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The Eponymous Cofactors in Cytochrome P460s from Ammonia-Oxidizing Bacteria are Iron Porphyrinoids whose Macrocycles are Dibasic

Meghan A. Smith and Kyle M. Lancaster

[†]Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, NY 14853

Abstract

The enzymes hydroxylamine oxidoreductase (HAO) and cytochrome (cyt) P460 contain related unconventional “heme P460” cofactors. These cofactors are unusual in their inclusion of non-standard cross-links between amino acid sidechains and the heme macrocycle. Mutagenesis studies performed on the *Nitrosomonas europaea* cyt P460 that remove its lysine-heme cross-link show that the cross-link is key to defining the spectroscopic properties and kinetic competence of the enzyme. However, exactly how this cross-link confers these features remains unclear. Here we report the 1.45 Å crystal structure of cyt P460 from *Nitrosomonas* sp. AL212 and conclude that the cross-link does not lead to a change in hybridization of the heme carbon participating in the cross-link, but rather enforces structural distortions to the macrocycle away from planarity. Time-dependent density functional theory (TDDFT) coupled to experimental structural and spectroscopic analysis suggest that this geometric distortion is sufficient to define the spectroscopic properties of the heme P460 cofactor and provide clues toward establishing a relationship between heme P460 electronic structure and function.

Graphical Abstract

Supporting Information

Synthetic *Nitrosomonas* sp. AL212 cyt P460 gene, expression and purification details, UV-vis absorption comparison figure, homology model figure, difference density from restrained cofactor refinement, Orca input files, input coordinates for TDDFT calculations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Cytochrome (cyt) P460s are small (~17–23 kDa) proteins found in diverse classes of organisms, including ammonia-oxidizing bacteria (AOB), methanotrophs, proteobacteria, planctomycetes, acidobacteria, and bacteroidetes.^{1–2} Proposed to act in the detoxification of hydroxylamine (NH₂OH)³ and/or nitric oxide (NO),⁴ cyt P460 from *Nitrosomonas europaea* has been shown to be a biological source of the potent greenhouse gas nitrous oxide (N₂O).³ Cyt P460 contains a unique porphyrinoid cofactor named “heme P460” due to the characteristic Soret maximum of the Fe^{II} form near 460 nm.

Heme P460 cofactors are unusual porphyrinoids found in both cyt P460 and hydroxylamine oxidoreductase (HAO), despite these two proteins bearing no sequence or structural homology (Figure 1).⁵ In addition to two covalent Cys cross-links characteristic of *c*-type hemes, hemes P460 feature additional cross-links to amino acid side chains. The active site of HAO contains a Tyr phenolate side chain which forms two covalent attachments to the heme at the 2' and 3' carbons (Figure 1a). The *N. europaea* cyt P460 cofactor bears a cross-link between Lys Ne and the 13' *meso* carbon (Figure 1b).^{4, 6–9} The formation of this cross-link is expected to be autocatalytic, as recombinant expression of cyt P460 in both *E. coli* and *P. aeruginosa* yield the correct Lys-heme attachment.⁶ Mutations to this Lys in *N. europaea* cyt P460 result in heme proteins with spectroscopic properties more typical of cyt *c* (i.e. a sharp Soret band with a maximum around 400 nm) and abolish the NH₂OH oxidase activity.^{6,10} Thus, the cross-link defines the unusual spectroscopic properties and reactivity of hemes P460.

Prior studies have argued that the heme P460 cofactor in HAO should be assigned as a phlorin.⁸ However, the assignment of HAO's active site cofactor as comprising an electron-rich, tribasic macrocycle was thought to be at odds with the role of HAO in oxidative chemistry. It is interesting to note that though the crosslink has been shown to be essential for NH₂OH oxidation activity in *N. europaea* cyt P460, the identity and placement of the cross-link seem unimportant as these attributes are different in cyt P460 and HAO. Both Tyr and Lys have similarly alkaline side-chain *pK_a* values, but it is unlikely that this relates to the ability of both enzymes to oxidize NH₂OH. However, cyt P460 and HAO seem to differ in the binding of NO to the ferric heme. The cyt P460 mechanism has been shown to include an {FeNO}⁶ (following Enemark-Feltham notation¹¹) intermediate,³ which is also proposed by computational models of HAO catalysis.¹² However, whereas the cyt P460 {FeNO}⁶ persists indefinitely in the absence of O₂ or NH₂OH, an HAO {FeNO}⁶ is believed to be short-lived.¹³ As NO was recently shown to be the enzymatic product of HAO,¹⁴ facile NO dissociation accords with the function of the enzyme. Since both HAO and cyt P460 from *N. europaea* are competent for NH₂OH dehydrogenation, only the presence but not the nature of the cross-link appears to be mandatory. However, the nature of the cross-link may influence Fe-NO binding affinities.

In a recent 2.1 Å crystal structure of HAO,¹⁵ it was concluded that the heme carbons participating in the crosslink (C2' and C3') are in fact sp³ hybridized, as evidenced by the elongation of the C—C bond distances of the heme. Although this structure remains consistent with a dibasic macrocycle, the aromaticity is disrupted. Consequently, the

cofactor was described as a tetrapyrrole and not a true porphyrin. In a 1.8 Å crystal structure of *N. europaea* cyt P460, the authors also assign the *meso* C participating in the crosslink as sp³ hybridized by analogy.⁴ If a single macrocycle C is truly sp³ hybridized, this necessitates either a tribasic phlorin or monobasic isoporphyrin configuration (Figure 2) for the cyt P460 cofactor, and thus is distinct from the dibasic HAO heme P460 macrocycle. This is at odds with the similarity of spectroscopic properties between the heme P460 cofactors of these two proteins.

Herein we report a 1.45 Å X-ray crystal structure of cyt P460 from *Nitrosomonas* sp. AL212. This structure, with higher resolution and greater completeness, advances the structural understanding of cyt P460s by providing a model of the polypeptide loop enclosing the cofactor binding site. Our structural analysis, coupled to UV/vis absorption spectroscopy and time-dependent density functional theory (TDDFT) indicate that the *meso* C involved in the cross-link remains sp² hybridized. Distortions in heme planarity and decreased symmetry, enforced by the crosslink, appear sufficient to red-shift the Soret absorption maximum to values characteristic of P460 centers.

Experimental

General Considerations

Milli-Q water (18.2 MΩ; Millipore) was used in the preparation of all buffers and solutions. UV-visible (UV-vis) absorption spectra were obtained using a Cary 60 UV-vis spectrometer. Data were fit using Igor Pro version 6.37 (WaveMetrics). For the generation of the {FeNO}⁶ species, the NO-donor disodium 1-(Hydroxyl-NNO-azoxy)-L-proline (PROLI-NONOate) was used, whereas for the generation of the {FeNO}⁷ species, the HNO-donor disodium diazen-1-ium-1,2,2 triolate (Na₂N₂O₃, Angeli's salt) was used; both were purchased from Cayman Chemicals. All other chemicals were purchased from VWR International.

Protein Overexpression and Purification

A codon-optimized gene for cyt P460 from *Nitrosomonas* sp. AL212 was synthesized and cloned into the *Nco*I and *Xho*I sites of pET-22b(+) vector by GenScript, Inc. The vector was designed to include a C-terminal His-tag found in the parent pET-22b(+) plasmid. This plasmid was co-transformed into *E. coli* BL21(DE3) cells along with a pEC86 plasmid containing the cyt *c* maturation genes ccmABCDEFGH¹⁶ (provided by H.B. Gray) and plated on LB agar supplemented with 100 μg mL⁻¹ each ampicillin and 37 μg mL⁻¹ chloramphenicol. Protein expression and purification followed the protocol for *N. europaea* cyt P460 expression and purification described previously.³ Briefly, cells were grown on a 5 L scale in Terrific Broth containing 0.5% glycerol (0.1% inoculum), 100 μg mL⁻¹ ampicillin, and 37 μg mL⁻¹ chloramphenicol. Cultures were grown at 30 °C for 18 hrs before protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Cells were harvested 6 to 8 hours post-induction and lysed via sonication and centrifuged for 45 min at 35,000 × g relative centrifugal force. The supernatant was applied to a HisTrap HP Ni affinity column (GE Lifesciences), equilibrated with 20 mM Tris (pH 8.0) containing 150 mM NaCl. Proteins were eluted on a gradient over 10 column volumes to 20 mM Tris (pH 8.0) containing 150 mM NaCl and 330

mM imidazole. Green-colored fractions were combined, concentrated and further purified to homogeneity using a HiLoad Superdex 75 size-exclusion column equilibrated with a running buffer of 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.2) containing 150 mM NaCl.

EPR Spectroscopy

X-band (9.40-GHz) EPR spectra were collected on a sample containing 170 μ M *Nitrosomonas* sp. AL212 cyt P460 in 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0 with 25% (v/v) glycerol. The measurements were obtained using a Bruker Elexsys-II spectrometer equipped with a liquid He cryostat maintained at 10.0 K. EPR data were simulated using SpinCount.¹⁷

Crystallization and Data Collection

Purified Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 was screened for crystallization conditions using the PACT Suite (Qiagen). Crystals suitable for diffraction were obtained using the sitting drop method. 3 μ L of 650 μ M *Nitrosomonas* sp. AL212 cyt P460 in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 8.0 were mixed with 1–4 μ L of a crystallization buffer containing 0.2 M sodium chloride, 0.1 M sodium acetate pH 5.0, and 17–22% (w/v) PEG 6000 and allowed to equilibrate above 250 μ L of this buffer. Crystals grew after 2–3 days at room temperature. To avoid oxidative damage, crystals used in diffraction experiments were grown, looped, and frozen in an anaerobic Coy chamber. Crystals were soaked in the aforementioned crystallization solution containing 30% ethylene glycol before being frozen in liquid N₂ for storage and transport.

X-ray diffraction experiments were conducted at beamline NE-CAT 24-ID-C of the Advanced Photon Source (APS). Crystals were irradiated at 100 K using X-rays with a wavelength (λ) of 0.979 Å. An initial model was generated in Phenix using the molecular replacement method and the cyt P460 structure from *N. europaea* (PDB ID 2JE2). The autosol function was then applied in Phenix¹⁸ to produce an initial working *Nitrosomonas* sp. AL212 cyt P460 model. Refinements and building to completion were then conducted using Phenix and Coot,¹⁹ respectively. PyMol²⁰ was used for figure-generation.

Calculations

Electronic structure calculations were performed using version 3.03 of the ORCA quantum chemical computation suite.²¹ Calculations were carried out on cofactors including amino acid sidechains serving as inner-sphere ligands to Fe where applicable. Amino acid residues in the second- and outer-sphere of the cofactor binding pocket were excluded. Calculations employed crystallographic coordinates whose H-atom positions were optimized using the BP86 density functional^{22, 23} and the scalar relativistically recontracted Ahlrich's def2-TZVP(-f)-ZORA functional on all atoms.²⁴ These calculations included the zeroth-order regular approximation (ZORA)²⁵ for relativistic corrections, and solvation was modeled using COSMO²⁶ with a dielectric of 36.6 (acetonitrile). TDDFT calculations using the Tamm-Dancoff approximation²⁷ were initiated from single point calculations that employed the B3LYP hybrid density functional,^{28, 29} the CP(PPP) basis set on Fe,³⁰ def2-TZVP(-f)-ZORA basis set on all other atoms, ZORA, and COSMO with a dielectric of 36.6

(acetonitrile). The RIJCOSX procedure was used to accelerate hybrid DFT calculations.^{31, 32} For energy diagrams, quasi-restricted orbitals (QROs)³³ were generated from unrestricted Kohn-Sham spinorbitals. Orbital images were generated using Chimera.³⁴

Heme out-of-plane distortions were analyzed using an online version of the normal-mode structural decomposition script developed following the normal-coordinate structural decomposition procedure originally outlined by Jentzen and Shelnutz.^{35–36}

Results

Spectroscopic Characteristics

Nitrosomonas sp. AL212 cyt P460 expressed in *E. coli* exhibits UV/vis absorption spectroscopic properties similar to *N. europaea*³ Resting, oxidized *Nitrosomonas* sp. AL212 cyt P460 features a broad Soret absorption band with $\lambda_{\text{max}} = 440 \text{ nm}$ ($\epsilon = 41 \text{ mM}^{-1} \text{ cm}^{-1}$) and Q-bands at 576 nm and 628 nm ($\epsilon = 3.5 \text{ mM}^{-1} \text{ cm}^{-1}$ and $3.9 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively) (Figure 3a, Figure S2). An {FeNO}⁶ can be generated by treating 5 μM Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 with 50 μM NO-donor PROLI-NONOate (Figure 3a). Similarly, an {FeNO}⁷ species can be formed by treatment of 5 μM Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 with 50 μM HNO-donor Na₂N₂O₃ (Angeli's salt) (Figure 3a). The continuous wave X-band (9.40 GHz) EPR spectrum of resting oxidized *Nitrosomonas* sp. AL212 cyt P460 is characteristic of a slightly rhombic high spin ($S = 5/2$) Fe^{III} system, with g_{eff} -values of 6.39, 5.13, 1.97, and an E/D value of 0.03 (Figure 3b). These features closely match those of the *N. europaea* Fe^{III} cyt P460, whose g_{eff} -values are 6.57, 5.09, and 1.97 with an E/D of 0.03.³

Crystal Structure

Diffraction quality, green, rod-like crystals of *Nitrosomonas* sp. AL212 Fe^{III} cyt P460 were grown via the sitting drop method in an anaerobic Coy chamber. These crystals tolerated soaking with cryoprotectant solution containing 30% ethylene glycol. X-ray diffraction data were obtained to high, 1.45 Å resolution using highly attenuated 0.979 Å X-rays at beamline 24-IDC at APS.

The structure of the resting Fe^{III} form of *Nitrosomonas* sp. AL212 cyt P460 was solved via molecular replacement using the structure of *N. europaea* cyt P460 (PDB ID 2JE2) as a model, and the electron density was anisotropically refined³⁸ to a resolution of 1.45 Å. Our *Nitrosomonas* sp. AL212 cyt P460 structure includes residues 37–194 for chain A (80.6% complete) and 35–194 for chain B (81.6% completeness). The absence of at least the first 27 residues may be attributable to cleavage of a periplasmic localization tag as suggested by SignalP.³⁹ Previous structures of cyt P460 from *N. europaea* include breaks in the electron density, resulting in missing loops around the heme P460 active site.⁴ Our structure has no breaks in electron density, and thus affords a model of the full distal heme pocket as well as a loop of residues 113–122 that wraps around from one monomer toward the heme site of another, partially blocking this site and exposing possible functional significance to the dimeric structure of the enzyme (Figure 4, Figure S3).

The *N. europaea* structure features a phosphate ion coordinated to Fe *trans* to His140,⁴ while HAO structures feature H₂O bound in this position.¹⁵ By contrast, the *Nitrosomonas* sp.

AL212 Fe is 5-coordinate. While we cannot directly rule out the possibility that photoreduction of cyt P460 occurred during data collection, no photoreduction was observed for the *N. europaea* cyt P460 crystal structure, which shows an almost identical heme structure.⁴ Moreover, prolonged exposure of Fe^{III} heme P460 cofactors to high-flux X-rays in Fe K-edge XAS experiments does not result in any spectral changes that would be consistent with Fe reduction.¹⁰ We considered the possibility that Phe76 in the *Nitrosomonas* sp. AL212 structure disfavors axial ligand coordination (Figure S3). Phe76 is part of the loop comprising residues 65–80 that is missing from the *N. europaea* structure. Based on mass spectrometry evidence, the absence of this loop was attributed to oxidative damage to cyt P460 that occurred during crystallization.⁴ Thus, it is possible that the *N. europaea* active site is similarly hindered from distal axial ligand coordination when the full loop is present. Structural homology modeling reveals that this loop is similar in both isozymes (Figure S3), including in the placement of the “capping” Phe sidechain distal to the heme cofactor. The similarity of g-values in the $S = 5/2$ EPR signals characteristic of resting Fe^{III} cyt P460 in both *N. europaea* and *Nitrosomonas* sp. AL212 and the similar UV-vis absorption profiles (Figure S2) of both proteins strongly suggests that both proteins are 5-coordinate in their resting Fe^{III} form.

Heme Geometry and Normal-Coordinate Structural Decomposition

Refinements of the Fe^{III} are 5-coordinate in their resting X-ray datasets were carried out with effectively no restraints imposed on cofactor structural parameters. Distances and angles about the Lys N-C13' cross-link resulting from these refinements are shown for the A subunit in Figure 6, with complete heme metrical parameters for cofactors in both subunit reproduced in Table 2. Metrical parameters about the cross-linked 13' *meso* C are inconsistent with sp³ hybridization. To further probe this geometry, we imposed restraints on these distances to enforce sp²-sp³-like distances ca. 1.5 Å. This lead to the generation of negative difference density in the $F_{\text{obs}} - F_{\text{calc}}$ omit map clearly visible at 2.5 σ (Figure S4), further supporting a model with sp²-sp² C-C distances (~1.39 Å)

The macrocycles in *Nitrosomonas* sp. AL212 and *N. europaea* cyt P460, as well as HAO are highly distorted away from planarity, as determined via normal-coordinate structural decomposition (NCSD)^{35, 36} It has been shown that the type of heme distortion is generally conserved among proteins with the same functionality.^{37,40} The cyt P460 Fe^{III} heme experiences almost equal distortion from saddling (B_{2u}) and ruffling (B_{1u}) (Table. 3).

Calculated Spectroscopy

TDDFT calculations were used to predict UV-vis absorption spectra corresponding to the heme P460 derived from the Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 crystal structure. Variants with a protonated, “forced” sp³ 13' *meso* C were generated by geometry optimization of H-atom positions using the BP86 functional and ZORA-def2-TZVP basis set on all atoms. The positions of non-H atoms were taken directly from the subunit A in the crystal structure, then H atoms were added as necessary to produce either a porphyrin, isoporphyrin, or phlorin configuration. Resultant structures were used as coordinates for the subsequent TDDFT calculations, which included sufficient roots to saturate the Soret region of the absorbance profile. The heme structure from horse heart metmyoglobin (PDBID:

1WLA⁴²) was also subjected to this procedure to facilitate comparisons. A key feature of the spectra calculated for the three cyt P460 macrocycle variants are absorption profiles that are highly red-shifted and diminished in intensity relative to that of metmyoglobin, in accord with the experimental spectra (Figure 7). These calculations predict an intense feature near 700 nm for the phlorin variant; while appreciable error is expected in the energy values calculated via TDDFT for these systems, the lack of any appreciable absorbance past the Q-band region in experimental Fe^{III} cyt P460 spectra (cf. Figure 3a) disfavors the phlorin assignment. We note that making a concrete assignment based on TDDFT is tenuous, nevertheless these data strongly suggest that sp³ hybridization of the 13 'C is not necessary to give rise to an absorption profile with a red-shifted, weak Soret characteristic of Fe^{III} cyt P460.

An additional factor that could perturb the heme P460 UV-vis absorption profile relative to canonical *c* type hemes is simply the presence of a *meso* substitution. In particular, Lys N lone pair admixture in the porphyrin ring π -system could profoundly influence the absorption spectrum. The influence of *meso* substitution was explored via calculation of spectra corresponding to two additional heme P460 variants: one whose cross-linking Lys N (of the 'meso C-N' variant) was replaced with a methylene (data for this structure hereby referred to as 'meso C-C') and a spectrum where the cross-link was removed entirely, leaving a standard *meso* unsubstituted porphyrin ('meso C-H'). These calculated spectra (Figure 8) show that the *meso* unsubstituted heme P460 variant Soret maximum is red-shifted by 20 nm relative to the calculated Soret maximum for metmyoglobin. Substitution by either a C- or N-based linkage leads to a larger, 35 nm red shift, with the N-cross-linked variant showing the broadest profile and the least intense Soret absorption. Thus, *meso*-substitution—whether *meso* C-C or *meso* C-N bonds— compounds the red-shift imposed by heme distortion from planarity.

These electronic structure calculations can be used further to explain the curiously broad Soret absorption profile of the Fe^{III} heme P460 cofactor. Following the four orbital model of porphyrin electronic structure defined by Gouterman and assuming—for convenience—rigorous D_{4h} symmetry (Figure 9),⁴³ Lys N lone pair mixing into the porphyrin ring π system is expected to most directly influence the composition and energetics of the a_{2u} -like orbital, causing it to increase in energy, which would result in a red-shifted Soret. This prediction is supported by the electronic structure calculations (Figure 10). Interestingly, the a_{2u} -like orbital's energy increase raises it to be nearly isoenergetic with the a_{1u} -like orbital; this accidental degeneracy should lead to a sharper Soret instead of the broad, split Soret that is observed for cyt P460. However, the Lys N lone pair also contributes to one of the e_g orbitals, breaking the degeneracy of the e_g -like orbitals, thus providing an explanation for the observed broadening of the cyt P460 Soret band.

Discussion

The structural parameters for the heme macrocycles in Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 are most consistent with those of canonical porphyrin structures, where typical C-C distances and angles are 1.39 Å and 125°, respectively.⁴⁴ For isoporphyrin or phlorin structures, typical distances about the sp hybridized C are elongated to approximately 1.5 Å.

^{45–46} The geometry about the cross-link also agrees with a more planar *meso* C, consistent with an sp²—and not sp³—hybridized C.

The 13 'C geometry indicates that the cyt P460 heme macrocycle is dibasic. Moreover, the TDDFT-predicted spectra show that a hybridization change of the 13 ' *meso* C is not necessary for the unusual spectroscopic characteristics of cyt P460. That is, an equally red-shifted Soret is predicted for all three macrocycle variants: porphyrin, isoporphyrin, and phlorin. The observed red-shift is thus best attributable to a combination of *meso* substitution and distortions of the macrocycle away from planarity. While the exact role of the cross-link remains unclear, a corrected electronic structural formulation now opens the possibility for detailed spectroscopic studies informed by high-level calculations.

We speculate that in addition to N lone pair participation in the porphyrin π system, another possible role for the cross-link is to enforce and/or promote the observed heme distortion. Distortions in the heme that lead to out-of-plane deformations are common in many heme-bearing proteins.⁴⁰ The most common types of deformation are ruffling (alternating rotations around the Fe-N bonds), saddling (movement of the pyrrole rings up and down), and doming (bending of all pyrrole rings in one direction).^{36,37,40} Ruffling has been observed in the P460 cofactors of both HAO and cyt P460. Many properties of certain heme enzymes have been attributed to ruffling, including a lower reduction potential³⁷ and stronger bonding between the Fe and axial ligand.⁴⁹ The very negative Fe^{II/III} reduction potentials (ca. -400 to -300 mV vs. normal hydrogen electrode) exhibited by the cyt P460 and the HAO active sites may be why these centers avoid auto-reduction upon NO binding via reductive nitrosylation.¹³ Moreover, maintaining coordination of axial histidine Fe during enzymatic turnover has been shown to be important to the activity of cyt P460.¹⁰ Thus, the common characteristics of ruffling seem to agree with the requirements of cyt P460 and HAO activity; however, there are problems with ascribing the properties of these enzymes entirely to ruffling, which is present in many enzymes besides cyt P460 and HAO (Table 3) that have not been shown to be competent for NH₂OH oxidation.

Ruffling has been offered as the reason for favoring {FeNO}⁶ over {FeNO}⁷ formation in the enzyme nitrophorin.⁴⁸ In certain blood-sucking insects, nitrophorin is used to release NO into the blood stream of the victim, leading to vasodilation and inhibition of platelet aggregation. This behavior relies on {FeNO}⁶ formation, as an {FeNO}⁷ species has a NO dissociation constant in the picomolar range (compared to micromolar for the {FeNO}⁶).⁴⁹ The ruffling observed in nitrophorin is suggested to stabilize the protein against reductive nitrosylation, favoring the formation of the {FeNO}⁶ necessary for release of NO in the victim, which is triggered by changes in pH or dilution of the concentration of NO around the protein.⁴⁸ The degrees of ruffling observed for cyt P460 and NO-bound nitrophorin are similar, so this agrees with the observation of an {FeNO}⁶ species formed by cyt P460, which persists in anaerobic solution unless NH₂OH is introduced or if NO is removed from solution. The appreciable difference in the ruffling of HAO vs. cyt P460 and nitrophorin may also explain differential NO binding affinities between these enzymes.³ Thus, not only the presence of ruffling, but the degree to which the heme is distorted out of the plane seem important to reactivity, and may be heavily influenced by the cross-link. These structure-

function relations will be explored in future work informed by enhanced definition of the cofactor afforded by our presently reported structure.

Conclusions

We have obtained the structure of *Nitrosomonas* sp. AL212 cyt P460, which includes a complete active site pocket absent in prior cyt P460 structures. The metrical parameters about the cross-link indicate that the cross-link does not impose any hybridization change on the 13' *meso* C from sp^2 to sp^3 . Our TDDFT-predicted spectra also show that such a hybridization change is not necessary to red shift the cyt P460 UV-vis spectra relative to a standard heme. The cyt P460 cofactor macrocycle is therefore best formulated as a dibasic porphyrin. Though the complete role of the cross-link remains undefined, we have shown that it subtly alters the heme electronic structure. This arises both by virtue of substituting the *meso* position C-H for a C-N linkage, and furthermore the cross-link contributes an anti-bonding interaction between the Lys N lone pair and the π system of the macrocycle. This interaction destabilizes key molecular orbitals comprising transitions that give rise to the Soret absorption feature. We hypothesize that the cross-link may promote and/or enforce heme distortions essential to function. These possibilities will be explored in future work employing our more complete structural analysis of the heme P460 cofactor that will seek to understand the exact effects of heme cross-links and distortions as they relate to Fe-nitrosyls and selective redox transformations of energetic substrates such as NH_2OH .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Heme P460 cofactors from *N. europaea* (a) hydroxylamine oxidoreductase (PDBID: 4FAS) and (b) cytochrome P460 (PDBID: 2JE3).

Figure 2.

A single sp^2 meso C in a porphyrin backbone can give rise to either isoporphyrin or phlorin electronic configurations.

Figure 3.

(a) UV-vis absorption spectra of resting Fe^{III} (black), {FeNO}⁶ (red), and {FeNO}⁷ (gray) forms of *Nitrosomonas* sp. AL212 cyt P460. (b) 10 K X-band (9.40 GHz) EPR spectrum of Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 (black) recorded at 633 μW microwave power. The corresponding SpinCount simulation is shown in red.

Figure 4. Crystal structure of *Nitrosomonas* sp. AL212 Fe^{III} cyt P460 (PDB ID 6AMG). Subunit A shown in cyan, subunit B shown in purple. The heme P460 cofactors are shown in green.

Figure 5. Cofactor binding pocket of *Nitrosomonas* sp. AL212 cyt P460 depicted using (a) sticks or (b) space-filling spheres. The $2F_o - F_c$ simulated annealing omit map at a level of 2.5σ is displayed for the loops that cover the distal side of the heme P460 cofactor. Subunit A shown in cyan, subunit B shown in purple. The heme P460 cofactors are shown in green.

Figure 6.
2F_o-F_c simulated annealing omit map shown at 2.5 σ and corresponding stick representation of the heme P460 cofactor of subunit A from *Nitrosomonas* sp. AL212 Fe^{III} cyt P460. The inset shows the 13' *meso* C participating in the cross-link with the Lys106 sidechain and corresponding metrical parameters about this atom.

Figure 7.

(a) Experimental and (b) TDDFT-calculated (B3LYP/ZORA-def2-TZVP(-f) with CP(PPP) on Fe) UV-vis absorption spectra of horse heart metmyoglobin and Fe^{III} *Nitrosomonas* sp. AL212 cyt P460. In (b), spectra are calculated for isoporphyrin, porphyrin, and phlorin macrocycle configurations.

Figure 8. TDDFT-calculated (B3LYP/ZORA-def2-TZVP(-f) with CP(PPP) on Fe) UV-vis absorption spectra of horse heart metmyoglobin and Fe^{III} *Nitrosomonas* sp. AL212 cyt P460 variants where the 13' meso C is bound to H, C (from RCH₂), or N (from RNH).

Figure 9.

(a) Simplified, top down depiction of the frontier molecular orbitals of a porphyrin π -system modified to show the *meso*-C cross-link and labeled with corresponding D_{4h} irreducible representations, (b) Quasi-restricted orbital (QROs) plots depicting the porphyrin π -system molecular orbitals of the Fe^{III} cyt P460 cofactor with Lys N lone-pair admixture. Calculations were carried out using B3LYP/ZORA-def2-TZVP(-f) with CP(PPP) on Fe, and orbitals are plotted at an isovalue of 0.03 au.

Figure 10.

Simplified energy diagram depicting frontier porphyrin π -system molecular orbitals of the Fe^{III} cyt P460 cofactor as a dibasic porphyrin with Lys cross-link (meso C-N) and dibasic porphyrin variants where the cross-link is modified to a meso C-C linkage or removed entirely (meso C-H). The red lines indicate molecular orbitals whose energies are influenced by orbital admixture from the cross-link.

Scheme 1.

Atom numbering used for the *Nitrosomonas* sp. AL212 cyt P460 cofactor.

Table 1.

X-ray data collection and crystal structure refinement statistics.

Fe^{III} cyt P460	
Wavelength (Å)	0.979
Temperature (K)	100
Space Group	P 2 ₁ 2 ₁ 2 ₁
a (Å)	47.267
b (Å)	80.138
c (Å)	108.962
α (deg)	90
β (deg)	90
γ (deg)	90
Reflections	475,694 (12,166)
Number of Reflections in <i>R</i>_{work} Set	66,399
Number of Reflections in <i>R</i>_{free} set	3,441
Resolution (Å)	64.62–1.45
<i>R</i>_{merge}(%)	4.1 (72.7)
CC_{1/2}	0.999 (0.748)
Completeness (%)	97.0 (90.4)
Redundancy	6.6 (4.6)
I/σ (I)	23.8 (1.4)
<i>R</i>_{work}	14.09 (25.04)
<i>R</i>_{free}	15.75 (23.4)
RMSD from Ideality	
Bonds (Å)	0.0 28
Angles (deg)	2.348
Average <i>B</i> Factors (Å ²)	29.24
Ramachandran Plot	
Allowed Regions (%)	100
Disallowed Regions (%)	0
PDBID	6AMG

Table 2.Geometric parameters obtained from *Nitrosomonas* sp. AL212 cyt P460 crystal structure.^b

C2-C3	1.34 Å (1.33 Å)
C3-C4	1.30 Å (1.32 Å)
C7-C8	1.35 Å (1.32 Å)
C8-C9	1.35 Å (1.31 Å)
C12-C13	1.39 Å (1.39 Å)
C13-C14	1.41 Å (1.40 Å)
C17-C18	1.39 Å (1.33 Å)
C18-C19	1.30 Å (1.35 Å)
C13-N_{Lys}	1.37 Å (1.37 Å)
∠ C2-C3-C4	133.4° (132.8°)
∠ C7-C8-C9	130.1° (135.5°)
∠ C12-C13-C14	123.0° (121.8°)
∠ C17-C18-C19	127.5° (123.6°)
∠ C12-C13-N_{Lys}	116.0° (118.1°)
∠ C14-C13-N_{Lys}	120.9° (118.9°)
Dihedral ∠C₁₃ plane-N_{Lys}	175.6° (166.7°)

^bValues outside of parentheses are given for the A subunit, values in parentheses correspond to the B subunit.

Table 3.

Normal-coordinate structural decomposition for representative heme cofactors.

	Saddling (b_{2u})	Ruffling (b_{1u})	PDBID	Reference
<i>Nitrosomonas</i> sp. AL212 Fe ^{III} cyt P460	0.82	−0.89	6AMG	This work
<i>N. europaea</i> cyt P460	0.78	−0.76	2JE3	This work
<i>N. europaea</i> HAO	0.14	2.36	4FAS	This work
Metmyoglobin	0.03	−0.20	1WLA	This work
Horse heart cyt C	−0.23	−1.01	1HRC	This work
Nitrophorin 4—H ₂ O	0.30	−0.55	1X8Q	Ref. 41
Nitrophorin 4—NO	0.37	−0.81	1X80	Ref. 41