.3 units/mg at room temperature. The optimum pH for NO reduction was at pH 6.0. The specific activity for $NO_2^-$ reduction was 0.87 ± 0.2 unit/mg.

*Inhibitors.* Several compounds were tested for the possible inhibitory effect on the activity of HAO. Sodium azide, methanol, sodium nitrite, and the chelating agent EDTA did not inhibit HAO activity. The addition of 35, 85, or 350 $\mu$M cyanide to the standard HAO assay resulted in 25%, 50%, and 95% inhibition, respectively. $H_2O_2$ also had a strong effect on the specific activity of the enzyme. Addition of 0.4, 4, and 40 $\mu$M $H_2O_2$ resulted in a loss of activity of 30%, 75%, and 90%, respectively. Hydrazine, which can also be used as a substrate for HAO, competed strongly with hydroxylamine. Addition of 15, 35, and 350 $\mu$M $N_2H_4$ to the standard HAO assay resulted in 15%, 50%, and 85% inhibition, respectively. The activity in the presence of 0.75%, 3%, and 8% SDS was reduced by 20%, 50%, and 95%, respectively.

*Product Analyses.* Batch experiments with HAO were performed to analyze the products when different substrates were used. The average rates in the 80 min test period were 2−4-fold lower than the initial rates calculated from spectroscopic HAO assays. The conversion of $NH_2OH$ by HAO resulted in the formation of NO and $N_2O$ (Figure 5). A small amount of $NO_2^-$ was measured, but this was probably due to the chemical reaction of NO. No other nitrogen com-

Table 3: Comparison of Hydroxylamine Oxidoreductase (HAO) from Various Microorganisms

| | Anammox | *N. europaea* | *P. denitrificans* | *Pseudomonas* PB16 |
|---|---|---|---|---|
| mol mass (kDa) | 183 ± 12 | 189 | 20 | 132 |
| MM subunit (kDa) | 58[b] | 63 | 20 | 68 |
| composition | $\alpha_3$ | $\alpha_3$ | $\alpha$ | $\alpha_2$ |
| 409/280 ratio | 4.5 | 3.3 | NR[a] | NR[a] |
| heme *c* | 26 ± 4 | 24 | none | none |
| heme type | P-468 | P-460 | | |
| p*I* | 5.5 | 5.3 | ND[a] | ND[a] |
| $V_{max}$ ($NH_2OH$) (units/mg) | 21 | 75 | 0.13 | 0.45 |
| $K_m$ ($NH_2OH$) ($\mu$M) | 26 | NR[a] | 10 | 37 |
| e-acceptor | PMS + MTT | PMS | cyt $c_{551}$ | $K_3Fe(CN)_6$ |
| reference | this paper | 10 | 12 | 20 |

[a] NR, not reported; ND, not determined. [b] Determined after heme removal.

pounds were detected. In the control experiment, only a small amount of $NH_2OH$ disappeared, but no other nitrogen compound could be detected in the assay mixture. $N_2H_4$ was solely converted into dinitrogen gas. In the control experiment without HAO, no hydrazine was converted. The conversion of NO by HAO resulted in the production of $N_2O$ only. The control experiment showed no biological conversion of NO. Reduction of nitrite in the presence of reduced methyl viologen resulted in the production of $N_2O$ and some NO (<5%). No other nitrogen compounds were detected. In the presence of ascorbate and PMS, no nitrite conversion was observed. HAO, reduced by either $NH_2OH$ or $N_2H_4$, could be oxidized by the addition of NO or $NO_2^-$, as monitored by UV/vis spectroscopy (data not shown).

*Amino Acid Sequence Analysis.* The N-terminal amino acid sequences of different peptide fragments from HAO, which were generated by trypsin or V8 protease digestions, were determined. The sequences are (E)HYWTPGSQNNE, (E)AFWQHGE, (K/R)SGIVTR, and (K/R)IGLEHK. Each fragment determined for HAO from Anammox had a unique sequence and was not present in the deduced amino acid sequence of HAO from *N. europaea* (*15*), nor did it show homology with other proteins in the SWISS-PROT database.

## DISCUSSION

The discovery of a new microbial pathway for the oxidation of ammonium under anaerobic conditions (Anammox) offers a challenge to unravel the unique biochemistry of this process (*8*). This paper reports on a novel hydroxylamine oxidoreductase isolated from an Anammox enrichment culture. The enzyme properties of this HAO will be compared to HAO from the aerobic, chemolithoautotrophic bacterium *N. europaea* (Table 3), which is a complex hemoprotein known to contain seven *c*-type hemes and an unusual iron-containing P-460 heme per subunit (*14*, *41*).

The molecular mass of the purified HAO from Anammox was difficult to determine, since complementary techniques yielded different results. However, after treatment of HAO with sulfonyl chloride, a single molecular mass of 58 kDa was obtained. This is very similar to the 59 kDa observed for the HAO subunit from *N. europaea* (*17*). Assuming a molecular mass of about 5 kDa for the eight hemes, this value corresponds quite well to the molecular mass of 63 kDa for a single HAO subunit of *N. europaea*, which was deduced from DNA sequence analysis (*15*). Since the elucidation of the crystal structure of HAO from *N. europaea* (*16*), it is known that HAO consists of three subunits with a total molecular mass of 189 kDa. The migration pattern on native PAGE, as depicted in Figure 2A, indicated that HAO from Anammox has a slightly smaller molecular mass compared to HAO from *N. europaea* or that it has a greater net negative charge. From these results, we propose that HAO from Anammox is also a trimer. On the basis of this assumption, in combination with the observed 58 kDa mass of a single subunit and the migration pattern on nondenaturing gel, the molecular mass of HAO from Anammox is estimated at 183 ± 12 kDa. This value agrees quite well with the results from the pyridine heme−chrome assay.

Significant differences between the HAO from Anammox and the HAO of *N. europaea* were found in the UV/vis spectra and the elution pattern on the nondenaturing gel (Table 3). The spectrum of reduced HAO from Anammox showed a unique absorption peak at 468 nm (Figure 3A), corresponding to a special *c*-type cytochrome, which is significantly different from the 463 nm peak for HAO in *N. europaea*. It was proposed that this P-460 chromophore is the substrate binding site (*38*). Incubation of HAO from Anammox with $H_2O_2$ resulted in a rapid inactivation, which was also observed for HAO from *N. europaea* (*38*). $H_2O_2$ bound selectively to the oxidized P-468 chromophore, which is shown in Figure 3B. These results suggest that $H_2O_2$ prevented the binding of hydroxylamine to the oxidized P-468 cytochrome. Indeed, when $H_2O_2$ was added in the presence of hydroxylamine, the enzyme was protected from inactivation. This indicates that P-468 is the substrate binding site, similar to the P-463 in HAO from *N. europaea*.

The EPR spectrum of HAO is a complex overlap of at least four low-spin heme signals. Signals of high-spin heme, e.g., from P-468, expected to display *g*-values around $g = 6$, were virtually absent in the oxidized and substrate-reduced enzyme, a phenomenon also noted for the HAO from *N. europaea* (*42*). Spin−spin coupling between different heme centers, fast relaxation, a negative value of the zero-field splitting parameter *D*, or a combination of these three factors may be responsible for the absence of a quantitative amount of the $g = 6$ signal. The closeness of the heme centers in HAO from *N. europaea* as revealed by X-ray crystallography (*16*) certainly suggests that spin−spin coupling may play a role in the comparable HAO from Anammox. With the exception of the $g_z = 2.37$ and 2.42 resonances, all *g*-values in the Anammox and *N. europaea* HAO (*42*) are very similar, indicating strong structural similarity between the two enzymes. The $g = 2.70$ resonance found in HAO from *N. europaea* is not represented in the enzyme from Anammox. Since this heme center represents about one heme and the overlapping signals at $g = 2.37$ and 2.42 also represent about one heme center, they may occupy comparable positions in the respective proteins, albeit that the environments must be quite different. Assuming that the catalytic core and electron-transfer pathways within the two enzymes are the most conserved parts, these hemes might function to accept electrons from soluble electron donors.

The HAO enzyme must have a great metabolic importance for the Anammox culture, since it has a high conversion rate

and represents up to 9% of the total soluble protein fraction (Table 1). The $V_{max}$ and $K_m$ values determined for HAO from Anammox were comparable to the values obtained for aerobic autotrophic nitrifiers, such as *N. europaea* (Table 3). As stated above, $N_2H_4$ could also be used as a substrate for HAO from Anammox, although at a much lower rate (Table 2). In addition, $N_2H_4$ strongly inhibits the oxidation of $NH_2OH$ by HAO. The turnover numbers of HAO from Anammox for $NH_2OH$ and $N_2H_4$ utilization are $3.9 \times 10^3$ and $2.0 \times 10^2$ $min^{-1}$, respectively (assuming a molecular mass of 183 kDa). This number is 10-fold lower than the turnover of HAO for $NH_2OH$ from *N. europaea* ($2.3 \times 10^4$ $min^{-1}$), calculated using a molecular mass of 189 kDa (*10*). This agrees well with the difference in specific ammonium oxidation rate of the two cell types. Anammox has a specific activity of 55 nmol $min^{-1}$ (mg of protein)$^{-1}$, whereas *N. europaea* has a rate of 500 nmol $min^{-1}$ (mg of protein)$^{-1}$ (*43*).

HAO from Anammox not only is capable of oxidizing $NH_2OH$ and $N_2H_4$ but can also reduce $NO_2^-$ and NO to $N_2O$. Ascorbate and PMS could not be used as the electron donor for nitrite reduction, although ascorbate partially reduces HAO. It is known for HAO from *N. europaea* that the redox potential of the eight different *c* types varies from −412 mV to +288 MV (*14*). $N_2H_4$, $NH_2OH$, or $MV_{red}$ is probably capable of reducing the low-potential cytochromes that donate electrons to nitrite whereas ascorbate/PMS cannot.

The physiological role of HAO in Anammox may therefore be different from that in *N. europaea*. It could be envisaged that under physiological conditions HAO from Anammox might catalyze the reverse reaction, namely, nitrite reduction to hydroxylamine (as shown in Figure 1). Preliminary studies in which HAO and nitrite were incubated with different electron donors did not show any reduction of nitrite to hydroxylamine. However, HAO was able to reduce nitrite to NO and $N_2O$ in the presence of reduced methyl viologen, although at a low rate (0.87 unit/mg). Since the oxidation of hydroxylamine by HAO is about 25 times faster than the reduction of $NO_2^-$, no accumulation of hydroxylamine would be expected. Instead of reduced MV, hydrazine could also be used as the electron donor for nitrite reduction, and therefore, it could be hypothesized that only one enzyme is responsible for both nitrite reduction and hydrazine oxidation. Alternatively, the role of HAO from Anammox might be solely the oxidation of hydrazine to dinitrogen gas. In addition, one could also envisage that not $NH_2OH$ but NO is a potential intermediate in the Anammox process (*43*). In such a mechanism $NO_2^-$ would first be reduced to NO and then subsequently reduced to $N_2H_4$ in the presence of $NH_4^+$, as suggested by Hooper (*14*). This reaction could be catalyzed by an enzyme, which may be similar to NO reductase or AMO. Clearly, more research is needed to unravel the exact physiological role of HAO in Anammox.

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