Nitrosomonas europaea cytochrome P460 is a direct link between nitrification and nitrous oxide emission

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Ammonia oxidizing bacteria (AOB) are major contributors to the emission of nitrous oxide (N₂O). It has been proposed that N₂O is produced by reduction of NO. Here, we report that the enzyme cytochrome (cyt) P460 from the AOB Nitrosomonas europaea converts hydroxylamine (NH₂OH) quantitatively to N₂O under anaerobic conditions. Previous literature reported that this enzyme oxidizes NH₂OH to nitrite (NO₂⁻) under aerobic conditions. Although we observe NO₂⁻ formation under aerobic conditions, its concentration is not stoichiometric with the NH₂OH concentration. By contrast, under anaerobic conditions, the enzyme uses 4 oxidizing equivalents (eq) to convert 2 eq of NH₂OH to N₂O. Enzyme kinetics coupled to UV/visible absorption and electron paramagnetic resonance (EPR) spectroscopy supports a mechanism in which an Fe₈–NH₂OH adduct of cyt P460 is oxidized to an (FeNO)₈ unit. This species subsequently undergoes nucleophilic attack by a second equivalent of NH₂OH, forming the N–N bond of N₂O during a bimolecular, rate-determining step. We propose that NO₂⁻ results when nitric oxide (NO) dissociates from the (FeNO)₈ intermediate and reacts with dioxygen. Thus, NO₂⁻ is not a direct product of cyt P460 activity. We hypothesize that the cyt P460 oxidation of NH₂OH contributes to NO and N₂O emissions from nitrifying microorganisms.

nitric oxide | nitrification | nitrous oxide | enzymology | bioinorganic chemistry

Nitrous oxide (N₂O) participates in ozone-layer depletion and possesses a global warming potential nearly 300-fold greater than carbon dioxide (1). Atmospheric N₂O concentrations have increased ~120% since the preindustrial era, largely due to the widespread use of fertilizers required to produce sustenance for humans and livestock. N₂O is a byproduct of the microbial metabolism of fertilizer components, including ammonia (NH₃) and nitrate (NO₃⁻); consequently, agricultural soils account for an estimated 60–75% of global N₂O emissions. The metabolic pathway by which microorganisms oxidize NH₃, nitrification, occurs in two phases, both of which are mediated by autotrophic microorganisms. In the first, NH₃-oxidizing bacteria (AOA) or archaea (AOA) oxidize NH₃ to nitrite (NO₂⁻). In the second, NO₂⁻ is subsequently oxidized to NO₃⁻ by NO₂⁻-oxidizing bacteria. NH₃-oxidizing microbes contribute substantially to global N₂O emissions, whereas NO₂⁻-oxidizing bacteria produce negligible N₂O (2, 3). AOB are proposed to emit N₂O either as a byproduct of the nitrification pathway or as a product of the nitrifier denitrification pathway (i.e., the reduction of NO₃⁻) (4–6).

Nitrification of NH₃ to NO₃⁻ occurs in two steps (7, 8). The first step is catalyzed by NH₃ monooxygenase, which uses copper (Cu) and dioxygen (O₂) to hydroxylate NH₃ to hydroxylamine (NH₂OH) (9). In AOB, the second step is thought to be the four-electron oxidation of NH₂OH to NO₃⁻ by NH₂OH oxidoreductase (HAO). HAO is a multitheme enzyme with eight c-type hemes per subunit: seven are electron transfer cofactors, and the eighth is the so-called P460 active site that contains a unique tyrosine cross-link to the heme ring. The enzyme (or enzymes) that AOA uses to oxidize NH₂OH is currently unknown (10).

AOB possess machinery for nitrifier denitrification that reduces NO₃⁻ to N₂O via a nitric oxide (NO) intermediate (11, 12). The archetypal AOB Nitrosomonas europaea possesses genes for a Cu-containing nitrite reductase (NirK) and a membrane-bound, heme-containing NO reductase (NorB). NirK reduces NO₃⁻ by one electron to NO, whereas NorB catalyzes the two-electron reduction of 2 eq of NO to N₂O. Nitrifier denitrification is thought to lead to the increased production of N₂O and NO by AOB in microaerophilic or anaerobic conditions (13). However, this pathway does not account for the total AOB N₂O emission under aerobic conditions.

Under aerobic conditions, N₂O emission from AOB is proposed to result from the incomplete oxidation of NH₂OH to either nitroxy (HNO) or NO. Two eq of HNO rapidly react to form N₂O (14), whereas NO is reduced by an NO reductase (12). In support of these hypotheses, both NO and N₂O have been observed to form during steady-state turnover of purified HAO under aerobic conditions (15). Although NorB could facilitate this N₂O production, a NorB knockout strain also produces N₂O at atmospheric O₂ concentration, consistent with the presence of an alternate N₂O-producing pathway (16).

In this study, we demonstrate that there indeed exists a direct enzymatic pathway from NH₂OH to N₂O, and that this pathway is mediated by cyt P460 (17), a constitutively expressed, soluble, periplasmic metalloenzyme originally isolated from N. europaea (18). N. europaea cyt P460 is a 36-kDa homodimeric protein in which each subunit bears a mono-His c-type heme with an N–C cross-link from the 13’ mesocarbon to the amine of Lys70 (19) (Fig. L4). Such cross-links alter porphyrin π-conjugation. Moreover, the heme P460 macrocycle exhibits significant ruffling, a common distortion mode for c-type hemes (20, 21). P460 hemes are named for the 460-nm Soret band observed in their ferrous state. The P460 center found in N. europaea HAO differs from cyt P460: it is doubly cross-linked by Tyr491 at the 5’ mesocarbon and an adjacent pyrrole α-carbon (Fig. 1B). Despite lacking homology to HAO (19, 22), the presence of a P460 cofactor in cyt P460 has implicated this enzyme in NH₂OH oxidation (23–25).

Significance

Nitrous oxide (N₂O) is a potent ozone-depleting greenhouse gas. This work identifies a means by which N₂O is generated during nitrification, or biological ammonia oxidation. Fertilizer use in agriculture stimulates nitrification, thus increasing the volume of N₂O emissions worldwide. The results presented herein will inform models and strategies toward optimized, sustainable agriculture. Moreover, these results highlight a rare example of biological N–N bond formation.
Cyt P460 was previously reported to oxidize NH$_2$OH to NO$_2^-$ (26), implicating all P460 centers in mediating four-electron oxidation of NH$_2$OH. However, an octaheme HAO-like protein from the anaerobic NH$_3$-oxidizing microorganism *Kuenenia stuttgartiensis* was shown to oxidize NH$_2$OH to NO instead of NO$_2^-$ (27). An X-ray crystal structure of the *K. stuttgartiensis* HAO-like enzyme features a tyrosine cross-linked active site and heme distribution that is identical to *N. europaea* HAO. The *K. stuttgartiensis* enzyme was proposed to yield NO due to the absence of a nearby tyrosine residue that is present in *N. europaea* HAO. The *N. europaea* cyt P460 active site also lacks the nearby tyrosine residue and is far more solvent-exposed (19). Herein, we show that N$_2$O is the enzymatic product of anaerobic NH$_2$OH oxidation by *N. europaea* cyt P460. We demonstrate that this pathway proceeds through an NH$_2$OH-bound form that is oxidized by three electrons to an Fe$^3+$NO species that then reacts with NH$_2$OH to form N$_2$O in the rate-determining step.

**Results and Discussion**

**Exclusive Conversion of NH$_2$OH to N$_2$O by Cyt P460 Under Anaerobic Conditions.** Consistent with previous work on the *Methylococcus capsulatus* cyt P460 (26), under aerobic conditions, cyt P460 reacts with NH$_2$OH in the presence of the oxidant phenazine methosulfate (PMS) to form NO$_2^-$, as detected by Griess diazotization assays ([SI Appendix, Fig. S1A](#)). However, the maximum stoichiometry achieved was 0.7 mol of NO$_2^-$ per mol of NH$_2$OH ([SI Appendix, Table S1](#)). Gas chromatography ([GC](#)) analysis reveals that the remainder of the NH$_2$OH is converted to N$_2$O (SI Appendix, Fig. S1A).

Under anaerobic conditions, no NO$_2^-$ is formed when cyt P460 is treated with NH$_2$OH and PMS. GC analysis ([SI Appendix, Fig. S1B](#)) reveals that in the presence of an oxidant (Ru(NH$_3$)$_5$Cl)$_3$, horse heart cyt c, or PMS), cyt P460 catalyzes the exclusive formation of N$_2$O from NH$_2$OH. In the absence of either enzyme or oxidant, only trace N$_2$O is formed ([SI Appendix, Fig. S1](#)). By monitoring the amount of N$_2$O produced under various NH$_2$OH or oxidant concentrations (Fig. 2A), a stoichiometry of 2 NH$_2$OH eq and 4 oxidizing eq producing 1 eq of N$_2$O was established (Eq. 1):

\[
2 \text{NH}_2\text{OH} \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} + 4 \text{e}^- + 4 \text{H}^+.
\]

**Steady-State Kinetics.** Steady-state activity assays of cyt P460 were performed under anaerobic conditions using a 2,6-dichlorophenolindophenol (DCPIP)/PMS coupled assay (23) and an N$_2$O-selective electrode. By monitoring the decay of the DCPIP absorbance at 605 nm ($\Delta A_{605}$), it was determined that 2 eq of DCPIP was reduced per 1 eq of N$_2$O produced under steady-state conditions ([SI Appendix, Fig. S2](#)). Because DCPIP is a two-electron oxidant, this stoichiometry is consistent with Eq. 1. The DCPIP/PMS assay therefore provides a convenient means of measuring steady-state N$_2$O production by cyt P460.

Steady-state activities of cyt P460 have been reported previously (23, 26), but to our knowledge, no studies have presented steady-state activity plots. Fig. 2B shows the steady-state activity plot exhibiting nonsaturating, linear behavior from 0.05 to 20 mM NH$_2$OH that spans turnover frequencies of 0.3–80 μM DCPIP consumed min$^{-1}$μM-enzyme$^{-1}$. Although this nonsaturating behavior precludes determination of $k_{cat}$ or $K_m$, the slope of the linear region suggests a $K_m$ of 5,000 M$^{-1}$min$^{-1}$ (28). However, the characterization of pathway intermediates suggests a multistep reaction mechanism that is inconsistent with classical Michaelis–Menten kinetics (vide infra).

![Fig. 1.](#) Ferric P460 cofactors in cyt P460 ([A, Protein Data Bank (PDB) ID code 2JE2](#)) and HAO ([B, PDB code 4N4N](#))

Characterization of Cyt P460 Fe$^{III}$$\cdot$NH$_2$OH. Recombinant expression and purification of cyt P460 was previously achieved by Elmore et al. (29). This method yields a British racing green protein with UV/visible absorption and electron paramagnetic resonance (EPR) spectra consistent with those reported for the enzyme isolated from *N. europaea* (18, 23, 30). The absorption spectrum of the as-isolated cyt P460 has a Soret band at 440 nm with a shoulder at 414 nm and Q-band maxima at 570 nm and 627 nm (Fig. 3). The corresponding EPR spectrum (Fig. 4A) is characteristic of an $S = \frac{5}{2}$ Fe$^{III}$, with g-values of 6.57, 5.09, and 1.97 ($E_g = 0.03$). The crystal structure of the as-isolated cyt P460 (19, 29) (Fig. 4A) shows phosphate ligated to the Fe center. These crystallization conditions used 2 M phosphate buffer; we expect that H$_2$O will occupy this site under our experimental conditions, and therefore assign the as-isolated protein as an Fe$^{III}$$\cdot$H$_2$O heme center.

The addition of NH$_2$OH to cyt P460 produces a species with a UV/visible absorption spectrum distinct from Fe$^{III}$$\cdot$H$_2$O (Fig. 3). Within the time of manual mixing, the 414-nm shoulder of the Fe$^{III}$$\cdot$H$_2$O spectrum disappears concomitant with a shift in the Soret band to 445 nm and broadening of the Q-bands. The simplest interpretation is that NH$_2$OH substitutes for H$_2$O at the Fe site (i.e., Fe$^{III}$$\cdot$NH$_2$OH). Cyt P460 was titrated with NH$_2$OH, and the resulting series of spectra ([SI Appendix, Fig. S3A](#)) show an isosbestic point at 438 nm, which suggests a one-step conversion from Fe$^{III}$$\cdot$H$_2$O to the putative Fe$^{III}$$\cdot$NH$_2$OH. To determine the NH$_2$OH dissociation constant ($K_{d(\text{NH}_2\text{OH})}$), the absorption at 414 nm ($A_{414}$) was plotted against NH$_2$OH concentration. Fitting the data to a hyperbolic binding curve resulted in a $K_{d(\text{NH}_2\text{OH})}$ of $9 \pm 1$ mM ([SI Appendix, Fig. S3A, Inset](#)).

Treatment of cyt P460 with 100 mM NH$_2$OH results in the disappearance of the $S = \frac{5}{2}$ Fe$^{III}$$\cdot$H$_2$O signal and the appearance of two rhombic $S = \frac{1}{2}$ EPR signals (Fig. 4B). The first...
signal has g-values of 2.75, 2.28, and 1.54, and is consistent with a low-spin Fe$^{III}$. The EPR spectra of cyt P460 treated with increasing concentrations of NH$_2$OH (SI Appendix, Fig. S3B) corroborate the $K_{(NH_2OH)}$ value determined with UV-vis spectrometry. The conversion from a high-spin to low-spin Fe$^{III}$ is consistent with the binding of NH$_2$OH to the Fe center; thus, we assign this $S = \frac{1}{2}$ as a rare example of a stable heme Fe$^{III}$-NH$_2$OH complex (31).

The second rhombic $S = \frac{1}{2}$ signal has g-values of 2.10, 2.02, and 2.01, and $^{14}$N hyperfine coupling values of 50 MHz, 57 MHz, and 45 MHz. This second signal represents less than 5% of the total spin as determined by spin quantitation and is consistent with a five-coordinate heme Fe$^{II}$-NO species (32), or {FeNO}$^-$ in Enemark–Feltham notation (33). This {FeNO}$^-$ species can be independently generated by treating cyt P460 with the HNO donor, disodium diazen-1-ium-1,2,2 triolate (Na$_2$N$_2$O$_3$, Angell’s salt). This species is stable in the presence of NH$_2$OH or oxidant (SI Appendix, Fig. S4). The low yield observed in the EPR spectrum and the lack of reactivity suggest that the {FeNO}$^-$ is an off-path product and does not contribute to the productive N$_2$O-generating cyt P460 pathway. At present, we are unsure why this oxidized species appears in the absence of O$_2$, oxidant, or NO. One possibility is that the samples are exposed to a small amount of O$_2$ during freezing, which would oxidize Fe$^{III}$-NH$_2$OH to {FeNO}$^-$. The persistence of the 455-nm intermediate in the presence of excess oxidant suggests that, under turnover conditions, the decay of the 455-nm intermediate is the rate-determining step. The increase in decay rate at higher NH$_2$OH concentrations implies a bimolecular reaction between the 455-nm intermediate and NH$_2$OH. To confirm this relationship, the formation and decay of the 455-nm intermediate at various NH$_2$OH concentrations were monitored (SI Appendix, Fig. S8A). The sum of two exponentials (SI Appendix, Eq. S1) was fit to the $A_{455}$ traces, providing observed rate constants ($k_{obs}$) for both the formation [$k_{obs(1)}$] and decay [$k_{obs(2)}$] of the 455-nm intermediate. At higher NH$_2$OH concentrations, $k_{obs(1)}$ was too fast to fit accurately, but, qualitatively, it increases with increasing NH$_2$OH concentration. The $k_{obs(2)}$ parameter showed a linear dependence on NH$_2$OH, consistent with a bimolecular reaction of NH$_2$OH and the 455-nm intermediate. A linear fit to a plot of $k_{obs}$ versus NH$_2$OH concentration provided a second-order rate constant of 0.07 mM$^{-1}$min$^{-1}$ (SI Appendix, Fig. S8B).

The 455-nm intermediate was trapped for EPR characterization by freezing the reaction of 170 μM cyt P460 with 2 mM NH$_2$OH and 2 mM DCPIP within 1 min. The EPR spectrum of the trapped 455-nm intermediate lacks the signals associated with Fe$^{III}$-H$_2$O and Fe$^{III}$-NH$_2$OH, suggesting that the protein is completely converted to the 455-nm intermediate or to another EPR-silent species (Fig. 4C). The only EPR signal observed is an $S = \frac{1}{2}$ signal with hyperfine structure that can be attributed to Mn$^{2+}$ contamination in the protein sample. The lack of an Fe-based EPR signal implies that the 455-nm intermediate is either a diamagnetic or non-Kramer’s (integer spin greater than 0) species.

**Fig. 4.** EPR spectra of species on the proposed cyt P460 NH$_2$OH oxidase pathway. Cyt P460 at 170 μM (A) was treated with 100 mM NH$_2$OH (B), with 2 mM NH$_2$OH and 2 mM DCPIP (C), or with 45 mM NH$_2$OH and 2 mM DCPIP (D) and incubated for 10 min. Black traces are spectra simulated with the parameters listed in SI Appendix, Table S2. Spectra were collected at 10 K and 633 μW or at 20 K and 63 μW. A 5% impurity of an {FeNO}$^-$ species is indicated by a single asterisk. An Mn$^{2+}$ EPR signal is indicated by double asterisks. dy/gh, derivative of magnetic susceptibility vs. magnetic induction.
The decay product was prepared by treating 150 μM cyt P460 with 45 mM NH3·OH and 2 mM DCPIP, followed by incubation for 10 min at room temperature. The EPR spectrum of this sample shows signals for FeII–NH3·OH and (FeNO)2−/3 (Fig. 4D). The combined spins account for the total Fe concentration in the sample, indicating the quantitative conversion of the 455-nm intermediate to either FeIII–NH3·OH (80 μM) or (FeNO)2+ (70 μM), following the depletion of oxidant.

**Identification of the 455-nm Intermediate as an (FeNO)2+.** The need for oxidant to form the 455-nm intermediate suggests that it is an oxidized form of FeIII–NH3·OH. Bari and coworkers (34) have postulated several oxidized species in their proposed mechanism of NH3·OH oxidation by HAO, including an (FeNO)3−, an (FeNO)2−, and a ferrous-nitrous acid (FeII–ONOH) species. Other possibilities include a ferric hydroxylamine radical (FeIII–NH2·OH), which has been proposed as an intermediate in the enzymatic pathway of P450 NO reductase (35, 36) and FeIII–ONOO·, which has been characterized in only one biological system, the myoglobin–HNO complex (37, 38). Finally, because NO2 is the product of NH3·OH oxidation by cyt P460 under our experimental conditions, an Fe–N2O complex is also possible. We propose that our experimental data are most consistent with assignment of the 455-nm intermediate as an (FeNO)2− species (vide infra).

The number of oxidizing equivalents required to convert FeIII–NH3·OH to the 455-nm intermediate was determined by treating cyt P460 with substoichiometric NH3·OH and excess cyt (SI Appendix, Fig. S9). The reduction of cyt c was monitored by the increase in absorbance at 550 nm (ε550 = 19,600 M−1 cm−1 (39). These experiments show that 3 electrons are required to oxidize FeII–NH3·OH to the 455-nm intermediate, suggesting an (FeNO)2− or FeII–ONOO· species (33). Consistent with this observation, treatment of FeII–NH3·OH with NO supplied either as the gas or via the NO donor PROLI-NONOate [1-hydroxy-NNO-azoxy]-l-proline] produces an EPR-silent species with an absorption spectrum identical to the absorption spectrum of the 455-nm intermediate (Fig. 3). The lack of an EPR signal precludes assignment of the 455-nm intermediate as an (FeNO)3−, because these species typically exhibit S = 1/2 EPR signals (32). The 455-nm intermediate generated in this way persists for ca. 1 h and is stable to excess DCPIP (SI Appendix, Fig. S10). This lack of reactivity is consistent with the hypothesis of Bari and coworkers (34) that the hydrolysis of an (Fe–NO)3− generates an FeII–ONOO adduct that is poised for proton-coupled, one-electron oxidation to NO3− because cyt P460 does not produce NO3− under anaerobic conditions. Taken together, these data support the assignment of the 455-nm intermediate as an (FeNO)2−.

The stability of the (FeNO)2− intermediate in the absence of NH3·OH and the NH3·OH dependence of its decay suggests that (FeNO)2− reacts with NH3·OH to form NO2. A so-called shunted (FeNO)2− species was prepared by adding 2 eq of NO supplied via PROLI-NONOate to cyt P460. The addition of 2 mM NH3·OH results in the decay of (FeNO)2− to FeIII–NH3·OH (SI Appendix, Fig. S11). Consumption of the shunted (FeNO)2− exhibits the same NH3·OH concentration dependence, 0.07 ± 0.01 mM−1 min−1, as the (FeNO)2− that accumulates under anaerobic conditions.

To confirm the production of NO2 in the above reaction, cyt P460 was treated with varying concentrations of NO and excess NH3·OH. The amount of NO2 produced was measured by GC/mass spectrometry (MS). There is a clear 1:1 stoichiometry of N2O produced versus NO added (SI Appendix, Fig. S12A). This result is consistent with NO binding to cyt P460 to form the (FeNO)2− intermediate and subsequent reaction with NH3·OH to form NO2. The same experiment was performed with NO and isotopically labeled 15NH3·OH. The mass shift from 44 atomic mass units (amu) to 45 amu (SI Appendix, Fig. S12B) in the presence of cyt P460 clearly demonstrates that NO is coupled to NH3·OH via cyt P460. The reaction of NO with NH3·OH in the absence of cyt P460 does not result in N2O production.

**Mechanism for N2O Formation from NH3·OH by Cyt P460.** Given the above results, we propose the mechanism described in Fig. 5 for the NH3·OH oxidase activity of cyt P460. The catalytic cycle initiates from the S = 1/2 FeIII–OH2, which binds NH3·OH to form an S = 1/2 FeIII–NH3·OH species that is stable in the absence of oxidant. In the presence of oxidant, FeIII–NH3·OH is rapidly oxidized by 3 electrons to the EPR-silent (FeNO)2−, which undergoes nucleophilic attack by a second equivalent of NH3·OH to yield N2O and H2O. We propose that the Fe-containing product of this reaction is an FeII species that is rapidly oxidized and converted to the starting FeIII–OH2 species.

Several other intermediates can be envisioned in the conversion of FeIII–OH2 to (FeNO)2−. One-electron (K3[Fe(CN)6], CuI–azurin, and [Ru(NH3)6]Cl2) and two-electron (DCPIP and PMS) oxidants can access (FeNO)2−, which suggests that rapid, subsequent one-electron oxidation steps occur in the conversion of FeIII–NH3·OH to (FeNO)2−. Although (FeNO)2− appeared under certain conditions (i.e., after complete consumption of oxidant), it is not a catalytically competent intermediate. The EPR spectrum of this species is consistent with a five-coordinate (FeNO)2− (32), however, a six-coordinate (FeNO)3− could be on the reaction pathway. Other possible one-electron oxidized intermediates include FeIII–NH2·OH, FeIII–ONOH, or (Fe(NH)(HO))3− (40). To date, we have found no evidence to exclude any of these possibilities, and the characterization of these possible intermediates is the subject of future investigations in our laboratory.

The rate-determining step of the anaerobic oxidation of NH3·OH by cyt P460 is the bimolecular reaction of NH3·OH with (FeNO)2− that results in N2O formation. The intensities of the UV/visible absorption spectral features of FeII–OH2 treated with NO identical to the intensities observed for the (FeNO)2− intermediate formed using NH3·OH and oxidant (Fig. 3). Combined with the lack of Fe-based EPR signals in these samples, these data suggest that under both conditions, all Fe sites are quantitatively converted to the (FeNO)2− species. This quantitative accumulation is consistent with the nucleophilic attack by NH3·OH being the rate-determining step of the catalytic cycle. There is precedent for NH3·OH reacting with [Fe(CN)5(NO)]2−, a classic example of an (FeNO)2−, to form NO with stoichiometry matching the stoichiometry above for cyt P460 (41).

We propose that NH3·OH reacts with (FeNO)2− to form an FeII species, which is rapidly oxidized to return the enzyme to FeIII–OH2. This proposal is based on the precedent that [Fe(CN)5NO]2− reacts with NH3·OH to form [Fe(CN)5(OH)2]3−. However, the characteristic 463-nm UV/visible absorption peak of FeII cyt P460 was never observed in our experiments as an intermediate or an end product. The FeII cyt P460 is expected to react rapidly with other species present during turnover (oxidant, NH3·OH, or NO), thereby precluding its observation (vide infra). The reaction of FeIII cyt P460 with NO produces the same inactive (FeNO)2− species observed when FeIII–OH2 is treated with HNO (SI Appendix, Fig. S4). FeII cyt P460 reacts with NH3·OH to form FeIII–OH2 via an intermediate with a UV/visible absorption feature at 663 nm that has not been observed in any of our other experiments (SI Appendix, Fig. S13). This 663-nm intermediate forms and disappears within 3 s; assignment of this 663-nm intermediate will require rapid-mixing techniques. Critically, the reaction of FeIII cyt P460 with NH3·OH does not inactivate the enzyme.

Stopped-flow UV/visible absorption spectroscopy provided insight into why inactivation of the enzyme by NO is avoided during turnover (SI Appendix, Fig. S14). FeII cyt P460 is quantitatively oxidized to FeIII–OH2 by [Ru(NH3)6]Cl2 within the time of mixing.
(<3 ms), placing a lower limit on \( k_{\text{obs}} \) of 1.100 s\(^{-1}\). The reactions with NH\(_3\)OH and NO exhibit \( k_{\text{obs}} \) of 1.3 s\(^{-1}\) and 2.8 s\(^{-1}\), respectively. These results show that oxidation of Fe\(^{III}\) cyt P460 to Fe\(^{III-}\)OH\(_2\) is much faster than the reaction with NH\(_3\)OH or NO, thereby avoiding the production of inactive \( \{\text{FeNO}\}\)\(^{-}\). After the oxidant is completely consumed, Fe\(^{III}\) cyt P460 will react with either NH\(_3\)OH or NO. The reaction of Fe\(^{III}\) cyt P460 with NH\(_3\)OH forms Fe\(^{III-}\)OH\(_2\) or, in the case of high NH\(_3\)OH concentration, Fe\(^{III-}\)NO\(_2\), whereas the reaction of Fe\(^{II}\) cyt P460 with NO results in the formation of inactive \( \{\text{FeNO}\}\)\(^{-}\). Therefore, these reactions with Fe\(^{II}\) cyt P460 likely account for the products observed in the EPR spectrum of the \( \{\text{FeNO}\}\)\(^{-}\) intermediate decay sample (Fig. 5D).

The King–Altman method (42) was used to derive a steady-state equation based on the minimal mechanism shown in Fig. 5. In this simplified model, NH\(_3\)OH binding is treated as reversible, with association \( k_1 \) and dissociation \( k_{-1} \) rate constants. Both the Fe\(^{III-}\)NH\(_3\)OH oxidation \( k_2 \) and the subsequent reaction of \( \{\text{FeNO}\}\)\(^{-}\) with NH\(_3\)OH \( k_3 \) are assumed to be irreversible. The resulting model (Eqs. 2–4) indicates that \( k_2 \)\( [\text{ox}] \) influences both \( k_{\text{cat}} \) and \( K_m \):

\[
\frac{\text{velocity}}{[\text{cyt P460}]_0} = \frac{k_2[\text{ox}][\text{NH}_3\text{OH}]_0}{K_m + [\text{NH}_3\text{OH}]_0} \tag{2}
\]

\[
K_m = \frac{k_{-1}k_3 + k_2[\text{ox}](k_1 + k_3)}{k_2k_3} \tag{3}
\]

\[
k_{\text{cat}} = k_2[\text{ox}] \tag{4}
\]

There are two limiting regimes of the derived steady-state model in which either \( k_2[\text{ox}] \) or \( k_2[\text{ox}](k_1 + k_3) \) dominates the numerator of the \( K_m \) term. In the former, \( K_m \) should resemble \( K_{\text{cat}}/[\text{NH}_3\text{OH}]_0 \) which was determined to be 9 mM. The steady-state activity plot lacks any clear curve despite the inclusion of activity measurements in up to 20 mM NH\(_3\)OH. This lack of curve suggests that the second regime, which is highly dependent on \( k_2[\text{ox}] \), contributes substantially under the assay conditions, thereby increasing the observed \( K_m \). Currently, we have an estimate of only \( k_1 \) (0.07 mM\(^{-1}\) min\(^{-1}\)). Given the assumptions made, we attribute the absence of saturation in the steady-state activity plot to a large \( K_m \) which suggests a large second-order rate constant for Fe\(^{III-}\)NH\(_3\)OH oxidation.

\( \text{N. europaea} \) tolerates at least 10 mM NH\(_3\)OH without showing inhibited O\(_2\) uptake (43). Under the steady-state conditions studied, we estimate the rate of N\(_2\)O production at 10 mM NH\(_3\)OH to be 23 mM N\(_2\)O min\(^{-1}\). The properties of the oxidant heavily influence \( k_2 \) and, by extension, the rate at which N\(_2\)O is produced by cyt P460. Thus, lacking the identity of the native electron transfer partner, we cannot verify that this rate is the physiologic rate.

The observation that NO binding accesses a shunt in the cyt P460 catalytic pathway provides evidence for the hypothesis that cyt P460 contributes to NO detoxification in the cell (11). Furthermore, if NO binding is reversible, there may be an alternate cyt P460 NH\(_3\)OH oxidation pathway that results in NO as the product. At low NH\(_3\)OH concentrations, the bimolecular reaction with \( \{\text{FeNO}\}\)\(^{-}\) should be slow and NO dissociation may outpace N\(_2\)O formation. This alternate NO-forming pathway could also be responsible for the observation of NO\(_2\) formation under aerobic conditions, as NO reacts with O\(_2\) in aqueous solution to form N\(_2\)O. To test this hypothesis, \( \{\text{FeNO}\}\)\(^{-}\) was generated by treating Fe\(^{III-}\)OH\(_2\) with 1 eq of NO generated from PROLI NONOate. Exposure of the resulting \( \{\text{FeNO}\}\)\(^{-}\) species to O\(_2\) results in the return to Fe\(^{III-}\)OH\(_2\) and generation of N\(_2\)O (SI Appendix, Fig. S15). We therefore propose that the NO\(_2\) observed as an aerobic product is not directly formed by cyt P460, but rather is a byproduct resulting from NO dissociation from the \( \{\text{FeNO}\}\)\(^{-}\) intermediate. A detailed kinetic analysis will determine the partitioning between the N\(_2\)O\(^{-}\)- and N\(_2\)O-forming pathways.

Cell-free extracts of \( \text{N. europaea} \) were previously shown to oxidize NH\(_3\)OH to N\(_2\)O and NO without formation of NO\(_2\) (44). Furthermore, purified HAO was shown to react with NH\(_3\)OH and PMS or DCPIP under aerobic conditions to form a mixture of products, including NO, N\(_2\)O, NO\(_2\), and NO\(_3\) (15). Additionally, a stable \( \{\text{FeNO}\}\)\(^{-}\) species on the H\(_{2}\)AO P460 cofactor was observed after Fe\(^{III-}\)OH\(_2\) was allowed to react with NO in the absence of NH\(_3\)OH (25). Given this evidence, our results, and the recent report that an HAO-like protein oxidizes NH\(_3\)OH to NO (27), we suggest that the biochemistry of HAO be revisited to determine if NO\(_2\) is indeed its terminal, enzymatic product.

**Outlook: Environmental Consequences.** AOB are major contributors to N\(_2\)O emissions from wastewater treatment plants (WWTPs), at which \( \text{N. europaea} \) is the dominant AOB species (45). There are two proposed methods for N\(_2\)O emission from AOB: The first is as a product in the nitrifier denitrification pathway, and the second is as a byproduct in incomplete NH\(_3\)OH oxidation. The results of our study demonstrate that the constitutively expressed cyt P460 is a direct link between NH\(_3\)OH oxidation and the emission of N\(_2\)O from \( \text{N. europaea} \), thus establishing an alternative oxidative pathway to N\(_2\)O. We have established through GC and kinetic analysis a strict stoichiometry of 2 eq of NH\(_3\)OH and 4 oxidizing equivalents to produce 1 eq of N\(_2\)O. Due to the reactivity of NH\(_3\)OH with biological electrophiles, \( \text{N. europaea} \) likely has a detoxifying role at high NH\(_3\)OH concentrations. Previously, cyt P460 was thought to oxidize NH\(_3\)OH and NO to NO\(_2\) (46). However, we suggest herein an alternative role in NH\(_3\)OH detoxification through the production of N\(_2\)O (vide supra) (11).

We have demonstrated the production of N\(_2\)O from NH\(_3\)OH oxidation by cyt P460 under anaerobic conditions, thereby establishing a direct enzymatic link between nitrification and N\(_2\)O formation via NH\(_3\)OH. The identification of the source of N\(_2\)O emission helps to explain the production of N\(_2\)O in WWTPs under conditions of low dissolved O\(_2\) concentrations and high NH\(_3\) concentrations, conditions in which the nitrifier denitrification pathway would not dominate (47). The influx of high concentrations of NH\(_3\) in WWTPs causes an increase in NH\(_3\) oxidation rates, which, in turn, increases intracellular NH\(_3\)OH concentration (4). Studies using activated sludge from WWTPs with high concentrations of NH\(_3\) have shown that transitioning from an aerobic environment to an anoxic environment increases the amount of N\(_2\)O released. Isotopic labeling studies show that the N\(_2\)O produced under these conditions originates from the NH\(_3\)OH oxidation pathway rather than the nitrifier denitrification pathway (48). Our study pinpoints direct N\(_2\)O production via an enzymatic, anaerobic NH\(_3\)OH oxidation mechanism. Identifying...
the chemical source of this emission should aid in the design and operation of WWTPs with curtailed N₂O emission.

Experimental Procedures

Materials. Cyt P460 was prepared as previously described (29). PROLI-NONOate was purchased from Cayman Chemicals. DCPIP was purchased from Alfa Aesar, PMS from Bean Town Chemical, and NH₂OH·HCl from TCI Chemicals.

Spectroscopy. X-band (9.40-GHz) EPR spectra were obtained using a Bruker Elexys-II spectrometer equipped with a liquid He cryostat maintained at 10 or 20 °K. Temperatures and microwave powers are listed in the figure legends. Unidirectional absorption spectra were obtained using a Cary 60 UV-visible absorption spectrometer.

N₂O Quantification. All reactions were prepared and sealed in 5-mL headspace GC vials (Wheaton). The final N₂O concentration was analyzed with GC (Agilent), GC/MS (GC-MATE II; JEOL), or an N₂O microsensor housed within a septum-piercing needle (Unisense). For GC experiments, the headspace was inserted through the septum and into the solution. Calibration standards for all experiments were made either by diluting an N₂O-saturated solution into water or by decomposing Na₂N₂O₅ in Hesper buffer, pH 8.0, in a sealed headspace vial.

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