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Formation and Reactivity of New Isoporphyrins: Implications for Understanding the Tyr-His Cross-Link Cofactor Biogenesis in Cytochrome *c* Oxidase

Melanie A. Ehudin[†], Laura Senft[‡], Alicja Franke[‡], Ivana Ivanovi -Burmazovi [‡], and Kenneth D. Karlin^{*,†}

[†]Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, United States

[‡]Department of Chemistry and Pharmacy, Friedrich-Alexander University Erlangen-Nuremberg, 91058 Erlangen, Germany

Abstract

Cytochrome *c* oxidase (CcO) catalyzes the reduction of dioxygen to water utilizing a heterobinuclear active site composed of a heme moiety and a mononuclear copper center coordinated to three histidine residues, one of which is covalently cross-linked to a tyrosine residue via a post-translational modification (PTM). Although this tyrosine—histidine moiety has functional and structural importance, the pathway behind this net oxidative C—N bond coupling is still unknown. A novel route employing an iron(III) *meso*-substituted isoporphyrin derivative, isoelectronic with Cmpd-I ((Por^{•+})Fe^{IV}=O), is for the first time proposed to be a key intermediate in the Tyr-His cofactor biogenesis. Newly synthesized iron(III) *meso*-substituted isoporphyrins were prepared with azide, cyanide, and substituted imidazole functionalities, by adding nucleophiles to an iron(III) π -dication species formed via addition of trifluoroacetic acid to F₈Cmpd-I (F₈ = (tetrakis(2,6-difluorophenyl)porphyrinate)). Isoporphyrin derivatives were characterized at cryogenic temperatures via ESI-MS and UV—vis, ²H NMR, and EPR spectroscopies. Addition of 1,3,5-trimethoxybenzene or 4-methoxyphenol to the imidazole-substituted isoporphyrin led to formation of the organic product containing the imidazole coupled to aromatic substrate via a new C—N bond, as detected via cryo-ESI-MS. Experimental evidence for the formation of an imidazole-substituted isoporphyrin and its promising reactivity to form the imidazole—phenol coupled product yields viability to the herein proposed pathway behind the PTM (i.e., biogenesis) leading to the key covalent Tyr-His cross-link in CcO.

Graphical Abstract

*Corresponding Author karlin@jhu.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b01791.

Synthetic and analytical details (methodologies and UV—vis, ESI-MS, EPR, and NMR spectra) (PDF)

The authors declare no competing financial interest.

Proposed CcO Tyr-His Cofactor Biogenesis Pathway via Isoporphyrin Intermediate

INTRODUCTION

Cytochrome *c* oxidase (CcO), found in the mitochondria electron transport chain, catalyzes the reduction of dioxygen to two molecules of water through the use of a heterobinuclear active site composed of a histidine axially ligated high-spin heme a_3 (Fe a_3) and a mononuclear copper center, Cu_B, in the distal heme pocket (Figure 1).¹⁻⁶ This exergonic redox reaction is coupled to the membrane translocation of protons, both of which drive the synthesis of the chemical energy source adenosine triphosphate (ATP).^{1,4,7-10} In the active site of heme-copper oxidases (HCOs), three histidine (His) residues chelate Cu_B, one of which is covalently cross-linked to a highly conserved tyrosine (Tyr) residue^{2,6,11-13} biosynthesized via a post-translational modification (Figure 1).^{2,4,5,10,14-17} It has been established that the tyrosine–histidine moiety acts as a crucial redox site for the enzyme’s oxidase activity (donating a proton and electron during the catalytic cycle) and is structurally significant, ensuring the proper configuration of the Cu_B ion in the active site to be able to interact with heme a_3 .^{4,14,15,18-22}

Investigations with myoglobin (Mb)-engineered systems mimicking HCOs, by Lu and co-workers, have highlighted the functional significance behind the tyrosine–histidine cross-link binding to a Cu_B site,²³⁻²⁸ as systems without the tyrosine or lacking the cross-link were more susceptible to deleterious selfoxidation reactions resulting in decreased turnovers of O₂ reduction to water. Further, they observed significantly greater selectivity toward catalyzing O₂ reduction to water (vs harmful reactive oxygen species formation pathways) and an increased number of turnovers using a protein construct incorporated with the unnatural amino acid imiTyr, which mimics the Tyr-His cross-link (versus without the cross-link).²⁵ Due to difficulty in isolating the pro-enzyme state of natural HCOs, an inactive precursor without a Tyr-His cross-link, understanding the pathway behind its formation is challenging.^{1,15} This may be attributed to the instability of the enzyme without the cross-link and its importance in ensuring proper protein folding and the relative geometry of metal centers (*vide infra*).^{4,23-25,30} However, the HCO literature consensus is that the His-Tyr cross-link may form during the initial turnovers of the catalytic cycle.^{1,24} Here, we employ a synthetic heme and develop new reaction chemistry demonstrating how a C–N bond might form to give a cross-link like that in CcO (*vide infra*); as befits a scientific model approach,^{31,32} this chemistry occurs without being able to fully reproduce the enzymatic environment or aqueous, physiological conditions relevant to the protein.

In spectroscopic studies by Rousseau and co-workers involving CcO from *Rhodobacter sphaeroides*, site-directed mutagenesis of Tyr288 (to Phe, i.e., Y288F) that normally is cross-linked to the Cu_B ligand His284 resulted in lack of oxidase function.³⁰ The heme *a*₃ moiety, usually high-spin, was instead found to be a low-spin six-coordinate iron(III) species. The authors suggested that when the Tyr-His cross-link is not present, His284 can bind to the iron metal center (Figure 2A).³⁰ Proper protein folding and copper binding (in the Cu_B site) are hindered without the cross-link (only 30% occupancy of Cu in the Y288F mutant), thus suggesting that the cross-link may be formed before Cu enters the active site (i.e., the cross-link formation is possibly heme-only catalyzed).^{1,30}

This is further corroborated by investigations utilizing the HCO in *Escherichia coli*, cytochrome *bo*. This enzyme has a Tyr-His cross-link in the active site with proton/electron donor capabilities at the distal side of heme *a*₃ (heme *o* in cytochrome *bo*) synonymous with CcO.³⁴ A biosynthetically modified cytochrome *bo* with a deuterated tyrosine (Tyr-*d*₄) residue at the heterobinuclear site showed reduced oxidase activity (versus the native enzyme).^{1,34} Kitagawa and co-workers showed that this active site Tyr-*d*₄ caused a distorted geometry of the Cu_B metal center relative to heme *o*, and thus a decrease in proper protein folding resulting in a decreased amount of enzyme containing the Tyr-His cross-link.³⁴ It was proposed that the isotopically modified tyrosine residue inhibited cross-link formation due to the strengthened C–D bond (vs the native C–H bond) on the Tyr residue, and the subsequent decrease in oxidase function again substantiated the key role of the Tyr-His cross-link at the dioxygen reduction site.³⁴ Again, this suggests that the cross-link is heme-only catalyzed, as its formation is needed to properly orient the Cu_B ion to interact with the heme moiety to facilitate O₂ reduction in the mature and active enzyme.

Role of Cmpd-I (like) Species Responsible for Biosynthesis of Functionally Important Cross-Links in Heme Enzymes.

It is the premise of the present study and it is demonstrated below that critical chemistry emanating from a Cmpd-I-like (e.g., (Por^{•+})Fe^{IV}=O or an isoelectronic form, *vide infra*) species (which forms at the CcO active site) leads to formation of the functionally critical Tyr-His cross-link. In fact, high-valent heme iron(IV) oxo species have been proposed as reactive intermediates for formation of covalently cross-linked amino acid post-translational modifications in other enzymes.^{15-17,34-36} The environment wherein post-translational modifications occur likely influences any course of reaction (i.e., mechanism) responsible for these modifications. For instance, the environment may dictate the identity of the reactive intermediate, the orientation/juxtaposition with which species/substrates may interact, and the basicity/protonation state or valence tautomer state of species/substrates.

The heme-containing enzymes catalase–peroxidases (KatG), which possess both peroxidase and catalase activities, post-translationally incorporate a unique cofactor with two covalent cross-links formed between tryptophan (Trp), tyrosine, and methionine (Met) residues on the distal side of the catalytic heme center (Figure 2B and Scheme S1).^{16,17,37,38} However, unlike for CcO, the pro-enzyme of KatGs, without the cross-links present, is able to be isolated and studied.³⁸ Cross-link formation is observed upon addition of peroxyacetic acid to the oxidized pro-enzyme, suggesting that a high-valent (Cmpd-I) intermediate may be

responsible, through initial one-electron oxidation of both Tyr and Trp residues (an overall two-electron oxidation), facilitating radical coupling and thus C–C bond formation (Scheme S1).^{16,38,39} Further oxidation of the Tyr-Trp moiety mediated by a second equivalent of Cmpd-I allows for nucleophilic attack by the sulfur of the nearby Met residue, facilitating C–S bond formation (Scheme S1).^{16,38} Cross-link biogenesis is dependent on the heme and the presence of exogenous H₂O₂ to form Cmpd-I, while the catalytic function of the mature enzyme is dependent on the cross-links.^{17,39}

There are many documented cases of heme enzymes possessing AA/AA or AA/heme cross-links (AA = amino acid), such as for AA Tyr, Cys, His, Trp, or lysine (Lys).^{15-17,40-44} Detailed mechanisms for the formation of these heme moieties with new O–C,^{42,45} S–C,^{38,39,46} or N–C^{11,12,47,48} bonds are generally not known; however, Cmpd-I species are implicated as part of the new bond-forming reactions.^{15-17,34-36} The Cmpd-I species may be derived either from conditions of (i) dioxygen + reductant (such as occurs in cyt. P450 monooxygenases^{5,49}) (Scheme 1A) or (ii) hydrogen peroxide plus ferric heme⁵⁰⁻⁵² (Scheme 1B). In fact, an iron(IV) oxo species is observed during the catalytic cycle of CcO (Scheme 1A).¹⁻⁵ Since KatG and CcO both employ highly oxidizing ferryl intermediates in their catalytic cycles, it is theorized that the Tyr-His cross-link may possibly be formed by such a species. This can be especially true for heme-based enzymes, which commonly utilize many different isoelectronic forms of Cmpd-I to carry out various biochemical transformations including iron(IV) oxo complexes with a porphyrin π -cation radical, iron(IV) oxo complexes with a nearby amino acid radical, iron(III) porphyrin *N*-oxide species, iron(III) porphyrin dication complexes, or iron(III) isoporphyrin complexes (Figure S1).^{41,53}

RESULTS AND DISCUSSION

Proposed Pathway of Cross-Link Formation in CcO: Heme-Only Catalyzed via Isoporphyrin Reactive Intermediate.

Fujii and co-workers have found that addition of 20 equiv of trifluoroacetic acid (TFA) to F₂₀Cmpd-I (F₂₀ = tetrakis(pentafluorophenyl)porphyrinate) in dichloromethane (DCM) at –90 °C led to oxo ligand protonation, release of water, and formation of an iron(III) π -dication complex (Scheme 2).⁵⁴ Subsequent addition of two equivalents of tetrabutylammonium chloride (TBACl) leads to *meso* position chloride nucleophilic attack and generation of an iron(III) *meso*-chloro-isoporphyrin complex. This species is isoelectronic to Cmpd-I (Scheme 2 and Figure S1B), and the authors demonstrated for the first time that this can also be highly oxidizing.^{41,54} For instance, they were able to selectively and cleanly chlorinate aromatic and olefin substrates (e.g., 1,3,5-trimethoxybenzene, anisole, cyclohexene) by adding them to the iron(III) *meso*-chloro-isoporphyrin, effecting the transfer of a chlorine atom via electrophilic aromatic substitution (Scheme 2).⁵⁴

Isoporphyrins are NH tautomers of porphyrins in which one proton migrates such that the *meso*-carbon hybridization changes from sp² to sp³ (Figure S1).^{41,55,56} Isoporphyrin species are essential intermediates in heme oxidation and chlorophyll biosynthesis.^{41,57-61} In the enzyme heme oxygenase (HO), iron(III) *meso*-hydroxy-isoporphyrin may be a key intermediate in the degradation of heme proteins to biliverdin, carbon monoxide, and free

iron and thus is important to iron homeostasis and metabolism.^{58,60-66} Isoporphyrin-like intermediates have also been found to be catalytically active in bacterial denitrification pathways, specifically hydroxylamine oxidation to nitric oxide via hydroxylamine oxidoreductases (HAOs) or to nitrous oxide via cytochrome P460.^{41,45,47,48,67} In HAOs, the active heme moiety is cross-linked to a nearby tyrosine residue at both the *meso* position and a pyrrole carbon (Figure S2A), while in cytochrome P460 the heme is cross-linked to a nearby Lys residue (Figure 2B). These cross-links are thought to ruffle the heme moiety, stabilizing a ferric nitrosyl intermediate in the catalytic pathway (Figure S2)^{41,45,47,48,67}

Due to the disruption in the conjugation of the macrocycle (and thus the aromatic current), there is a smaller energy gap between the HOMO and LUMO of an isoporphyrin complex, resulting in characteristic UV-vis features, such as a weak, split Soret band and two intense low-energy Q-bands (700–1000 nm).^{41,68-70} These UV-vis features have been previously reported for various synthetic metalloporphyrins,^{41,54,59,68,71-76} including iron(III) *meso*-isoporphyrins incorporating a *tert*-butylperoxy, hydroxy, or chloro functionality on the *meso* carbon (Scheme 2, Figure S3, and Table S1).^{41,54,68,72} Their strong absorption in the near-IR region allows them to be promising photosensitizers for photodynamic therapy (cancer treatment).^{41,77,78}

Thus, a novel proposal for the cofactor biogenesis in CcO is presented herein (Scheme 3): Cmpd-I first forms via O₂ reduction chemistry or oxidation of the ferric heme by cellular H₂O₂. Subsequently, tautomerization to the isoelectronic iron(III) π -dication species⁷⁹ allows for consequent His residue nucleophilic attack on the heme (Scheme 3). This results in the formation of an electrophilic His moiety, an iron(III) *meso*-histidine-isoporphyrin species, which can be readily attacked by the aromatic Tyr residue nucleophile, forming the Tyr-His cross-link (Scheme 3). As delineated above, the presence of the cross-link allows the Cu ion to enter the active site and correctly orient; Cu_B now has the proper structural and electronic/redox properties to effect normal dioxygen binding and reduction.

There are some other aspects of discussion, related to our proposal, worth discussing: (i) It is possible that hydrogen-bonding interactions with interstitial waters (to protonate and thus weaken the iron(IV) oxo bond) may aid the enzyme in driving the formation of the iron(III) π -dication species. Further, the proximal axial histidine ligand present in CcO would allow the iron(IV) oxo moiety to be more basic than the oxo in our synthetically prepared Cmpd-I with F₈ (F₈ = tetrakis(2,6-difluorophenyl)porphyrinate); thus, the formation of the iron(III) π -dication species in the enzyme would also be facilitated. A weaker acid such as a nearby tyrosine residue can be utilized (Scheme 3) instead of the strong trifluoroacetic acid that was needed to synthetically prepare the iron(III) π -dication species in this work (*vide infra*). Also, a *proximity effect* should greatly facilitate His imidazole direct nucleophilic addition to the close-by heme *meso* position in Cmpd-I even with only the presence of a weak acid (e.g., a water or Tyr residue) to protonate the oxo O atom. (ii) It is also possible that the cross-link in CcO could form via the same mechanism as proposed for part of the KatG cross-link, wherein Cmpd-I oxidizes the proximal Trp and Tyr residues, giving radicals, which is followed by their coupling (Figure 2). Other mechanisms for the cross-link formation in CcO are not discounted, but herein we suggest a new (novel) idea that has not been previously considered based on a synthetic model study (*vide infra*) supporting the

comparable oxidative capabilities of an iron(III) *meso*-isoporphyrin complex (vs an iron(IV) oxo complex with a porphyrin π -cation radical). Addition of a substituent to an aromatic moiety (e.g., like an electron-rich phenol in a Tyr residue) can of course proceed by well-known aromatic electrophilic substitution chemistry; chlorination of tryptophan residues by such a mechanism to give C–Cl bonds is well known for flavin-dependent halogenases.^{80,81}

Formation of Iron(III) *meso*-4,5-Dimethylimidazole-Isoporphyrin Complex.

To support this postulated pathway, methods established by Fujii and co-workers⁵⁴ (Scheme 2) were employed in this work to synthetically generate the postulated key reactive intermediate, a new compound, an iron(III) *meso*-imidazole-isoporphyrin (Scheme 4). A synthetic Cmpd-I complex was generated, and 20 equiv of TFA was added, generating an iron(III) π -dication complex. Various imidazoles, organic analogues to the histidine residue, were then added in an attempt to yield the desired isoporphyrin derivative. Substituted phenols, to resemble the protein tyrosine residue, were reacted with the iron(III) *meso*-imidazole-isoporphyrin to form coupled C–N bond products.⁸² Our spectroscopic and mass spectrometric (cryo ESI-MS) supporting data and explanations follow.

F₈Cmpd-I (F₈ = tetrakis(2,6-difluorophenyl)porphyrinate) was prepared utilizing previously established methods.⁵⁸ The ferric precursor, F₈Fe^{III}SbF₆, was cooled to –90 °C in DCM, and 2 equiv of the oxidant *meta*-chloroperbenzoic acid (mCPBA) was added, after which the reaction mixture changed from brown to typical emerald green for these types of complexes (Scheme 4A). Characteristic iron(IV) oxo π -cation radical spectral features were observed, such as the diminished intensity of the Soret band (Figure S4, top) and the intense low-energy bands centered around 600 nm (Figure 3A).^{53,54,58,83-87} Further, cryogenic electrospray ionization mass spectrometry (ESI-MS) measurements identified a peak at 828.0838 (M⁺) corresponding to the ferryl species (Figure 3B). Addition of 20 equiv of TFA to this F₈Cmpd-I complex resulted in the color change of the reaction mixture from green to purple and the formation of a stable (>3 h) iron(III) π -dication species (Scheme 4B and Figures 3A, S4, and S5). Attempts to use lesser amounts of TFA failed to lead to formation of a clean isoporphyrin species in subsequent steps, substantiating the need for 20 equiv of TFA as reported by Fujii and co-workers.⁵⁴ The reaction was monitored by UV–vis and EPR spectroscopy, wherein an immediate blue shift was observed (Figures 3A and S4) and typical high-spin ferric signals at *g*=6 and 2 (Figure S5).

To confirm that the iron(III) π -dication complex had been formed successfully in our system (F₈ vs F₂₀), 2 equiv of TBACl was added (Schemes 2 and 5A), resulting in similar UV–vis and cryo-ESI-MS spectra (Figure S6) to those reported by Fujii and co-workers⁵⁴ for the iron(III) *meso*-chloro-isoporphyrin. Further, addition of the strong but weakly basic nucleophiles, TBACN and TBAN₃, to the iron(III) π -dication complex (Scheme 5B and C) similarly yielded the respective iron(III) isoporphyrin complexes, with either an azide or cyanide functionality at the *meso* carbon (Figures S7 and S8). The formation of the newly synthesized iron(III) *meso*-cyanide-isoporphyrin and iron(III) *meso*-azido-isoporphyrin complexes was confirmed by the observed diagnostic spectral features of isoporphyrin complexes, a weak split Soret band around 374 and 409 nm and two intense low-energy bands around 800 and 900 nm (Scheme 5B and C, Figures S7A,B and S8A,B, and Table

S1). Interestingly, titration experiments via UV-vis spectroscopy, in which TBAN₃ or TBACN was added incrementally to the iron(III) π -dication complex, indicated that 6 equiv was needed to fully form the isoporphyrin complex (indicated by no further spectral change upon addition of more equivalents of either nucleophile). Further, the ferric oxidation state was confirmed by the typical high-spin ferric signals at $g=6$ and 2 (Figures S7C and S8C), which was similar to the previously reported EPR spectra of iron(III) *meso*-chloro-isoporphyrin.⁵⁴ These results indicate that the methodology established by Fujii and co-workers⁵⁴ is quite versatile, in that various nucleophiles (not just TBACl) can be employed to attack the electrophilic iron(III) π -dication complex, to generate numerous substituted isoporphyrin complexes with promising oxidative capabilities.

A variety of substituted imidazoles were added to the iron(III) π -dication species (Scheme 4C and Figure 4A); however, we found that only 4,5-dimethylimidazole successfully formed a stable isoporphyrin complex with intense diagnostic isoporphyrin spectral features (Figure 4B and Table S1), such as the split Soret at 373 and 410 nm and the intense double low-energy bands at 840 and 933 nm. Typical and distinctive ferric high-spin signals at $g=6$ and 2 were detected via EPR spectroscopy for the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin complex (Figure S9), thus confirming its iron(III) oxidation state, and the EPR spectrum were similar to the previously reported EPR spectrum of iron(III) *meso*-chloro-isoporphyrin.⁵⁴ It was determined that for successful formation of an iron(III) *meso*-imidazole-isoporphyrin complex, the imidazole had to be nucleophilic, not too basic, and could not have unfavorable steric interactions with the aryl groups on the periphery of the porphyrin ring in order to successfully react with the iron(III) π -dication species (Figure 4A). Titration experiments, monitored by UV-vis spectroscopy, showed that 14 equiv of 4,5-dimethylimidazole were necessary to fully form the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin, even though a 1:1 adduct is formed, as evidenced by lack of further formation of the characteristic low-energy bands at 840 and 933 nm (Figure 4B) upon adding the 15th equivalent. Along with direct cryo-ESI-MS detection (*vide infra*), this provides evidence that 4,5-dimethylimidazole is bound to the heme porphyrin, in that sequential addition of the imidazole forms a greater amount of the desired complex, which can be similarly said for the CN and N₃ isoporphyrin derivatives.

Cryo-ESI-MS was utilized to further support the chemical formula of the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin complex (Figures 5A, S10, and S11). Parent peaks pertaining to two iron complexes were observed in the mass spectrum, with m/z values of 1020.1364 and 1022.1521 (Figures 5A and S10). The peak at 1020.1364 m/z is assigned to [(F₈-Im)Fe^{III}(OC(O)CF₃)]⁺, the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin with an axially bound trifluoroacetate ligand, supporting that the imidazole is covalently bound to the porphyrin (Figures 5A, S10, and S11). The complex cannot be a ferric species with anionic 4,5-dimethylimidazolate and trifluoroacetate bound axially, since such a species would have an overall -1 charge (Figure S11C) and thus would not be detected in the positive mode ESI-MS. The peak at 1022.1521 m/z is assigned to [F₈Fe^{III}(Im)(HOC(O)CF₃)]⁺, a minor ferric impurity with neutral 4,5-dimethylimidazole and trifluoroacetic acid bound axially (see the SI and Figure S10 for further explanation).

The overall transformations from $F_8Fe^{III}SbF_6$ to the resulting iron(III) *meso*-X-isoporphyrin complexes, where X = azide, cyanide, and 4,5-dimethylimidazole, were also monitored by 2H NMR spectroscopy at $-90\text{ }^\circ C$, utilizing the deuterated pyrrole analogue of F_8 (d_8 - $F_8Fe^{III}SbF_6$) (Figures 5B, S12, and S13). An upfield shift from 41.5 to -86.8 ppm, the latter ascribed to F_8 Cmpd-I, was observed upon addition of 2 equiv of *m*CPBA to 5 mM $F_8Fe^{III}SbF_6$ in DCM at $-90\text{ }^\circ C$, identical to the previously reported⁵⁴ F_{20} Cmpd-I detected at -86.8 ppm via 2H NMR spectroscopy in DCM at $-80\text{ }^\circ C$. Addition of 20 equiv of TFA to F_8 Cmpd-I resulted in a downfield shift from -86.8 to 62.7 ppm and the formation of the iron(III) π -dication complex. Subsequent addition of 6 equiv of $TBAN_3$, 6 equiv of TBACN, or 14 equiv of 4,5-dimethylimidazole resulted in a comparable 2H NMR signature to that reported for the iron(III) *meso*-chloro-isoporphyrin complex by Fujii and co-workers,⁵⁴ wherein four peaks were identified in the 140–110 ppm region (Figures 5B, S12, and S13). Substitution at the *meso* carbon results in the pyrrole signals around the ring being inequivalent, and thus four peaks are observed. Further, the paramagnetic signals of the iron(III) *meso*-X-isoporphyrin complexes are in a region typical for high-spin ferric complexes,^{2,58,88-90} additionally confirming the ferric oxidation state.

Reactivity of the Iron(III) *meso*-4,5-Dimethylimidazole-Isoporphyrin Complex.

Phenolic substrates were added to the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin to experimentally examine whether such a complex was capable of facilitating 2 e^- oxidation, forming a phenolimidazole coupled product and therefore mimicking the Tyr-His cross-link (Scheme 4D). Addition of phenols that did not have a methoxy substituent (i.e., electron-donating groups) did not cause any UV–vis spectral change; however, addition of 50 to 100 equiv of 2-*tert*-butyl-4-methoxyphenol, 3,5-dimethoxyphenol, 4-methoxyphenol, or the aromatic substrate 1,3,5-trimethoxybenzene to the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin complex resulted in the disappearance of the intense low-energy isoporphyrin peaks via UV–vis spectroscopy (Figures 6 and S14).⁹¹ The resulting UV–vis signature at the end of the reactions (Figures 6 and S14) was comparable to the UV–vis spectrum of the starting ferric precursor, $F_8Fe^{III}SbF_6$ (Figure 3A), suggesting the imidazole was transferred to the phenolic substrate; however, it is possible that water, imidazole, or TFA is weakly coordinated to the ferric ion. The resulting UV–vis spectra (in brown in Figures 6 and S14) were also comparable to the resulting UV–vis spectra reported by Fujii and co-workers⁵⁴ after iron(III) *meso*-chloro-isoporphyrin reacted with various aromatic or olefin substrates, wherein they reported generating $F_{20}Fe^{III}Cl$ as the final product. EPR spectra of the reaction mixtures confirmed the resulting inorganic product was a ferric species, as typical high-spin ferric signals $g=6$ and $g=2$ were detected (Figure S15). Interestingly, it was observed by 2H NMR spectroscopy that the four weak peaks of the imidazole isoporphyrin complex coalesced into one fairly intense peak upon addition of 50 equiv of 4-methoxyphenol or 1,3,5-trimethoxybenzene to 5 mM of the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin in DCM at $-90\text{ }^\circ C$ (Figures S16 and S17). This further indicates the imidazole isoporphyrin complex reacted with the substrates and that the final inorganic product's typical paramagnetic signal in the region around 123 ppm suggests it is ferric (the difference between the 2H NMR of the starting material (Figure 5B) and the resulting inorganic product (Figures S16 and S17) suggests possibly water, imidazole, TFA, etc., is coordinated).^{2,58,90}

Cryo-ESI-MS of the reaction mixtures from adding 50 equiv of 4-methoxyphenol or 1,3,5-trimethoxybenzene to the iron-(III) *meso*-4,5-dimethylimidazole-isoporphyrin in DCM at –90 °C successfully detected the resulting inorganic and organic products (Scheme 4D and Figures 7, S18, and S19). Consistent with the UV-vis, EPR, and ^2H NMR data, the naked ferric complex, $\text{F}_8\text{Fe}^{\text{III}}$, and the corresponding species coordinated to one imidazole, (4,5-dimethylimidazole) $\text{F}_8\text{Fe}^{\text{III}}$, were identified. Also, the C–N bond coupled products between 4,5-dimethylimidazole with 4-methoxyphenol or with 1,3,5-trimethoxybenzene were identified by cryo-ESI-MS (Figure 7). Similar to Fujii and co-workers,⁵⁴ two-electron oxidation occurred even with the aromatic substrate 1,3,5-trimethoxybenzene. Taken together, these results suggest that the phenol or aromatic substrate have acted as nucleophiles and attacked the porphyrin-bound imidazole (via an electrophilic aromatic substitution reaction, Figure 6), resulting in C–N bond formation concomitant with rearomatization of the porphyrin ring to form the starting ferric species, possibly coordinated to imidazole, water, or TFA (Figures 6, 7, S18, and S19). Thus, experimentally, we have demonstrated with a synthetic model of an iron(III) *meso*-histidine-isoporphyrin the proposed reactive intermediate in the Tyr-His biogenesis in CcO is potentially oxidatively capable (Scheme 3).

CONCLUSION

While the Tyr-His cross-link present in the active site of CcO is of critical structural and catalytic importance to the enzymatic reduction of O_2 to water, the pathway behind the biosynthesis of this post-translational modification is still unknown. Site-directed mutagenesis studies of CcO may yield further insights into the Tyr-His cross-link formation. Herein, a novel pathway is proposed for the C–N bond coupling in which it is suggested that it is heme-only catalyzed and further that the key reactive intermediate is an iron(III) *meso*-histidine-isoporphyrin species (a species isoelectronic to Cmpd-I, which should have just as promising oxidative capabilities). It is postulated that the heme moiety in the active site of CcO may adopt an isoporphyrin conformation, which would give it different electronic and physical properties than in the resting state heme enzyme. Future promising studies to further investigate the mechanism proposed herein may include modifications of the following: the porphyrin substituents, the presence of an imidazole axial ligand, the imidazole substrate, the phenol substrates, and the solvent medium, all in order to use more biologically relevant moieties and/or local environment.

In this work, it is suggested that the heme moiety in CcO first forms Cmpd-I and then subsequently tautomerizes to an iron(III) π -dication species. It is indicated that this electrophilic complex can then be attacked by a nearby histidine residue to form the key intermediate, an iron(III) *meso*-histidine-isoporphyrin. Subsequent nucleophilic attack from the Tyr residue yields the cross-link, and thus, it is suggested that only then (after cross-link formation) can the Cu_B ion enter the active site to interact appropriately with the heme to bind O_2 and begin the catalytic cycle.

Experimentally, a synthetic model of the proposed iron(III) *meso*-histidine-isoporphyrin intermediate was successfully generated, employing 4,5-dimethylimidazole, yielding the iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin complex. The ability to generate this

reactive intermediate is a feat and provides support for the viability of an imidazole-substituted isoporphyrin intermediate to be able to form in the active site of CcO. Further, we examined the reactivity of the imidazole isoporphyrin complex with various phenols, and the successful identification of coupled C–N bond products, for instance, 4-methoxyphenol coupled to 4,5-dimethylimidazole, supports that this reactive intermediate potentially is oxidatively capable of facilitating the net two-electron oxidative coupling of the key Tyr and His residues in CcO.

This work highlights the already well-known importance of post-translational modifications in enzymes and the need for further investigations to understand how they come about (i.e., the mechanism of their biogenesis) and their structural/functional implications for enzyme function. We wonder, or speculate, about the possible involvement of electrophilic amino acid isoporphyrins for other situations (in other enzymes) where post-translational modification involves formation of cross-links to aromatic amino acids.

EXPERIMENTAL SECTION

General Procedures.

All reagents and solvents purchased and used were of commercially available quality except as noted. Dichloromethane was purified according to literature methods⁹² and deoxygenated with Ar before use. Solvent deoxygenation was achieved by bubbling Ar through the desired solvent for 45 min via an addition funnel connected to a receiving Schlenk flask. All solvents were stored in amber bottles under 4 Å sieves. The oxidant, 3-chloroperbenzoic acid, and trifluoroacetic acid were purified according to published procedures.⁹² The $\text{F}_8\text{Fe}^{\text{III}}\text{SbF}_6$ and $d_8\text{-F}_8\text{Fe}^{\text{III}}\text{SbF}_6$ were synthesized as previously described.^{58,90,93,94} Air-free manipulations were performed in a vacuum atmosphere OMNILAB drybox or under an argon atmosphere using standard Schlenk techniques. Low-temperature UV–vis spectroscopy experiments were carried out by using a Cary-50 Bio spectrophotometer equipped with a Unisoku USP-203A cryostat with a modified Schlenk cuvette with a 1 cm path quartz cell. The spectrometer was equipped with Cary WinUV Scanning Kinetics software. All NMR spectra were recorded in 9 in., 5 mm o.d. NMR tubes on a Bruker 300 NMR instrument equipped with a tunable deuterium probe to enhance deuterium detection. The ^2H chemical shifts are calibrated to natural abundance deuterium solvent peaks. EPR spectra were collected with an ER 073 magnet equipped with a Bruker ER041 X-Band microwave bridge and a Bruker EMX 081 power supply: microwave frequency = 9.42 GHz, microwave power = 0.201 mW, attenuation = 30 db, modulation amplitude = 10 G, modulation frequency = 100 kHz, temperature = 10 K.

Spectroscopic Sample Preparations.

UV-Vis Spectroscopy.— $\text{F}_8\text{Cmpd-I}$ was synthesized as previously described.⁵⁸ The high-valent complex was generated by taking a dichloromethane solution containing 3.0 mL (0.01 or 0.1 mM) of $\text{F}_8\text{Fe}^{\text{III}}\text{SbF}_6$ in a 10 mm path length quartz Schlenk cuvette that was prepared in a glovebox and sealed with a rubber septum before being cooled in the cryostat chamber to $-90\text{ }^\circ\text{C}$ for 10 min. The iron(III) complex was subjected to 2 equiv of *m*CPBA in dichloromethane solution at $-90\text{ }^\circ\text{C}$, forming the $\text{F}_8\text{Cmpd-I}$. Subsequently, 20 equiv of

trifluoroacetic acid in dichloromethane was added to F₈Cmpd-I, resulting in the formation of an iron(III) π -dication species. This was then subjected to either 2 equiv of TBACl, 6 equiv of tetrabutylammonium azide, 6 equiv of tetrabutylammonium cyanide, or 14 equiv of 4,5-dimethylimidazole to generate the iron(III) *meso*-X-isoporphyrin species (where X = chloro, azide, cyanide, or 4,5-dimethylimidazole). Preliminary reactivity studies involved the addition of 50–100 equiv of phenolic or aromatic substrate (e.g., 4-methoxyphenol, etc.) to the isoporphyrin derivatives, and the resulting mixture was allowed to react until there was no further spectral change (typically the reactions would take hours).

²H NMR Spectroscopy.—Samples were prepared similarly to the UV–vis spectroscopy section; however, the pyrrole-deuterated porphyrin, *d*₈-F₈Fe^{III}SbF₆, complex was utilized at 5.0 mM concentrations in dichloromethane with a total volume of 0.6 mL at –90 °C (acetone/liquid N₂ bath) in a 9-in., 5 mm, rubber septum-capped NMR tube. After addition of a new reagent, Ar was bubbled through the reaction mixture to adequately mix the resulting solution (immediate color changes were observed upon addition of each reagent). ²H NMR spectra were acquired on a Bruker AVANCE 300 MHz NMR spectrometer at 46.072 MHz. Experiments were carried out at –90 °C using a recycle delay of 0.01 s, and a total of 5120 scans were collected. The peaks were referenced to the dichloromethane solvent peak at 5.32 ppm.

EPR Spectroscopy.—Samples were prepared similarly to the UV–vis; however, samples were made at 1 mM concentrations in dichloro-methane with a total volume of 0.6 mL at –90 °C (acetone/liquid N₂ bath) in a 5 mm outer diameter quartz EPR tube. After complexes were generated, tubes were frozen in N₂(liq), and all spectra were recorded at 10 K.

Cryogenic ESI-MS.—Samples were prepared similarly to the UV–vis spectroscopy section. Cryospray-ionization mass spectrometry measurements were performed on a UHR-TOF Bruker Daltonik maXis Plus, an ESI-quadrupole time-of-flight mass spectrometer capable of a resolution of at least 60,000 (fwhm), which was coupled to a Bruker Daltonik Cryospray unit. Detection was in positive ion mode; the source voltage was 2.5 kV. The flow rate was 240 μ L/h. The drying gas (N₂), to achieve solvent removal, was held at –90 °C, and the spray gas was also held at –90 °C. The temperature of the samples injected was also at –90 °C. The mass spectrometer was calibrated prior to every experiment via direct infusion of an Agilent ESI-TOF low concentration tuning mixture, which provided an *m/z* range of singly charged peaks up to 2700 Da in both ion modes. Simulated spectra were generated in DataAnalysis from Bruker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Heterobinuclear CcO active site where O₂ binding and reduction occurs. The Cu_B ion is ligated by three His residues, one of which is covalently cross-linked to the Tyr244 residue (bovine numbering, PDB: 5B1B). This diagram was generated using PyMOL (www.pymol.org).²⁹

Figure 2.

(A) When Tyr288 (*Rhodobacter sphaeroides* numbering, same as Tyr244 in bovine) is mutated to Phe288, the cross-link does not form, and it is proposed that His284 binds to heme a_3 , giving a low-spin ferric heme. (B) The cofactor in catalase–peroxidases consists of covalently cross-linked Trp, Tyr, and Met residues via post-translational modification, while the cofactor in cytochrome *c* oxidase consists of covalently cross-linked Tyr and His. Also, see the text.

Figure 3.

(A) UV-vis spectra monitoring the formation of $F_8Cmpd-I$, upon addition of 2 equiv of *m*CPBA to 0.1 mM $F_8Fe^{III}SbF_6$ in DCM at $-90^\circ C$ (from brown to pink). Addition of 20 equiv of TFA generates the iron(III) π -dication species (from pink to green). (B) Cryogenic ESI-MS (acquired in positive ion mode) of $F_8Cmpd-I$ in DCM at $-90^\circ C$, detected at 828.0838, in accordance with the calculated m/z . See Figure S4 for an enlarged Figure 3B.

Figure 4.

(A) Proposed pathway for the formation of the imidazole isoporphyrin complex, wherein X is a weakly coordinated ligand and B is a base, both of which may be water, trifluoroacetate, trifluoroacetic acid, 4,5-dimethylimidazole, etc. UV-vis spectra following the formation of iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin in DCM at -90°C at 0.01 mM (B) and 0.1 mM (C) upon addition of 14 equiv of 4,5-dimethylimidazole to the iron(III) π -cation complex (green to blue). Note the characteristic spectral features of isoporphyrin complexes including the split Soret (373 and 410 nm) and the intense low-energy bands (840 and 933 nm).

Figure 5.

(A) Cryogenic ESI-MS (acquired in positive ion mode) of iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin in dichloromethane at -90°C , detected at 1020.1364, in accordance with the calculated m/z . See text and Supporting Information for further details. (B) ^2H NMR spectra following the formation (top-to-bottom) of iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin in dichloromethane at -90°C .

Figure 6.

UV-vis spectra monitoring the disappearance of iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin upon addition of 50 equiv of 4-methoxyphenol in DCM at -90°C (from blue to brown), wherein X is possibly a weakly coordinating axial ligand which may be water, trifluoroacetate, trifluoroacetic acid, 4,5-dimethylimidazole, etc. Note the disappearance of the diagnostic isoporphyrin low-energy bands at 840 and 933 nm and the formation of the ferric species at 510 nm.

Figure 7. Cryogenic ESI-MS (acquired in positive ion mode) or resulting coupled C-N organic products from the addition of (A) 4-methoxyphenol or (B) 1,3,5-trimethoxybenzene to iron(III) *meso*-4,5-dimethylimidazole-isoporphyrin in dichloromethane at $-90\text{ }^{\circ}\text{C}$, detected at 219.1112 or 263.1372 m/z , respectively, in accordance with the calculated m/z . See text and Supporting Information for further details.

Scheme 1.

(A) Portion of the Catalytic Cycle of CcO, Demonstrating That Cmpd-I Intermediates May Form upon O–O Cleavage;^a (B) Cmpd-I Species May Also Be Formed from Cellular Hydrogen Peroxide Reaction with Oxidized Enzyme

^aThe species in brackets have not been experimentally observed in the enzyme.

Scheme 2.

Formation of an Iron(III) *meso*-Chloro-Isoporphyrin Complex and Subsequent Chlorination of Various Substrates, Which Was Previously Reported by Fujii and Co-workers⁵⁴

Scheme 3.

Postulated Tyr-His Cofactor Biogenesis Pathway in CcO via an Isoporphyrin Intermediate^a

^a Water is assumed to be a general acid/base for the proposed mechanism.

This work

Scheme 4.
Formation of the Iron(III) *meso*-Imidazole-Isoporphyrin Species and Its Subsequent Reactivity

Scheme 5.

Addition of Various Nucleophiles (Nuc) Such as 2 Equiv of TBACl, 6 Equiv of TBACN, or 6 Equiv of TBAN₃ to the Iron(III) π -Dication Complex Generating Novel Iron(III) *meso*-Substituted-Isoporphyrin Species, Incorporating (A) Chloro, (B) Cyanide, or (C) Azide Functionality^a

^aX is a possible weakly coordinating anionic ligand such as trifluoroacetate, chloride, cyanide, or azide.