Abstract

Ammonia-oxidizing bacteria (AOB), ubiquitous chemoautotrophic bacteria, convert ammonia (NH₃) to nitrite (NO₂⁻) via hydroxylamine as energy source. Excessive growth of AOB, enhanced by
applying large amounts of ammonium-fertilizer to the farmland, leads to nitrogen leaching and nitrous oxide gas emission. To suppress these unfavorable phenomena, nitrification inhibitors, AOB specific bactericides, are widely used in fertilized farmland. However, new nitrification inhibitors are desired because of toxicity and weak-effects of currently used inhibitors. Toward development of novel nitrification inhibitors that target hydroxylamine oxidoreductase (HAO), a key enzyme of nitrification in AOB, we established inhibitor evaluation systems that include simplified HAO purification procedure and high-throughput HAO activity assays for the purified enzymes and for the live AOB cells. The new assay systems allowed us to observed distinct inhibitory responses of HAOs from beta-proteobacterial AOB (βAOB) *Nitrosomonas europaea* (NeHAO) and gamma-proteobacterial AOB (γAOB) *Nitrosococcus oceani* (NoHAO) against phenylhydrazine, a well-known suicide inhibitor for NeHAO. Consistently, the live cells of *N. europaea*, *Nitrosomonas* sp. JPCCT2 and *Nitrospira multiformis* of βAOB displayed higher responses to phenylhydrazine than those of γAOB *N. oceani*. Our homology modeling studies suggest that different inhibitory responses of βAOB and γAOB are originated from different local environments around the substrate-binding sites of HAOs in these two classes of bacteria due to substitutions of two residues. The results reported herein strongly recommend inhibitor screenings against both NeHAO of βAOB and NoHAO of γAOB to develop HAO-targeting nitrification inhibitors with wide anti-AOB spectra.
Keywords:
nitrification inhibitor; inhibitor screening; hydroxylamine oxidoreductase; ammonia-oxidizing bacteria; phenylhydrazine

Abbreviations:
HAO, hydroxylamine oxidoreductase; NeHAO, HAO from *Nitrosomonas europaea*; NoHAO, HAO from *Nitrosococcus oceani*; NmHAO, HAO from *Nitrosospira multiformis*; AOB, ammonia-oxidizing bacteria; βAOB, beta-proteobacterial AOB; γAOB, gamma-proteobacterial AOB; AMO, ammonium monooxygenase

Introduction

Ammonia-oxidizing bacteria (AOB), ubiquitous chemoautotrophic bacteria that convert ammonia (NH₃) to nitrite (NO₂⁻) as their sole energy source, are important not only for global nitrogen cycle but also for agriculture. In farmlands, large amounts of ammonium-fertilizer are applied, and excess-grown AOB cause emission of nitrous oxide (N₂O), a powerful greenhouse gas, and leaching of up to 70% of applied nitrogen from farmlands to water bodies. Therefore, nitrification inhibitors, specific bactericides for AOB, are used for increasing crop yields and/or decreasing applied nitrogen fertilizer (see review by Subbarao *et al*. [1]). Typical studies have shown that nitrification inhibitors decreased
63% of nitrogen leaching [2] and 38% of N₂O emissions [3]. However, commercial nitrification inhibitors possess particular problems such as toxicity and weak effects. Thus, next-generation nitrification inhibitors are demanded.

Ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are key enzymes of nitrification pathway in AOB, and catalyze ammonia (NH₃) to nitrate (NO₂⁻) via hydroxylamine (NH₂OH) by following chemical reactions [4]:

\[
\text{AMO: } \text{NH}_3 + 2\text{H}^+ + \text{O}_2 + 2\text{e}^- \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O},
\]

\[
\text{HAO: } \text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 5\text{H}^+ + 4\text{e}^-.
\]

In this pathway, four electrons are generated by HAO; half of the electrons are transferred back to the AMO as the catalytic energy source, and the remaining half are consumed as a growth energy through generating NADPH⁺ and ATP. Therefore, both of the enzymes, AMO and HAO, are potential targets of nitrification inhibitors. Actually, AMO is believed to be a molecular target of commercial nitrification inhibitors such as nitrpyrin [5]. However, rational design of AMO-targeting inhibitor is difficult, because valuable assay methods and structure information of AMO are not available yet.

HAO is a newly-emerging target of nitrification inhibitors. Recently, Wu et al. [6] have reported that the activity of soil nitrification was suppressed by organo-hydrazines that act as suicide
inhibitors for HAO [7]. Crystal structures of naturally purified \( \beta \)AOB *Nitrosomonas europaea* HAO (NeHAO) have been resolved [8–10], and they allow us to develop HAO inhibitors by structure-guided drug design. Therefore, we believe that HAO is more suitable as a nitrification-inhibitor target than AMO.

For developing nitrification inhibitors that possess wide anti-AOB spectra against a variety of uncultured soil AOB, proper model AOB strains must be selected. Okano et al. [11] showed that *N. europaea* is adequate to screening of nitrification inhibitors by using common nitrification inhibitors, including AMO-targeting inhibitor nitrapyrin, and \( \beta \)AOB strains. However, \( \gamma \)AOB, which was believed to inhabit only the sea and salt lake, has been detected in soil by metagenomic analysis, recently [12]. We thus need to determine which AOB strains are adequate to screening of HAO-targeting nitrification inhibitors. In addition, a simple but effective protein purification system and high-throughput inhibitor screening/evaluation assay methods are also required to assess inhibitor response of HAO.

Here, we show different inhibitor responses of HAOs from \( \beta \) and \( \gamma \)AOB by using optimized inhibitor evaluation systems including simplified HAO purification procedure and high-throughput HAO activity assay. Our results will assist developing effective nitrification inhibitors with wide anti-AOB spectra, and will lead to promotion of sustainable agriculture.
Material and methods

Homology modeling

Homology modeling of βAOB *Nitrosospira multiformis* HAO (NmHAO) and γAOB *Nitrosooccus oceani* HAO (NoHAO) was performed using Molecular Operating Environment (MOE) software (version 2015.1001, Chemical Computing Group) with the following parameters: forcefield of Amber10:EHT, amino acid sequences of NmHAO (Uniprot: Q2YA36) and NoHAO (Q3JCP2), induced fit includes hemes and waters on the iron in heme P460. Crystal structure of NeHAO (PDB:4n4n) was used as a template. The graphical representations were prepared by PyMOL (version 1.7.0.1, Schrödinger, LLC.).

Cell lines and cell cultures

*N. europaea* strain NBRC 14298 (= ATCC 19718) and *Nitrosomonas* sp. NBRC 108559 (=JPCCT2) were purchased from National Institute of Technology and Evaluation, Biological Resource Center (NBRC) (Chiba, Japan). *N. oceani* ATCC 19707 and *N. multiformis* ATCC 25196 were purchased from ATCC (Manassas, Virginia, USA). NBRC Medium No.829 [13] was used for the culture of *N. europaea*, *N. multiformis*, and *N. sp JPCCT2*. The culture medium for *N. oceani* was prepared according to previous report [14]. Stocked culture stored at 4°C was inoculated to 40 mL medium, and incubated for 1 week at 26 °C with gently shaking (~80 rpm). Then, the pre-culture was inoculated to
the 20 L medium in a 20-L plastic carboy (Nalgene® Round Polycarbonate Clearboy® with Spigot, Thermo Fisher Scientific, Waltham, MA, USA), and was incubated for 2 weeks at room temperature (25 °C) with air ventilation (2.5 L/min) until phenol red turned to yellow (pH ~6.0). For the use of purification of HAO, the cells were harvested by centrifuge with 7000 × g, and were stored at −80 °C until purification. For the use of live cell inhibitor assay, the culture is stored at 4 °C and used within 2 weeks. The yields of the each AOB strain cells were approximately 1.5 g by 20-L cultures.

Purification of NeHAO and NoHAO

Frozen cell pellet (1.5 g of *N. europaea* or *N. ocean*) was suspended in 40 mL buffer A (pH 7.5, 50 mM Tris-HCl) and sonicated. The suspension was then centrifuged at 40 000 × g for 40 min at 4 °C. The supernatant was applied to an anion-exchange column system composed of three tandemly connected HiTrap™ Q HP 5 mL columns (GE Healthcare, Buckinghamshire, England, UK), which were equilibrated with buffer A. A linear gradient of NaCl was performed with 0–500 mM NaCl in 100 mL buffer A. Post-elution cleaning was performed using 1 M NaCl. The eluted HAO fractions were subsequently applied to a HiLoad™ 26/600 Superdex™ 200 pg gel-filtration column (GE Healthcare) equilibrated with buffer B (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl). Then, the eluted HAO fractions were applied to hydroxyapatite column composed of 5 mL CHT™ Ceramic Hydroxyapatite media (Type 1, 20 μm particle size, Bio-Rad, Hercules, CA, USA) packed to 10 mm
inner diameter column that was equilibrated with buffer C (pH 7.5, 20 mM potassium phosphate buffer). The elution was performed by linear gradient of 20–500 mM potassium-phosphate buffer (pH 7.5). This hydroxyapatite chromatography step is not necessary for purification of NoHAO. Thereafter, the eluted HAO fractions were applied to a high-performance anion-exchange column Mono Q® 10/100 GL (GE Healthcare). The elution was performed using a linear gradient of NaCl in buffer A (0–500 mM, 100 mL). Post-elution cleaning was performed using 1 M NaCl. The eluted HAO fractions were then stored at 4 °C until further experiments.

All chromatography procedures were performed using the ÄKTAexplore 100 system (GE Healthcare). All eluted fractions containing HAO were detected by two methods: 1) wavelength monitoring by ÄKTAexplore (protein: 280 nm; c-type hemes in HAO: 409 nm) and 2) SDS-PAGE analysis with coomassie brilliant blue stain.

Characterizations of purified proteins

N-terminal sequences of the purified proteins were analyzed by Procice® 491HT Protein Sequencing System (Applied Biosystems, Foster, CA, USA). Crystallization of NeHAO was performed as described by Maalcke et al. [10]. Protein concentration was determined by Qubit® protein assay kit (Thermo Fisher Scientific) with Quick Start® Bovine Serum Albumin Standard Set (Bio-Rad, Hercules, CA, USA). The UV-vis spectra were measured from 220 to 800 nm with 0.2 nm bandwidth and 0.1
nm s⁻¹ scan rate at 25 °C using a Cary 400 Bio spectrophotometer (Varian, Zug, Switzerland). The measurement buffer was 20 mM Tris-HCl with or without 50 mM dithionite. The dithionite was prepared as a 1 M solution, and was added to the protein solution immediately before measurement. Tris-Glycine-SDS-PAGE was performed on 5–20% gradient polyacrylamide precast-gel (e-PAGEL® E-R520L, ATTO, Tokyo, Japan) with EzRunC+ electrophoresis buffer (ATTO). To detect low molecular mass proteins, Tris-Tricine-SDS-PAGE was performed on 15% polyacrylamide precast-gel (e-PAGEL® E-R15S, ATTO) with the electrophoresis buffer containing 100 mM Tris, 50 mM Tricine, and 0.1% (w/v) SDS. The samples were boiled at 100 °C for 3 min with same volume of the Laemmli sample buffer composed of 125 mM Tris-HCl, 0.004% (w/v) bromophenol blue, 4% (w/v) SDS, 20% (w/v) glycerol, 10 mg/mL dithiothreitol (DTT), pH 6.8. The sample buffer was stocked in a −20 °C freezer in single-use volumes because air oxidization of DTT leads to diffusion of the HAO bands. The boiled samples in 10 μL were applied to the gel. BenchMark™ Protein Ladder (Thermo Fisher Scientific) was used as a protein marker. The gel was stained by coomassie brilliant blue solution EzStain AQua (ATTO).

**HAO activity assay**

HAO activity was detected by increase of 550 nm absorbance of cytochrome-c reduced coupled with hydroxylamine oxidation by HAO [15]. 100 μL reaction mixture for one assay contained 50 μM
hydroxylamine, 50 μM horse-heart cytochrome-c (Nacalai Tesque, Kyoto, Japan), several buffer types, pH, NaCl concentration, and additives. The reaction was started by addition of hydroxylamine. The absorbance was measured by using Infinite® M1000 PRO microplate reader (Tecan) on a 384-well non-binding clear microplate (Greiner bio-one, Kremsmünster, Austria). The reactions were performed at room temperature (25 °C). The relative activity was calculated by defining highest absorbance after incubation as 100% and initial absorbance as 0%. Optimization for pH and buffer was performed using following conditions; 20 mM citric acid buffer (pH 2.6–4.1, 8 point), 20 mM citrate-phosphate buffer (pH 3.1–7.5, 28 points), potassium-phosphate buffer (pH 5.5–8.1, 24 points), HEPES-NaOH buffer (pH 6.6–8.1, 10 points), 20 mM Tris-HCl buffer (pH 6.8–8.7, 15 points), CAPSO buffer (pH 9.0–9.8, 4 points), CAPS buffer (pH 10.1–10.8, 6 points), n = 4, total 384 assays. Optimization for salt concentration was performed by NaCl varied with 0, 12.5, 25, 50, 100, 200, and 400 mM in 50 mM HEPES-NaOH (pH 7.0) buffer. HAO assay validation and inhibition assays were performed with 100 μL solution containing 50 mM HEPES-NaOH (pH7.0), 50 mM NaCl, 50 μM hydroxylamine, 50 μM horse heart cytochrome-c, 0.01% Triton X-100, and 0.5 μL DMSO in which certain concentration of phenylhydrazine was dissolved.

Nitrification activity assay for AOB cells

AOB cells transferred to same volume of fresh medium were divided into 100 μL to each wells of 384
deep well plate (BIO-BIK, Osaka, Japan). 0.5 μL DMSO solutions containing certain concentration of phenylhydrazine were added to each wells. The plate was sealed with air-exchange film (AeraSeal™, EXCEL Scientific, Victorville, CA, USA), and incubated 18 hours at 25 °C. Then, 1 μL of the media in each wells were transferred to 384-well non-binding clear microplate (Greiner) filled with 100 μL Greiss Reagent in each wells. After 20 min incubation at room temperature (25 °C), 545 nm absorbance was measured by using Infinite® M1000 PRO microplate reader (Tecan). The Greiss Reagent was optimized to be composed of same volumes of stock solution A {2% (w/w) sulfanilamide, 12% (v/v) phosphoric acid} and stock solution B {0.2% (w/w) N-(1-naphthylethyl)enediamine} according to Giustarini et al. [16] with slight modifications. The stock solutions A and B were stored at 4 °C, and mixed just before measurements. 50% inhibition concentration (IC₅₀) was calculated by GraphPad Prism6 software (GraphPad Software, La Jolla, CA, USA).

Results and Discussion

1. Comparison of HAOs by sequence analysis

A lot of uncultured ammonia-oxidizing bacteria (AOB) live in soil, and nitrification inhibitors should be effective to all of these AOB. Identity tree of HAO in isolated AOB (Fig. 1A) reflects classification
of AOB, which are divided into two classes of beta-proteobacterial AOB (βAOB) and gamma-proteobacterial AOB (γAOB), and βAOB are further divided into two families, *Nitrosomonas* and *Nitrospira*. We compared amino acid residues around the substrate binding pocket of HAO between βAOB and γAOB (Figs. 1B–E). Six residues arranged around the pocket on the catalytic heme P460 in the NeHAO crystal structure (Fig. 1C) are completely conserved among three βAOB species (Fig. 1B). Figure 1D shows that all of these six residues in the NmHAO model structure is nearly superimposable to those in the NeHAO crystal structure. On the other hand, sequence alignment and homology modeling revealed that two residues in γAOB (Phe\textsuperscript{367}–Asn\textsuperscript{368} in NoHAO) are varied from βAOB (Asn\textsuperscript{357}–Tyr\textsuperscript{358} in NeHAO). These results suggest that HAO inhibitor responses are similar in the classes, but may be different between the two classes. To verify this statement, we compared inhibitory responses of NeHAO and NoHAO, as representatives of βAOB and γAOB, to phenylhydrazine, a well-known NeHAO inhibitor, after optimization of inhibitor evaluation systems.

2. Purification of NeHAO and NoHAO by new methods

We report new integrated purification methods for NeHAO and NoHAO. Our methods only employ a series of chromatography purification steps instead of cumbersome steps such as ammonium-precipitation, buffer exchange, dilution and concentration used in the previously reported conventional methods [10,14,17–19]. Purification procedures and results are shown in Figure 2. Purity of the HAOs
eluted gel-filtration column may be enough for enzymatic assay; hydroxyapatite and MonoQ column chromatography steps are optional to remove minor contaminations (Figs. 2D and 2G). The hydroxyapatite column step is not necessary for NoHAO. The purified proteins were identified as NeHAO and NoHAO using a protein sequencer (determined sequences are shown in Fig. S1). Yields were 0.8 mg for NeHAO and 0.6 mg for NoHAO from approximately 1.5 g of wet cell pellets.

Quality of the purified HAOs were analyzed by absorbance measurement (Fig. 2D and H). Judging from the 409/280 nm absorbance ratios of 3.39 and 3.94 respectively for the oxidized NeHAO and NoHAO, our purified proteins possess comparable or even higher purity than the corresponding proteins prepared by the conventional purification methods [22]. Quality of the HAOs were also checked by crystallization. Crystals of NeHAO were appeared within 2 weeks by using the condition described by Maalcke et al. [10] (Fig. S2). Crystallization of NoHAO is underway.

3. Optimization of HAO activity assay method

Many activity assays and inhibitor assays have been performed for HAO [7,15,19], but none of them could not be used for high-throughput screening and evaluation as they are. The most commonly used assay method is that the HAO activity is determined by monitoring the absorbance change at 550 nm due to the reduction of an electron acceptor cytochrome-c coupled with hydroxylamine oxidation by HAO [15]. We optimized this method for high-throughput HAO inhibitor screening and evaluation. In
particular, the assay method was optimized for the 384-well microplate assay by re-profiling conditions of the buffer, pH, salt and additives. Figures 3A and 3B show that NeHAO and NoHAO displayed almost same pH-dependent activity profiles although the optimum pH values were slightly different, pH 9.4 for NeHAO and 10.0 for NoHAO. As an optimized buffer condition, we select a pH 7.0, HEPES-NaOH buffer based upon the following reasons. (1) Since nitrous oxide produced during the hydroxylamine oxidation by HAO decreases pH of the assay solution, the HAO activity measured at an initial pH above 7.5 is too sensitive against pH decrease accompanied by hydroxylamine oxidation. (2) The activities at pH 7.0, 15% for both HAOs, are large enough for the inhibitor screening and evaluation. (3) The HAO activities measured at pH 7.0 in four different buffers were essentially the same. In addition, we used HEPES-NaOH buffer as the *N. europaea* culture medium. As an optimized salt concentration, we selected a 50 mM NaCl with which NeHAO and NoHAO displayed highest activities (Figs. 3C and 3D). Furthermore, we confirmed that no significant changes in the NeHAO and NoHAO activities by addition of 0.5% DMSO and 0.01% Triton X-100 (Fig. 3E). DMSO is frequently used as a solvent to dissolve chemicals in screening libraries, and Triton X-100 is a detergent to prevent chemical aggregation of compounds and non-specific binding of enzymes to plastic wares [20]. Finally, measurements of the NeHAO and NoHAO reaction time courses under the optimized conditions allowed us to employ the activity value at 30 min for evaluating inhibitory response (Fig. 3F).
To assess our assay method quantitatively, we estimated a statistical parameter termed \( Z' \)-factor that determines the suitability of an assay for high-throughput screening based on the signal dynamic range and the data variability [21]. A score of \( 1 > Z' \geq 0.5 \) indicates an excellent assay, \( Z' = 1 \) an ideal assay, \( Z' < 0 \) essentially impossible for screening. Calculated \( Z' \)-factors were 0.73 and 0.88 for the NeHAO and NoHAO assays, respectively, indicating that our method is suitable for high-throughput screening and inhibitor evaluation. Other validation statistics were summarized in Table S1.

4. Comparison of inhibitory response between NeHAO of \( \beta \)AOB and NoHAO of \( \gamma \)AOB

NeHAO of \( \beta \)AOB and NoHAO of \( \gamma \)AOB are characteristically different from each other in several points of views. NeHAO was purified as a complex with its partner protein NE1300 while NoHAO was purified as an isolated protein without any associate proteins (Fig. 4A). As shown in Figure 4B, the dithionite-reduced UV-vis spectra measured under the same conditions showed a marked difference of the P460 absorption peak positions between NeHAO (461.2 nm) and NoHAO (466.2 nm), suggesting different local environment around the substrate-binding sites of these two proteins as expected by our homology modeling study that showed different arrangements of the pocket-forming residues between the \( \beta \)AOB HAO and \( \gamma \)AOB HAO. This is further supported by the results of inhibition assays performed on the purified HAO enzymes and the live AOB cells with
phenylhydrazine, a well-known suicide inhibitor that binds to the P460 in NeHAO (Fig. 4C).

Phenylhydrazine inhibited about 60% of the NeHAO activity at 10 μM while the NoHAO activity was scarcely inhibited at the same concentration (Fig. 4D). Consistently, the live cell assay showed higher phenylhydrazine response for βAOB, *N. europaea*, *N. sp. JPCCT2* and *N. multiformis*, (IC₅₀ = 0.9–1.2 μM) than βAOB *N. oceani* (2.9 μM) (Fig. 4E).

Our studies clearly showed that βAOB are more sensitive to inhibition by phenylhydrazine than γAOB due to different local environments around the substrate-binding sites of HAOs in these two classes of bacteria. Different inhibitory responses between βAOB and γAOB may be emerged not only by phenylhydrazine but also by other inhibitors that bind to the active center in HAO. Even small difference in inhibitor sensitivity increases a risk for appearance of drug-resistant bacteria. The findings reported herein indicate that effects of inhibitors should be examined for at least two types of HAOs purified from βAOB and γAOB. Selection of NeHAO and NoHAO is the best choice for discovery of novel nitrification inhibitors targeting HAO with wide anti-AOB spectra due to the established purification and high-throughput screening methods and the long phylogenetically distance between *N. europaea* of βAOB and *N. oceani* of γAOB.

Acknowledgements
We would like to thank Dr. Wataru Tsuchiya (NARO) for technical advice, Dr. Mayumi Kuroiwa (The University of Tokyo) for protein sequence analysis. The MOE software was provided by the Agriculture, Forestry and Fisheries Research Information Technology Center (AFFRIT) of the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan. This work was in part supported by Japan Society for the Promotion of Science KAKENHI Grant 26310317 and the NIAS Strategic Research Fund to TY, and the Sasakawa Scientific Research Grant from the Japan Science Society and Kurita Water and Environment Foundation (KWEF) research grant to YN.
References


[18] D.M. Arciero, C. Balny, A.B. Hooper, Spectroscopic and rapid kinetic studies of reduction of cytochrome c554 by hydroxylamine oxidoreductase from *Nitrosomonas europaea*,


Legends to Figures

Figure 1. Comparison of NeHAO with other HAOs.

(A) Identity tree for HAO. (B) Multiple alignments for active-site residues in HAOs. Full-length sequence alignment is represented in Figure S1. (C) Overall structure and close-up view of the around active site of NeHAO (PDB:4n4n). Comparison of active-site residues in the NeHAO crystal structure with those in NmHAO (D) and NoHAO (E) models. “Wat” is represented a water molecule that binds on the iron in heme P460. The identity tree was calculated by average distance clustering algorithm using identity of the full-length HAO alignment by using MOE software version 2015.1001 (Chemical Computing Group).

Figure 2. Integrated purification methods for NeHAO (A–D) and NoHAO (E–H). Purification schemes of NeHAO (A) and NoHAO (E). Elusion profiles of HiTrap Q ion-exchange, Superdex 200 gel-filtration, CHT ceramic hydroxyapatite (NeHAO only), and MonoQ ion-exchange chromatography of NeHAO (B) and NoHAO (F). SDS-PAGE analysis of HAO containing fractions at each chromatography steps of NeHAO (C) and NoHAO (G). UV-vis spectra for air-oxidized and dithionite reduced NeHAO (D) and NoHAO (H).
Figure 3. Optimization of high-throughput HAO activity assay method. Effect of pH and buffer types for activity of NeHAO (A) and NoHAO (B). Effect of salt concentration for activity of NeHAO (C) and NoHAO (D). (E) Effect of additives (0.5 % DMSO and 0.01% Triton X-100) for activity of NeHAO and NoHAO. (F) Time courses of activity measurements for NeHAO and NoHAO.

Figure 4. Comparison of βAOB HAO and γAOB HAO. (A) SDS-PAGE analysis for concentrated NeHAO/NE1300 complex and NoHAO (each ~2 μg). (B) Comparison of UV-vis spectra around the heme P460 in NeHAO and NoHAO. The spectral data are the same as those of Figures 2D and 2H, and the absorbance intensities were adjusted so as to overlap the heme-c peaks of the two proteins. (C) Structure of phenylhydrazine, an HAO specific inhibitor. (D) Inhibition effects of phenylhydrazine against purified NeHAO and NoHAO. (E) Determination of 50% inhibition (IC₅₀) concentrations of phenylhydrazine against nitrification activity of live AOB cells.
Fig. 1

A

\[ \beta\text{-AOB} \]

- Nitrosomonas europaea (NeHAO)
- Nitrosomonas eutropha
- Nitrosospira multiformis (NmHAO)
- Nitrosococcus watsoni
- Nitrosococcus oceanii (NoHAO)
- Nitrosococcus halophilus

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2. NE1300

D

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- N357
- Y358
- F447
- D291
- Y491
- N357
- E480
- H302
- H292
- D301
- D291
- Heme P460
- Y491
- Y500
- Wat

- NeHAO
- NoHAO
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<td>HAO_Nhalo</td>
</tr>
<tr>
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<tr>
<td><strong>E</strong>588 (NeHAO)</td>
<td><strong>E</strong>588 (NeHAO)</td>
<td><strong>E</strong>588 (NeHAO)</td>
<td><strong>E</strong>588 (NeHAO)</td>
<td><strong>E</strong>588 (NeHAO)</td>
<td><strong>E</strong>588 (NeHAO)</td>
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</table>

**Figure S1.** Multiple alignment and identity matrix for HAOs from AOB. (A) Multiple alignment of HAOs from several βAOB and γAOB. Heme binding motifs (-CxxCH-) and tyrosine residues covalently cross-linked to heme P460 are colored in red. Active-site residues are colored in green. N-terminal sequences of NeHAO and NoHAO determined by the protein sequencer were underlined. Signal sequences are shown in italic. (B) All-against-all pairwise identity matrix. Percent sequence identities were calculated dividing the number of identical residues between protein in the row and column by the total number of residues in the column protein. All calculations were performed using MOE software version 2015.1001. Pretty printing of the alignment was performed by BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html). The amino acid sequence of HAO in N. sp. JPCCT2, which was used for the inhibition assay in Fig. 4E, has not been determined yet.
Figure S2. Crystals of NeHAO purified by our method.
<table>
<thead>
<tr>
<th>Benchmark</th>
<th>formula</th>
<th>NeHAO</th>
<th>NoHAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Average (Av100%)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>-</td>
<td>0.72</td>
<td>0.82</td>
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<td>100% Standard deviation (SD100%)</td>
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<td>0.0275</td>
<td>0.0084</td>
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<tr>
<td>Background Average (Av0%)&lt;sup&gt;a,c&lt;/sup&gt;</td>
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<td>0.38</td>
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<tr>
<td>Background Standard deviation (SD0%)</td>
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<td>0.0092</td>
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<td>Coefficient value (CV %)</td>
<td>SD100%/Av100% &lt;sup&gt;c&lt;/sup&gt;×100</td>
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<tr>
<td>Signal Background ratio (S/B)</td>
<td>Av100%/Av0%</td>
<td>2.01</td>
<td>2.17</td>
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<tr>
<td>Z'-factor&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 - (\frac{3 \times SD_{100%} + 3 \times SD_{0%}}{Av_{100%} - Av_{0%}})</td>
<td>0.73</td>
<td>0.88</td>
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</tbody>
</table>

<sup>a</sup>n = 4.

<sup>b</sup>550 nm absorbance measured at 30 min (without inhibitors).

<sup>c</sup>550 nm absorbance measured at 0 min (without inhibitors).

<sup>c</sup>Referred to Zhang et al. [22].