Characterization of a nitrite-reducing octaheme hydroxylamine oxidoreductase that lacks the tyrosine cross-link

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The hydroxylamine oxidoreductase (HAO) family consists of octaheme proteins that harbor seven bis-His ligated electron-transferring hemes and one 5-coordinate catalytic heme with His axial ligation. Oxidative HAOs have a homotrimeric configuration with the monomers covalently attached to each other via a unique double cross-link between a Tyr residue and the catalytic heme moiety of an adjacent subunit. This cross-linked active site heme, termed the P460 cofactor, has been proposed to modulate enzyme reactivity toward oxidative catalysis. Conversely, the absence of this cross-link is predicted to favor reductive catalysis. However, this prediction has not been directly tested. In this study, an HAO homolog that lacks the heme-Tyr cross-link (HAOr) was purified to homogeneity from the nitrite-dependent anaerobic ammonium-oxidizing (anammox) bacterium Kuenenia stuttgartiensis, and its catalytic and spectroscopic properties were assessed. We show that HAOr reduced nitrite to nitric oxide and also reduced nitric oxide and hydroxylamine as nonphysiological substrates. In contrast, HAOr was not able to oxidize hydroxylamine or hydrazine supporting the notion that cross-link-deficient HAO enzymes are reductases. Compared with oxidative HAOs, we found that HAOr harbors an active site heme with a higher (at least 80 mV) midpoint potential and a much lower degree of porphyrin ruffling. Based on the physiology of anammox bacteria and our results, we propose that HAOr reduces nitrite to nitric oxide in vivo, providing anammox bacteria with NO, which they use to activate ammonium in the absence of oxygen.

Cytochrome c proteins are among the most common redox tools in nature and are ubiquitous in all domains of life (1). The electron configuration of elemental iron in combination with both the electrochemical properties of the heme porphyrin and the structural and functional contributions from the protein backbone renders heme c cofactors suitable for catalysis and electron transfer. This versatility is further enhanced by the arrangement of two or more heme cofactors within a protein as seen in the multiheme cytochromes c (MCCs). This class of proteins is involved in prokaryotic energy metabolism ranging from intra- or extracellular electron transfer to substrate conversion (2–5). Within the MCCs, the hydroxylamine oxidoreductase (HAO) protein family constitutes the largest group of octaheme cytochromes c (OCCs) next to the octaheme nitrite and tetrahionate reductases (ONRs and OTRs, respectively) (6–10).

The HAO family comprises enzymes that harbor eight CX$_{2-4}$CH heme-binding motifs, but otherwise exhibits low primary structure similarity (10, 11). HAOs harbor seven bis-His ligated electron-transferring hemes and one 5-coordinate catalytic heme with a His axial ligation (12–14). In vitro, isolated HAOs are catalytically versatile and can perform hydrazine (N$_2$H$_4$) and hydroxylamine (NH$_2$OH) oxidation, as well as nitrite (NO$_2^-$), nitric oxide (NO), and NH$_2$OH reduction, albeit at varying catalytic efficiencies (2, 15–17). Under physiological conditions, however, three of the four structurally characterized representatives perform oxidative catalysis with high catalytic efficiencies (0.1–12 s$^{-1}$ μM$^{-1}$) (14, 18, 19). Both the HAO from the aerobic ammonium-oxidizing bacteria—Nitrosomonas europaea (NeHAO) (12, 16, 18)—and the HAO from the anaerobic ammonium-oxidizing (anammox) bacteria—Kuenenia stuttgartiensis (KsHAO) (14)—catalyze NH$_2$OH oxidation to NO, while hydrazine dehydrogenase from K. stuttgartiensis (KsHDH) oxidizes N$_2$H$_4$ to dinitrogen gas (N$_2$) and is, interestingly, inhibited by both NO and NH$_2$OH at low μM concentrations (19, 20).

Oxidative HAOs display high tertiary and quaternary similarity, all adopting a homotrimeric configuration (14) (or multiples thereof) (20), with the monomers covalently attached to each other via a unique double cross-link: The C3 and the phenolate oxygen atoms of a Tyr are attached to the C5 and C4 atoms of the catalytic heme moiety of an adjacent subunit. This cross-link results in a diagnostic Soret...
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absorption feature of the reduced protein in the wavelength range of 463 to 475 nm (14, 19, 21), which lends the prosthetic group its name—the P460 cofactor (also see cytochrome P460) (22). The Tyr cross-link in the P460 active site of HAO has long been hypothesized to modulate enzyme reactivity toward oxidative catalysis; accordingly, its absence is predicted to favor reduction (11, 23).

Four HAO genes from eProteobacteria (eHAOs) that lack the critical Tyr residue were recombinantly expressed and shown to reduce NO2− and NH2OH in vitro (Vmax = 1–180 U mg protein−1), while their NH2OH-oxidizing activity was negligible (highest Vmax = 0.07 ± 0.05 U mg protein−1) (24). Additionally, the structure of an HAO—the gene product of kustc0458 from K. stuttgartiensis—that is predicted to be reductive based on physiological experiments (23) and proteomic analyses (25), was recently resolved (26). The structure of Kustc0458 revealed the absence of the heme-Tyr cross-link but otherwise highlighted the high structural conservation within the HAO family. Kustc0458 crystallized as a heterododecamer (ααββ) with a diheme cytochrome c (Kustc0457; DH), resulting in a total of 60 hemes for the Kustc0458/7 protein complex (26). Based on the structure, the heterohexamer (ααββ) presents the largest possible heme circuit within the complex and complies with the heme ring arrangement that is seen in all oxidative HAOs. In this case, the heme ring is modified by the addition of two 6-coordinate hemes, contributed by the DH subunits, at each HAO surface heme (heme 1). In analogy with the oxidative HAOs, the Kustc0458/7 catalytic protomer (αβ) comprises nine hemes with saturated coordination—seven HAO bis-His and two DH His/Met ligated hemes—and one 5-coordinate HAO His-ligated heme (heme 4). Kustc0458 is highly transcribed and expressed in K. stuttgartiensis cells under standard laboratory growth conditions (27) and has been localized within the anammoxosome (28), a bacterial organelle where the main anammox catabolism takes place (29, 30).

The anammox pathway proceeds through NO2− reduction to NO, subsequent comproportionation of ammonium (NH4+) and NO by hydrazine synthase (HJS), and the final oxidation of N2H4 to N2 by HDH (Reactions 1–3) (19, 27, 31). The low-potential electrons released from the oxidation of N2H4 are used for cell carbon fixation and other cellular anabolic reactions (23).

\[ \text{NO}_2^- + 2H^+ + e^- \rightarrow \text{NO} + \text{H}_2\text{O} (E^0 = +380 \text{ mV}) \]  

\[ \text{NO} + \text{NH}_4^+ + 2H^+ + 3e^- \rightarrow \text{N}_2\text{H}_4 + \text{H}_2\text{O} (E^0 = +60 \text{ mV}) \]  

\[ \text{N}_2\text{H}_4 \rightarrow \text{N}_2 + 4H^+ + 4e^- (E^0 = -750 \text{ mV}) \]  

In the absence of nitrite, K. stuttgartiensis can grow on NO and NH4+ instead (25). During this NO-dependent anaerobic NH4+ oxidation, transcription of both Kustc0458 and Kustc0457 was strongly downregulated (28- and 39-fold, respectively). Additionally, the only NO-forming nitrite reductase identified in the K. stuttgartiensis genome (cd3NiR homolog) had low abundance in both transcriptomes and proteomes obtained under standard and NO-dependent growth conditions (25, 27). Furthermore, neither cd1 cytochrome (cd1NiR) nor copper-containing (CuNiR) nitrite reductase homologs are conserved among anammox genomes, while the orthologs of Kustc0458 are present in all known anammox genera (26). Based on in vivo observations from K. stuttgartiensis, Kustc0458 was implicated in the reduction of nitrite to NO, the first step of the anammox pathway (23). In this study, we purified the native Kustc0458/7 complex from K. stuttgartiensis biomass, assessed its catalytic potential involving physiologically relevant nitrogen species and showed that this protein reduces nitrite to NO. Our findings are examined in the context of the HAO protein family, and the physiological relevance of Kustc0458/7 is discussed.

Results

Kustc0458 was consistently purified from K. stuttgartiensis single-cell enrichment cultures in an equimolar complex with Kustc0457. Native and sodium dodecyl sulfate (SDS)-denaturing polyacrylamide gel electrophoresis (PAGE) migration profiles indicated a stable, noncovalent dodecameric composition for the complex in solution that is in agreement with the crystallographically resolved structure (26). The electronic absorption spectra of the fully reduced protein revealed the absence of the characteristic P460 absorption feature (Fig. 1), in line with the absence of the Tyr cross-link. Fully oxidized (as isolated) Kustc0458/7 displayed a Soret maximum at 409 nm, which broadened and shifted to 415 nm upon partial reduction by ascorbate. Full reduction by dithionite resulted in a sharp Soret at 419.5 nm, a subtle shoulder at 425 nm, and Q band maxima at 524 and 553 nm. Notably, despite the Met axial ligation of both DH heme iron centers (26), no charge transfer band at 695 nm was observed.

Kinetic properties

To test whether the Tyr cross-link is necessary for oxidative catalysis, we probed the capacity of Kustc0458/7 to oxidize NH3OH and N2H4 spectroscopically. Depending on the sample preparation, the measured rates were ranging from zero to about 0.020 U mg−1 for both substrates, which is 100- to 1000-fold slower compared with the anammox oxidative HAOs and NeHAO, respectively. Notably, both anammox oxidative HAOs are among the most abundant proteins in K. stuttgartiensis cells (27) and hence, even a minute (i.e., ~0.5%) contamination of the Kustc0458/7 sample with either KsHAO or KsHDH (for NH3OH or N2H4 oxidation, respectively) could explain the measured rates, strongly suggesting that Kustc0458/7 is not an oxidative catalyst. Reductive reactivity of Kustc0458/7 toward NO2−, NO, and NH3OH was investigated by monitoring the oxidation of the methyl viologen monocation radical (MVred) (32). At 50 μM substrate concentration, NO2− reduction proceeded at 0.1 U mg−1 while
NO and NH₃OH were consistently faster (2.8 and 4.6 U mg⁻¹, respectively). Similar kinetic trends have been observed for the ammonia-producing respiratory cytochrome c nitrite reductase (ccNiR) and were explained by the presumed higher activation entropy for hydroxylamine reduction (33).

Colorimetric determination of ammonia after completion of each reductive assay routinely returned the same substrate-to-activation entropy for hydroxylamine reduction (33). The stoichiometric nitrogen imbalance observed for nitrite conversion is presumably due to the release of at least one catalytic intermediate from the Kustc0458/7 active site during catalysis in vitro. Given the reducing environment in the assay achieved by excess MVred and the higher catalytic rates of Kustc0458/7 for the downstream reactions at relevant concentrations, i.e., NO and NH₂OH reduction, this observation could only be attributed to finely tuned binding equilibria that could possibly be affected by redox modulations of the extended 9-heme network per active site.

In order to determine whether Kustc0458/7 performed its predicted physiological function of nitrite reduction to NO, different artificial electron carriers were used with nitrite as the substrate. The reducing environment created by excess MVred and the reactivity of NO prevented the direct measurement of NO when MVred was used as the electron carrier. To circumvent this problem, phenazine ethosulfate, which has a much higher midpoint potential (E’₀ = +55 mV) compared with MV (E’₀ = −440 mV), was used as an electron carrier to directly measure NO production in membrane-inlet mass spectrometry (MIMS) experiments. Here, when the reaction was initiated with the addition of nitrite, stable and reproducible NO production was detected at a rate of 0.52 mU mg⁻¹ (Fig. 2). Colorimetric nitrite measurements at the end of these experiments showed that all nitrite reduction could be accounted for by the produced NO and no other nitrogenous species such as hydroxylamine or ammonium were produced.

Based on these results, we conclude that the Kustc0458/7 protein complex is a NO-producing reductive HAO that is incompetent for oxidative catalysis. Whereas HAOs from K. stuttgartiensis and HAOs from Epsilonproteobacteria all lack appreciable in vitro oxidative activity (24), oxidative HAO complexes, conversely, have been shown to act as reductases in vitro, albeit with diverse rates. Both NeHAO and KsHAO can reduce NO₂⁻, and NeHAO can also reduce NO and NH₂OH, demonstrating that the HAO active site architecture is inherently competent for substrate reduction (14–16). In fact, it is the oxidation state of the heme Fe in NeHAO that presumably dictates the redox direction toward hydroxylamine as well as the observed rates. By comparing hydroxylamine reductase activity among NeHAO, myoglobin, and catalase, Kostera et al. (16) showed that a reduced active site led to conversion rates 100-fold greater than those observed for the ferric enzyme, concluding that the redox equilibrium during catalysis heavily determines the reactivity of the enzyme.

**Effects of substrate additions**

Nitrite titration (1 μM–2.2 mM) of reduced HAO (Fig. 3A) first resulted in the oxidation of the residual dithionite—most likely by nonenzymatic reduction of NO₂⁻ to NO. Subsequently, a feature peaking at 354 nm, probably indicative of nitrite (π→π* transition) (34), grew in. Notably, free NO gas also features a transition in the same wavelength range and so does the heme porphyrin (35) as exemplified in the spectrum of fully oxidized HAO (N band maximum at 355 nm; Fig. 1). This overlap of multiple absorbing species restricts any direct conclusions, but the presence of free NO in the cuvette, released from a putative iron-nitrosyl intermediate during partial nitrite turnover, remains possible.
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When fully oxidized HAO was incubated with excess NH$_2$OH, the protein became partially reduced with a new redshifted Soret maximum, distinct Q bands, and a narrow shoulder around 420 nm (Blue line, Fig. 3B). Interestingly, addition of excess NH$_2$OH to fully reduced HAO resulted in a similar spectrum with a more pronounced 420 feature (Pink line, Fig. 3B), which could be indicative of a Fe$^{II}$-NH$_2$OH adduct.

Potentiometric redox titration

The sheer heme count in each HAO catalytic protomer (αβ) along with the absence of any spectroscopic handle for the active site heme 4 poses limitations to the direct determination of the midpoint potentials of individual heme centers. To better inform our hypotheses, however, we performed electrochemical redox titrations of HAO$\alpha$ coupled to UV/Vis spectroscopy. The complete redox transition of HAO occurred in a range over 600 mV versus the standard hydrogen electrode (versus SHE; this reference is used throughout the paper) and Nernst global fittings of the spectra discerned five redox transitions: $E_{m1} = +305$ mV; $E_{m2} = +92$ mV; $E_{m3} = -41$ mV; $E_{m4} = -175$ mV; $E_{m5} = -315$ mV (Fig. 4A). The first two transitions ($E_{m1}$ and $E_{m2}$) exhibited Soret maxima at 422 and 420 nm, respectively, and their corresponding α bands were centered around 554 nm (Fig. 4B). In the spectrum corresponding to the third transition ($E_{m3} = -41$ mV), the Soret peaked at 422 nm and the α band was split with maxima at 552 and 556 nm, respectively. The fourth transition ($E_{m4} = -175$ mV) featured a wide and redshifted Soret peaking at 420 and 425 nm, while the spectrum of the last redox transition ($E_{m5} = -315$ mV) presented two Soret maxima at 421 and 426 nm, respectively, as well as a split α band peaking at 552 and 557 nm. The red-shifted Soret maxima of the latter two transitions imply the growth of a low-energy Soret species that is usually indicative of high-spin Fe$^{II}$ heme centers (36) and could therefore correspond to the HAO$\alpha$ active site heme. However, the relative intensities at longer wavelengths imply complete reduction of this high-spin species already at potentials higher than -175 mV.

Since not all five transitions contributed equally to the total redox change of the system, we used difference spectra corresponding to each redox transition to unravel the number of hemes contributing to each transition. We assumed a total contribution of ten redox centers for the Soret and nine for the α band, excluding the structurally resolved HAO$\alpha$ heme 4 that has a His/H$_2$O iron coordination and is expected to have low extinction at the Q region of the spectrum. The sum of the differences between the heme count from the Soret area and the α band, respectively, corresponds to the single unaccounted heme, i.e., the catalytic heme. Based on this approach, transitions 1, 3, and 4 collectively accounted for the bulk of this difference, corroborating the higher redox potential of HAO$\alpha$ heme 4 compared with its oxidative counterparts that range between −260 and −420 mV (Fig. 4C) (14, 19, 37).

Based on the NeHAO kinetic studies discussed above (16), oxidative HAOs essentially require reduced active sites for efficient NH$_2$OH reduction, which is a nonphysiological reactivity for all biochemically characterized homologs (14, 16, 17, 19). Harboring a catalytic heme with a low redox potential substantially increases the possibility of the heme Fe always being oxidized under physiological conditions and, thus, favoring oxidative catalysis. To the contrary, reductive HAOs, represented by HAO$\alpha$, are catalytically competent only for substrate reduction, which requires reductive conditions. They, therefore, feature a higher redox potential catalytic heme that can afford physiological catalysis without any side reactions.

Low reduction potentials of heme cofactors have been associated with increased heme ruffling (38–40), a structural out-of-plane (OOP) distortion of the porphyrin that results in the absence of any overlap between the Fe d-orbitals manifold.
and the porphyrin π system of the heme (41). Subjecting all four structurally resolved HAO homologs in a quantitative analysis assessing different heme distortions (42, 43) underlined another difference between HAOr and the three oxidative HAOs (Fig. 5 and Table S1). The HAOr catalytic heme exhibits a lower overall OOP distortion, with the most substantial difference in the degree of ruffling that is about 50% lower compared with oxidative HAOs.

Heme ruffling enforces an electronic ground state of iron that results in lower reduction potentials (41, 44, 45) and appears to promote the stability of [FeNO]6 adducts against reductive nitrosylation, which could lead to stable off-pathway intermediates (22, 44–46). Interestingly, while the dissociation of NO from fully reduced b- and c-type heme nitrosyls is usually very slow (10^{-3}–10^{-5} s^{-1}), cd_{1}NiR releases NO from its unique Fe^{II} d_{1} active site about 1000-fold faster (47), presumably due to an orchestrated and dynamic hydrogen-bonding network established by the protein scaffold (48). NO dissociation from the putative iron-nitrosyl intermediate of HAOr that was formed during nitrite reduction in vitro

Figure 4. Potentiometric redox titration of 50 μM Kustc0458/7 performed in both reductive and oxidative directions. Global spectra Nernst fitting discerned five redox transitions; the midpoint potentials of each (with one standard deviation) and the color code used for the transitions are shown in panel C. A, ΔAbsorbance values corresponding to the Soret (420–404 nm) and the α band (552–544 nm) are independently plotted against the applied potentials and fitted to a Nernst equation with five components and fixed potentials—as discerned by the global spectra fits. Grayscale indicates different data series in either oxidizing or reductive direction during the titration, illustrating the stability of the electrochemical system throughout the experiment. Nernst fittings are shown in black lines and the points corresponding to the discerned E_{m} values are shown as crosses. B, difference spectra corresponding to each of the five redox transitions. C, the mathematical areas corresponding to the Soret and the α bands (411–434 nm and 540–570 nm, respectively) were calculated for all discerned transitions and for the total. Assuming contribution of ten hemes to the total Soret area change and nine to the α band, the number of hemes contributing to each redox transition was calculated, based on both the Soret and the α band changes (color-coded bars and numbers therein). The difference between the heme count from the Soret and the α band (Δ% bar) corresponds to a fraction of one unaccounted heme, i.e., the catalytic heme.
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Indeed, HAOr isolated from the anammox bacterium *K. stuttgartiensis* reduced nitrite to NO when phenazine ethosulfate was used as the electron carrier. The observed low rate of NO production *in vitro* is probably due to suboptimal redox equilibria and the overall disruption of a tightly coupled intracellular multicomponent system, as has been seen before for hydrazine synthesis by HZS (27). Next to the direct measurement of NO production from nitrite reduction with HAOr, and the features of the HAOr active site, the physiology of anammox bacteria strongly suggests that HAOr functions as an NO-producing nitrite reductase in *vivo*. The other reductive catalytic reactions performed by HAOr (reduction of nitrite, hydroxylamine, and NO to ammonium) measured *in vitro* using the much stronger reducing agent methyl viologen cannot be reconciled with anammox catabolism. Under standard growth conditions with limiting nitrite and excess of millimolars of ammonium, the high expression of an ammonia-producing nitrite reductase by anammox bacteria would be unnecessary and energetically inefficient (23). Furthermore, as anammox bacteria use NO to activate ammonium into hydrazine with hydroxylamine as an enzyme-bound intermediate, having a highly abundant enzyme that rapidly reduces both NO and hydroxylamine back to ammonium would be detrimental to hydrazine production, and consequently to cell growth and activity in general as hydrazine synthesis and oxidation are the central pillars of anammox catabolism (25, 27, 31). Indeed, all cellular ammonium oxidation activity stops completely when hydrazine synthesis is blocked by inhibitors or NO scavengers (27). Together with the core catabolic proteins unique to anammox bacteria, hydratase synthase, and hydrazine dehydrogenase, HAOr is conserved throughout anammox genera including those that lack a conventional nitrite reductase. In addition, when *K. stuttgartiensis* is grown with NO and ammonium, in the absence of nitrite, the transcription of the HAOr complex is strongly downregulated, implicating HAOr in NO production from nitrite *in vivo* (25). Consequently, based on our results and earlier physiological observations, we propose that the first step of the anammox pathway, the reduction of nitrite to NO, is carried out by HAOr.

**Figure 5. Out-of-plane (OOP) heme displacements.** (Sad: saddling; dom: doming; ruf: ruffling) were calculated for NeHAO (blue), KsHAO (pink), KsHDH (green), and Kustc0458/7 (orange). The inset shows a schematic representation of the heme porphyrin with each OOP distortion, where the **dark circles** represent atomic positions above and the **light circles** below the heme plane. The inset is adapted with permission from Jentzen et al. (43). Copyright 1997 American Chemical Society. The complete output can be found in the Table S1.

could account for the measured discrepancy in the nitrogen balance during ammonia determination (60% N-NH₃). Under the heavily reducing conditions of the *in vitro* assay (MVred excess), it is conceivable that the HAOr active site is in a super-reduced state. Upon one-electron reduction of nitrite to NO, the latter dissociates from a reduced heme at rates comparable to the rate of overall electron consumption. If this holds true under physiological conditions, HAOr presents another exception of on-pathway [FeNO]² intermediates, next to cd₁NiR.

**Discussion**

Pentaheme ccNiR, OTR, and ONR can all reduce nitrite, albeit with different catalytic efficiencies, and share similar active site architectures, with the most remarkable being the Lys proximal ligand of the heme Fe (6, 9, 49). Interestingly ccNiR and ONR code for this Fe-ligating Lys within a conserved CXXCK heme-binding sequence, while OTR harbors the typical CXXCH motif instead. Both ccNiR and ONR feature a catalytic triad composed of tyrosine, histidine, and arginine at the distal side of their catalytic heme that appears to tune the proton-coupled redox steps during nitrite turnover (50, 51). A point mutation of this Tyr in the ccNiR from *Wolinella succinogenes* resulted in an over 90% decrease of the produced ammonia during nitrite reduction *in vitro*, while the conversion rates appeared unaffected (52). One-electron reduction of nitrite and subsequent release of NO from the mutated ccNiR active site could offer a plausible explanation for these observations and further emphasize how binding equilibria of catalytic intermediates define the observed enzymatic reactivity. Given the catalytic behavior of HAOr during the spectrophotometric nitrite reduction *in vitro* and the considerably different active site architecture compared with the ancestral ccNiR template, it appears that the HAOr houses an active site that is tuned for NO release.
simultaneously preventing reductive side-reactivity. Oxidative HAOs appear designed to kinetically favor hydroxylamine oxidation under physiologically relevant conditions even when reduction to ammonia is thermodynamically favorable (16). The oxidative module includes the distinct Tyr-heme cross-link, resulting in a substantially more ruffled P460 cofactor, with a lower midpoint potential compared with the reductive HAOs. Intriguingly, it appears that the information about the direction of catalysis for each HAO homolog is highly conserved on the genomic level, encoded as the absence or presence of the Tyr residue.

**Experimental procedures**

**General considerations**

All chemicals used were purchased from Sigma-Aldrich, unless stated otherwise. High-performance liquid chromatography (HPLC)-grade chemicals were purchased from Baker. In total, 18.2 MΩ cm water was used to prepare all buffers and solutions. All protein purification steps took place in ambient air and at 4 °C.

**Protein purification**

Cells were harvested from a 10-l single-cell continuous membrane bioreactor containing *K. stuttgartiensis* (~95% pure) (53) and cell lysis proceeded as described previously (54). HAOOr was brought to homogeneity in a two-step liquid chromatography procedure using an Äkta Purifier (GE Healthcare). Columns used were packed at a flow rate of 10 ml-min⁻¹ (XK 26/20 column, GE Healthcare) and eluted at 5 ml-min⁻¹. The eluate was monitored at 280 nm. Cell-free extract was applied to a column packed with 60 ml of Q Sepharose XL (GE Healthcare) and equilibrated with 20 mM Tris-HCl buffer, pH 8.0. After washing with two column volumes (CV) of 400 mM NaCl in Tris-HCl buffer, pH 8.0, the sample of interest was eluted iso-
cratically with 550 mM NaCl in the same buffer. This fraction was desalted with 20 mM potassium phosphate buffer (KPi), pH 7.0 and concentrated with 100-kDa molecular mass cutoff spin filters (Vivaspin 20; Sartorius Stedim Biotech). The concentrated fraction was loaded onto a 40-ml column packed with Ceramic membranes bioreactor containing...
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MOPS buffer, pH 7.0 at room temperature ($E_m = +280$ mV versus SHE (60)). The assay mixture contained 50 μM as isolated HAO or in 50 mM MOPS, pH 7.0, containing 50 mM KCl, 40 mM glucose, 10 U glucose oxidase, 5 U catalase, and a cocktail of mediators (20 μM final concentration for each: ferrocene ($E'_0 = +640$ mV); ferricyanide ($E'_0 = +430$ mV); 1,4-benzoquinone ($E'_0 = +280$ mV); 2,5-dimethyl-1,4-benzoquinone ($E'_0 = +180$ mV); 1,2-naphthaquinone ($E'_0 = +145$ mV); phenazine methosulfate ($E'_0 = +80$ mV); 1,4-naphthaquinone ($E'_0 = +60$ mV); phenazine ethosulfate ($E'_0 = +55$ mV); 5-hydroxy-1,4-naphthaquinone ($E'_0 = +30$ mV); 1,2-dimethyl-1,4-naphthaquinone ($E'_0 = 0$ mV); 2,5-dihydroxy-p-benzoquinone ($E'_0 = −60$ mV); 5,8-dihydroxy-1,4-naphthaquinone ($E'_0 = −145$ mV), 9,10-anthraquinone ($E'_0 = −184$ mV), 9,10-anthraquinone-2-sulfonate ($E'_0 = −225$ mV); benzyl viologen ($E'_0 = −350$ mV); and methyl viologen ($E'_0 = −440$ mV/−772 mV)).

Titrations were performed from −600 mV to +650 mV (versus SHE) at room temperature, in both reductive and oxidative directions, with potential increments of 25 mV. Equilibration time was 10 min per step. HAO or was cycled several times between redox states with no detectable spectral alterations. Spectral changes were evaluated by global fitting of the spectra as a function of potential, using the “mft-nernst” function in the QSoas software (61) (version 2.1, build 2017.06.27), taking into account six oxidation states (A–F). Using the raw spectra corresponding to each of the six redox states (A–F), the difference spectra corresponding to each of the five transitions were calculated, e.g., the change from state A to state B corresponds to transition 1. The difference spectrum of the total transition (from state A to state F), corresponding to the fully reduced minus fully oxidized states, was also calculated. The mathematical area corresponding to the Soret and the α bands (411–434 nm and 540–570 nm, respectively) was calculated for all discerned transitions (1–5) and for the total transition, using Origin 2020 (OriginLab). Assuming contribution of ten hemes to the total Soret area change and nine to the alpha band, the number of hemes contributing to each redox transition was calculated, based on both the Soret and the α band changes. For each transition, the difference between the heme count from the Soret area and the α band, respectively, corresponded to the single unaccounted heme, i.e., the catalytic heme.

Heme normal coordinate structural decomposition

The heme OOP distortions can be quantitatively assessed by assuming planar porphyrin with D4h symmetry and calculating the distance of specific atoms from this plane. The crystallographic coordinates of each catalytic heme of interest were retrieved from the PDB files and were used as input for the online calculation of heme OOP distortions (41, 43). For NeHAO, chain A, chain C, and chain E were calculated separately, and the average is shown in Figure 1. The same was done for KsHDH, where chain G, chain H, and chain I were used. For KsHAO and HAO the available monomeric structures were used. The complete output can be found in the Table S1.

Miscellaneous

Ammonia concentration was measured with the colorimetric method described by Taylor et al. (62). Nitrite was measured with the Griess reaction as described by Kartal et al. (63). Protein concentration was measured with the Bio-Rad protein assay based on the method of Bradford (64), using bovine serum albumin as standard. The identification of the isolated protein complex was confirmed by MALDI-TOF mass spectrometry (56). Each spectrum (900–4000 m/z) was analyzed using the Mascot Peptide Mass Fingerprint (Matrix Science) against the K. stuttgartiensis database, allowing methionine oxidation as variable modification, 0.2-Da peptide tolerance, and at most one trypsin mis cleavage.

Data availability

Data that are not contained within this article are to be shared upon request.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: HAO, hydroxylamine oxidoreductase; MCC, multiheme cytochrome; MIMS, membrane-inlet mass spectrometry; OCC, octaheme cytochrome; ORN, octaheme nitrite reductase; OTR, octaheme tetrahionate reductase; OTTLE, optically transparent thin-layer electrochemical cell; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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