Structural Basis of Biological NO Generation by Octaheme Oxidoreductases

Wouter J. Maalcke, Andreas Dietl, Sophie J. Marriott, Julea N. Butt, Mike S. M. Jetten, Jan T. Keltjens, Thomas R. M. Barends, and Boran Kartal

Background: Multiheme proteins have crucial roles in diverse nitrogen cycle processes.

Results: The kustc1061 octaheme protein from anaerobic ammonium-oxidizing (anammox) bacteria specifically oxidizes hydroxylamine to NO.

Conclusion: Enzyme specificity derives from subtle amino acid changes near the P460 catalytic heme.

Significance: The presence of kustc1061 homologs in anammox and other bacteria enables the detoxification of hydroxylamine, thereby generating NO for respiratory purposes.

Nitric oxide is an important molecule in all domains of life with significant biological functions in both pro- and eukaryotes. Anaerobic ammonium-oxidizing (anammox) bacteria that contribute substantially to the release of fixed nitrogen into the atmosphere use the oxidizing power of NO to activate inert ammonium into hydrazine (N₂H₄). Here, we describe an enzyme from the anammox bacterium Kuenenia stuttgartiensis that uses a novel pathway to make NO from hydroxylamine. This new enzyme is related to octaheme hydroxylamine oxidoreductase, a key protein in aerobic ammonium-oxidizing bacteria. By a multiphasic approach including the determination of the crystal structure of the K. stuttgartiensis enzyme at 1.8 Å resolution and refinement and reassessment of the hydroxylamine oxidoreductase structure from Nitrosomonas europaea, both in the presence and absence of their substrates, we propose a model for NO formation by the K. stuttgartiensis enzyme. Our results expand the understanding of the functions that the widespread family of octaheme proteins have.

Nitric oxide has a multitude of roles in cell-cell communication, in microbial defense, and as a metabolite in nitrogen cycle microorganisms, where it is an intermediate in the reduction and oxidation of inorganic nitrogen compounds (1–7). Anaerobic ammonium-oxidizing (anammox) bacteria use the oxidizing power of NO to activate inert ammonium into hydrazine (N₂H₄) (8). In all these processes, NO is synthesized by specific enzymes. Here, we describe an enzyme previously (8) identified as kustc1061 from the anammox bacterium Kuenenia stuttgartiensis that makes NO from hydroxylamine (NH₂OH) (Equation 1). This enzyme is related to hydroxylamine oxidoreductase (HAO) of aerobic ammonium-oxidizing bacteria (9). In aerobic ammonium-oxidizing bacteria, HAO catalyzes the oxidation of hydroxylamine to nitrite (NO₂⁻), the second step in the aerobic oxidation of ammonium (Equation 2). Several properties of this enzyme have been elucidated including the determination of a crystal structure of the Nitrosomonas europaea protein (NeHAO) (9–13). Each subunit of the homotrimeric NeHAO harbors eight c-type hemes for catalysis and electron transfer, which are involved in the oxidation of its substrate. A distinguishing feature of NeHAO is the covalent attachment of the catalytic heme (fourth) of one subunit to a tyrosine residue (Tyr₄⁹¹) of the adjacent subunit in the trimer (11, 13).

\[ \text{NH}_2\text{OH} \rightarrow \text{NO} + 3\text{H}^+ + 3e^- \quad (E'_o = -0.030 \text{ V}) \]  
(Eq. 1)

\[ \text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 5\text{H}^+ + 4e^- \quad (E'_o = +0.065 \text{ V}) \]  
(Eq. 2)

HAO-like proteins are not unique to aerobic ammonium-oxidizing bacteria. A BlastP search reveals hundreds of octaheme proteins from a wide range of microorganisms that are homologous to NeHAO. Anammox bacteria, such as K. stuttgartiensis, which were shown to contribute substantially to nitrogen release into the atmosphere (14), harbor no less than...
10 divergent HAO paralogs. In fact, one of these (kustc0694 in *K. stuttgartiensis*) is dedicated to the oxidation of hydrazine, the *N₂*-forming reaction in these organisms (8). Octaheme proteins from other organisms reduce nitrite to ammonium (15, 16). Apparently, various types of HAO are capable of taking the nitrogen atom through all its oxidation states between −3 (ammonium) and +3 (nitrite), both in oxidative and reductive directions. The presence of the tyrosine-dependent covalent linkage between different subunits seems to be a distinctive feature in governing reactions into an oxidative direction (17). These diverse catalytic potentials could very well make HAO-like proteins central but thus far overlooked catalysts in biogeochemical nitrogen cycle processes. In addition to the primary question of which reactions are performed by various HAO-related proteins, an even more intriguing issue is how these variants are tuned to their specific functions.

Presently, it is unknown which structural features determine the differences in reaction specificities of HAO-like proteins. Furthermore, these differences cannot be explained by sequence analyses and structural modeling, which are hampered by the lack of structural and biochemical information. To address these questions, we studied the catalytic and redox properties of the HAO-like protein kustc1061 of *K. stuttgartiensis*. Further, we determined the high resolution structure of both the as-isolated kustc1061 and crystals soaked with substrates. In parallel and independent from a recent refinement (12, 13), we reassessed the structure of NeHAO not only in the absence but also in presence of substrates. The results of this study shed light on determinants in a widespread group of homologs about which very little is known at the biochemical level.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification and Identification—**Kustc1061 was purified from a *K. stuttgartiensis* enrichment culture (95% pure) that was grown continuously as planktonic cells in a 10-liter membrane bioreactor using methods described by Kartal et al. (18). Purification typically used 4 liters of cells (A₆₀₀ = 1) that were collected by centrifugation (8,000 × g, 15 min). This and all following steps, except for FPLC, were done at 4 °C. Pelleted cells were resuspended in 5 ml of 20 mM potassium Pi buffer (pH 7) and broken by three subsequent passages through a French pressure cell operating at 138 MPa. The lysate was incubated with 1% deoxycholate (w/v) on a rotating incubator (20 rpm) for 1 h. After incubation, cell debris was removed by centrifugation (3,000 × g, 15 min), and the supernatant was subjected to ultracentrifugation (150,000 × g, 1 h) in a Discovery 100 ultracentrifuge equipped with a T-1270 rotor (Sorvall, Newton, CT) to pellet cell membranes. The deep red dark supernatant represented the crude extract. Kustc1061 was brought to homogeneity in a two-step FPLC procedure using an Äkta purifier (GE Healthcare). Columns were eluted by isocratic washing with 200 mM NaCl in the Tris-HCl buffer. This fraction was desalted in the above potassium Pi buffer and concentrated to 5 ml using 100-kDa molecular mass cutoff spin filters (Vivaship 20; Sartorius Ste dim Biotech, Aubagne, France). The concentrated fraction was loaded onto 10-ml column packed with Ceramic Hydroxyapatite (Bio-Rad) that had been equilibrated by washing with five column volumes of potassium Pi buffer. After application of the sample, the column was eluted with a 120-ml linear gradient of 20–500 mM potassium Pi buffer (pH 7). Hydroxylamine oxidizing activity eluted as a prominent bright red symmetrical peak at 80 mM potassium Pi buffer (pH 7). This peak (12 ml) was collected, desalted in 20 mM potassium Pi buffer (pH 7), and concentrated as above to ~2.8 mg of protein ml⁻¹. Purity was checked by nondenaturating PAGE and SDS-PAGE (19). The identity of kustc1061 was established by MALDI-TOF analysis (see below). By the procedure outlined, kustc1061 was purified 5.3-fold in 31% yield. Both the yield and purification factor will likely be underestimates because crude extract contained substantial hydroxylamine oxidizing activity derived from hydrazine synthase (8).

*N. europaea* ATCC 19718 was cultured, and NeHAO was purified essentially as described by Hooper and Nason (20). 110 liters of a *N. europaea* culture were grown at room temperature in mineral medium (ATCC medium 2265) in 20-liter carboys, which were vigorously sparged with air. The pH of the medium was regularly adjusted to 8 by adding 20% (w/v) Na₂CO₃. The cells were harvested after 6 days (12 g of wet weight), frozen in liquid nitrogen, and stored at −80 °C. The cells were resuspended in 50 mM Tris-HCl (pH 7.5; buffer A) and disrupted by sonication, and the cell-free extract was fractionated by ammonium sulfate precipitation following by subsequent DEAE-Sepharose (Merck) and ceramic hydroxyapatite (Bio-Rad) liquid chromatography. After exchange to buffer A using a PD-10 desalting column (GE Healthcare), the 35–70% ammonium sulfate fraction was loaded onto a 30-ml DEAE-Sepharose column, which was eluted with a linear gradient of 0–400 mM NaCl in buffer A. The red fractions eluting at 200 mM NaCl were combined, buffer-exchanged to 20 mM potassium Pi buffer (pH 7.5), and loaded onto a 5-ml hydroxyapatite column, which was eluted with a gradient of 20–500 mM potassium phosphate (pH 7.5). NeHAO and Ne1300 co-eluted at ~250 mM potassium phosphate. These fractions were concentrated and buffer-exchanged to 25 mM HEPES-KOH (pH 7.5) containing 25 mM KCl. The identity of the proteins was confirmed by MALDI-TOF mass spectrometry after tryptic digest from SDS-PAGE gel slices.

**Analytical Ultracentrifugation—**Sedimentation velocity and equilibrium ultracentrifugation was performed in a Beckman XL-I Proteomelab ultracentrifuge, using 1.2-cm path length cells at 20 °C. The protein was dissolved in 25 mM HEPES-KOH (pH 7.5), 25 mM KCl to an absorbance at 280 nm of less than 0.5 (for 1.2 cm path length). The speeds used were 9.000 and 12.000 rpm in an An60Ti rotor. Equilibrium data were analyzed using SEDFIT (21).

**Spectrophotometric Enzyme Assays—**By routine, reactions were followed at 37 °C by measuring the reduction of bovine cytochrome *c* at 550 nm (Δεᵣᵣ = 19,600 M⁻¹ cm⁻¹) (22) in a Cary 50 spectrophotometer (Agilent, Santa Clara, CA). Reac-
### TABLE 1
Data collection, phasing, and refinement statistics

The values in parentheses are for highest resolution shell.

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<th></th>
<th>KustC1061 native HgCl₂</th>
<th>KustC1061 native initial model</th>
<th>KustC1061 native high resolution (4N4J)</th>
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<th>KustC1061 phenyl hydrazine soak (4N4L)</th>
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<td>P2₂,3</td>
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<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
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tion rates were determined from the initial linear portion of the progress curves employing the Cary 50 software package. For enzyme kinetics, initial reaction rates were fitted by nonlinear regression analysis (Origin 8.5.1; OriginLab Corporation, Northampton, MA) applying Michaelis-Menten equations.

Reaction mixtures (0.4 ml) in potassium Pi buffer contained 50 μM cytochrome c and an appropriate amount of enzyme. After following the absorption at 550 nm for 1 min, reactions were started by the addition of substrate (hydroxylamine or hydrazine) in the requested concentration from 100 μM anoxic stock solutions. Potential inhibitors (90 μM NO, 5 mM phenyl hydrazine) were added prior to the enzyme. An NO stock solution (0.9 mM) was prepared by sparging anoxic potassium Pi buffer with He/NO (50%:50%, v/v) in a butyl-rubber capped serum vial for 10 min. For assays in the direction of reduced cytochrome c oxidation, a stock solution was prepared by mixing 50 μM cytochrome c with 20 μM ascorbic acid, giving 40 μM reduced cytochrome c.

Nitrite reduction with reduced methyl viologen as electron donor was followed by recording methyl viologen oxidation at 600 nm (ε_{600} = 13,700 M⁻¹ cm⁻¹) (23). Reduced methyl viologen monocation radical was made by zinc reduction of a 1 mM stock solution of the oxidized compound (24). The preparation of reduced methyl viologen and of the assay mixtures and subsequent enzymatic reactions were carried out in an anaerobic glove box (97% cytochrome, 3% H₂; O₂ < 0.2 ppm). Reduced methyl viologen was added to an A_{500} of 1 (73 μM), and reactions were started by the addition of enzyme.

Spectrofluorometric NO Measurements—Nitric oxide production was measured by two different fluorescence-based methods. Fluorescence was recorded in 4-ml rubber-stoppered fluorescence cuvettes (Hellma, Müllheim, Germany) in a Cary Eclipse fluorometer.

**FIGURE 1. Hydroxylamine and hydrazine oxidation by kustc1061 and NeHAO.** A, cytochrome c reduction (50 μM) catalyzed by kustc1061 (200 ng) in the presence of 1.5 μM hydroxylamine (blue) or 1.5 μM hydrazine (red). The oxidation of hydroxylamine (1.5 μM) coupled to the reduction of cytochrome c (50 μM) by NeHAO (30 ng) is represented by the green curve. Reactions were followed and quantified by measuring the increase in the absorption at 550 nm (Δε_{550} = 19,600 M⁻¹ cm⁻¹) as the result of cytochrome c reduction. B, hydroxylamine oxidation (9 μM) by 1.4 μg of kustc1061 (blue) and hydrazine oxidation (9 μM) by 20 μg of kustc1061 (red) recorded by measuring the reduction of cytochrome c at 550 nm (Δε_{550} = 19,600 M⁻¹ cm⁻¹, right axis). C, NO production from hydroxylamine (8 μM) by kustc1061 (150 ng) in the presence of 50 μM cytochrome c. NO formation was followed by recording the increase in fluorescence as the result of the formation of the nitrosylated derivative of Cu(II)FL2E and was quantified from a calibration curve prepared from known NO stock solutions. D, hydroxylamine oxidation (2 μM) recorded by the increase in fluorescence resulting from the formation of a nitrosylated derivative of the NO-probe 4-amino-5-methylamino-2',7'-difluorofluorescein (black) and cytochrome c reduction under the same conditions (blue), showing a 1:3 stoichiometry of NO production versus cytochrome c reduction. E, accumulation of 35N₂ during [15N]hydrazine oxidation. 15N15N (open squares) was produced upon addition of 7.5 μM H^15N-15N H₂ to 2 ml of 20 mM potassium Pi buffer containing 1.3 μg of kustc1061 and 50 μM oxidized cytochrome c. The concentration of reduced cytochrome c was determined as in A. NO in C and D was quantified from calibration curves prepared from known NO stock solutions.
NO Generation by Octaheme Oxidoreductases

TABLE 2

Catalytic and structural properties of kustc1061 from K. stuttgartiensis, characterized orthologues from B. anammoxidans, and anamox strain KSU1 and hydroxylamine oxidoreductase from N. europaea

<table>
<thead>
<tr>
<th>Property</th>
<th>K. stuttgartiensis</th>
<th>B. anammoxidans</th>
<th>KSU-1</th>
<th>N. europaea</th>
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<tr>
<td>( V_{\text{max}} ) ( \text{NH}_2\text{OH} ) (( \mu \text{mol min}^{-1} \text{mg}^{-1} ))</td>
<td>4.8</td>
<td>21</td>
<td>9.6</td>
<td>28.5</td>
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<tr>
<td>( K_{m} ) ( \text{NH}_2\text{OH} ) (s(^{-1}))</td>
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<td>64</td>
<td>19</td>
<td>95</td>
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<tr>
<td>( K_{m} ) ( \text{NH}_2\text{OH} ) (( \mu \text{M} ))</td>
<td>4.4</td>
<td>26</td>
<td>33</td>
<td>3.6</td>
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<tr>
<td>( K_m/K_m ) ( \text{NH}_2\text{OH} ) (s(^{-1} \mu \text{M}^{-1} ))</td>
<td>3.4</td>
<td>2.5</td>
<td>0.57</td>
<td>26</td>
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<tr>
<td>( V_{\text{max}} ) ( \text{N}_2\text{H}_4 ) (( \mu \text{mol min}^{-1} \text{mg}^{-1} ))</td>
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<td>1</td>
<td>0.54</td>
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<td>1.1</td>
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<tr>
<td>( K_m ) ( \text{N}_2\text{H}_4 ) (( \mu \text{M} ))</td>
<td>54</td>
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<td>4</td>
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<td>( K_m/K_m ) ( \text{N}_2\text{H}_4 ) (s(^{-1} \mu \text{M}^{-1} ))</td>
<td>0.1</td>
<td>0.19</td>
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<td>( \text{NO}_2^- ) reduction (( \mu \text{mol min}^{-1} \text{mg}^{-1} ))</td>
<td>0.18(^a)</td>
<td>0.87(^a)</td>
<td>NR</td>
<td>6.18(^b)</td>
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<td>468</td>
<td>468</td>
<td>463</td>
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\(^a\) Specific activity with 0.1 mM nitrite.
\(^b\) Specific activity with 2.5 mM nitrite.
\(^c\) \( V_{\text{max}} \).

The identity of proteins was established by MALDI-TOF analysis of the trypsin-cleaved protein prepared from the gels as detailed elsewhere (8, 30). Spectra were evaluated by the Mascot Peptide Mass Fingerprint search program (Matrix Science, London, UK) against the K. stuttgartiensis and the N. europaea protein databases. In case of kustc1061, 12 of 43 predicted peptides were retrieved (Mowse identification score, >50) (supplementary Fig. S1).

**FIGURE 2. Electronic absorbance spectra of kustc1061.** Spectra are the following: as-isolated (fully oxidized) kustc1061 (80 \( \mu \)g ml\(^{-1} \)) (gray), dithionite-reduced kustc1061 (red), and the as-isolated enzyme incubated either with 100 \( \mu \)M hydroxylamine (dark blue) or with 100 \( \mu \)M hydrazine (light blue).

The identity of proteins was established by MALDI-TOF analysis of the trypsin-cleaved protein prepared from the gels as detailed elsewhere (8, 30). Spectra were evaluated by the Mascot Peptide Mass Fingerprint search program (Matrix Science, London, UK) against the K. stuttgartiensis and the N. europaea protein databases. In case of kustc1061, 12 of 43 predicted peptides were retrieved (Mowse identification score, >50) (supplementary Fig. S1).

**Electronic Absorbance Spectra—UV-visible spectra** were measured in 1-ml quartz cuvettes (path length, 1 cm). Samples contained 80 \( \mu \)g ml\(^{-1} \) of as-isolated fully oxidized kustc1061 in potassium \( \text{P}_4 \) buffer. Spectra of the reduced kustc1061 were recorded immediately after adding few crystals of sodium dithionite to the solution containing oxidized enzyme. Spectra in the presence of substrates were made after preincubating oxidized or reduced kustc1061 solution with 100 \( \mu \)M of the compound to be tested (5 \( \mu \)M for phenyl hydrazine). NO (final concentration, 90 \( \mu \)M) was added from a stock solution prepared as described above.

**Spectroelectrochemistry**—Heme reduction potentials were measured through changes in the electronic absorbance of kustc1061 that had been adsorbed on an optically transparent SnO\(_2\) electrode and then equilibrated at 4 °C at a series of defined potentials following the protocol previously published.
FIGURE 3. Protein film electrochemistry of kustc1061. A, full spectra of the oxidative titration from \(-490\) to \(+230\) mV versus standard hydrogen electrode (SHE) in 20-mV steps. B, table showing the \(E_m\) values (\(\pm 10\) mV) calculated for the successive oxidations and reductions of the c-type hemes in kustc1061 and the comparison to those reported for NeHAO (53, 54). Numbers in parentheses refer to the corresponding hemes in NeHAO. Note that the heme c midpoint redox potentials in kustc1061 are generally lower, but the method applied does not allow the assignment to specific hemes, except for the catalytic heme 4. C, spectral details of the Soret region. D, signals at 418 nm normalized to 1 and plotted against the applied potentials. E, spectral details of the heme c-\(\alpha\) and -\(\beta\) bands. F, signals at 551 nm normalized to 1 and plotted against the applied potentials. G, spectral details of the P460 region. H, signals at 468 nm normalized to 1 and plotted against the applied potentials. Squares in D, F, and H represent experimental values. Solid lines in these graphs are Nernstian fits. The data in D and F were well described by the sum of seven Nernstian contributions from isolated, single-electron \((n = 1)\) redox centers having the \(E_m\) values listed in B (all \(\pm 10\) mV). It was assumed that P460 spectral properties did not interfere significantly with the spectral changes of the Soret (C) and \(\alpha\) and \(\beta\) bands of the His/His ligated hemes; clear isosbestic points at 435 nm and at 505 and 561 nm, respectively, supported this assumption. The variation in absorbance at 468 nm had contributions from redox transformation of the P460 co-factor in addition to those of low spin His/His ligated hemes. The changes in absorbance caused by the P460 co-factor were readily identified through their larger magnitude and the appearance of a peak at low potential with an isosbestic point at 455 nm. The plot of normalized \(A_{468\ \text{nm}} - A_{455\ \text{nm}}\) was well described by a fit to the Nernst equation for an \(n = 1\) center with \(E_m = -300\) mV.
NO Generation by Octaheme Oxidoreductases

The electrode was composed of a mesoporous, nanocrystalline layer of SnO₂ coated on a glass slide (32). The electrode (∼0.7-cm² geometric area) was covered with 5 μL of 3.5 mg ml⁻¹ kustc1061 in potassium P₃ buffer and incubated on ice for 10 min. After removal of excess protein solution, the electrode was rinsed with 50 mM MOPS buffer (pH 7) and placed in an anaerobic 3-ml quartz cuvette (path length, 1 cm) filled with 50 mM MOPS buffer (pH 7). A platinum wire and an AgCl-coated silver wire served as counter and reference (Eₐ = +400 mV versus standard hydrogen electrode) electrodes, respectively.

The cuvette containing the kustc1061-coated SnO₂ electrode was placed in a spectrophotometer (V650; Jasco Analytical Instruments, Easton, MD) and connected to a potentiostat (PGSTAT20; Autolab, Utrecht, The Netherlands). To minimize the spectral contribution from scattering by the SnO₂ particles, a cuvette containing a SnO₂ electrode that had not been exposed to kustc1061 was placed in the reference beam of the spectrometer during measurements. UV-visible spectra were recorded after setting the potential applied to the kustc1061-coated electrode to the desired value and allowing the protein to equilibrate with that potential. Voltage-dependent spectral changes were fully reproducible for at least five cycles between −600 and +400 mV (20 mV steps) in reductive and oxidative directions. Changes in absorbance at 551 and 418 nm derived from the heme directions. Changes in absorbance at 551 and 418 nm derived from the heme and 30% (w/v) sucrose and then flash-cooled in liquid cryoprotected in reservoir solution with 25% (v/v) ethylene glycol.

Another, cubic crystal form diffracting to much higher resolution was observed upon annealing (33, 34) the flash-cooled crystals by interrupting the cryostream once for 15 s.

The cuvette containing the kustc1061-coated SnO₂ electrode was placed in a spectrophotometer (V650; Jasco Analytical Instruments, Easton, MD) and connected to a potentiostat (PGSTAT20; Autolab, Utrecht, The Netherlands). To minimize the spectral contribution from scattering by the SnO₂ particles, a cuvette containing a SnO₂ electrode that had not been exposed to kustc1061 was placed in the reference beam of the spectrometer during measurements. UV-visible spectra were recorded after setting the potential applied to the kustc1061-coated electrode to the desired value and allowing the protein to equilibrate with that potential. Voltage-dependent spectral changes were fully reproducible for at least five cycles between −600 and +400 mV (20 mV steps) in reductive and oxidative directions. Changes in absorbance at 551 and 418 nm derived from the heme directions. Changes in absorbance at 551 and 418 nm derived from the heme and 30% (w/v) sucrose and then flash-cooled in liquid cryoprotected in reservoir solution with 25% (v/v) ethylene glycol.

Another, cubic crystal form diffracting to much higher resolution was observed upon annealing (33, 34) the flash-cooled crystals by interrupting the cryostream once for 15 s.

Data Collection and Structure Solution—Diffraction data from kustc1061 were collected at the X10SA Beamline of the Swiss Light Source (Villigen, CH) at 100 K and processed with XDS (35, 36). Statistics are reported in Table 1. A highly redundant, 3.5 Å resolution SAD data set was collected from a hexagonal crystal at a wavelength of 1.738 Å, in which AutoSHARP (37) detected seven heavy atom sites per asymmetric unit using SHELDX (38). Phasing and density modification using SOLOMON (39) resulted in an excellent electron density map calculated from 1.8 Å resolution data is overlaid at a 2 σ contour level (blue mesh). The figures were prepared in PyMOL (47).

Protein Crystallization and Crystal Handling—Kustc1061 was concentrated by ultrafiltration to A₂₈₀,₁ cm = 20 (A₄₀₉,₁ cm = 95) in 25 mM HEPES-KOH buffer (pH 7.5), containing 25 mM KCl. Needle-shaped, hexagonal crystals of kustc1061 grew from hanging drops equilibrated against 1.2 M ammonium sulfate, 1 M sodium phosphate buffer (pH 7.4), and the detergent additive cyclodextrin-N-hydroxymethylglucamide. These crystals were cryoprotected in reservoir solution containing 25% (v/v) ethylene glycol and 10 mM HgCl₂ before flash-cooling in liquid nitrogen.

No crystal form diffracting to much higher resolution grew from 1.3 M ammonium sulfate, 0.05–0.1 M sodium phosphate buffer (pH 7.4), and the detergent additive cyclodextrin-N-hydroxymethylglucamide. These crystals were cryoprotected in reservoir solution containing 25% (v/v) ethylene glycol and 30% (w/v) sucrose and then flash-cooled in liquid nitrogen.

Protein Crystallization and Crystal Handling—Kustc1061 was concentrated by ultrafiltration to A₂₈₀,₁ cm = 20 (A₄₀₉,₁ cm = 95) in 25 mM HEPES-KOH buffer (pH 7.5), containing 25 mM KCl. Needle-shaped, hexagonal crystals of kustc1061 grew from hanging drops equilibrated against 1.2 M ammonium sulfate, 0.05–0.1 M sodium phosphate buffer (pH 7.4), and the detergent additive cyclodextrin-N-hydroxymethylglucamide. These crystals were cryoprotected in reservoir solution containing 25% (v/v) ethylene glycol and 10 mM HgCl₂ before flash-cooling in liquid nitrogen.

Another, cubic crystal form diffracting to much higher resolution was observed upon annealing (33, 34) the flash-cooled crystals by interrupting the cryostream once for 15 s.
MAC (41) resulted in an excellent model with good $R$ factors (Table 1). Geometric parameters for the modified heme co-factor were obtained from semiempirical calculations at the PM-3 level carried out using HyperChem 8.0 (Hypercube Inc., Gainesville, FL). Crystals of protein-ligand complexes were obtained by soaking cubic crystals in artificial mother liquor containing the respective compounds (100 mM hydroxylammonium chloride, 10 min; 5 mM hydrazinium sulfate, 10 min; and 50 mM phenyl hydrazine, 30 min) followed by flash-cooling in liquid nitrogen. Soaking with NO was done by incubating crystals in thoroughly degassed and cytochrome-saturated buffers, and cryoprotectants were placed inside an anaerobic glove box for 15 min at room temperature with 4 mM of the NO donor 1-(N,N-diethylamine)diazen-1-ium-1,2-diolate.  

NeHAO crystals of either crystal form were annealed as described above, and native data were collected at PETRA-III using a Pilatus 6 M detector at 100 K and processed with XDS (35, 36). Molecular replacement with PHASER (43) using the structure from Igarashi et al. (11) (Protein Data Bank 1FGJ) as the search model. During iterative rebuilding in COOT (40) and refinement with REFMAC (41), an extra polypeptide chain became visible in the electron density maps. This protein was confirmed to be Ne1300 by peptide mass fingerprinting and was built accordingly. A ligand-coordinated structure of NeHAO was obtained by performing the dehydration protocol in the presence of 100 mM hydroxylamine. Data were collected at the X10SA Beamline of the Swiss Light Source (Villigen, CH) at 100 K. Difference electron density maps clearly displayed extra density connected to the position where a water molecule is bound in the native structure, clearly showing that a larger molecule had bound to the P$_{460}$ co-factor.

**Bioinformatics**—Protein sequence homology searches were performed against nonredundant protein databases using the BlastP program (44) at the NCBI website. N-terminal signal cleavage sites were predicted with SignalP 4.1 (45). Multiple amino acid sequence alignments were made with the ClustalW program (46) at the EMBL-EBI website. All structural figures were prepared in PyMOL (47).

**RESULTS**

**Kustc1061 Is a Specific NO-forming Hydroxylamine Oxidase**—Previously, we purified an abundant HAO-like protein from *K. stuttgartiensis* and identified this as kustc1061 (8). This protein performed the three-electron oxidation of hydroxylamine, resulting in the stoichiometric formation of NO and three molecules of reduced cytochrome $c$ (Fig. 1, A–D, and Equation 1). In contrast and as expected, the purified NeHAO catalyzed a four-electron oxidation of hydroxylamine in accordance with Equation 2 (Fig. 1A). Hydroxylamine oxidation by kustc1061 followed Michaelis-Menten kinetics (apparent $K_m = 4.4 \pm 0.9$ $\mu$M, $V_{max} = 4.8 \pm 0.2$ $\mu$mol min$^{-1}$ mg protein$^{-1}$). NO in concentrations up to 80 $\mu$M did not have any effect on this reaction.

![Figure 5. Views of kustc1061 and NeHAO along the 3-fold axis of the trimers.](image-url)
Further, kustc1061 was also able to catalyze the four-electron oxidation of hydrazine to N\textsubscript{2} (Fig. 1, A and E), albeit with lower affinity and maximum rate (apparent $K_m = 54 \pm 3.3$ $\mu$M, $V_{max} = 1.60 \pm 0.05$ $\mu$mol min\textsuperscript{-1} mg protein\textsuperscript{-1}), i.e., with a 34-fold lower catalytic efficiency ($k_{cat}/K_m$) (Table 2). Kustc1061 was capable of nitrite reduction with an artificial reductant, reduced methyl viologen, but only with a very low rate (0.18 $\mu$mol min\textsuperscript{-1} mg protein\textsuperscript{-1}), which is also comparable to nitrite reduction by NeHAO (Table 2). In contrast to what was reported for NeHAO (48), we could not measure any hydroxylamine disproportionation to ammonium and NO, nitrite, N\textsubscript{2}O, or N\textsubscript{2}. These findings strongly suggested that the oxidation of hydroxylamine to NO was the distinctive physiological reaction of kustc1061. The kustc1061 catalytic constants were in the same range as those reported for not well defined, homologous enzymes of the anammox bacteria Brocadia anammoxidans and strain KSU-1 (Table 2) (49, 50). NeHAO oxidized hydroxylamine and hydrazine at 6- and 9-fold higher maximal rates, respectively.

**Kustc1061 Is a Multiheme Protein with a Low Redox Potential $P_{460}$ Chromophore**—Kustc1061 was purified as a protein with a molecular mass of 184.20 kDa calculated for a homotrimeric gene product, after cleavage of a predicted signal peptide, harboring 24 heme $c$ molecules in total. The resolution of the crystal structure described below confirmed the presence of three identical subunits, each binding eight $c$-type hemes.

The UV-visible spectrum of the as-isolated kustc1061 exhibited the characteristic features of low spin ferric $c$-heme, namely, a pronounced maximum at 408 nm in the Soret region and a broad feature of lower intensity between 500 and 600 nm (Fig. 2). Upon addition of dithionite, these features of the fully oxidized enzyme changed, giving rise to maxima at 418, 522, and 551 nm and at 468 nm. The maxima at 418, 522, and 551 nm were indicative of (His/His ligated) low spin ferrous $c$-hemes, whereas that at 468 nm was reminiscent of a ferrous heme with covalent modification by tyrosine as found for the $P_{460}$ catalytic heme in NeHAO (11, 13, 53). Cross-linking of kustc1061 subunits by covalent modification of the heme was supported by the failure of the three subunits to dissociate upon denaturing SDS-PAGE (data not shown) and confirmed by x-ray crystallography (see below). Using protein film spectroelectrochemistry, the low spin His/His-ligated hemes could be reversibly reduced in seven consecutive one-electron reduction steps.

**FIGURE 6. Structural similarity of the catalytic features of kust1061 and NeHAO.** A, stereo view of a superposition of the active sites of kustc1061 (light gray) and NeHAO (dark gray) soaked in hydroxylamine. B, stereo view of a superposition of kustc1061 and NeHAO based on Cα-carbons, showing only the heme groups. The two proteins show a virtually identical arrangement of hemes.
showing midpoint redox potentials at pH 7 ($E_m$) ranging from $-10$ to $-410$ mV versus the standard hydrogen electrode (Fig. 3). This potential window encompassed six $E_m$ values for the His/His-ligated hemes in NeHAO (53, 54). The $P_{460}$ chromophore in kustc1061 displayed an $E_m$ of $-300 \pm 10$ mV compared with $E_m = -260$ mV for NeHAO.

When as-isolated kustc1061 was incubated with its substrate hydroxylamine, the protein became partly reduced (Fig. 2). Comparison with protein film spectroelectrochemistry results suggested the reduction of two hemes such that hydroxylamine would undergo a two-electron oxidation upon binding to the enzyme. Incubation with hydrazine also resulted in a spectrum indicative of two-electron reduction. In contrast, NO had no effect on the absorption spectrum of oxidized kustc1061, but it reoxidized the reduced enzyme completely.

**Kustc1061 and NeHAO Share a Common Architecture**—The high resolution x-ray crystal structure of kustc1061 was predominantly $\alpha$-helical, with long helices running approximately parallel to the 3-fold symmetry axis of the trimer and surrounding a central, water-filled cavity (Figs. 4 and 5). The subunits were linked by two covalent bonds between Tyr$^{451}$ of one subunit and heme 4 of an adjacent subunit. Heme 4 was ligated by a single amino acid (His$^{227}$) and could be assigned as the $P_{460}$ co-factor identified by UV-visible spectroscopy. The additional seven $c$-type hemes had His/His ligation, so that together they readily accounted for the additional ferric and ferrous heme features observed in the UV-visible spectra.

Despite a sequence identity of only 26% to NeHAO (supplemental Fig. S1), the structures of kustc1061 and NeHAO were similar and could be superimposed to a root mean square positional difference of 1.7 Å for 377 $C\alpha$ atoms. This conservation also applied to the position of the heme moieties (Figs. 4–6). One may note that the same heme configuration was observed in other octaheme proteins and in pentaheme nitrite reductase (NrfA) in which the five hemes (1–5) superimpose with hemes 4–8 of the octaheme proteins (14, 15, 55, 56). However, in the trimeric kustc1061 and NeHAO, hemes were arranged in a ring. It was proposed earlier that such a ring-like arrangement of single electron carriers would enable electron transfer between the subunits, thus facilitating the extraction of two electrons from the substrate in rapid succession (54), which could also be essential for the oxidation of NH$_2$OH to NO catalyzed by kustc1061.

Apart from this structural similarity between kustc1061 and NeHAO, we observed distinguishing differences between these two proteins, notably in their N- and C-terminal parts. Kustc1061 had a negatively charged N-terminal domain (N1; amino acids 36–80 of the translated protein) that clung to a positively charged amino acid stretch of an adjacent subunit (Fig. 5). This domain was absent in NeHAO. Instead, and in full agreement with the structure recently published by Cedervall et al. (13), the subunit interface in our native and complexed NeHAO structures was occupied by a polypeptide of 69 amino acids, which was identified as a fragment of the hypothetical protein Ne1300 (101 amino acids). However, these overall structural differences between the two proteins did not immediately explain why in kustc1061 the oxidation of NH$_2$OH stops at NO, whereas in NeHAO the reaction continues to NO$_2$.

Kustc1061 and NeHAO Have Structurally Similar $P_{460}$ Co-factors—We determined kustc1061 crystal structures soaked with substrates, observing binding to heme 4 (the $P_{460}$
co-factor), which was apparently the active site, as in NeHAO (11, 13). Considering the difference in reaction specificity, it was surprising that at first glance the active sites of kustc1061 and NeHAO appeared highly similar as well (Figs. 6 and 7A). In both proteins, heme 4 was covalently linked to a tyrosine from another subunit, causing the co-factor to be substantially ruffled. This ruffling was proposed to support electron abstraction from the substrate by the heme (11, 54). In the 1.8 Å resolution structure of kustc1061, we observed two covalent bonds between Tyr451 and the P460 co-factor, between a Tyr451 C and the heme meso-carbon C5, as well as between the Tyr451 O and the heme carbon atom C4 (Figs. 4C, 6A, 7, A–C, and 8, A–C). Although only the latter of these two was built in the previous, 2.8 Å resolution structural model of NeHAO (11), both in our higher resolution structures and in the recent refinement (2.1 Å) (13), two bonds were clearly present (Figs.

FIGURE 8. X-ray structures of the kustc1061 and NeHAO catalytic sites in crystals soaked with substrates. Close-ups of the active sites of kustc1061 soaked with hydroxylamine (A), hydrazine (B), and phenyl hydrazine (C) and of NeHAO soaked with hydroxylamine (D) are shown.
A, 7D, and 8D), excluding the possibility that the different reaction specificities result from differences in tyrosine binding. Similarly, the direct vicinity of the substrate-binding site on the P₄₆₀ co-factor did not immediately explain reaction specificities either. In as-isolated kustc1061, a water molecule was present as the sixth (distal) ligand to the iron in the P₄₆₀ co-factor (Fig. 4C). Also in NeHAO, a distal water molecule was bound to the catalytic P₄₆₀ co-factor, as deduced from our native (2.2 Å) and recent (2.2 Å) resolution structures. This feature could not be discerned originally because of limited resolution (11).

Substrates Bind to P₄₆₀ in Both Kustc1061 and in NeHAO—In kustc1061, the water bound to the iron in the P₄₆₀ co-factor was replaced by a larger molecule after soaking crystals with hydroxylamine (Figs. 6A, 7A, and 8A). In the resulting 2.2 Å structure of kustc1061, this compound was modeled as an NO species coordinating at an angle of ~107° with respect to the heme-iron bond. Considering that hydroxylamine underwent a two-electron oxidation upon binding, this structure most likely represented a {FeNO}⁷ nitrosyl derivative, which was consistent with the observed angle between the NO molecule and the iron-nitrogen bond. Interestingly, a similarly sized molecule bound with comparable orientation to the P₄₆₀ was observed in the 1.9 Å electron density maps of a hydrazine-soaked kustc1061 crystal, possibly representing an iron-bound diazene (HN=NH) (Figs. 7B and 8B). Such a diatomic molecule was not observed in crystals incubated anaerobically with the NO donor 1-(N,N-diethylaminodiazene-1-ium-1,2-diolate. In the hydroxylamine-soaked kustc1061 structure, the putative nitrosyl group bound to the P₄₆₀ heme iron atom contacted the conserved aspartate (Asp²⁶²) and histidine (His²⁶³) residues. The structure of the kustc1061 hydrazine soak was very similar, except that Asp²⁶² had rotated away from the binding site. We also determined the structure of a complex of kustc1061 with a phenyl radical at 2.1 Å resolution by soaking a crystal in phenyl hydrazine (Figs. 7C and 8C). In this structure, the loop carrying the Asp²⁶²/His²⁶³ pair moved by several Ångstrōms, which further suggested flexibility in this region. In our structure of NeHAO soaked with hydroxylamine, a diatomic molecule was found as well (Figs. 6A, 7D, and 8D). Again, this molecule was bound to the heme iron at an angle of 118°, and it was coordinated by the Asp²⁹¹/His²⁹² pair, which corresponded to Asp²⁶²/His²⁶³ in kustc1061. Probably, the aspartate/histidine pair served to abstract protons from hydroxylamine (Equations 1 and 2) as was proposed for the orthologous residues in NeHAO (11, 54, 57). Given the flexibility of this region observed in kustc1061 and its proximity to the central, water-filled cavity, these residues could shuttle the protons into this cavity, from where they could be transferred to the solvent. Indeed, in our native NeHAO structure, two chains of water molecules were observed leading from Asp²⁹¹ to the central cavity, which could assist in the proposed proton transfer (Fig. 9).

Ultimately, only one significant structural difference between the kustc1061 and NeHAO active sites was apparent. Close to the NeHAO active site, a tyrosine (Tyr³⁵⁸) was present at the distal side of the porphyrin ring (Fig. 10A). As judged from the amino acid sequence (supplemental Fig. S1), an
orthologous tyrosine (Tyr$^{320}$) was present in kustc1061. However, a two-amino acid contraction in kustc1061 resulted in Tyr$^{320}$ being moved away from the active site, where it was replaced by a hydrophobic methionine (Met$^{323}$) (Fig. 10).

**DISCUSSION**

Here, we functionally and structurally characterized the octaheme protein kustc1061 from the anammox bacterium *K. stuttgartiensis*. Despite the limited sequence identity, kustc1061 shared multiple characteristics with HAO from *N. europaea*. Both proteins were organized into covalently bound homotrimers harboring eight c-type hemes per monomer. The arrangement of these hemes, as well as their comparable midpoint redox potentials, and the presence of the P$_{460}$-type catalytic centers were similar. Both kustc1061 and NeHAO use hydroxylamine as their substrate, but whereas kustc1061, and presumably many other HAO-like proteins mentioned below, oxidizes the substrate to NO, nitrite is the (major) product of NeHAO activity. This resulted in the question of what determined the reaction specificity.

Crystals both of kustc1061 and NeHAO soaked with hydroxylamine showed the presence of a diatomic molecule in the active site that could be modeled as a {FeNO}$^7$ nitrosyl (Figs. 6A; 7A and D; and 8A and D). This would imply that these two proteins follow a common catalytic cycle (57) at least up to this point, involving the abstraction of two protons and two electrons (Fig. 10). Considering that these electrons have to be shuttled one at a time through the wire of His/His-ligated cytochrome c one-electron carriers, it is possible to speculate that the tyrosine bound to the catalytic heme assists in electron transfer by temporarily storing one of the electrons as a tyrosine radical. The subsequent one-electron oxidation of the {FeNO}$^7$ nitrosyl would then yield Fe(III)-bound NO ({FeNO}$^6$) as proposed for NeHAO in Ref. 57 (Fig. 11). Apparently, in kustc1061 this would be followed by the dissociation of NO and the rebinding of water, regenerating the resting state. Although it is known that NO is a minor product of the reaction catalyzed by NeHAO (60), its oxidation reaction has to continue to the nitrite state. To reach this state, the NeHAO reaction requires the addition of an oxygen atom, which derived from a water molecule (57), to the substrate. Comparing the active
sites of kustc1061 and NeHAO, it seems that the reaction specificity relies on facilitating or preventing this step. In NeHAO, the hydrophilic $O_2^-$ hydroxyl of the Tyr$^{358}$ side chain is perfectly positioned to bind and orient a water molecule to attack a substrate bound to the P$_{460}$ iron (Fig. 10A). Importantly, in kustc1061 this particular tyrosine was replaced by the hydrophobic Met$^{323}$ (Fig. 10B), which would make the binding of a water molecule in this position unlikely, thus preventing the reaction from proceeding to nitrite.

Hydroxylamine is a potent inhibitor of the N$_2$-forming reaction catalyzed by the HAO-like hydrazine dehydrogenase (kustc0694 in K. stuttgartiensis) (8). The presence of kustc1061 provides these microorganisms a means for hydroxylamine detoxification, at the same time generating NO, a substrate for hydrazine synthesis (8). Indeed, kustc1061 homologs are found in all anammox genomes sequenced so far (supplemental Fig. S1), in which close paralogs may even be observed, such as kust0043 in K. stuttgartiensis. Furthermore, hydroxylamine is a toxic product of ammonium co-metabolism of the particulate methane monoxygenase, the key enzyme in the highly diverse anaerobically relevant methanotrophic bacteria. Many of these organisms harbor at least one HAO-like protein that has an elusive role. It was suggested that these HAO-like proteins could either oxidize hydroxylamine to nitrite or NO or alternatively reduce nitrite to NO (61, 62). Despite substantial sequence divergence, HAO-like proteins from methane oxidizers have two prominent features in common with kustc1061 and its homologs in anammox bacteria: they do contain the tyrosine involved in the covalent binding of the catalytic heme that favors oxidative reactions and lack the tyrosine present in NeHAO (Tyr$^{358}$; supplemental Fig. S1), which we hypothesize to be involved in the addition of water for nitrite formation. This suggests that methanotroph HAOs are also NO producing, which would then be reduced to N$_2$O by a respiratory cNOR-like protein that is commonly found in methane oxidizers. Through these reactions, methanotrophs might even benefit from ammonium co-metabolism, explaining the enigmatic presence of HAO-like proteins in these microorganisms.

In conclusion, here we present a detailed characterization of kustc1061 from K. stuttgartiensis including the first crystal structure of an enzyme from an anammox organism. Despite remarkable similarity to the related NeHAO enzyme, the kustc1061 enzyme converts NH$_2$OH into NO rather than NO$_2$ because of second shell effects steering reaction specificity so to prevent the addition of a water-derived oxygen atom. Anammox bacteria and possibly methane oxidizers take advantage of this novel way of making NO to detoxify hydroxylamine, thereby generating electrons and substrate for respiration.

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NO Generation by Octaheme Oxidoreductases

SUPPLEMENTAL INFORMATION

Structural basis of biological NO generation by octaheme oxidoreductases

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SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. Multiple protein sequence alignment of kustc1061, hydroxylamine oxidoreductase from N. europaea (Neuro_HAO, NeHAO) and related proteins from anammox bacteria and methane oxidizers. Sequence alignments were made for the protein sequences of the kustc1061 and NeHAO structures and omitting predicted N-terminal signal sequences from the other proteins. Amino acids are numbered according to their sequence in the N-terminal cleaved products; their numbering in the translated gene products is mentioned in parentheses. The CXXCH binding motifs of the heme c molecules are printed in red; red primed numbers represent the histidines proximal to the respective heme c molecules as deduced from the kustc1061 and NeHAO crystal structures. The tyrosine involved in the covalent binding to the catalytic heme 4 (P460) in kustc1061 (Y491) and NeHAO is printed in pink. The aspartate, histidine and tyrosine moieties (Y358 in NeHAO) near the catalytic site are printed in blue. Note that the tyrosine is apparently conserved in most proteins. However, in kustc1061 the tyrosine is moved away from the catalytic site by several Ångströms by a two-amino acid contraction and it is replaced at that position by a methionine (M323). This same contraction is found in other proteins. Peptide sequences identified for kustc1061 by MALDI-TOF analyses are underlined. We never detected predicted peptides derived from the 36-amino acids N-terminal signal sequence, nor from sequences involved in the covalent binding of the heme c molecules nor from stretches related with the covalent binding between subunits. Protein identifiers and abbreviations represent the following: kust, K. stuttgartiens; KSU-1_HAO, hydroxylamine oxidoreductase (HAO) from anammox enrichment culture KSU-1 (WP_007222832); BROSI, anammox bacterium Brocadia sinica (these sequences were kindly provided by Dr. Mamuro Oshiki); scal, HAOs from anammox bacterium Scalindua profunda (Taxon Object IDs 2017108002 and 2022004002 at JGI); M.oxyfera_HAO, HAO from the anaerobic methane oxidiser Methylomirabilis oxyfera (YP_003207347); M.capsul_HAO, HAO from Methylococcus capsulatus strain Bath (YP_113436); M.inferno_HAO, HAO from Methylacidiphilum infernorum V4 (YP_001941085).
Structural Basis of Biological NO Generation by Octaheme Oxidoreductases
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