Correction

BIOCHEMISTRY
Correction for “Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase,” by Jonathan D. Caranto and Kyle M. Lancaster, which was first published July 17, 2017; 10.1073/pnas.1704504114 (Proc Natl Acad Sci USA 114:8217–8222).
The authors note that on page 8221, left column, expression [3] should instead appear as:

\[ 2 \text{NH}_2\text{OH} + \frac{3}{2} \text{O}_2 \rightarrow 2 \text{NO} + 3 \text{H}_2\text{O}; \Delta G^\circ = -58.7 \text{ kcal mol}^{-1} \text{N} \]

As a result, the preceding sentence, starting with “Furthermore,” should instead appear as, “We note that termination at NO allows for addition free energy to be withdraw via subsequent oxidation to NO\(\text{2}^-\) by an as-yet-unidentified enzyme (vide infra).”

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Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase

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Ammonia (NH3)-oxidizing bacteria (AOB) emit substantial amounts of nitric oxide (NO) and nitrous oxide (N2O), both of which contribute to the harmful environmental side effects of large-scale agriculture. The currently accepted model for AOB metabolism involves NH3 oxidation to nitrite (NO2−) via a single obligate intermediate, hydroxylamine (NH2OH). Within this model, the multiheme enzyme hydroxylamine oxidoreductase (HAO) catalyzes the four-electron oxidation of NH2OH to NO2−. We provide evidence that HAO oxidizes NH2OH by only three electrons to NO under both anaerobic and aerobic conditions. NO2− observed in HAO activity assays is a nonenzymatic product resulting from the oxidation of NO by O2 under aerobic conditions. Our present study implies that aerobic NH3 oxidation by AOB occurs via two obligate intermediates, NH2OH and NO, necessitating a mediator of the third enzymatic step.

Significance

The enzymatic reactions that occur during nitrification are nature’s means to use ammonia as cellular fuel. Complete understanding of nitrification and related processes are vital to sustainable agriculture and renewable energy technologies. The prevailing view of the first phase of nitrification is that ammonia oxidizing bacteria use two enzymes, ammonia monooxygenase and hydroxylamine oxidoreductase, to oxidize ammonia to nitrite via hydroxylamine as an obligate intermediate. Our work reveals nitric oxide as an additional obligatory intermediate. The presented findings necessitate revision of a key biogeochemical process, identify a new bioenergetic role for nitric oxide, predict participation of a third enzyme in the biological oxidation of ammonia to nitrite, and will inform models toward sustainable agriculture.

Author contributions: K.M.L. designed research; J.D.C. performed research; J.D.C. and K.M.L. analyzed data; and J.D.C. and K.M.L. wrote the paper.

The authors declare no conflict of interest.

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O₂ precludes the final oxidation step to form NO₃⁻, allowing for NO and HNO to dissociate from HAO pathway intermediates, resulting in the observed NO and N₂O.

Recent studies of additional enzymes with HAO-like active sites support the conflicting view that NO₂⁻ is not the enzymatic product of HAO activity. Kartal and coworkers (20) purified kust1061, an octaheme oxidoreductase homologous to HAO, from the anammox bacterium *Kuenenia stuttgartiensis*. Kust1061 stoichiometrically oxidizes NH₂OH by three electrons to NO, not NO₂⁻. Additionally, an unrelated monoheme enzyme called cytochrome (cyt) P460, so named because it bears an active-site cofactor similar to the heme P460 center found in HAO, was also reported to oxidize NH₂OH to NO₂⁻ (22–25). We recently revised the cyt P460 activity, demonstrating that NO₂⁻ is the enzymatic product under anaerobic conditions (21). Mechanistic studies showed that cyt P460 catalyzes the oxidation of iron-bound NH₂OH to a ferric-nitrosyl [[Fe(NO)]₅] Enemark–Feltham notation (26). Nucleophilic attack on the cyt P460 (FeNO)₆ by a second molecule of NH₂OH results in the formation of N₂O. Under aerobic cyt P460 turnover conditions, at most, 70% of NH₂OH was converted to NO₂⁻, with the remaining NH₂OH being converted to N₂O. We proposed that NO dissociation from the (FeNO)₆ intermediate competes with attack by NH₂OH and that the nonenzymatic oxidation of disso-

ciated NO by O₂ accounts for the observed NO₂⁻. The similar substoichiometric NH₂OH oxidation to NO₂⁻ observed for both cyt P460 and HAO prompted us to revisit the activity of HAO. The work described herein uses NO scavenging assays as evidence that NO, not NO₂⁻, is the product of NH₂OH oxidation by *N. europaea* HAO under both aerobic and anaerobic conditions.

**Results and Discussion**

**HAO Does Not Produce NO₂⁻ in the Absence of O₂**

To firmly establish conditions under which NO₂⁻ is produced by HAO, we repeated several previously reported HAO turnover experiments (15, 17, 18). Under aerobic turnover conditions, HAO oxidizes NH₂OH to a mixture of NO₂⁻ and NO₃⁻ (Fig. 2 and SI Appendix, Table S1). We treated 150 nM HAO with 5 μM phenazine methosulfate (PMS) and various concentrations of NH₂OH at pH 8.0. The samples were incubated at room temperature for 30 min. On the time scale of the experiment, O₂ rapidly oxidizes reduced PMS (k = 180 M⁻¹ s⁻¹) (27) and therefore can be used in catalytic concentrations. All of the NH₂OH was consumed within 30 min. The NO₂⁻ concentration was determined via the Griess dia-

zotization assay. The combined NO₂⁻ and NO₃⁻ concentrations were determined by quantifying NO₂⁻ in parallel samples pre-

treated with NO₃⁻ reductase. Observed NO₂⁻ concentrations consis
tently accounted for ~40% of the added NH₂OH (SI Appendix, Fig. S1). Almost all of the remaining NH₂OH was accounted for as NO₃⁻.

Omitting PMS or HAO from the reaction mixture causes NH₂OH oxidase activity to decrease substantially (Fig. 2 and SI Appendix, Table S1). In the absence of HAO, less than 5% of the NH₂OH is consumed over a prolonged 5-h incubation time. Fur-

thermore, final NO₂⁻ and NO₃⁻ concentrations are negligible, which is consistent with the necessity of HAO for NH₂OH oxidase activity. When PMS is omitted, only ~15% of the NH₂OH was consumed over 5 h, most of which was converted to NO₂⁻. This slower NH₂OH consumption rate is consistent with the previously reported HAO activity with O₂ as the sole oxidant (18).

Under anaerobic conditions, neither NO₂⁻ nor NO₃⁻ are produced. In an anaerobic glovebox, 150 nM HAO was treated with 200 μM PMS and 100 μM NH₂OH at pH 8.0 for 30 min at room temperature. Under these conditions, NH₂OH is completely consumed and negligible concentrations of NO₂⁻ or NO₃⁻ were ob-

served (Fig. 2 and SI Appendix, Table S1), which is consistent with previous reports (15). The absence of NO₂⁻ or NO₃⁻ indicated that their production is O₂ dependent.

The low stoichiometry of NO₂⁻ produced from NH₂OH strongly suggests that NO₂⁻ is not the product of HAO activity. Indeed, more NO₃⁻ is produced than NO₂⁻, which is inconsistent with the assignment of the latter species as the product of HAO. The observed mixture of NO₂⁻ and NO₃⁻ formed during HAO oxidation of NH₂OH under aerobic conditions is consistent with previous work (17, 18). At low O₂ concentration, the reaction mixture is more complex; Hooper and coworkers (17) showed that under these conditions, four products are observed: NO₂⁻, NO₃⁻, NO, and NO. These observed product mixtures could be the result of compet-

ing reactions of a common reactive species, which is the true HAO enzymatic product. The observation of NO among the four products at low O₂ concentration strongly suggests that NO is this reactive molecule. Reactions of NO that produce N₂O, NO₂⁻, or NO₃⁻ are well characterized; the latter two products require O₂ for their formation (28). We propose that HAO oxidizes NH₂OH to NO and that NO₂⁻ is not a direct enzymatic product but is instead, a result of nonenzymatic oxidation of NO by O₂. Consistent with this hypothesis, neither NO₂⁻ nor NO₃⁻ is observed as a product of NH₂OH oxidation by HAO under anaerobic conditions (Fig. 2 and SI Appendix, Table S1).

In the presence of O₂, NO has a short lifetime and thus, would be difficult to detect as a product of HAO. Aqueous NO reacts with O₂ to form NO₃⁻ (4k = 8 × 10⁶ M⁻¹ s⁻¹) (29). Despite this reaction being second order with respect to NO, the half-life of aqueous NO is 5.6–56 s when the NO concentration is 10–100 μM under air-saturated conditions (230 μM O₂). O₂ has been reported to react with reduced PMS to form superoxide (O₂⁻) (27). The subsequent reaction of O₂⁻ with NO to form NO₃⁻ is above the diffusion-controlled limit (k = 4.3 × 10¹⁴ M⁻¹ s⁻¹) (30). Even without O₂, NO could still be difficult to detect; Pucheco and coworkers reported that NO reacts with reduced HAO (k = 1.5 × 10¹⁰ M⁻² s⁻¹) (31); this could account for the N₂O produced under anaerobic or low O₂ conditions (17). In light of these potential side reactions, we quantified NO produced during
Evidence that reduction resulting from the addition of NH$_3$ indicates time point of NH$_3$. To test for NO as an HAO product, an NO seques-
tration system was used (32). In this assay, catalase rapidly binds NO (Fig. S2A). Upon treatment of catalase with NO, the Soret band shifts to 433 nm and poorly resolved absorbance features between 500 and 700 nm (Fig. S2A, solid black trace). Upon treatment of catalase with NO, the Soret band shifts to 433 nm and two well-resolved features at 542 and 579 nm appear (Fig. S2A, black dashed trace). NO can be quantified from the change in absorption at 433 nm ($\Delta A_{433}$) resulting from NO binding to cata-
lase ($\Delta A_{433} = 47.9$ nm$^{-1}$·cm$^{-1}$) (32).

Samples containing catalase and HAO in anaerobic turnover conditions exhibit features consistent with NO binding to cata-
lase (SI Appendix, Fig. S2). Samples were prepared containing 150 nM HAO, 100 μM 2,6-dichlorophenolindophenol (DCPIP), 10 μM catalase, and 200 μM NH$_3$OH at pH 8.0 under anaerobic conditions. UV-vis absorption spectra collected after incubating these samples for 5 min exhibited a decrease in intensity of the catalase 403 nm Soret band and appearance of absorption features consistent with NO binding to catalase (SI Appendix, Fig. S2A). These absorption features are absent when HAO is ex-
cluded from the reaction mixture (SI Appendix, Fig. S2B), indicating that NO production is HAO dependent. Together, these results indicate that NO is at least one product of NH$_3$OH oxid-
ation by HAO under anaerobic conditions.

To quantify the amount of NO released under anaerobic conditions, similar sample conditions were used and the NH$_3$OH concentra-
tion was varied with excess catalase present. The re-
actions were monitored at 433 nm. Addition of NH$_3$OH initiated an increase in the 433 nm absorbance (Fig. 3A). This absorbance increase was not observed in the absence of HAO (SI Appendix, Fig. S3A). Using $\Delta A_{433}$ to quantify the amount of NO released, we found that 88–100% of the NH$_3$OH was converted to NO (Table 1).

Parallel reactions were performed in the absence of catalase to determine the oxidant stoichiometry. UV-vis absorption spec-
troscopy was used to monitor and quantify the reduction of DCPIP or horse heart cyt c at 605 ($\Delta A_{605} = 20.3$ nm$^{-1}$·cm$^{-1}$) or 550 nm ($\Delta A_{550} = 19.6$ nm$^{-1}$·cm$^{-1}$), respectively (Fig. 3A). At several NH$_3$OH concentrations, 1.5–1.7 mol DCPIP or 2.8–3.0 mol cyt c were reduced per 1 mol NH$_3$OH (Table 1). Together, these data are consistent with the quantitative three-electron oxida-
tion of NH$_3$OH to NO by HAO under anaerobic conditions.

HAO Stoichiometrically Oxidizes NH$_3$OH to NO Under Anaerobic Conditions. To test for NO as an HAO product, an NO sequestration system developed by Pacheco and coworkers was used (32). In this assay, catalase rapidly binds NO ($k_{cat} = 1.3 \times 10^7$ M$^{-1}$·s$^{-1}$) (33) with concomitant appearance of defined UV-visible (UV-vis) absorption features (SI Appendix, Fig. S2A). Catalase exhibits a Soret band at 403 nm and poorly resolved absorbance features between 500 and 700 nm (SI Appendix, Fig. S2A, solid black trace). Upon treatment of catalase with NO, the Soret band shifts to 433 nm and two well-resolved features at 542 and 579 nm appear (SI Appendix, Fig. S2A, black dashed trace). NO can be quantified from the change in absorption at 433 nm ($\Delta A_{433}$) resulting from NO binding to cata-
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tion of NH$_3$OH to NO by HAO under anaerobic conditions.

HAO Oxidizes NH$_3$OH to NO Under Aerobic Conditions. Under the canonical model of HAO activity (Fig. 1, NH$_3$OH obligate inter-
mediate model), HAO requires O$_2$ to complete the oxidation of NH$_3$OH to NO$_2^-$. Therefore, the observed quantitative oxidation of NH$_3$OH to NO could result from the lack of O$_2$. To demonstrate that NO remains the product of HAO catalysis in the presence of O$_2$, we used ferrous-O$_2$ hemoglobin (oxyHb) to simultaneously scavenge and quantify NO under aerobic conditions. In this assay, NO reacts with oxyHb to form ferric-hemoglobin (metHb) and
NO$_3^-$. The oxyHb to metHb conversion can be monitored from the resulting UV-vis absorption increase at 421 nm and concomitant decrease at 401 nm ($\Delta_{421}$ and $\Delta_{401}$, respectively). The difference in $\Delta_{421}$ and $\Delta_{401}$ provides the concentration of oxyHb converted to metHb ($\Delta_{421-401} = 77.3$ nm$^{-1}$ cm$^{-1}$) (34) and indirectly, the concentration of NO released during HAO turnover.

The results of this assay showed that HAO produced NO under aerobic turnover conditions. Samples were prepared containing 150 nM HAO, 20 μM oxyHb, 25 μM DCPIP, and varying NH$_2$OH concentrations at pH 8.0. In the presence of HAO, the absorption at 401 nm decreased over 30 s with a concomitant increase in absorption at 421 nm (Fig. 3B). These absorption changes were not observed in the absence of HAO, once again indicating that NO production is HAO dependent (*SI Appendix, Fig. S3B*).

$\Delta_{421-401}$ for samples containing HAO was consistent with the production of 2.4 and 2.9 μM NO when the reaction was initiated with either 3 or 4 μM NH$_2$OH, respectively (Table 1). At each of these concentrations, NO accounted for ~75% of the NH$_2$OH added to the reaction mixture.

Although the full complement of NO was not detected, the oxidant stoichiometry with respect to NH$_2$OH was consistent with its full conversion to NO. Samples without oxyHb were prepared containing 150 nM HAO, 20 μM cyt c, and 3.4 μM NH$_2$OH at pH 8.0. By monitoring cyt c as described above, we found that 2.91 (0.05) mol cyt c were reduced per 1 mol NH$_2$OH (Table 1); this is consistent with the three-electron oxidation of NH$_2$OH to NO and suggests that conversion to NO is quantitative. This result in turn suggests that the NH$_2$OH is completely converted to NO but not all of it reacts with oxyHb. A fraction of the NO could react with O$_2$ to form NO$_2^-$. In this scenario, the expected NO$_2^-$ concentrations for these experiments were beneath the limit of detection of the Griess assay (<1 μM). However, NO$_2^-$ formation was observed under similar conditions at higher NH$_2$OH concentrations (see below). The combined data provide evidence that HAO-driven NH$_2$OH oxidation terminates at NO even under aerobic conditions.

**NO$_2^-$ is a Nonenzymatic Product of NO Oxidation by O$_2$.** Our results thus far suggest that NO, not NO$_2^-$, is the product of enzymatic NH$_2$OH oxidation by HAO, implying that NO$_2^-$ results from the nonenzymatic reaction of the NO with O$_2$. By this model, the NO$_2^-$ concentration should decrease in the presence of an NO scavenger. To test this hypothesis, we reacted 150 nM HAO with 5 μM PMS at various NH$_2$OH concentrations under aerobic conditions in the absence or presence of the NO scavenger oxyHb. Samples containing oxyHb showed a 66–75% decrease in NO$_2^-$ formation (Fig. 3C and *SI Appendix, Table S4*). This loss of NO$_2^-$ is consistent with our hypothesis. The remaining NO$_2^-$ in the presence of oxyHb suggests that some NO reacts with O$_2$. This competition between the NO reactions with oxyHb and O$_2$ also likely accounts for the lower-than-expected conversion of oxyHb to metHb discussed above (Fig. 3B and Table 1).

### Table 1. Stoichiometries of NO production and oxidant consumption vs. NH$_2$OH concentration under anaerobic and aerobic HAO turnover conditions

<table>
<thead>
<tr>
<th>NH$_2$OH, μM</th>
<th>NO$_2^-$final μM</th>
<th>% Conversion to NO</th>
<th>[cyt c]$_{reduced}$/[NH$_2$OH]</th>
<th>[DCPIP]$_{reduced}$/[NH$_2$OH]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaerobic conditions: Catalase NO-sequestering method</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.95 (0.04)</td>
<td>98 (2)</td>
<td>3.0 (0.3)</td>
<td>1.5 (0.1)</td>
</tr>
<tr>
<td>4</td>
<td>4.1 (0.1)</td>
<td>103 (3)</td>
<td>2.8 (0.1)</td>
<td>1.5 (0.1)</td>
</tr>
<tr>
<td>6</td>
<td>5.3 (0.2)</td>
<td>88 (3)</td>
<td>1.7 (0.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Aerobic conditions: OxyHb NO-scavenging method†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.2 (0.2)</td>
<td>75 (5)</td>
<td>2.91 (0.05)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.9 (0.1)</td>
<td>74 (4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are reported as averages of at least three replicates with SDs shown in parentheses. Expanded oxidant consumption data are presented in *SI Appendix, Tables S2 and S3*.

Reactions contained 150 nM HAO, 8 μM catalase, and 25 μM DCPIP or 20 μM cyt c in degassed 250 mM Na$_2$HPO$_4$, pH 8.0.

*Reactions contained 150 nM HAO, 20 μM OxyHb, and 25 μM DCPIP or 20 μM cyt c in air-saturated 50 mM Na$_2$HPO$_4$, pH 8.0.

NO is an Obligate Ammonia Oxidation Intermediate. NO production by HAO has been previously observed in the absence of O$_2$ but was attributed to incomplete NH$_2$OH oxidation when oxidant or O$_2$ concentrations were insufficient (17, 18, 31, 35). As discussed above, the detection of NO as the HAO product was likely obscured by several rapid, nonenzymatic NO reaction pathways, especially when O$_2$ was present. The present work quantified the NO produced under both anaerobic and aerobic conditions and demonstrates that NO$_2^-$ is not an enzymatic product of HAO.

The revised *N. europaea* HAO product is consistent with that of the *K. stuttgartiensis* kustc1061 protein that Kartal and coworkers (20) purified and characterized; in their paper, the authors proposed that a tyrosine above the active site pocket (Y358) present in *N. europaea* HAO but absent in *K. stuttgartiensis* kustc1061 facilitates the (FeNO)$^{18}$ reaction with H$_2$O to form NO$_2^-$. However, our results indicate that the *N. europaea* HAO and kustc1061 NH$_2$OH oxidation products are identical and, therefore, Y358 does not promote NO$_2^-$ formation. We briefly note that in this same paper, the authors reported that *N. europaea* HAO oxidized NH$_2$OH by four electrons. However, no evidence was presented demonstrating NO$_2^-$ production or the absence of NO formation by *N. europaea* HAO. We conclude based on our results that the NH$_2$OH oxidation products of *N. europaea* HAO and *K. stuttgartiensis* kustc1061 enzymes are both NO. The consolidation of the activities of these proteins suggests a similar physiological function of heme P460-containing octaheme proteins in aerobic and anaerobic ammonia-oxidizing bacteria.

The oxidation of NH$_2$OH to NO instead of NO$_2^-$ implies that AOB require three steps to oxidize NH$_3$ to NO$_2^-$, with both NH$_2$OH and NO acting as obligate intermediates (Fig. 1, NH$_2$OH/NO obligate intermediate model). One possibility accounting for the total conversion of NH$_3$ to NO$_2^-$ in aerobic cell culture is that NO is the terminal product of the enzymatic NH$_3$ oxidation pathway, and free NO is nonenzymatically oxidized to NO$_2^-$ by O$_2$. However, our results confirm those of previous studies showing that NO$_3^-$ is a significant by-product of in vitro NH$_2$OH oxidation by HAO and appears only under aerobic conditions (Fig. 2). This outcome suggests that, much like NO$_2^-$, NO$_3^-$ results from a side reaction of NO under aerobic conditions.

There is no evidence for a NO$_3^-$ reductase in the genome of *N. europaea* (36). Therefore, the lack of observed NO$_3^-$ formation by intact AOB (18) suggests that the NH$_3$ oxidation pathway requires an unidentified component that rapidly transforms NO to NO$_2^-$ to outcompete side reactions that produce NO$_3^-$ or N$_2$O. Such an agent would also avoid nonenzymatic oxidation of NO to NO$_2^-$, thereby permitting the full complement of four electrons to be removed from NH$_2$OH for entry into cellular electron transport chains. One possibility for this third step is the Cu NO$_2^-$ reductase NirK. NirK generally functions to reduce NO$_2^-$ to NO in denitrification or nitrifier denitrification pathways.
NirK deletion variants of *N. europaea* show no disruption in *N*₂*O* production under anaerobic conditions, which suggests that NirK is unnecessary for nitrifier denitrification in these organisms (37, 38). However, these variant strains exhibit considerably decreased *NO₂*⁻ production. Given these observations, we hypothesize that NirK catalyzes the oxidation of *NO* produced by HAO to form *NO₂*⁻, for which there is precedent in the literature (39). The combined, emergent picture strongly implicates a three-step oxidation of *NH₂OH* to *NO₂*⁻ by AOB.

Whether enzymatic *NH₂OH* oxidation terminates at *NO* or proceeds via a third enzymatic step to *NO₂*⁻, energy transduction remains productive. In the currently accepted model, hydroxyl-lation of *NH₂OH* to *NH₂O* requires two electrons for turnover of AMO (6). These reducing equivalents are believed to be furnished from the pool of electrons liberated from *NH₂OH*. Termination of enzymatic *NH₂OH* oxidation at NO liberates three electrons; after cycling AMO, one net electron remains for cellular respiration. Furthermore, we note termination at NO yields more free energy per mol N, assuming cellular respiration involving *O₂* as a terminal electron acceptor (40):

\[
2 \text{NH}_2\text{OH} + \frac{3}{2} \text{O}_2 \rightarrow 2 \text{NO} + 3 \text{H}_2\text{O}; \quad \Delta G^\circ = -117.4 \text{ kcal mol}^{-1} \text{ N} [3]
\]

\[
\text{NH}_2\text{OH} + \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+; \quad \Delta G^\circ = -70 \text{ kcal mol}^{-1} \text{ N} [4]
\]

The identification of HAO as an NO source may provide insights into the environmental impacts of AOB. *N*₂*O* emissions from AOB are largely attributed to nitrifier denitrification (8). However, this pathway activates under anaerobic conditions, and AOB have been shown to emit *N*₂*O* under aerobic conditions. Although we have recently identified a source of *N*₂*O* originating from *NH₂OH* (21), *N*₂*O* is usually the result of NO reduction. Our results identify HAO as a major source of NO under aerobic conditions. We propose that *N*₂*O* production under aerobic conditions arises when the rate of NO production outcompetes the rate of its oxidation to NO₂⁻. This would result in higher intracellular NO concentrations that lead to NO emission or its conversion to *N*₂*O* by the respiratory nitric oxide reductase, NorBC. Alternatively, NO could be scavenged by cyt P460 or cyt c₅₅₃ to form *N*₂*O* (35). A decrease in the rate of NO oxidation to NO₂⁻ might be expected at low *O₂* concentrations, and this may help explain the observed increase in *N*₂*O* emissions at low *O₂* or during oxic to anoxic transitions (41, 42). The presently afforded revision of HAO’s activity should facilitate understanding of the sources and rates of NO and *N*₂*O* emissions from ammonia oxidizing bacteria.

**Materials and Methods**

**General Considerations.** Purified *N. europaea* HAO was a generous gift from Prof. Sean Elliott, Boston University, Milli-Q water (18.2 MΩ; Millipore) was used in the preparation of all buffers and solutions. Hydroxylamine hydrochloride (*NH₂OH•HCl*) was purchased from Sigma Aldrich. Catalase and Ferrous hemoglobin were purchased from Sigma-Aldrich. All other chemicals were purchased from VWR International and used as obtained. UV-vis absorption measurements were performed using a Flame spectrophotometer (Ocean Optics) or a Cary 60 spectrophotometer (Agilent Technologies). *NO₂*⁻ was quantified using the Griess diazotization assay (Cayman Chemical) and measuring the resulting absorbance at 542 nm (*ε*₅₄₂ = 50 M⁻¹•cm⁻¹). The combined *NO₂*⁻ and *NO₃*⁻ concentrations were determined by treating samples with nitrate reductase and cofactor solution (Cayman Chemical) at room temperature for 1 h followed by *NO₂*⁻ quantitation. DCPIP (ε₂₅₄ = 20.3 M⁻¹•cm⁻¹), oxyHb (*ε*₄₁₅ = 131 M⁻¹•cm⁻¹) (34), catalase (*ε*₄₀₅ = 120 M⁻¹•cm⁻¹) (32, 34), cyt *c* (*ε*₅₅₃ = 28.0 M⁻¹•cm⁻¹), and phenazine methosulfate (PMS; ε₃₈₅ = 26.3 M⁻¹•cm⁻¹) (43) were quantified by UV-vis absorption spectroscopy. *NH₂OH* stocks were quantified by the method of Frear and Burrell (44). NO was generated from disodium 1-(hydroxyl-NNO-azoxy)-l-proline (PROLIONODate; Cayman Chemical) where the NO was quantified by Fe²⁺EDTA as previously reported (21).

**Stoichiometry of *NO₂*⁻ and *NO₃*⁻ by HAO Under Various Conditions.** Solutions used for *NO₂*⁻ and *NO₃*⁻ determination under aerobic HAO conditions contained 150 nM HAO and 5 μM PMS in 1 mL of air-saturated 50 mM Na₂HPO₄, pH 8.0. The reaction was initiated by addition of an appropriate volume of an *NH₂OH* stock solution in water. For samples treated with oxyHb, the conditions were identical except that the samples were incubated in the presence of 30 μM oxyHb.

Anaerobic samples were prepared in an MBraun glovebox maintained under an *N*₂ atmosphere. The conditions after mixing were 150 nM HAO, 100 μM *NH₂OH*, and 200 μM PMS in 1 mL of degassed 250 mM Na₂HPO₄, pH 8.0. The reaction was initiated by addition of an appropriate volume of an *NH₂OH* stock solution in water. All samples were incubated at room temperature for 30 min and then analyzed for *NO₂*⁻ and *NO₃*⁻ as described in Materials and Methods.

**Stoichiometry of *NH₂OH* Oxidation to NO by HAO Under Anaerobic Conditions.** NO production under anaerobic conditions was monitored by using a catalase NO-binding assay previously described by Pacheco and coworkers (32). To determine the stoichiometry of *NH₂OH* oxidation to NO, anaerobic solutions containing 150 nM HAO, 8 μM catalase, and 25 μM DCPIP in 1 mL of degassed 250 mM Na₂HPO₄, pH 8.0, were prepared in an anaerobic glovebox. All reactions were performed at room temperature and initiated by addition of an appropriate volume of an anaerobic stock of *NH₂OH* dissolved in water. The reactions were monitored at 433 nm by a UV-vis absorption spectrophotometer. The amount of NO generated was determined from the change in absorption at 433 nm and using the extinction coefficient of *ε*₄₃₃ = 47.9 M⁻¹•cm⁻¹. The stoichiometry of *NH₂OH* to reduced DCPIP was monitored in parallel reactions under identical reaction conditions except that catalase was omitted. The concentration of DCPIP reduced was determined from the change in absorption at 605 nm and using the extinction coefficient *ε*₆₀₅ = 20.3 M⁻¹•cm⁻¹. To determine the stoichiometry of *NH₂OH* to cyt *c* reduction, the reactions were simultaneously monitored at 401 and 421 nm by a UV-vis absorption spectrometer. The amount of NO generated was determined via the change in absorption at 421 nm minus the change in absorbance at 401 nm and using the extinction coefficient of *ε*₄₂₁⁻₄₀₁ = 77.3 M⁻¹•cm⁻¹. The stoichiometry of *NH₂OH* to cyt *c* reduction was performed under identical reaction conditions except the oxyHb was omitted. Cyt *c* reduction was quantified as described above for the anaerobic experiments.

**Stoichiometry of *NH₂OH* Oxidation to NO by HAO Under Aerobic Conditions.** To determine the stoichiometry of *NH₂OH* oxidation to NO, solutions were prepared containing 150 nM HAO, 20 μM oxyHb, and 25 μM DCPIP in 1 mL of air-saturated 50 mM Na₂HPO₄, pH 8.0. Reactions were initiated by addition of an appropriate volume of a *NH₂OH* stock solution. The reactions were simultaneously monitored at 401 and 421 nm by a UV-vis absorption spectrometer. The amount of NO generated was determined via the change in absorption at 421 nm minus the change in absorbance at 401 nm and using the extinction coefficient of *ε*₄₂₁⁻₄₀₁ = 77.3 M⁻¹•cm⁻¹. The stoichiometry of *NH₂OH* to cyt *c* reduction was performed under identical reaction conditions except the oxyHb was omitted. Cyt *c* reduction was quantified as described above for the anaerobic experiments.

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