V-C Bioinorganic Chemistry and Structural Biology of Heme Proteins

One of research activities of my group is directed toward developing a rigorous, quantitative understanding of the biochemical function of heme proteins such as oxygenases, peroxidases and oxidases by characterization of their structural and functional properties. We use different experimental strategies including protein engineering, spectroscopic characterization of the molecular structure of the active centers, measurements of dynamics of substrates and inhibitor binding, and X-ray crystallography.

My current heme protein projects include (1) elucidation of the catalytic mechanism of heme oxygenase, one of the essential components of the heme catabolism and biosynthesis of carbon monoxide, a versatile physiological messenger molecule, (2) elucidation of the mechanism of controlling reactivity of hemoglobin and myoglobin, and (3) determination of heme sensing mechanism of Bach1, a heme-dependent transcription factor which regulates heme oxygenase gene expression. Effective clues to delineate the detailed active site structure have been obtained by X-ray crystallography, resonance Raman and magnetic resonance studies. The synergy of site-directed mutagenesis, structural biology, and spectroscopic techniques has revealed the specific roles of amino acids located in the active centers of heme proteins. Ligands and substrates binding measurements complement the structural data for our understanding functional properties displayed by heme proteins at the molecular level.

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

DAVYDOV, Roman[1]; MATSUI, Toshitaka[2]; FUJII, Hiroshi; IKEDA-SAITO, Masao[3]; HOFFMAN, Brian M.[1]
([1]Northwestern Univ.; [2]Tohoku Univ.; [3]IMS and Tohoku Univ.)

[*J. Am. Chem. Soc.* **125**, 16208–16209 (2003)]

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 both as 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-α-*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** to **h** conversion, using enzyme prepared in H_2O/D_2O buffers to measure the solvent KIE (*solv*-KIE), and the secondary KIE (*sec*-KIE) associated with deuteration of the heme *meso*-carbons. This approach is unique in that KIEs measured by monitoring the rate-limiting step are not susceptible to masking by KIE's of other processes, and these results represent the first direct measurement of the KIE's 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 attack of 'OH^+' on the α-*meso* carbon to form a tetrahedral hydroxylated-heme intermediate. Subsequent rearrangement and loss of H_2O then generates **h**.

V-C-2 FeNO Structure in Distal Pocket Mutants of Myoglobin Based on Resonance Raman Spectroscopy

COYLE, Candace M.[1]; VOGEL, Kathleen M.[1]; RUSH, Thomas S. III[1]; KOZLOWSKI, Pawel M.[1]; WILLIAMS, Robert[1]; SPIRO, Thomas, G.[1]; DOU, Yi[2]; IKEDA-SAITO, Masao[3]; OLSON, John S.[4]; ZGIERSKI, Marek Z.[5]
([1]Princenton Univ.; [2]Case Western Reserve Univ.; [3]IMS and Tohoku Univ.; [4]Rice Univ.; [5]Natl. Res. Council Canada)

[*Biochemistry* **42**, 4896–4903 (2003)]

FeNO vibrational frequencies were investigated for a series of myoglobin mutants using isotopeedited resonance Raman spectra of $^{15/14}$NO adducts, which reveal the FeNO and NO stretching modes. The latter give rise to doublet bands, as a result of Fermi resonances with coincident porphyrin vibrations; these doublets were analyzed by curve-fitting to obtain the νNO frequencies. Variations in νNO among the mutants correlate with the reported νCO variations for the CO adducts of the same mutants. The correlation has a slope near unity, indicating equal sensitivity of the NO and CO bonds to polar influences in the heme pocket. A few mutants deviate from the correlation, indicating that distal interactions differ for the NO and CO adducts, probably because of the differing distal residue geometries. In contrast to the strong and consistent νFeC/νCO correlation found for the CO adducts, νFeN correlates only weakly with νNO, and the slope of the correlation depends on which residue is being mutated.

This variability is suggested to arise from steric interactions, which change the FeNO angle and therefore alter the Fe–NO and N–O bond orders. This effect is modeled with Density Functional Theory (DFT) and is rationalized on the basis of a valence isomer bonding model. The FeNO unit, which is naturally bent, is a more sensitive reporter of steric interactions than the FeCO unit, which is naturally linear. An important additional factor is the strength of the bond to the proximal ligand, which modulates the valence isomer equilibrium. The FeNO unit is bent more strongly in MbNO than in protein-free heme-NO complexes because of a combination of a strengthened proximal bond and distal interactions.

V-C-3 The Crystal Structures of the Ferric and Ferrous Forms of the Heme Complex of HmuO, a Heme Oxygenase of *Corynebacterium diphtheriae*

HIROTSU, Shoko[1]; CHU, Grace C.[2]; UNNO, Masaki[1]; LEE, Dong-Sun[3]; YOSHIDA, Tadashi[4]; PARK, Sam-Yong[3]; SHIRO, Yoshitsugu[3]; IKEDA-SAITO, Masao[5]
([1]Tohoku Univ.; [2]Case Western Reserve Univ.; [3]RIKEN Harima Inst./SPring-8; [4]Yamagata Univ.; [5]IMS and Tohoku Univ.)

[*J. Biol. Chem.* **279**, 1197–11947 (2004)]

Crystal structures of the ferric and ferrous heme complexes of HmuO, a 24 kDa heme oxygenase of *Corynebacterium diphtheriae*, have been refined to 1.4 and 1.5 Å resolution, respectively. The HmuO structures show that the heme group is closely sandwiched between the proximal and distal helices. The imidazole group of His20 is the proximal heme ligand, which closely eclipses the β and δ meso axis of the porphyrin ring. A long range hydrogen bonding network is present, connecting the iron-bound water ligand to the solvent water molecule. This enables proton transfer from the solvent to the catalytic site, where the oxygen activation occurs. In comparison to the ferric complex, the proximal and distal helixes move closer to the heme plane in the ferrous complex. Together with the kinked distal helix, this movement leaves only the α-meso carbon atom accessible to the iron-bound dioxygen. The heme pocket architecture is responsible for stabilization of the ferric hydroperoxo active intermediate by preventing premature heterolytic O–O bond cleavage. This allows the enzyme to oxygenate selectively at the α-meso carbon in HmuO catalysis.

V-C-4 Crystal Structure of the Dioxygen-Bound Heme Oxygenase from *Corynebacterium diphtheriae*: Implications for Heme Oxygenase Function

UNNO, Masaki[1]; MATSUI, Toshitaka[1]; CHU, Grace C.[2]; COUTURE, Manon[3]; YOSHIDA, Tadashi[4]; ROUSSEAU, Denis L.[3]; OLSON, John S.[5]; IKEDA-SAITO, Masao[6]
([1]Tohoku Univ.; [2]Case Western Reserve Univ.; [3]Albert Einstein College Med.; [4]Yamagata Univ.; [5]Rice Univ.; [6]IMS and Tohoku Univ.)

[*J. Biol. Chem.* **279**, 21055–21061 (2004)]

HmuO, a heme oxygenase of *Corynebacterium diphtheriae*, catalyzes degradation of heme using the same mechanism as the mammalian enzyme. The oxy form of HmuO, the precursor of the catalytically active ferric hydroperoxo species, has been characterized by ligand binding kinetics, resonance Raman spectroscopy, and X-ray crystallography. The oxygen association and dissociation rate constants are 5 μM^{-1} s^{-1} and 0.22 s^{-1}, respectively, yielding an O_2 affinity of 21 μM^{-1} that is ~20 times greater than that of mammalian myoglobins. However, the affinity of HmuO for CO is only 3 to 4-fold greater than that for mammalian myoglobins, implying the presence of strong hydrogen bonding interactions in the distal pocket of HmuO that preferentially favor O_2 binding. Resonance Raman spectra show that the Fe–O_2 vibrations are tightly coupled to porphyrin vibrations, indicating a highly bent Fe–O–O geometry that is characteristic of the oxy forms of heme oxygenases. In the crystal structure of the oxy form, the Fe–O–O angle is 110°; the O–O bond is pointed toward the heme α-*meso*-carbon by direct steric interactions with Gly135 and Gly139; and hydrogen bonds occur between the bound O_2 and the amide nitrogen of Gly139 and a distal pocket water molecule, which is a part of an extended hydrogen bonding network that provides the solvent protons required for oxygen activation. In addition the O–O bond is orthogonal to the plane of the proximal imidazole side chain, which facilitates hydroxylation of the porphyrin α-*meso*-carbon by preventing premature O–O bond cleavage.

V-C-5 Heme Regulates Gene Expression by Triggering Crm1-Dependent Nuclear Export of Bach1

SUZUKI, Hiroshi[1]; TASHIRO, Satoshi[1]; HIRA, Shusuke[2]; SUN, Jiying[1]; YAMAZAKI, Chikara[1]; ZENKE, Yukari[1]; IKEDA-SAITO, Masao[3]; YOSHIDA, Minoru[4]; IGARASHI, Kazuhiko[1]
([1]Hiroshima Univ.; [2]Tohoku Univ.; [3]IMS and Tohoku Univ.; [4]RIKEN)

[*EMBO J.* **23**, 2544–2553 (2004)]

Bach1 is a transcriptional repressor of heme oxygenase-1 and β-globin genes, both of which are known to be transcriptionally induced by heme. To test the hypothesis that heme regulates the activity of Bach1, we expressed wild-type and mutated versions of Bach1 together with or without its heterodimer partner MafK in human 293T and GM02063 cells and examined their subcellular localization. Inhibition of heme synthesis enhanced the nuclear accumulation of Bach1 whereas treating cells with hemin resulted in nuclear exclusion of Bach1. While the cadmium-inducible nuclear export signal of Bach1 was dispensable for the heme response, a region containing two of the heme-binding motifs were found to be critical for the heme-induced nuclear exclusion. This region functioned as a heme-regulated nuclear export signal dependent on the exporter Crm1. These results extend the regulatory roles for heme in protein sorting, and suggest that Bach1 transduces metabolic activity into gene expression.

V-D Pro-Oxidants-Induced Iron Release from the Fe-S Cluster of Mitochondrial Aconitase and Its Prevention by Flataxin

Pro-oxidants, such as hydrogen peroxide and superoxide anion, are highly toxic for many living organisms. One of the adverse effects of pro-oxidants is modulation of mitochondrial respiration. Using EPR spectroscopy, we have identified that pro-oxidants deactivates mitochondrial aconitase, the key enzyme in citrate cycle, by releasing one of Fe from the 4Fe-4S cluster, and that mitochondria is surprisingly equipped a recovery mechanism to restore the active 4Fe-4S cluster. We have recently discovered that this restoration is achieved by an iron insertion from frataxin, an iron storage protein in mitochondria, which functions as an iron chaperon protein. We are in the process of elucidation of the inter protein iron transfer mechanism from frataxin to aconitase at molecular level.

V-D-1 Redox-Dependent Modulation of Aconitase Activity in Intact Mitochondria

BULTEAU, Anne-Laure[1]; IKEDA-SAITO, Masao[2]; SZWEDA, Luke I.[1]
([1]Case Western Reserve Univ.; [2]IMS and Tohoku Univ.)

[*Biochemistry* **42**, 14846–14855 (2003)]

It has previously been reported that exposure of purified mitochondrial or cytoplasmic aconitase to superoxide ($O_2^{\bullet-}$) or hydrogen peroxide (H_2O_2) leads to release of the Fe-α from the enzyme's $[4Fe–4S]^{2+}$ cluster and to inactivation. Nevertheless, little is known regarding the response of aconitase to pro-oxidants within intact mitochondria. In the present study, we provide evidence that aconitase is rapidly inactivated and subsequently reactivated when isolated cardiac mitochondria are treated with H_2O_2. Reactivation of the enzyme is dependent on the presence of the enzyme's substrate, citrate. EPR spectroscopic analysis indicates that enzyme inactivation precedes release of the labile Fe-a from the enzyme's $[4Fe–4S]^{2+}$ cluster. In addition, as judged by isoelectric focusing gel electrophoresis, the relative level of Fe-α release and cluster disassembly does not reflect the magnitude of enzyme inactivation. These observations suggest that some form of posttranslational modification of aconitase other than release of iron is responsible for enzyme inactivation. In support of this conclusion, H_2O_2 does not exert its inhibitory effects by acting directly on the enzyme, rather inactivation appears to result from interaction (s) between aconitase and a mitochondrial membrane component responsive to H_2O_2. Nevertheless, prolonged exposure of mitochondria to steady-state levels of H_2O_2 or ($O_2^{\bullet-}$) results in disassembly of the $[4Fe–4S]^{2+}$ cluster, carbonylation, and protein degradation. Thus, depending on the pro-oxidant species, the level and duration of the oxidative stress, and the metabolic state of the mitochondria, aconitase may undergo reversible modulation in activity or progress to $[4Fe–4S]^{2+}$ cluster disassembly and proteolytic degradation.

V-D-2 Frataxin Acts as an Iron Chaperone Protein To Modulate Mitochondrial Aconitase Activity

BULTEAU, Anne-Laure[1]; O'NEILL, Heather A.[2]; KENNEDY, Mary Claire[3]; IKEDA-SAITO, Masao[4]; ISAYA, Grazia[2]; SZWEDA, Luke I.[1]
([1]Case Western Reserve Univ.; [2]Mayo Clinic College Med.; [3]Gannon Univ.; [4]IMS and Tohoku Univ.)

[*Science* **305**, 242–245 (2004)]

Friedreich's ataxia is a genetic disorder characterized by a deficiency in frataxin, the mitochondrial iron-binding protein. We have identified a role for frataxin as an iron chaperone protein that is required for the reversible modulation of mitochondrial aconitase activity in response to pro-oxidants. By protecting Fe-S clusters from disassembly, frataxin can prevent iron accumulation and production of the highly reactive and toxic hydroxyl radical. Alterations in the level, structure, and chaperone function of frataxin may participate in the progression of degenerative disorders associated with declines in aconitase and mitochondrial activity.