X-C Electronic Structure and Reactivity of Active Sites of Metalloproteins

Metalloproteins are a class of biologically important macromolecules that have various functions such as oxygen transport, electron transfer, oxidation, and oxygenation. These diverse functions of metalloproteins have been thought to depend on the ligands from amino acid, coordination structures, and protein structures in immediate vicinity of metal ions. In this project, we are studying the relationship between the structures of the metal active sites and functions of metalloproteins.

X-C-1 A Salen Iron Complex as a Model for Non-Heme Iron Enzymes; Electronic Structure and Reactivity of the Two-Electron-Oxidized State

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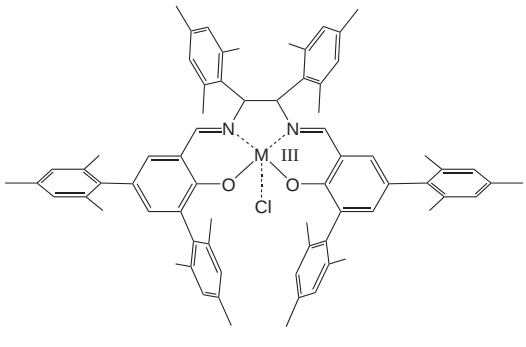
High-valent iron-oxo species are proposed to be the key reactive intermediate in oxidation reactions catalyzed by both heme and non-heme iron enzymes. Although oxo-iron (IV) porphyrin π-cation radical and related model species have been extensively investigated in the field of heme chemistry, non-heme counterparts are not well understood. We employ the salen ligand as a non-heme iron model complex, and attempt to isolate a transient oxidizing intermediate. Herein, we describe spectroscopic properties of oxidizing intermediates and discuss the structure-reactivity relationship. We synthesize the salen iron complex **1** having bulky mesityl substituents to avoid an undesirable dimmerization. A low-temperature electrochemical oxidation generates one and two-electron oxidized forms of the complex **1**. Spectroscopic investigation by use of UV-Vis, CV, EPR, Mössbauer, and ESI mass spectroscopy indicates that the phenoxy radical iron complex is formed. The reactivity of the oxidized intermediate is also investigated. In contrast, the salen manganese complex **2** exhibits different spectroscopic behavior. In this study, the electronic structure of the oxidized intermediate from **2** is also discussed.



1 M = Fe; **2** M = Mn

Figure 1. Structure of Sterically Hindered Salen Complexes prepared in this study.

X-C-2 A Superoxo-Ferrous State in a Reduced Oxy-Ferrous Hemoprotein and Model Compounds

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Cryoreduction of the $[FeO_2]^6$ (n = 6 is the number of electrons in 3d orbitals on Fe and π* orbitals on O_2) dioxygen-bound ferroheme through (irradiation at 77 K generates an $[FeO_2]^7$ reduced oxy-heme. Numerous investigations have examined $[FeO_2]^7$ centers that have been characterized as peroxo-ferric centers, denoted $[FeO_2]^7_{per}$, in which a ferriheme binds a dianionic peroxo-ligand. The generation of such an intermediate can be understood heuristically if the $[FeO_2]^6$ parent is viewed as a superoxo-ferric center and the injected electron localizes on the O–O moiety. We here report EPR/ENDOR experiments which show quite different properties for the $[FeO_2]^7$ centers produced by cryoreduction of monomeric oxy-hemoglobin (oxy-GMH3) from *Glycera dibranchiata*, which is unlike mammalian globins in having a leucine in place of the distal histidine, and of frozen aprotic solutions of oxy-ferrous octaethyl porphyrin and of the oxy-ferrous complex of the heme model, cyclidene. These $[FeO_2]^7$ centers are characterized as superoxo-ferrous centers, ($[FeO_2]^7_{sup}$), with nearly unit spin density localized on a superoxo moiety which is end-on coordinated to a low-spin ferrous ion. This assignment is based on their g tensors and ^{17}O hyperfine couplings, which are characteristic of the superoxide ion coordinated to a diamagnetic metal ion, and on the absence of detectable endor signals either from the in-plane ^{14}N ligands or from an exchangeable H-bond proton. Such a center would arise if the electron that adds to the $[FeO_2]^6$ superoxo-ferric parent localizes on the Fe ion, to make a superoxo-ferrous moiety. Upon annealing to T > 150 K the $[FeO_2]^7_{sup}$ species recruit a proton and converts to peroxo/hydroperoxo-ferric ($[FeO_2H]^7$) intermediates. These experiments suggest that the primary reduction product is $[FeO_2]^7_{sup}$, and that the internal redox transition to $[FeO_2]^7_{per}/[FeO_2H]^7$ states is driven at least in part by Hbonding/proton donation by the environment.

X-C-3 Synthesis and Characterization of Copper(I)-Nitrite Complexes as a Model for a Reaction Intermediate of Copper Nitrite Reductase

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Cu-containing nitrite reductase (NiR) contains a pair of Cu, a type1 Cu ion and a type2 Cu ion, which catalyze the reduction of NO_2^- to NO. In the catalytic reduction, type 2 Cu site receives one electron from type 1 Cu site, and reduce Cu-bound NO_2^- ion to NO with addition of two protons. In this study, we synthesized Cu(I)-nitrite complexes with various tridentate ligands, $TACN^{iPr}Cu(NO_2)$ (**1**), $TIC^{iPr}Cu(NO_2)$ (**2**), $TPM^{iPr}Cu(NO_2)$ (**3**), and $TPM^{Me}Cu(NO_2)$ (**4**), as a model for a reaction intermediate of NiR. Reaction of **1** ~ **3** with acetic acid or trifuluoroacetic acid afforded Cu(II) species, which characterized by UV-Vis and ESR. NO gas generated in the reaction was trapped with Fe(II)-octaethylporphyrinate and detected as a NO-Heme complex by ESR. In addition, two equivalent of proton was required for quantitative generation of NO. Therefore, the complexes **1** ~ **3** produce NO upon addition of two protons and one-electron to the Cu-bound NO_2^-, similar to native NiR. The rate of NO evolution was accelerated by the use of trifuluoroacetic acid instead of acetic acid, suggesting that the rate-determining step is the protonation of Cu-bound NO_2^-.

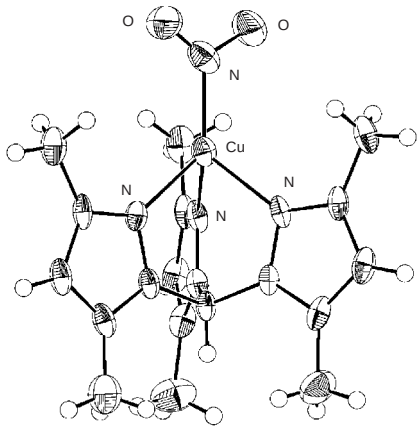

Figure 1. X-ray Crystal Structure of Copper(I)-Nitrite Complex.

X-D Molecular Mechanism of Heme Degradation and Oxygen Activation by Heme Oxygenase

Heme oxygenase (HO), an amphipathic microsomal protein, catalyzes the regiospecific oxidative degradation of iron protoporphyrinIX (heme) to biliverdinIXa, carbon monoxide, and iron in the presence of NADPH-cytochrome P-450 reductase, which functions as an electron donor. Heme oxygenase reaction is the biosynthesis processes of bile pigments and CO, which is a possible physiological messenger. Recent development in the bacterial expression of a soluble form of heme oxygenase has made it possible to prepare in the large quantities for structural studies. In this project, we are studying the molecular mechanism of heme degradation and the oxygen activation by heme oxygenase using various spectroscopic methods.

X-D-1 Kinetic Isotope Effects on the Rate-Limiting Step of Heme Oxygenase Catalysis Indicate Concerted Proton Transfer/Heme Hydroxylation

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Heme oxygenase (HO) catalyzes the O_2 and NADPH/cytochrome P450 reductase-dependent conversion of heme to biliverdin, free iron ion, and CO through a process in which the heme participates as both dioxygen-activating prosthetic group and substrate. We earlier confirmed that the first step of HO catalysis is a monooxygenation in which the addition of one electron and two protons to the HO oxy-ferroheme produces ferric-meso-hydroxyheme (**h**). Cryoreduction/EPR and ENDOR measurements further showed that hydroperoxo-ferri-HO converts directly to **h** in a single kinetic step without formation of a Compound I. We here report details of that rate-limiting step. One-electron 77 K cryoreduction of human oxy-HO and annealing at 200 K generates a structurally relaxed hydroperoxo-ferri-HO species, denoted **R**. We here report the cryoreduction/annealing experiments that directly measure solvent and secondary kinetic isotope effects (KIEs) of the rate-limiting **R** → **h** conversion, using enzyme prepared with meso-deuterated heme and in H_2O/D_2O buffers to measure the solvent KIE (solv-KIE), and the secondary KIE (sec-KIE) associated with the conversion. This approach is unique in that KIEs measured by monitoring the rate-limiting step are not susceptible to masking by KIEs of other processes, and these results represent the first direct measurement of the KIEs of product formation by a kinetically competent reaction intermediate in any dioxygen-activating heme enzyme. The observation of both solv-KIE(298) = 1.8 and sec-KIE(298) = 0.8 (inverse) indicates that the rate-limiting step for formation of h by HO is a concerted process: proton transfer to the hydroperoxo-ferri-heme through the distal-pocket H-bond network, likely from a carboxyl group acting as a general acid catalyst, occurring in synchrony with bond formation between the terminal hydroperoxo-oxygen atom and the α-meso carbon to form a tetrahedral hydroxylated-heme intermediate. Subsequent rearrangement and loss of H_2O then generates **h**.

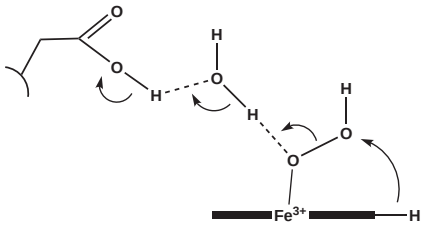

Figure 1. Mechanism of a proton transfer.

X-D-2 Essential Amino Acid Residues Controlling the Unique Regioselectivity of Heme Oxygenase in *Pseudomonas aeruginosa*

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Heme oxygenase (HO), an amphipathic microsomal protein, catalyzes the oxygen-dependent degradation of heme (iron-protoporphyrinIX) to -biliverdin, CO, and free iron ion. Interestingly, all of HO regiospecifically oxidize the α-meso position of the heme to form-biliverdin isomer while nonenzymatic heme degradation forms all four possible α-, β-, γ-, δ-biliverdin isomers at nearly identical yield. Recently, an interesting example has been found in HO (PigA) of the Gram-negative bacterium Pseudomonas aeruginosa, which does not produce α-biliverdin at all, but forms the mixture of β- and δ-biliverdins at a ratio of 3:7. While studying the mechanism of the unique regioselectivty of PigA, we found essential amino acid residues, Lys34, Lys132, and Phe189, controlling the unique regioselectivity of PigA. In this communication, we show that Lys34 and Lys132 are essential amino acid residues to hold the rotated heme in the active site of PigA via hydrogen-bonding interaction with the heme propionate and that Phe189 controls the product ratio of β- and δ-biliverdins *via* steric interaction with heme substituents. These interactions place the β- or δ-meso position of the heme at the oxidation site of PigA, leading to the unique regioselectivity.

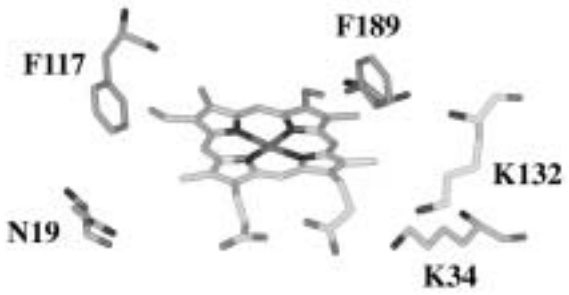

Figure 1. Active Site Structure of PigA-HO proposed in this study.