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Structural Basis of Biological NO Generation by Octaheme Oxidoreductases

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Received for publication, October 11, 2013, and in revised form, November 26, 2013 Published, JBC Papers in Press, December 3, 2013, DOI 10.1074/jbc.M113.525147

Background: Multitheme 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 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 crystal structure of the Nitrosomonas europaea 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)5 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).

\[
\text{NH}_2\text{OH} \rightarrow \text{NO} + 3\text{H}^+ + 3\text{e}^- \quad (E_0^f = -0.030 \text{ V})
\]  
\[
\text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 5\text{H}^+ + 4\text{e}^- \quad (E_0^f = +0.065 \text{ V})
\]

(HA0-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, were shown to contribute substantially to nitrogen release into the atmosphere (14), harbor no less than

8 This work was supported by Netherlands Organization for Scientific Research Darwin Grants 142.16.1201 (to W. J. M.) and VENI 863.11.003 (to B. K.), by European Research Council Advanced Grant 232937 (to M. S. M. J.), by the Max-Planck Society, and by UK Biotechnology and Biological Sciences Research Council Grant BB/G009228 (to S. J. M.).
9 The atomic coordinates and structure factors (codes 4N4J, 4N4K, 4N4L, 4N4M, 4N4N, and 4N4O) have been deposited in the Protein Data Bank (http://wwwpdb.org/).
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† Both authors contributed equally to this work.
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|| To whom correspondence may be addressed. Tel.: 31243652557; Fax: 31243652830; E-mail: kartal@science.ru.nl.
5 The abbreviations used are: HAO, hydroxylamine oxidoreductase; NeHAO, N. europaea HAO; FL2E, 2-(4,5-bis(6-(2-ethoxy-2-oxoethoxy)-2-methylquinolin-8-ylamino)methyl)-6-hydroxy-3-oxo-3H-xanthen-9-y]benzoic acid.
10 divergent HAO paralogs. In fact, one of these (kustc0694 in \textit{K. stuttgartiensis}) is dedicated to the oxidation of hydrazine, the N\textsubscript{2}-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 \textit{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 \textit{K. stuttgartiensis} enrichment culture ($\sim$95% pure) that was grown continuously as planktonic cells in a 10-liter membrane bioreactor using methods described by Kartal et al. (18). Purification typically used 4 liters of cells ($\sim$18 g wet weight), frozen in liquid nitrogen, and stored at $-80^\circ$C. The cells were resuspended after 6 days (12 g of wet weight), frozen in liquid nitrogen, and stored at $-80^\circ$C. The cells were resuspended 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 8,000 and 12,000 rpm in a Beckman X-100 ultracentrifuge rotor. Equilibrium data were analyzed using SEDFIT (21).

**Analytical Ultracentrifugation**—Sedimentation velocity and equilibrium ultracentrifugation was performed in a Beckman XL-1 X-Traneulab 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 speed used were 8,000 and 12,000 rpm in an X-A60Ti 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 \textit{c} at 550 nm ($\Delta_{550} = 19,600$ m$^{-1}$ cm$^{-1}$) (22) in a Cary 50 spectrophotometer (Agilent, Santa Clara, CA). React-
### TABLE 1
Data collection, phasing, and refinement statistics

The values in parentheses are for highest resolution shell.

<table>
<thead>
<tr>
<th></th>
<th>KustC1061</th>
<th>KustC1061</th>
<th>KustC1061</th>
<th>KustC1061</th>
<th>KustC1061</th>
<th>Native</th>
<th>NeHAO</th>
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<tr>
<td></td>
<td>native HgCl₂</td>
<td>native initial</td>
<td>native high resolution (4N4J)</td>
<td>NH₂OH soak (4N4K)</td>
<td>N₂H₄ soak (4N4L)</td>
<td>phenyl hydrazine soak (4N4M)</td>
<td>NeHAO NH₂OH soak (4N4O)</td>
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<td>P6₁</td>
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<td>P₂₁,3</td>
<td>P₂₁,3</td>
<td>P₂₁,3</td>
<td>P₂₁,2²</td>
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<td>96.4, 96.4, 118.0</td>
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<td>130.0, 130.0, 130.0</td>
<td>130.0, 130.0, 130.0</td>
<td>140.7, 140.0, 106.7</td>
<td>140.1, 142.0, 106.5</td>
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<td>90, 90, 120</td>
<td>90, 90, 90</td>
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<td>30.0-1.8 (1.9-1.8)</td>
<td>30.0-2.2 (2.3-2.2)</td>
<td>30.0-1.9 (2.0-1.9)</td>
<td>30.0-2.1 (2.2-2.1)</td>
<td>30.0-2.2 (2.3-2.2)</td>
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<tr>
<td>I/</td>
<td>I</td>
<td></td>
<td>0.083 (0.151)</td>
<td>0.081 (0.479)</td>
<td>0.084 (0.387)</td>
<td>0.076 (0.356)</td>
<td>0.100 (0.464)</td>
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<td>99.9 (100)</td>
<td>100.0 (100.0)</td>
<td>100.0 (99.9)</td>
<td>100.0 (99.5)</td>
<td>100.0 (99.3)</td>
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<td>7.2 (7.4)</td>
<td>7.5 (7.6)</td>
<td>6.6 (6.9)</td>
<td>13.4 (13.7)</td>
<td>18.0 (3.9)</td>
<td>5.6 (5.5)</td>
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<td>Resolution (Å)</td>
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<td>46-2.2</td>
<td>46-1.9</td>
<td>46-2.1</td>
<td>46-2.1</td>
<td>50-2.2</td>
<td>50-2.5</td>
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<td>35,645</td>
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<td>0.154/0.191</td>
<td>0.148/0.163</td>
<td>0.165/0.202</td>
<td>0.179/0.213</td>
<td>0.179/0.213</td>
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<td>13,314</td>
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<td>3,962</td>
<td>3,930</td>
<td>3,930</td>
<td>13,323</td>
<td>13,314</td>
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<td>Ligand/ion</td>
<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
<td>344 (8 heme)</td>
<td>1034 (24 heme)</td>
<td>1034 (24 heme)</td>
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<td>368</td>
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<td>993</td>
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<td>17.9</td>
<td>28.9</td>
<td>28.9</td>
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<td>Protein</td>
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<td>21.9</td>
<td>17.6</td>
<td>23.5</td>
<td>22.7</td>
<td>51.0</td>
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<tr>
<td>Ligand/ion</td>
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<td>33.7</td>
<td>24.6</td>
<td>33.1</td>
<td>34.7</td>
<td>32.3</td>
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<tr>
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<td>1.086</td>
<td>1.042</td>
<td>1.025</td>
<td>0.917</td>
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<tr>
<td>Bond lengths (Å)</td>
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<td></td>
<td></td>
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<tr>
<td>Bond angles (°)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
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 (ε600 = 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 A₆₀₀ = 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 fluorom
**NO Generation by Octaheme Oxidoreductases**

**TABLE 2**

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

Kinetic values are given for mammalian cytochrome c as electron acceptor. Reduced methyl viologen was used as electron donor for NO$_3^-$ reduction. NR, not reported.

<table>
<thead>
<tr>
<th>Property</th>
<th>K. stuttgartiensis</th>
<th>B. anammoxidans</th>
<th>KSU-1</th>
<th>N. europaea</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$, NH$_2$OH (μmol min$^{-1}$ mg$^{-1}$)</td>
<td>4.8</td>
<td>21</td>
<td>9.6</td>
<td>28.5</td>
</tr>
<tr>
<td>$K_m$, NH$_2$OH (s$^{-1}$)</td>
<td>15</td>
<td>64</td>
<td>19</td>
<td>95</td>
</tr>
<tr>
<td>$K_m$, NH$_2$OH (μM)</td>
<td>4.4</td>
<td>26</td>
<td>33</td>
<td>3.6</td>
</tr>
<tr>
<td>$k_{cat}/K_m$, NH$_2$OH (s$^{-1}$ μM$^{-1}$)</td>
<td>3.4</td>
<td>2.5</td>
<td>0.57</td>
<td>26</td>
</tr>
<tr>
<td>$V_{max}$, N$_2$H$_4$ (μmol min$^{-1}$ mg$^{-1}$)</td>
<td>1.6</td>
<td>1.1</td>
<td>0.54</td>
<td>14</td>
</tr>
<tr>
<td>$K_m$, N$_2$H$_4$ (s$^{-1}$)</td>
<td>3.4</td>
<td>1.1</td>
<td>1.1</td>
<td>47</td>
</tr>
<tr>
<td>$K_m$, N$_2$H$_4$ (μM)</td>
<td>54</td>
<td>18</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>$k_{cat}/K_m$, N$_2$H$_4$ (s$^{-1}$ μM$^{-1}$)</td>
<td>0.1</td>
<td>0.19</td>
<td>0.04</td>
<td>12</td>
</tr>
<tr>
<td>NO$_2^-$ reduction (μmol min$^{-1}$ mg$^{-1}$)</td>
<td>0.18</td>
<td>0.87</td>
<td>NR</td>
<td>6.18</td>
</tr>
<tr>
<td>Total size (kDa)</td>
<td>184</td>
<td>183</td>
<td>118</td>
<td>290</td>
</tr>
<tr>
<td>Subunit size (kDa)</td>
<td>61.5</td>
<td>58</td>
<td>53</td>
<td>67</td>
</tr>
<tr>
<td>Subunit composition</td>
<td>$\alpha_3$</td>
<td>$\alpha_3$</td>
<td>$\alpha_2$</td>
<td>$\alpha_2$</td>
</tr>
<tr>
<td>Catalytic heme optical maximum (nm)</td>
<td>468</td>
<td>468</td>
<td>468</td>
<td>463</td>
</tr>
<tr>
<td>References</td>
<td>This paper</td>
<td>50</td>
<td>20</td>
<td>51, 52</td>
</tr>
</tbody>
</table>

* Specific activity with 0.1 mM nitrite.
* Specific activity with 2.5 mM nitrite.
* $V_{max}$.

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](#)).

**Electronic Absorbance Spectra**—UV-visible spectra were measured in 1-ml quartz cuvettes (path length, 1 cm). Samples contained 80 μg ml$^{-1}$ of as-isolated fully oxidized kustc1061 in potassium $P$_{1} 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 μM of the compound to be tested (5 mM for phenyl hydrazine). NO (final concentration, 90 μM) 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 (±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-α and -β 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 ±10 mV). It was assumed that P460 spectral properties did not interfere significantly with the spectral changes of the Soret (C) and α and β (E) bands of the His/His ligated hemes; clear isoebestic 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 isoebestic 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.
The electrode was composed of a mesoporous, nanocrystalline layer of SnO₂ coated onto 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 (E₀' = +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 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 nitrogen.

Protein Crystallization and Crystal Handling—Kustc1061 was concentrated by ultrafiltration to A₂₈₀,₁ cm⁻¹ = 20 (A₄₂₀,₁ 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 mM ammonium sulfate, 0.1 mM sodium phosphate buffer (pH 7.4), and the detergent additive cyclohexybutanoyl-N-hydroxyethylglucamide. These crystals were soaked 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 grew from 1.3 mM ammonium sulfate, 0.05-0.1 mM sodium phosphate buffer (pH 7.4), and the detergent additive additive 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 c α and Soret bands, respectively, and at 468 nm of the P₄₆₀ chromophore were normalized and evaluated by the three monomers are shown in different colors. The 24 heme groups are shown in red, B, the same views of the kustc1061 trimers, showing the outline of the protein and the ring-like arrangement of heme groups (red). C, stereo figure, showing a close-up of the active site of kustc1061. The P₄₆₀ co-factor consists of a heme c moiety (white sticks) covalently bound in two places to the Tyr₃⁴⁴ side chain of an adjacent protein monomer (blue). The conserved Asp₃⁵⁵/His₃⁵⁶ is shown, as well as His₃⁵⁷, which coordinates the heme iron as the proximal ligand. A water molecule (red sphere) forms the distal ligand. The final, refined 2m₀₀₀ 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).

FIGURE 4. X-ray crystal structure of kustc1061 from K. stuttgartiensis. A, views of the kustc1061 trimer perpendicular to (left panel) and along the 3-fold symmetry axis (right panel). The three monomers are shown in different colors. The 24 heme groups are shown in red, B, the same views of the kustc1061 trimers, showing the outline of the protein and the ring-like arrangement of heme groups (red). C, stereo figure, showing a close-up of the active site of kustc1061. The P₄₆₀ co-factor consists of a heme c moiety (white sticks) covalently bound to the Tyr₃⁴⁴ side chain of an adjacent protein monomer (blue). The conserved Asp₃⁵⁵/His₃⁵⁶ is shown, as well as His₃⁵⁷, which coordinates the heme iron as the proximal ligand. A water molecule (red sphere) forms the distal ligand. The final, refined 2m₀₀₀ 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).
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.
Further, kustc1061 was also able to catalyze the four-electron oxidation of hydrazine to N₂ (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$^{-1}$ mg protein$^{-1}$), 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 µmol min$^{-1}$ mg protein$^{-1}$), 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, N₂O, or N₂. 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**

P₄₆₀ 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 P₄₆₀ 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.
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 ± 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 5A). The subunits were linked by two covalent bonds between Tyr451 of one subunit and heme 4 of an adjacent subunit. Heme 4 was ligated by a single amino acid (His227) 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 Cα 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 \(\text{NH}_2\text{OH}\) 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 \(\text{NH}_2\text{OH}\) stops at NO, whereas in NeHAO the reaction continues to \(\text{NO}_2^-\).

**Kustc1061 and NeHAO Have Structurally Similar \(P_{460}\) Co-factors**—We determined kustc1061 crystal structures soaked with substrates, observing binding to heme 4 (the \(P_{460}\) FIGURE 7. Stereo views of \(F_o - DFc\) electron density maps for the substrate soaks, calculated prior to the inclusion of the ligands in the model. Positive difference density (green) is contoured at 4 \(\sigma\), and negative difference density (red) is contoured at −4 \(\sigma\). A, kustc1061 soaked in \(\text{NH}_2\text{OH}\). B, kustc1061 soaked in hydrazine. C, kustc1061 soaked in phenyl hydrazine. D, Nitrosomonas HAO soaked in \(\text{NH}_2\text{OH}\).
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 Tyr\(^{451}\) and the P\(_{460}\) co-factor, between a Tyr\(^{451}\) C\(_\alpha\) and the heme meso-carbon C5, as well as between the Tyr\(^{451}\) O\(\eta\) 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. 8A–C).
6A, 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) (13) 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-diethylamino)diazene-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 Ångströms, 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. 10B).

**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$_{400}$-type catalytic centers were similar. Both kustc1061 and NeHAO use hydroxylamine as their substrate, but 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; 7, A and D; and 8, A 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 $\text{O}_7$ hydroxyl of the Tyr$^{1058}$ side chain is perfectly positioned to bind and orient a water molecule to attack a substrate bound to the $\text{P}_460$ iron (Fig. 10A). Importantly, in kustc1061 this particular tyrosine was replaced by the hydrophobic Met$^{123}$ (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 $\text{N}_2$-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 kusta0043 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 an environmentally 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$^{1058}$; 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 $\text{N}_2\text{O}$ 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 $\text{NH}_3\text{OH}$ into NO rather than $\text{NO}_2$ because of second shell effects steering reaction specificity so as 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.

Acknowledgments—The Dortmund-Heidelberg data collection team and the staff of Beamline X10SA at the Swiss Light Source of the PSI in Villigen are acknowledged for help and facilities. Portions of this research were carried out at the light source PETRA III at DESY, a member of the Helmholtz Association. We thank Anja Burkhardt, Saravanan Panneerselvam, and Alke Meents for assistance in using Beamline P11 and Marion Gradl for assistance with MALDI-TOF analyses. T. R. M. B. is grateful to Ilme Schlichting for continuous support.

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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).
Neuro_HAO        ETLKEVG
Minferno_HAO      ELLQEVS
kustc1061         LG---S
6'                (P460) Heme4      Heme5  2'
*: **             .: **    *  **  :
Mcapsul_HAO       EPLKEVG
Moxyfera_HAO      ---AKVY
scal01317         AAEQVVG
BROSI_A1461       TEKGVVG
scal01317         AAEQVVG
BROSI_A2677       PVEGTVG
Heme2          1'           Heme3
*: **   .*  .  *. * *.
Neuro_HAO
Hung_LAO
Minferno_HAO      DVLNYVWKDQFQFSPSNRATISPEDVRRKLTMHDVWASFRPQELAVSKASEMGD 180 (220)
Mcapsul_HAO       -------------------------------------------------------- GETD 4   ( 54)
Moxyfera_HAO
scal04133
BROSI_A2677       ---------------------------------EGPTFQDVASQVFGQPDNQVFGFQVDFPNDGTGLY 27 (65)
scal01317         ALLPMNFCSAGDAAPTQFDVASSISYQGVQDQQSSENVFVV 42 (80)
BROSI_A1461       EEKIESAKPEVEERITAPAGPAKKEVTASHPDKRTIQIQISEITEGKDSNGDLYH 73 (100)
kusta0043         EKPEKEAKPSASEKQEEKHAPVKVEKMDATHPKDAKTQIQQISEITGEKPDSNGDGVYH 114 (143)
BROSI_A2485       QLELQKDNVSPLAEPTTTATKAPPRITGRNLPRFMMAELGEKPDGVPPDGSDDYVNH 53 (85)
sca104133         EDTSPLEATIETTTSTSVIGSMMKQYEMMAELLERGVPPDDSNNH 50 (80)
Moxyfera_HAO
Mcapsul_HAO       DVLNYVWKDQFQFSPSNRATISPEDVRRKLTMHDVWASFRPQELAVSKASEMGD 180 (220)
Minferno_HAO      DVLNYVWKDQFQFSPSNRATISPEDVRRKLTMHDVWASFRPQELAVSKASEMGD 180 (220)
ksz1_HAO         LG---S
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
ksz1_HAO         FG-LTAKYTQPEYVE-GRGPYKSFLKFLP-SIRWYDPEHYWT-----PGSQNEGVFKNEE 79 (115)
8' Heme6 DH

5' Heme7

5' Y358 Heme8

7' Y491