RESEARCH ACTIVITIES X
Okazaki Institute for Integrative Bioscience

X-A Bioinorganic Chemistry of Heme-Based Sensor Proteins

Heme-based sensor proteins show a novel function of the heme prosthetic group, in which the heme acts as an active site for sensing the external environmental signal such as diatomic gas molecules and redox change. Heme-based O_2, NO, and CO sensor proteins have now been found in which these gas molecules act as a signaling factor that regulates the functional activity of the sensor proteins. Our research interest focuses on the elucidation of structure-function relationships of CO sensor protein (CooA), O_2 sensor protein (HemAT), and redox sensor protein (DcrA).

X-A-1 Effect of Mutation on the Dissociation and Recombination Dynamics of CO in Transcriptional Regulator CooA: A Picosecond Infrared Transient Absorption Study

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The CO ligation process in a mutant (H77G) of CooA, the CO-sensing transcriptional regulator in *Rhodospirillum rubrum*, is studied with femtosecond time-resolved transient absorption spectroscopy in the mid-infrared region. Following photolyzing excitation, a transient bleach in the vibrational region of bound CO due to the CO photodissociation is detected. In contrast to the spectra of the wild-type (WT) CooA, the transient bleach spectra of H77G CooA show a bimodal shape with peaks shifting to the lower frequency during spectral evolution. The CO recombination dynamics show single-exponential behavior, and the CO escaping yield is higher than that of the WT CooA. A reorientation process of CO relative to the heme plane during recombination is revealed by anisotropy measurements. These phenomena indicate changes in the protein response to the CO ligation and suggest an alteration to the CO environment caused by the mutation. On the basis of these results, the role of His77 in the CO-dependent activation of CooA and a possible activation mechanism involving collaborative movement of the heme and the amino residues at both sides of the heme plane are discussed.

X-A-2 Evidence for Displacements of the C-Helix by CO Ligation and DNA Binding to CooA Revealed by UV Resonance Raman Spectroscopy

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[*J. Biol. Chem.* **281**, 11271–11278 (2006)]

The UV and visible resonance Raman spectra are reported for CooA from Rhodospirillum rubrum, which is a transcriptional regulator activated by growth in a CO atmosphere. CO binding to heme in its sensor domain causes rearrangement of its DNA-binding domain, allowing binding of DNA with a specific sequence. The sensor and DNA-binding domains are linked by a hinge region that follows a long C-helix. UV resonance Raman bands arising from Trp-110 in the C-helix revealed local movement around Trp-110 upon CO binding. The indole side chain of Trp-110, which is exposed to solvent in the CO-free ferrous state, becomes buried in the CO-bound state with a slight change in its orientation but maintains a hydrogen bond with a water molecule at the indole nitrogen. This is the first experimental data supporting a previously proposed model involving displacement of the C-helix and heme sliding. The UV resonance Raman spectra for the CooA-DNA complex indicated that binding of DNA to CooA induces a further displacement of the C-helix in the same direction during transition to the complete active conformation. The Fe–CO and C–O stretching bands showed frequency shifts upon DNA binding, but the Fe–His stretching band did not. Moreover, CO-geminate recombination was more efficient in the DNA-bound state. These results suggest that the C-helix displacement in the DNA-bound form causes the CO binding pocket to narrow and become more negative.

X-A-3 Crystallization and Preliminary X-Ray Analysis of CooA from *Carboxydothermus hydrogenoformans*

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[*Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **62**, 471–473 (2006)]

CooA, a homodimeric haem-containing protein, is responsible for transcriptional regulation in response to carbon monoxide (CO). It has a b-type haem as a CO sensor. Upon binding CO to the haem, CooA binds promoter DNA and activates expression of genes for CO metabolism. CooA from *Carboxydothermus hydrogeno-*

formans has been overexpressed in *Escherichia coli*, purified and crystallized by the vapour-diffusion method. The crystal belongs to space group $P2_1$, with unit-cell parameters a = 61.8, b = 94.7, c = 92.8 angstroms, β = 104.8 degrees. The native and anomalous difference Patterson maps indicated that two CooA dimers are contained in the asymmetric unit and are related by a translational symmetry almost parallel to the c axis.

X-A-4 Specific Hydrogen-Bonding Networks Responsible for Selective O$_2$ Sensing of the Oxygen Sensor Protein HemAT from *Bacillus subtilis*

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[*Biochemistry* **45**, 8301–8307 (2006)]

HemAT from *Bacillus subtilis* (HemAT-Bs) is a heme-based O_2 sensor protein that acts as a signal transducer responsible for aerotaxis. HemAT-Bs discriminates its physiological effector, O_2, from other gas molecules to generate the aerotactic signal, but the detailed mechanism of the selective O_2 sensing is not obvious. In this study, we measured electronic absorption, electron paramagnetic resonance (EPR), and resonance Raman spectra of HemAT-Bs to elucidate the mechanism of selective O_2 sensing by HemAT-Bs. Resonance Raman spectroscopy revealed the presence of a hydrogen bond between His86 and the heme propionate only in the O_2-bound form, in addition to that between Thr95 and the heme-bound O_2. The disruption of this hydrogen bond by the mutation of His86 caused the disappearance of a conformer with a direct hydrogen bond between Thr95 and the heme-bound O_2 that is present in WT HemAT-Bs. On the basis of these results, we propose a model for selective O_2 sensing by HemAT-Bs as follows. The formation of the hydrogen bond between His86 and the heme propionate induces a conformational change of the CE-loop and the E-helix by which Thr95 is located at the proper position to form the hydrogen bond with the heme-bound O_2. This stepwise conformational change would be essential to selective O_2 sensing and signal transduction by HemAT-Bs.

X-A-5 Recognition and Discrimination of Gases by the Oxygen-Sensing Signal Transducer Protein HemAT as Revealed by FTIR Spectroscopy

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[*Biochemistry* **45**, 7763–7766 (2006)]

The determination of ligand binding properties is a key step in our understanding of gas sensing and discrimination by gas sensory proteins. HemAT is a newly discovered signal transducer heme protein that recognizes O_2 and discriminates against other gases such as CO and NO. We have used FTIR spectroscopy on CO- and NO-bound sensor domain HemAT and sensor domain distal mutants Y70F, T95A, R91A, and L92A to gain insight into the structure of the iron-bound ligand at ambient temperature. These mutations were designed to perturb the electrostatic field near the iron-bound gaseous ligand and also allow us to investigate the communication pathway between the distal residues of the protein and the heme. We show the formation of both H-bonded and non-H-bonded conformations in the CO-bound forms. In addition, we report the presence of multiple conformations in the NO-bound forms. Such distal H-bonding is crucial for ligand binding and activation by the heme. The comparison of the O_2, NO, and CO data demonstrates that Thr95 and Tyr70 are crucial for ligand recognition and discrimination and, thus, for specific sensing of gases, and L92 is crucial for controlling the conformational changes of the Thr95 and Tyr70 residues upon NO binding.

X-A-6 Biophysical Properties of a *c*-Type Heme in Chemotaxis Signal Transducer Protein DcrA

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[*Biochemistry* **44**, 15406–15413 (2005)]

Chemotaxis signal transducer protein DcrA from a sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough was previously shown to contain a *c*-type heme in its periplasmic domain (DcrA-N) for sensing redox and/or oxygen, which is the first example of a heme-based sensor protein containing a *c*-type heme as a prosthetic group. Optical absorption and resonance Raman spectroscopies indicates that heme *c* in DcrA-N shows a redox-dependent ligand exchange. Upon reduction, a water molecule that may be the sixth ligand of the ferric heme *c* is replaced by an endogenous amino acid. Although the reduced heme in DcrA-N is six-coordinated with two endogenous axial ligands, CO can easily bind to the reduced heme to form CO-bound DcrA-N. Reaction of the reduced DcrA-N with molecular oxygen results in autoxidation to form a ferric state without forming any stable oxygen-bound form probably due to the extremely low redox potential of DcrA-N (–250 mV). Our study supports the initial idea by Fu *et al.* that DcrA would act as a redox and/or oxygen sensor, in which the ligand exchange between water and an endogenous amino acid is a trigger for signal transduction. While the affinity of CO to DcrA-N (K_d = 138 μM) is significantly weak compared to those of other heme proteins, we suggest that CO might be another physiological effector molecule.

X-B Bioinorganic Chemistry of a Novel Heme Enzyme that Catalyzes the Dehydration Reaction

Aldoxime dehydratases from various bacteria catalyze the dehydration reaction of aldoxime to produce nitrile under mild conditions. Aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) exists in a monomer of a 40-kDa polypeptide containing a protoheme. The heme in OxdB is thought to be the active site for the dehydration reaction. OxdB is the first example of a hemeprotein catalyzing the dehydration reaction physiologically, although many functions of hemeproteins have been elucidated, including oxygen storage/transport, electron transfer, gas molecule sensor, and redox catalysis of various substrates. We are working on OxdB and OxdRE (aldoxime dehydratase from *Rhodococcus* sp. N-771) to elucidate the structure-function relationships of these novel heme enzymes.

X-B-1 Spectroscopic and Substrate Binding Properties of Heme-Containing Aldoxime Dehydratases, OxdB and OxdRE

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[*J. Inorg. Biochem.* **100**, 1069–1074 (2006)]

Phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1 (OxdB) catalyzes the dehydration of Z-phenylacetaldoxime (PAOx) to produce phenylacetonitrile. OxdB contains a protoheme that works as the active center of the dehydration reaction. The enzymatic activity of ferrous OxdB was 1150-fold higher than that of ferric OxdB, indicating that the ferrous heme was the active state in OxdB catalysis. Although ferric OxdB was inactive, the substrate was bound to the ferric heme iron. Electron paramagnetic resonance spectroscopy revealed that the oxygen atom of PAOx was bound to the ferric heme, whereas PAOx was bound to the ferrous heme in OxdB via the nitrogen atom of PAOx. These results show a novel mechanism by which the activity of a heme enzyme is regulated; that is, the oxidation state of the heme controls the coordination structure of a substrate-heme complex, which regulates enzymatic activity. Rapid scanning spectroscopy using stopped-flow apparatus revealed that a reaction intermediate (the PAOx-ferrous OxdB complex) showed Soret, , and bands at 415, 555, and 524 nm, respectively. The formation of this intermediate complex was very fast, finishing within the dead time of the stopped-flow mixer (3 ms). Site-directed mutagenesis revealed that His-306 was the catalytic residue responsible for assisting the elimination of the hydrogen atom of PAOx.

X-C Reaction Mechanism of Metalloenzymes Related to Oxygen Activation

Oxygen is quite important molecule for most organisms. Oxygen is utilized for various physiological functions such as ATP synthesis, defense mechanism, oxidation reactions, and signal transduction. These diverse functions are realized by many metalloenzymes. In this project, we are studying molecular mechanisms of these metalloenzymes.

X-C-1 Oxidizing Intermediates from the Sterically Hindered Salen Iron Complexes Related to the Oxygen Activation by Nonheme Iron Enzymes

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[*Inorg. Chem.* **44**, 8156–8166 (2005)]

Oxidizing intermediates are generated from nonheme iron(III) complexes to investigate the electronic structure and the reactivity, in comparison with the oxoiron(IV) porphyrin π-cation radical (compound I) as a heme enzyme model. Sterically hindered salen iron complexes, bearing a fifth ligand Cl (**1**), OH_2 (**2**), OEt (**3**) and OH (**4**), are oxidized both electrochemically and chemically. Stepwise one-electron oxidation of **1** and **2** generates iron(III)–mono- and diphenoxyl radicals, as revealed by detailed spectroscopic investigations, including UV-Vis, EPR, Mössbauer, resonance Raman, and ESIMS spectroscopies. In contrast to the oxoiron(IV) formation from the hydroxoiron(III) porphyrin upon one-electron oxidation, the hydroxo complex **4** does not generate oxoiron(IV) species. Reaction of **2** with *m*CPBA also results in the formation of the iron(III)–phenoxyl radical. One-electron oxidation of **3** leads to oxidative degradation of the fifth EtO ligand to liberate acetaldehyde even at 203 K. The iron(III)–phenoxyl radical shows high reactivity for alcoxide on iron(III), but exhibits virtually no reactivity for alcohols including even benzyl alcohol without a base to remove an alcohol proton. The present study explains unique properties of mononuclear nonheme enzymes with Tyr residues, and also a poor epoxidation activity of Fe salen compared to Mn and Cr salens.

X-C-2 A Trigonal-Bipyramidal Geometry Induced by an External Water Ligand in a Sterically Hindered Iron Salen Complex, Related to the Active Site of Protocatechuate 3,4-Dioxygenase

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[*Inorg. Chem.* in press]

A unique distorted trigonal-bipyramidal geometry observed for the nonheme iron center in protocatechuate 3,4-dioxygenase (3,4-PCD) was carefully examined utilizing a sterically hindered iron salen complex, which well reproduces the endogenous His_2Tyr_2 donor set with water as an external ligand. X-ray crystal structures of a series of iron model complexes containing bis(3,5-dimesitylsalicylidene)-1,2-dimesitylethylenediamine indicate that a distorted trigonal-bipyramidal geometry is achieved upon binding of water as an external ligand. The extent of a structural change of the iron center from a preferred square-pyramidal to a distorted trigonal-bipyramidal geometry varies with the external ligand that is bound in the order Cl << EtO < H_2O, which is consistent with the spectrochemical series. The distortion in the model system is not due to steric repulsions, but electronic interactions between the external ligand and the iron center, as evidenced from the X-ray crystal structures of another series of iron model complexes with a less-hindered bis(3-xylylsalicylidene)-1,2-dimesitylethylenediamine ligand, as well as by DFT calculations. Further spectroscopic investigations indicate that a unique distorted trigonal-bipyramidal geometry is indeed maintained even in solution. The present model study provides a new viewpoint that a unique distorted trigonal-bipyramidal iron site might not be preorganized by a 3,4-PCD protein, but could be electronically induced upon the binding of an external hydroxide ligand to the iron(III) center. The structural change induced by the external water ligand is also discussed in relation to the reaction mechanism of 3,4-PCD.

X-C-3 O_2- and H_2O_2-Dependent Verdoheme Degradation by Heme Oxygenase: Reaction Mechanisms and Potential Physiological Roles of the Dual Pathway Degradation

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[*J. Biol. Chem.* **280**, 36833–36840 (2005)]

Heme oxygenase (HO) catalyzes catabolism of heme to biliverdin, CO and a free iron through three successive oxygenation steps. The third oxygenation, oxidative degradation of verdoheme to biliverdin, has been the least understood step in spite of its importance to regulate the HO activity. We have thoroughly examined degradation of a synthetic verdoheme IXα complexed with rat HO-1. Our major findings include: (1) HO degrades verdoheme through a dual pathway using either O_2 or H_2O_2; (2) the newly found H_2O_2 pathway is approximately 40-fold faster than the O_2-dependent degradation; (3) both reactions are initiated by the binding of O_2 or H_2O_2 to allow the first direct obser-

vation of degradation intermediates of verdoheme; and (4) Asp[140] in HO-1 is critical for the verdoheme degradation regardless of the oxygen source. On the basis of these findings, we propose that the HO enzyme activates O_2 and H_2O_2 on the verdoheme iron with the aid of a nearby water molecule linked with Asp[140]. These mechanisms are similar to a well-established mechanism of the first oxygenation, *meso*-hydroxylation of heme, and thus, HO can utilize a common architecture to promote the first and third oxygenation steps of the heme catabolism. We also point out a possible involvement of the H_2O_2-dependent verdoheme degradation *in vivo*, and propose potential roles of the dual pathway reaction of HO against oxidative stress.

X-C-4 ^{13}C and ^{15}N NMR Studies of Iron-Bound Cyanides of Heme Proteins and Related Model Complexes: Sensitive Probe for Detecting Hydrogen Bonding Interactions at the Proximal and Distal Sides

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[*Inorg. Chem.* **45**, 6816–6827 (2006)]

Studies of the ^{13}C and ^{15}N NMR paramagnetic shifts of the iron bound cyanides in the ferric cyanide forms of various heme proteins containing the proximal histidine and related model complexes are reported. The paramagnetic shifts of the ^{13}C and ^{15}N NMR signals of the iron bound cyanide are not significantly affected by the substitution of the porphyrin side chains. On the other hand, the paramagnetic shifts of both ^{13}C and ^{15}N NMR signals decrease with an increase in the donor effect of the proximal ligand and the ^{13}C NMR signal is more sensitive to a modification of the donor effect of the proximal ligand than the ^{15}N NMR signal. With the tilt of the iron–imidazole bond, the paramagnetic shift of the ^{13}C NMR signal increases whereas that of the ^{15}N NMR signal decreases. The hydrogen bonding interaction of the iron bound cyanide with a solvent decreases the paramagnetic shift of both ^{13}C and ^{15}N NMR signals, and the effect is more pronounced for the ^{15}N NMR signal. Data on the ^{13}C and ^{15}N NMR signals of iron bound cyanide for various heme proteins are also reported and analyzed in detail. Substantial differences in the ^{13}C and ^{15}N NMR shifts for the heme proteins can be explained based on the results for the model complexes and structures around the heme in the heme proteins. The findings herein show that the paramagnetic shift of the ^{13}C NMR signal of the iron bound cyanide is a good probe to estimate the donor effect of the proximal imidazole and that the ratio of ^{15}N/^{13}C NMR shifts allows estimating the hydrogen bonding interaction on the distal side.

X-C-5 Roles of the Heme Distal Residues of FixL in O_2 Sensing: A Single Convergent Structure of the Heme Moiety Is Relevant to the Downregulation of Kinase Activity

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[*Biochemistry* **45**, 2515–2523 (2006)]

FixL is a heme-based O_2 sensor, in which the autophosphorylation is regulated by the binding of exogenous ligands such as O_2 and CN^-. In this study, mutants of the heme distal Arg200, Arg208, Ile209, Ile210, and Arg214 residues of *Sm*FixL were characterized biochemically and physicochemically, because it has been suggested that they are significant residues in ligand-linked kinase regulation. Measurements of the autoxidation rate, affinities, and kinetics of ligand binding revealed that all of the above residues are involved in stabilization of the O_2-heme complex of FixL. However, Arg214 was found to be the only residue that is directly relevant to the ligand-dependent regulation of kinase activity. Although the wild type and R214K and R214Q mutants exhibited normal kinase regulation, R214A, R214M, R214H, and R214Y did not. ^{13}C and ^{15}N NMR analyses for $^{13}C^{15}N^-$ bound to the truncated heme domains of the Arg214 mutants indicated that, in the wild type and the foregoing two mutants, the heme moiety is present in a single conformation, but in the latter four, the conformations fluctuate possibly because of the lack of an interaction between the iron-bound ligand and residue 214. It is likely that such a rigid conformation of the ligand-bound form is important for the downregulation of histidine kinase activity. Furthermore, a comparison of the NMR data between the wild type and R214K and R214Q mutants suggests that a strong electrostatic interaction between residue 214 and the iron-bound ligand is not necessarily required for the single convergent structure and eventually for the downregulation of FixL.

X-C-6 ^{17}O NMR Study of Oxo Metalloporphyrin Complexes: Correlation with Electronic Structure of M=O Moiety

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[*J. Inorg. Biochem.* **100**, 533–541 (2006)]

^{17}O NMR spectroscopy of oxo ligand of oxo metalloporphyrin can be considered as an excellent means to derive information about structure, electronic state, and reactivity of the metal bound oxo ligand. To show the utility of ^{17}O NMR spectroscopy of oxo ligand of oxo metalloporphyrin, ^{17}O-NMR spectra of oxo ligands of dioxo ruthenium(VI), oxo chromium(IV), and oxo titanium(IV) porphyrins are measured. For all oxo metalloporphyrins, well-resolved ^{17}O NMR signals are detected in far high frequency region. The ^{17}O NMR signal of the metal bound oxo ligand shifts high frequency in order of Ru(VI) < Ti(IV) < Cr(IV), thus the ^{17}O NMR chemical shift does not directly correlate with the oxo-transfer reactivity, Ti(IV) < Cr(IV) < Ru(VI). On the other hand, the ^{17}O NMR shift of oxo ligand correlates with the bond strength of metal–oxo bond. This suggests that the ^{17}O NMR signal of metal bound oxo ligand is a sensitive

probe to study the nature of metal–oxo bond in oxo metalloporphyrin. The effect of the electron-withdrawing meso-substituent on the ^{17}O NMR shift of the oxo ligand is also investigated. With increase in the electron-withdrawing effect of the meso-subsituent, the ^{17}O NMR signal of the oxo ligand of oxo chromium(IV) porphyrin shifts high frequency while that of dioxo ruthenium(VI) porphyrin hardly change resonance position. The changes in metal–oxo bonds induced by the electron-withdrawing meso-substituent are discussed on the basis of the ^{17}O NMR shifts, the strengths of the metal–oxo bonds, and the oxo-transfer reaction rates.

X-D Reaction Mechanism of Metalloenzymes Related to Global Nitrogen Cycle

For all organisms, organic nitrogen and ammonia are required as a constituent part of the cell. In order to keep the environment of the earth constant, the organic nitrogen, fixed nitrogen, must be completely reconverted into dinitrogen gas. The reverse process of the nitrogen fixing is called denitrification process. In this process, nitrate or nitrite ion is reduced to nitrogen gas *via* nitric oxide and nitrous oxide by many metalloenzymes, nitrate reducatse, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. In this project, we are studying the molecular mechanism of these metalloenzymes relating to the denitrification process.

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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[*Angew. Chem., Int. Ed.* **45**, 1089–1092 (2006)]

Reduction of nitrite (NO_2^-) to gaseous nitric oxide (NO) is one of key processes in the global nitrogen cycle and carried out by bacterial copper-containing nitrite reductases (NiR). The enzymes contain two copper ion centers: the type 1 copper site for electron transfer and the type 2 copper site for the catalytic nitrite reduction. Crystallographic and spectroscopic studies of Nir have proposed the mechanism of the nitrite reduction at the type 2 copper site. The enzyme reaction is initiated by the binding of the nitrite to the reduced form of the type 2 copper site to yield a copper(I) nitrite complex. Subsequently, the copper bound nitrite is reduced to NO and water with intramolecular one electron transfer from the type 2 copper(I) ion and two protons from a conserved aspartic acid placed near the type 2 site. During the course of synthetic study of the copper(I) nitrite complexes, we found that the rapid mixing of copper(I) nitrite complex with trifluoroacetic acid (TFA) with stopped flow at low temperature allows to detect new reaction intermediates in the reduction process. Here, we report detection and characterization of new reaction intermediates in the nitrite reduction. This study shows that two protons required for the reaction are not provided to the copper bound nitrite simultaneously but stepwise and that the intramolecular electron transfer from the copper(I) ion to the copper bound nitrite occurs in the second protonation step.

X-D-2 ^{63}Cu NMR Spectroscopy of Copper(I) Complexes with Various Tridentate Ligands: CO as a Useful ^{63}Cu NMR Probe for Sharpening ^{63}Cu NMR Signals and Analyzing the Electronic Donor Effect of a Ligand

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[*Inorg. Chem.* in press]

^{63}Cu NMR spectroscopic studies of copper(I) complexes with various N-donor tridentate ligands are reported. As has been previously reported for most copper(I) complexes, ^{63}Cu NMR signals, when acetonitrile is coordinated to copper(I) complexes of these tridentate ligands, are extremely broad or undetectable. However, when CO is bound the above tridentate copper(I) complexes, the ^{63}Cu NMR signals become much sharper and show a large downfield shift, compared to those for the corresponding acetonitrile complexes. Temperature dependence of ^{63}Cu NMR signals for these copper(I) complexes show that a quadrupole relaxation process is much more significant to their ^{63}Cu NMR line widths than a ligand exchange process. Therefore, an electronic effect of the copper bound CO makes the ^{63}Cu NMR signal sharp and easily detected. The large downfield shift for the copper(I) carbonyl complex can be explained by a paramagnetic shielding effect induced by the copper bound CO, which amplifies small structural and electronic changes that occur around the copper ion to be easily detected in their ^{63}Cu NMR shifts. This is evidenced by the correlation between the ^{63}Cu NMR shifts for the copper(I) carbonyl complexes and their $\nu(C\equiv O)$ values. Furthermore, the ^{63}Cu NMR shifts for copper(I) carbonyl complexes with imino type tridentate ligands show a different correlation line with those for amino type tridentate ligands. On the other hand, ^{13}C NMR shifts for the copper bound ^{13}CO for these copper(I) carbonyl complexes do not correlate with the $\nu(C\equiv O)$ values. The X-ray crystal structures of

these copper(I) carbonyl complexes do not show any evidence of a significant structural change around the Cu–CO moiety. The findings herein show that CO has great potential as a probe in ^{63}Cu NMR spectroscopic studies for characterizing the nature of the environment around copper ions in copper complexes.

X-E Biomolecular Science

Elucidation of a structure-function relationship of metalloproteins and structural chemistry of amyloid fibril are current subjects of this group. The primary technique used for the first project is the stationary and time-resolved resonance Raman spectroscopy excited by visible and UV lasers. Various model compounds of active site of enzymes are also examined with the same technique. IR-microscope dichroism analysis is the main techniques for the second project. The practical themes that we want to explore for the first project are (1) mechanism of oxygen activation by enzymes, (2) mechanism of active proton translocation and its coupling with electron transfer, (3) structural mechanism of signal sensing and transmission by heme-based sensory proteins, (4) higher order protein structures and their dynamics, and (5) reactions of biological NO.

In category (1), we have examined a variety of terminal oxidases in the respiratory chain, cytochrome P450s (including AOS), and peroxidases, and also treated their reaction intermediates by using the mixed-flow transient Raman apparatus and the Raman/absorption simultaneous measurement device. For (2) the third-generation UV resonance Raman (UVRR) spectrometer was constructed in this laboratory and applied to a giant protein like cytochrome *c* oxidase with M_r = 210,000, particularly to explore the oxidation state of Tyr244 in the P_M intermediate. Furthermore, a model complex of the Cu_B–His(240)–Tyr(244) and its Tyr-radical state were investigated in detail by UVRR spectroscopy. Recently, we succeeded in pursuing protein folding of apomyoglobin by combining the UV time-resolved Raman and rapid mixing techniques. With IR spectroscopy we determined the spectrum of carboxylic side chains of bovine cytochrome oxidase which undergo protonation/deprotonation changes and hydrogen-bonding status changes in response with electron transfers between metal centers or with ligand dissociation from heme a_3. In (3) we are interested in a mechanism of ligand recognition specific to CO, NO or O_2 and a communication pathway of the ligand binding information to the functional part of the protein. Several gas sensor heme proteins including DOS, HemAT, CooA, and NAPS2 were extensively treated in this year. For (4) we developed a novel technique for UV resonance Raman measurements based on the combination of the first/second order dispersions of gratings and applied it successfully to 235-nm excited RR spectra of several proteins including mutant hemoglobins and myoglobins.

Nowadays we can carry out time-resolved UVRR experiments with sub-nanosecond time resolution to discuss protein dynamics. With the system, we have succeeded in isolating the spectrum of tyrosinate in ferric Hb M Iwate, which was protonated in the ferrous state, and the deprotonated state of Tyr244 of bovine cytochrome *c* oxidase. The study is extended to a model of Tyr244, that is, imidazole-bound *para*-cresol coordinated to a metal ion, was synthesized and its UV resonance Raman was investigated. For (5) we purified soluble guanylate cyclase from bovine lung and observed its RR spectra in the presence of allosteric effector, YC-1. The CO and NO adducts in the presence of YC-1 were examined. To further investigate it, we are developing an expression system of this protein. For the amyloid study, we examined FTIR spectra of β_2-microglobulin and its fragment peptides of #11-21, K3, and K3-K7 which form a core part of amyloid fibril of β_2-microglobulin. The effect of seed upon the fibril structure and the chemical interactions of peptides in the growth process of amyloid fibril were focused in this year.

X-E-1 Kinetics and DFT Studies on the Reaction of Copper(II) Complexes and H_2O_2

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[*J. Biol. Inorg. Chem.* **10**, 581–590 (2005)]

Copper(II) complexes supported by bulky tridentate ligands $L1^H$ (*N*,*N*-bis(2-quinolylmethyl)-2-phenylethylamine) and $L1^{Ph}$ (*N*,*N*-bis(2-quinolylmethyl)-2,2-diphenylethylamine) have been prepared and their crystal structures as well as some physicochemical properties have been explored. Each complex exhibits a square pyramidal structure containing a coordinated solvent molecule at an equatorial position and a weakly coordinated counter anion (or water) at an axial position. The copper(II) complexes reacted readily with H_2O_2 at a low temperature to give mononuclear hydroperoxo copper(II) complexes. Kinetics and DFT studies have suggested that, in the initial stage of the reaction, deprotonated hydrogen peroxide attacks the cupric ion, presumably at the axial position, to give a hydroperoxo copper(II) complex retaining the coordinated solvent molecule ($\mathbf{H}^R$·S). $\mathbf{H}^R$·S then loses the solvent to give a tetragonal copper(II)-hydroperoxo complex ($\mathbf{H}^R$), in which the -OOH group may occupy an equatorial position. The copper(II)-hydroperoxo complex $\mathbf{H}^R$ exhibits a relatively high O–O bond stretching vibration at 900 cm^{-1} compared to other previously reported examples.

X-E-2 Detection of the His-Heme Fe^{2+}–NO Species in the Reduction of NO to N_2O by *ba*$_3$-Oxidase from *Thermus thermophilus*

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[*J. Am. Chem. Soc.* **127**, 15161–15167 (2005)]

Reaction pathways in the enzymatic formation and cleavage of the N–N and N–O bonds, respectively, are difficult to verify without the structure of the intermediates, but we now have such information on the

heme a_3^{2+}-NO species formed in the reaction of ba_3-oxidase with NO from resonance Raman spectroscopy. We have identified the His-heme a_3^{2+}-NO/Cu_B^{1+} species by its characteristic Fe–NO and N–O stretching frequencies at 539 and 1620 cm^{-1}, respectively. The Fe–NO and N–O frequencies in ba_3-oxidase are 21 and 7 cm^{-1} lower and higher, respectively, than those observed in Mb–NO. From these results and earlier Raman and FTIR measurements, we demonstrate that the protein environment of the proximal His384 that is part of the Q-proton pathway controls the strength of the Fe–His384 bond upon ligand (CO *vs* NO) binding. We also show by time-resolved FTIR spectroscopy that Cu_B^{1+} has a much lower affinity for NO than for CO. We suggest that the reduction of NO to N_2O by ba_3-oxidase proceeds by the fast binding of the first NO molecule to heme a_3 with high-affinity, and the second NO molecule binds to Cu_B with low-affinity, producing the temporal co-presence of two NO molecules in the heme-copper center. The low-affinity of Cu_B for NO binding also explains the NO reductase activity of the ba_3-oxidase as opposed to other heme-copper oxidases. With the identification of the His-heme a_3^{2+}-NO/Cu_B^{1+} species, the structure of the binuclear heme a_3-Cu_B^{1+} center in the initial step of the NO reduction mechanism is known.

X-E-3 Structural Studies Reveal that the Diverse Morphology of β_2-Microglobulin Aggregates is a Reflection of Different Molecular Architectures

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[*Biochim. Biophys. Acta* **1753**, 108–120 (2005)]

Amyloid deposition accompanies over 20 degenerative diseases in human, including Alzheimer's, Parkinson's, and prion diseases. Recent studies revealed the importance of other type of protein aggregates, *e.g.*, non-specific aggregates, protofibrils, and small oligomers in the development of such diseases and proved their increased toxicity for living cells in comparison with mature amyloid fibrils. We carried out a comparative structural analysis of different monomeric and aggregated states of β_2-microglobulin, a protein responsible for hemodialysis-related amyloidosis. We investigated the structure of the native and acid-denatured states, as well as that of mature fibrils, immature fibrils, amorphous aggregates, and heat-induced filaments, prepared under various *in vitro* conditions. Infrared spectroscopy demonstrated that the β-sheet compositions of immature fibrils, heat-induced filaments and amorphous aggregates are characteristic of antiparallel intermolecular β-sheet structure while mature fibrils are different from all others suggesting a unique overall structure and assembly. Filamentous aggregates prepared by heat treatment are of importance in understanding the *in vivo* disease because of their stability under physiological conditions, where amyloid fibrils and protofibrils formed at acidic pH depolymerize. Atomic force microscopy of heat-induced filaments represented a morphology similar to that of the low pH immature fibrils. At a pH close to the pI of the protein, amorphous aggregates were formed readily with association of the molecules in native-like conformation, followed by formation of intermolecular β-sheet structure in a longer time-scale. Extent of the core buried from the solvent in the various states was investigated by H/D exchange of the amide protons.

X-E-4 Biophysical Properties of a *c*-Type Heme in Chemotaxis Signal Transducer Protein DcrA

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[*Biochemistry* **44**, 15406–15413 (2005)]

Chemotaxis signal transducer protein DcrA from a sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough was previously shown to contain a *c*-type heme in its periplasmic domain (DcrA-N) for sensing redox and/or oxygen [Fu *et al. J. Bacteriol.* **176**, 344–350 (1994)], which is the first example of a heme-based sensor protein containing a *c*-type heme as a prosthetic group. Optical absorption and resonance Raman spectroscopies indicates that heme *c* in DcrA-N shows a redox-dependent ligand exchange. Upon reduction, a water molecule that may be the sixth ligand of the ferric heme *c* is replaced by an endogenous amino acid. Although the reduced heme in DcrA-N is six-coordinated with two endogenous axial ligands, CO can easily bind to the reduced heme to form CO-bound DcrA-N. Reaction of the reduced DcrA-N with molecular oxygen results in autoxidation to form a ferric state without forming any stable oxygen-bound form probably due to the extremely low redox potential of DcrA-N (–250 mV). Our study supports the initial idea by Fu *et al.* that DcrA would act as a redox and/or oxygen sensor, in which the ligand exchange between water and an endogenous amino acid is a trigger for signal transduction. While the affinity of CO to DcrA-N (K_d = 138 μM) is significantly weak compared to those of other heme proteins, we suggest that CO might be another physiological effector molecule.

X-E-5 Discovery of a Reaction Intermediate of Aliphatic Aldoxime Dehydratase Involving Heme as an Active Center

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[*Proc. Natl. Acad. Sci. U.S.A.* **103**, 564–568 (2006)]

Recently, we discovered an intriguing hemoprotein [aliphatic aldoxime dehydratase (OxdA)] that catalyzes the dehydration of aliphatic aldoximes [R–CH=N–OH] to the corresponding nitriles [R–CN] in the industrial

Pseudomonas chlororaphis B23 strain. Unlike the utilization of H_2O_2 or O_2 as a mediator of the catalysis by other heme-containing enzymes (*e.g.*, P450), OxdA is notable for the direct binding of a substrate to the heme iron, experimental evidence of which was obtained here by means of resonance Raman (RR) analysis with an isotope technique. We found that the addition of a large amount of butyraldoxime (final concentration, 200 mM) to ferrous OxdA with a low enzyme concentration (final concentration, 5 μM) yields a long-lived OxdA-substrate complex (named OS-II), whose UV-vis spectrum is different from the corresponding spectra of the OxdA-substrate complex I and CO-bound, ferrous, and ferric forms of OxdA. Intriguingly, the RR analysis demonstrated that OS-II includes a highly oxidized heme with strong bonding between a substrate and the heme iron, as judged from the heme oxidation state markerν_4 band at 1,379 cm^{-1} and the ^{15}N-isotope-substituted butyraldoxime sensitive band at 857 cm^{-1} in the RR spectra. It is noteworthy that OS-II has a highly oxidized heme like the ferryl–oxo heme species (*e.g.*, compound II) formed by some general hemoproteins, although the function of OxdA is different from those (transport of electrons, transport of oxygen, sensing of oxygen or carbon monoxide, and catalysis of redox reactions) of general hemoproteins.

X-E-6 Intramolecular Arene Hydroxylation *versus* Intermolecular Olefin Epoxidation by (μ-η^2:η^2-Peroxo)dicopper(II) Complex Supported by Dinucleating Ligand

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[*J. Am. Chem. Soc.* **128**, 3874–3875 (2006)]

A discrete (μ-η^2:η^2-peroxo)Cu(II)$_2$ complex, [Cu_2(O_2)(H-L)]$^{2+}$, is capable of performing not only intramolecular hydroxylation of a *m*-xylyl linker of a dinucleating ligand but also intermolecular epoxidation of styrene *via* electrophilic reaction to the C=C bond and hydroxylation of THF by H-atom abstraction.

X-E-7 Significance of the Molecular Shape of Iron Corrphycene in a Protein Pocket

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[*Inorg. Chem.* **45**, 4238–4242 (2006)]

The iron complex of a new type of corrphycene bearing two ethoxycarbonyl (-$CO_2C_2H_5$) groups on the bipyrrole moiety was introduced into apomyoglobin. The reconstituted ferric myoglobin has a coordinating water molecule that deprotonates to hydroxide with a pK_a value of 7.3 and exhibits 3–10-fold higher affinities for anionic ligands when compared with a counterpart myoglobin with the same substituents on the dipyrroethene moiety. In the ferrous state, the oxygen affinity of the new myoglobin was decreased to 1/410 of the native protein. The anomalies in the ligand binding, notably dependent on the side-chain location, were interpreted in terms of a characteristic core shape of corrphycene that produces the longer and shorter Fe–N(pyrrole) bonds. The spin-state equilibrium analysis of the ferric azide myoglobin containing the new iron corrphycene supported the nonequivalence of the Fe–N(pyrrole) bonds. These results demonstrate that the trapezoidal molecular shape of corrphycene exerts functional significance when the iron complex is placed in a protein pocket.

X-E-8 Sequential Reaction Intermediates in Aliphatic C–H Bond Functionalization Initiated by a Bis(μ-oxo)dinickel(III) Complex

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[*Inorg. Chem.* **45**, 2873–2885 (2006)]

Reaction of [$Ni_2(OH)_2(Me_2$-tpa$)_2$]$^{2+}$ (**1**) (Me_2-tpa = bis(6-methyl-2-pyridylmethyl)(2-pyridylmethyl)amine) with H_2O_2 causes oxidation of a methylene group of Me_2-tpa ligand to give N-dealkylated ligand and oxidation of a methyl group to afford ligand-based carboxylate and alkoxide as final oxidation products. A series of the sequential reaction intermediates produced in the oxidation pathways, a bis(μ-oxo)dinickel(III) ([$Ni_2(O)_2(Me_2$-tpa$)_2$]$^{2+}$(**2**)), a bis(μ-superoxo)dinickel(II) ([$Ni_2(O_2)_2(Me_2$-tpa$)_2$]$^{2+}$ (**3**)), a (μ-hydroxo)(μ-alkylperoxo)dinickel(II) ([Ni_2(OH)(Me_2-tpa)(Me-tpa-CH_2OO)]$^{2+}$ (**4**)), and a bis(μ-alkylperoxo)dinickel(II) ([Ni_2(Me-tpa-CH_2OO$)_2$]$^{2+}$ (**5**)), was isolated and characterized by various physicochemical measurements including X-ray crystallography and their oxidation pathways were investigated. Reaction of **1** with H_2O_2 in methanol at –40 °C generates **2**, which is extremely reactive with H_2O_2 to produce **3**. **2** was isolated only from disproportionation of **3** in the absence of H_2O_2 at –40 °C. Thermal decomposition of **2** under N_2 generated an N-dealkylated ligand Me-dpa ((6-methyl-2-pyridylmethyl)(2-pyridylmethyl)amine) and a ligand-coupling dimer (Me-tpa-CH_2)$_2$. The formation of (Me-tpa-CH_2)$_2$ suggests that a ligand-based radical Me-tpa-CH_2• is generated as a reaction intermediate produced probably by H-atom abstraction by the oxo group. Isotope labeling experiment revealed that intramolecular coupling occurs for the formation of the coupling dimer. The results indicates that rebound of oxygen to the Me-tpa-CH_2• is slower compared to those observed for various high-valent bis(μ-oxo)dimetal complexes. In contrast, decomposition of **2** and **3** in the presence of O_2 gave

carboxylate and alkoxide ligands (Me-tpa-COO$^-$ and Me-tpa-CH$_2$O$^-$) instead of (Me-tpa-CH$_2$)$_2$, indicating that the reaction of the Me-tpa-CH$_2$• with O$_2$ is faster than the coupling of Me-tpa-CH$_2$• to generate a ligand-based peroxyl radical Me-tpa-CH$_2$OO•. Although there is a possibility that the Me-tpa-CH$_2$OO• species could undergo various reactions, one of possible reactive intermediates, **4**, was isolated from the decomposition of **3** under O$_2$ at –20 °C. The alkylperoxo ligands in **4** and **5** can be converted to a ligand-based aldehyde by either homolysis or heterolysis of the O–O bond and disproportionation of the aldehyde gives carboxylate and alkoxide *via* the Cannizzaro reaction.

X-E-9 ^{17}O NMR Study of Oxo Metalloporphyrin Complexes: Correlation with Electronic Structure of M=O Moiety

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[*J. Inorg. Biochem.* **100**, 533–541 (2006)]

^{17}O NMR spectroscopy of oxo ligand of oxo metalloporphyrin can be considered as an excellent means to derive information about structure, electronic state, and reactivity of the metal bound oxo ligand. To show the utility of ^{17}O NMR spectroscopy of oxo ligand of oxo metalloporphyrin, ^{17}O NMR spectra of oxo ligands of dioxo ruthenium(VI), oxo chromium(IV), and oxo titanium(IV) porphyrins are measured. For all oxo metalloporphyrins, well-resolved ^{17}O NMR signals are detected in far high frequency region. The ^{17}O NMR signal of the metal bound oxo ligand shifts high frequency in order of Ru(VI) < Ti(IV) < Cr(IV), thus the ^{17}O NMR chemical shift does not directly correlate with the oxo-transfer reactivity, Ti(IV) < Cr(IV) < Ru(VI). On the other hand, the ^{17}O NMR shift of oxo ligand correlates with the bond strength of metal–oxo bond. This suggests that the ^{17}O NMR signal of metal bound oxo ligand is a sensitive probe to study the nature of metal–oxo bond in oxo metalloporphyrin. The effect of the electron-withdrawing meso-substituent on the ^{17}O NMR shift of the oxo ligand is also investigated. With increase in the electron-withdrawing effect of the meso-substituent, the ^{17}O NMR signal of the oxo ligand of oxo chromium(IV) porphyrin shifts high frequency while that of dioxo ruthenium(VI) porphyrin hardly change resonance position. The changes in metal–oxo bonds induced by the electron-withdrawing meso-substituent are discussed on the basis of the ^{17}O NMR shifts, the strengths of the metal–oxo bonds, and the oxo-transfer reaction rates.

X-E-10 Nonheme Iron(II) Complexes of Macrocyclic Ligands in the Generation of Oxoiron(IV) Complexes and the Catalytic Epoxidation of Olefins

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[*J. Inorg. Biochem.* **100**, 627–633 (2006)]

Mononuclear nonheme oxoiron(IV) complexes bearing 15-membered macrocyclic ligands were generated from the reactions of their corresponding iron(II) complexes and iodosylbenzene (PhIO) in CH$_3$CN. The oxoiron(IV) species were characterized with various spectroscopic techniques such as UV-vis spectrophotometer, electron paramagnetic resonance, electrospray ionization mass spectrometer, and resonance Raman spectroscopy. The oxoiron(IV) complexes were inactive in olefin epoxidation. In contrast, when iron(II) and oxoiron(IV) complexes are combined with PhIO in the presence of olefins, high yields of oxygenated products were obtained. These results indicate that in addition to the oxoiron(IV) species, there must be at least one more active oxidant (*e.g.*, Fe^{IV}-OIPh adduct and oxoiron(V) species) that is responsible for the olefin epoxidation. We have also demonstrated that the ligand environment of iron catalysts is an important factor in controlling the product selectivity as well as the catalytic activity in the catalytic epoxidation of olefins.

X-E-11 Resonance Raman Enhancement of Fe^{IV}=O Stretch in High-Valent Iron Porphyrins: An Insight from TD-DFT Calculations

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[*J. Inorg. Biochem.* **100**, 744–750 (2006)]

Density functional theory (DFT) has been applied to explain the origin of resonance Raman enhancement associated with the Fe^{IV}=O stretch observed in iron(IV) oxo porphyrins. To accomplish this electronic excitations of the Im–(Por) Fe^{IV}=O model were computed in the 1.5–4.0 eV spectral range using time-dependent DFT (TD-DFT). All electronic transitions having dominant $\pi \rightarrow \pi^*$ character were analyzed and assigned in terms of one-electron excitations. It was found that the most intense Soret band has a multi-component character, but the π (a_{2u}) $\rightarrow$ π^* (d_{xz},, d_{yz}) and π (a_{1u}) $\rightarrow$ π^* (d_{xz}, d_{yz}) electronic excitations are primarily responsible for observed resonance enhancement of the Fe^{IV}=O stretch.

X-E-12 Spectroscopic and Substrate Binding Properties of Heme-Containing Aldoxime Dehydratases, OxdB and OxdRe

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[*J. Inorg. Biochem.* **100**, 1069–1074 (2006)]

Aldoxime dehydratase (Oxd) is a novel hemeprotein that catalyzes the dehydration reaction of aldoxime to produce nitrile. In this study, we studied the spectroscopic and substrate binding properties of two Oxds, OxdB from *Bacillus* sp. strain OxB-1 and OxdRE from

Rhodococcus sp. N-771, that show different quaternary structures and relatively low amino acid sequence identity. Electronic absorption and resonance Raman spectroscopy revealed that ferric OxdRE contained a six-coordinate low-spin heme, while ferric OxdB contained a six-coordinate high-spin heme. Both ferrous OxdRE and OxdB included a five-coordinate high-spin heme to which the substrate was bound via its nitrogen atom for the reaction to occur. Although the ferric Oxds were inactive for catalysis, the substrate was bound to the ferric heme *via* its oxygen atom in both OxdB and OxdRE. Electronic paramagnetic resonance (EPR) and rapid scanning spectroscopy revealed that the flexibility of the heme pocket was different between OxdB and OxdRE, which might affect their substrate specificity.

X-E-13 Spectroscopic and DNA-Binding Characterization of the Isolated Heme-Bound Basic Helix-Loop-Helix-PAS-A Domain of Neuronal PAS Protein 2 (NPAS2), a Transcription Activator Protein Associated with Circadian Rhythms

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[*FEBS J.* **273**, 2528–2539 (2006)]

Neuronal PAS domain protein 2 (NPAS2) is a circadian rhythm-associated transcription factor with two heme-binding sites on two PAS domains. In the present study, we compared the optical absorption spectra, resonance Raman spectra, heme-binding kinetics and DNA-binding characteristics of the isolated fragment containing the N-terminal basic helix-loop-helix (bHLH) of the first PAS (PAS-A) domain of NPAS2 with those of the PAS-A domain alone. We found that the heme-bound bHLH-PAS-A domain mainly exists as a dimer in solution. The Soret absorption peak of the Fe(III) complex for bHLH-PAS-A (421 nm) was located at a wavelength 9 nm higher than for isolated PAS-A (412 nm). The axial ligand *trans* to CO in bHLH-PAS-A appears to be His, based on the resonance Raman spectra. In addition, the rate constant for heme association with apo-bHLH-PAS (3.3×10^7 $mol^{-1} \cdot s^{-1}$) was more than two orders of magnitude higher than for association with apo-PAS-A ($< 10^5$ $mol^{-1} \cdot s^{-1}$). These results suggest that the bHLH domain assists in stable heme binding to NPAS2. Both optical and resonance Raman spectra indicated that the Fe(II)–NO heme complex is five-coordinated. Using the quartz-crystal microbalance method, we found that the bHLH-PAS-A domain binds specifically to the E-box DNA sequence in the presence, but not in the absence, of heme. On the basis of these results, we discuss the mode of heme binding by bHLH-PAS-A and its potential role in regulating DNA binding.

X-E-14 Specific Hydrogen-Bonding Networks Responsible for Selective O_2 Sensing of the Oxygen Sensor Protein HemAT from *Bacillus subtilis*

YOSHIMURA, Hideaki; YOSHIOKA, Shiro; KOBAYASHI, Katsuaki[1]; OHTA, Takehiro[2]; UCHIDA, Takeshi[3]; KUBO, Minoru; KITAGAWA, Teizo; AONO, Shigetoshi
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[*Biochemistry* **45**, 8301–8307 (2006)]

HemAT from Bacillus subtilis (HemAT-Bs) is a heme-based O_2 sensor protein that acts as a signal transducer responsible for aerotaxis. HemAT-Bs discriminates its physiological effector, O_2, from other gas molecules to generate the aerotactic signal, but the detailed mechanism of the selective O_2 sensing is not obvious. In this study, we measured electronic absorption, electron paramagnetic resonance (EPR), and resonance Raman spectra of HemAT-Bs to elucidate the mechanism of selective O_2 sensing by HemAT-Bs. Resonance Raman spectroscopy revealed the presence of a hydrogen bond between His86 and the heme propionate only in the O_2-bound form, in addition to that between Thr95 and the heme-bound O_2. The disruption of this hydrogen bond by the mutation of His86 caused the disappearance of a conformer with a direct hydrogen bond between Thr95 and the heme-bound O_2 that is present in WT HemAT-Bs. On the basis of these results, we propose a model for selective O_2 sensing by HemAT-Bs as follows. The formation of the hydrogen bond between His86 and the heme propionate induces a conformational change of the CE-loop and the E-helix by which Thr95 is located at the proper position to form the hydrogen bond with the heme-bound O_2. This stepwise conformational change would be essential to selective O_2 sensing and signal transduction by HemAT-Bs.

X-E-15 Raman Evidence for Specific Substrate-Induced Structural Changes in the Heme Pocket of Human Cytochrome P450 Aromatase during the Three Consecutive Oxygen Activation Steps

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[*Biochemistry* **45**, 5631–5640 (2006)]

Specific substrate-induced structural changes in the heme pocket are proposed for human cytochrome P450 aromatase (P450arom) which undergoes three consecutive oxygen activation steps. We have experimentally investigated this heme environment by resonance Raman spectra of both substrate-free and substrate-bound forms of the purified enzyme. The Fe–CO stretching mode (ν_{Fe-CO}) of the CO complex and Fe^{3+}–S stretching mode (ν_{Fe-S}) of the oxidized form were monitored as a structural marker of the distal and proximal sides of the

heme, respectively. The ν_{Fe-CO} mode was upshifted from 477 to 485 and to 490 cm^{-1} by the binding of androstenedione and 19-aldehyde-androstenedione, substrates for the first and third steps, respectively, whereas ν_{Fe-CO} was not observed for P450arom with 19-hydroxy-androstenedione, a substrate for the second step, indicating that the heme distal site is very flexible and changes its structure depending on the substrate. The 19-aldehyde-androstenedione binding could reduce the electron donation from the axial thiolate, which was evident from the low-frequency shift of ν_{Fe-S} by 5 cm^{-1} compared to that of androstenedione-bound P450arom. Changes in the environment in the heme distal site and the reduced electron donation from the axial thiolate upon 19-aldehyde-androstenedione binding might stabilize the ferric peroxo species, an active intermediate for the third step, with the suppression of the formation of compound I (Fe^{4+}=O porphyrin$^{+\bullet}$) that is the active species for the first and second steps. We, therefore, propose that the substrates can regulate the formation of alternative reaction intermediates by modulating the structure on both the heme distal and proximal sites in P450arom.

X-E-16 Evidence for Displacement of the C-Helix by CO Ligation and DNA Binding to CooA Revealed by UV Resonance Raman Spectroscopy

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[*J. Biol. Chem.* **281**, 11271–11278 (2006)]

The UV and visible resonance Raman spectra are reported for CooA from Rhodospirillum rubrum, which is a transcriptional regulator activated by growth in a CO atmosphere. CO binding to heme in its sensor domain causes rearrangement of its DNA-binding domain, allowing binding of DNA with a specific sequence. The sensor and DNA-binding domains are linked by a hinge region that follows a long C-helix. UV resonance Raman bands arising from Trp-110 in the C-helix revealed local movement around Trp-110 upon CO binding. The indole side chain of Trp-110, which is exposed to solvent in the CO-free ferrous state, becomes buried in the CO-bound state with a slight change in its orientation but maintains a hydrogen bond with a water molecule at the indole nitrogen. This is the first experimental data supporting apreviously proposed model involving displacement of the C-helix and heme sliding. The UV resonance Raman spectra for the CooA-DNA complex indicated that binding ofDNA to CooA induces a further displacement of the C-helix in the same direction during transition to the complete active conformation. The Fe–CO and C–O stretching bands showed frequency shifts upon DNA binding, but the Fe–His stretching band did not. Moreover, CO–geminate recombination was more efficient in the DNA-boundstate. These results suggest that the C-helix displacement in the DNA-bound form causes the CO binding pocket to narrow and become more negative.

X-E-17 On the Relationship of Coral Allene Oxide Synthase to Catalase: A Single Active Site Mutation that Induces Catalase Activity in Coral Allene Oxide Synthase

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A heme domain of coral allene oxide synthase (cAOS) catalyzes the formation of allene oxide from fatty acid hydroperoxide. Although cAOS has a similar heme active site to that of catalase, cAOS is completely lacking in catalase activity. A close look at the hydrogen-bonding possibilities around the distal His in cAOS suggested that the imidazole ring is rotated by 180° relative to that of catalase because of the hydrogen bond between Thr-66 and the distal His-67. This could contribute to the functional differences between cAOS and catalase, and to examine this possibility, we mutated Thr-66 in cAOS to Val, the corresponding residue in catalase. In contrast to the complete absence of catalase activity in wild type (WT) cAOS, T66V had a modest catalase activity. On the other hand, the mutation suppressed the native enzymatic activity of the formation of allene oxide to 14% of that of WT cAOS. In the resonance Raman spectrum, whereas WT cAOS has only a 6-coordinate/high spin heme, T66V has a 5-coordinate/high spin heme as a minor species. Because catalase adopts a 5-coordinate/high spin structure, probably the 5-coordinate/high spin portion of T66V showed the catalase activity. Furthermore, in accord with the fact that the CN affinity of catalase is higher than that of WT cAOS, the CN affinity of T66V was 8-fold higher than that of WT cAOS, indicating that the mutation could mimic the heme active site in catalase. We, therefore, propose that the hydrogen bond between Thr-66 and distal His-67 could modulate the orientation of distal His, thereby regulating the enzymatic activity in cAOS.

X-E-18 Similarities and Differences between Cyclobutane Pyrimidine Dimer (CPD) Photolyase and (6-4) Photolyase as Revealed by Resonance Raman Spectroscopy: Electron Transfer from FAD Cofactor to UV-Damaged DNA

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Cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct, two major types of DNA damage caused by ultraviolet (UV) light, are repaired under illumination with near-UV/visible light by CPD photolyase and (6-4) photolyase, respectively. To understand the mechanism of DNA repair, we examined the resonance Raman spectra of complexes between damaged DNA and the neutral semiquinoid and oxidized forms of (6-4) and CPD photolyases. The marker band for a neutral semi-

quinoid flavin and band I of the oxidized flavin, which are derived from the vibrations of the benzene ring of FAD, were shifted to lower frequencies upon binding of damaged DNA by CPD photolyase but not by (6-4) photolyase, indicating that CPD interacts with the benzene ring of FAD directly but that (6-4) photoproduct does not. Bands II and VII of the oxidized flavin and the 1398/1391 cm^{-1} bands of the neutral semiquinoid flavin, which may reflect the bending of the U-shaped FAD, were altered upon substrate binding, suggesting that CPD and (6-4) photoproduct interact with the adenine ring of FAD. When substrate is bound, there is an upshifted 1528 cm^{-1} band of the neutral semiquinoid flavin in CPD photolyase, indicating a weakened hydrogen bond at N5-H of FAD, and in (6-4) photolyase, band X seems to be downshifted, indicating a weakened hydrogen bond at N3-H of FAD. These Raman spectra led us to conclude that the two photolyases have different electron transfer mechanisms as well as different hydrogen bonding environments, which account for the higher redox potential of CPD photolyase.

X-E-19 Structure of Interacting Segments in the Growing Amyloid Fibril of β_2-Microglobulin Probed with IR Spectroscopy

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[*J. Mol. Biol.* **362**, 355–364 (2006)]

Inter-segmental interaction at the growing tip of the amyloid fibril of β_2-microglobulin (β_2m) was investigated using IR microscopy. Cross-seeded fibril formation was implemented, in which the amyloid fibril of the #21-31 fragment of β_2m (fA[#21-31]) was generated on β_2m amyloid fibril (fA[β_2m]) as a seed. Differences between the IR spectra of the cross-seeded fibril and the seed were attributed to the contribution from the tip, whose structure is discussed. The results indicated that 6.5±1.0 out of 11 residues of the fA[#21-31] tip on fA[β_2m] are contained in a β-sheet at pH 2.5, which was smaller than the corresponding value (7.5±1.1 residues) of the spontaneous fA[#21-31] at pH 2.5. The tip was suggested to have a planar structure, indicating the planarity of the interacting segment. The N-terminal region of fA[#21-31] in the fibril is more exposed to the solvent than that in the tip, and *vice versa* for the C-terminal region. This is consistent with the different protonation levels of these regions, and from these results the direction of peptide in the fibrils is determined.

X-E-20 Pathway of Information Transmission from Heme to Protein upon Ligand Binding/ Dissociation in Myoglobin Revealed by UV Resonance Raman Spectroscopy

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Gas sensory heme proteins respond to their environment by binding a specific gas molecule to heme and transmitting this primary binding signal to the protein. How the binding signal is transmitted from the heme to the protein remains to be clarified. Using UV resonance Raman (UVRR) spectroscopy, we investigated this pathway in sperm whale myoglobin as a model gas sensory heme protein. Based on the UVRR data and the effects of deleting one of three important pathways (His93, 6-propionate, or 7-propionate), we determined the changes in the conformation of globin that occur upon binding of CO, NO, or O_2 to heme and how they are transmitted from heme to globin. The UVRR results show that heme discriminates different ligands, resulting in different conformations in the globin protein. Specifically, NO induces changes in the spectrum of Trp residues in the A-helix that are significantly different from those induced by O_2 or CO binding. On the other hand, binding of O_2 to heme produces changes in the Tyr residues of the H-helix that are different from those induced by CO or NO binding. Furthermore, we found that cleavage of the Fe–His93 covalent bond eliminates communication to the terminal region of the H-helix and that the 7-propionate hydrogen-bonding network is essential for transmitting the CO or NO binding signal to the N- and C-termini. Finally, the 6-propionate is important only for NO binding. Thus, the hydrogen-bonding network in the protein appears to be critical for intramolecular signal transduction in gas sensory heme proteins.

X-E-21 Characteristic Structure and Environment in FAD Cofactor of (6-4) Photolyase along Function Revealed by Resonance Raman Spectroscopy

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[*J. Phys. Chem. B* **110**, 16724–16732 (2006)]

A pyrimidine-pyrimidone (6-4) photoproduct and a cyclobutane pyrimidine dimer (CPD) are major DNA lesions induced by ultraviolet irradiation, and (6-4) photolyase, an enzyme with flavin adenine dinucleotide (FAD) as a cofactor, repairs the former specifically by light illumination. We investigated resonance Raman spectra of (6-4) photolyase from *Arabidopsis thaliana* having neutral semiquinoid and oxidized forms of FAD, which were selectively intensity enhanced by excitations at 568.2 and 488.0 nm, respectively. DFT calculations were carried out for the first time on the neutral semiquinone. The marker band of a neutral semiquinone at 1606 cm^{-1} in H_2O, whose frequency is the lowest among various flavoenzymes, apparently splits into two comparable bands at 1594 and 1608 cm^{-1} in D_2O, and similarly that at 1522 cm^{-1} in H_2O does into three bands at 1456, 1508, and 1536 cm^{-1} in D_2O. This D_2O effect was recognized only after being oxidized once and photoreduced to form a semiquinone again, but not by simple H/D exchange of solvent. Some Raman bands of

the oxidized form were observed at significantly low frequencies (1621, 1576 cm^{-1}) and with band splittings (1508/1493, 1346/1320 cm^{-1}). These Raman spectral characteristics indicate strong H-bonding interactions (at N5-H, N1), a fairly hydrophobic environment, and an electron-lacking feature in benzene ring of the FAD cofactor, which seems to specifically control the reactivity of (6-4) photolyase.

X-E-22 Time-Resolved Raman Evidence for Energy Funneling through Propionate Side Chains in Heme Cooling Upon Photolysis of Carbonmonoxy Myoglobin

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[*Chem. Phys. Lett.* **429**, 239–243 (2006)]

The heme “cooling” following CO photolysis was investigated with picosecond time-resolved anti-Stokes Raman spectroscopy on modified myoglobins (Mb), in which the 6- or 7-propionate is selectively replaced by a methyl group. The time constants of population decay of vibrationally excited states for two modified Mbs became significantly larger compared with those of native Mb. This is the first experimental evidence for appreciable contribution of each heme-propionate side chain to the vibrational energy transfer from the heme to the surroundings.

X-E-23 Characterization of the Phenoxyl Radical in Model Complexes for the Cu_B Site of Cytochrome *c* Oxidase: Steady-State and Transient Absorption Measurements, UV Resonance Raman Spectroscopy, EPR Spectroscopy, and DFT Calculations for M^{II}-BIAIP

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[*J. Am. Chem. Soc.* **128**, 14560–14570 (2006)]

Physicochemical properties of the covalently cross-linked tyrosine-histidine-Cu_B (Tyr-His-Cu_B) unit, which is a minimal model complex [M^{II}-**BIAIP**Br]Br (M = Cu^{II}, Zn^{II}) for the Cu_B site of cytochrome *c* oxidase, were investigated with steady-state and transient absorption measurements, UV resonance Raman (UVRR) spectroscopy, X-band continuous-wave electron paramagnetic resonance (EPR) spectroscopy, and DFT calculations. The pH dependency of the absorption spectra reveals that the pK_a of the phenolic hydroxyl is *ca.* 10 for the Cu^{II} model complex (Cu^{II}-**BIAIP**) in the ground state, which is similar to that of *p*-cresol (tyrosine), contrary to expectations. The bond between Cu^{II} and nitrogen of cross-linked imidazole cleaves at pH 4.9. We have successfully obtained UVRR spectra of the phenoxyl radical form of **BIAIP**s, and have assigned bands based on the previously reported isotope shifts of Im-Ph (2-(1-imidazoyl)-4-methylphenol) (Aki, M.; Ogura, T.; Naruta, Y.; Le, T. H.; Sato, T.; Kitagawa, T. *J. Phys. Chem. A* **106**, 3436–3444 (2002)) in combination with DFT calculations. The upshifts of the phenoxyl vibrational frequencies for 8*a* (C–C stretching), 7*a*’ (C–O stretching), and 19*a*, and the Raman-intensity enhancements of 19*b*, 8*b*, and 14 modes indicate that UVRR spectra are highly sensitive to imidazole-phenol covalent linkages. Both transient absorption measurements and EPR spectra suggest that the Tyr-His-Cu_B unit has only a minor effect on the electronic structure of the phenoxyl radical form, although our experimental results appear to indicate that the cross-linked Tyr radical exhibits no EPR. The role of the Tyr-His-Cu_B unit in the enzyme is discussed in terms of the obtained spectroscopic parameters of the model complex.

X-E-24 Ultraviolet Resonance Raman Evidence for Utilization of the Heme 6-Propionate Hydrogen Bond Network in Signal Transmission from Heme to Protein in *Ec* DOS Protein

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[*J. Am. Chem. Soc.* in press]

The direct oxygen sensor protein from *Escherichia coli* (*Ec* DOS) is a heme-based signal transducer protein responsible for phosphodiesterase (PDE) activity. Binding of either O_2 or CO molecule to a reduced heme enhances the PDE activity towards 3’,5’-cyclic diguanylic acid. We report ultraviolet resonance Raman (UVRR) spectroscopic investigations of the reduced, O_2- and CO-bound forms of heme-bound PAS domain (*Ec* DOSH) of *Ec* DOS. The UVRR results show that heme discriminates different ligands, resulting in altered conformations in the protein moiety. Specifically, the environment around Trp53 that contacts heme 2-vinyl group, shifts towards more hydrophobic upon O_2 binding, while towards more hydrophilic upon the CO-binding. In addition, the PDE activity of the O_2- and CO-bound forms for Trp53Phe is significantly decreased compared with that of WT, demonstrating the importance of Trp53 for the catalytic reaction. On the other hand, binding of O_2 or CO to the heme produces drastic changes in the Tyr126 of $I_β$-strand at the surface of the sensor domain. Furthermore, we found that Asn84 forms a hydrogen bond with Tyr126 either in the O_2- or CO-bound forms but not in the reduced form. Finally, the PDE activities of the ligand bound forms for Asn84Val and Tyr126Phe mutants are significantly reduced compared with that of WT, suggesting the importance of the hydrogen bonding network from heme 6-propionate to Tyr 126 through Asn84 in signal transmission.

X-E-25 Identification of an “End-on” Nickel-Superoxo Adduct, $[Ni(tmc)(O_2)]^+$

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[*J. Am. Chem. Soc.* in press]

An "end-on" Ni^{2+}-superoxo adduct has been prepared *via* two independent synthetic routes and its structure ascertained by spectroscopic and computational methods. The new structure type in nickel coordination chemistry is supported by resonance Raman and EPR spectroscopic features, the former displaying a high frequency ν(O–O) mode (1131 cm^{-1}) consistent with significant superoxo character. The Ni^{2+}-superoxo adduct reacts with PPh_3, thereby generating $OPPh_3$.

X-E-26 Regioselective Arene Hydroxylation Mediated by a (μ-Peroxo)diiron(III) Complex: A Functional Model for Toluene Monooxygenase

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[*J. Am. Chem. Soc.* in press]

Diiron(II) complexes, $[Fe_2(Ph_4\text{-tidp})(RCO_2)]^2$ (R = Ph or Ph_3CCO_2), react with dioxygen to generate peroxo–diiron(III) complexes $[Fe_2(Ph_4\text{-tidp})(RCO_2)(O_2)]^2$. Dioxygen affinity can be greatly controlled by the stereochemistry of the bridging carboxylates. Thermal decomposition of a peroxo complex with Ph_3CCO_2 resulted in regioselective hydroxylation of one of phenyl groups of the supporting ligand, which mimics toluene monooxygenase activity.

X-E-27 A Novel Mononuclear Ligand-Based Alkylperoxo Copper(II) Complex as a Reaction Intermediate in the Oxidation of the Methyl Group of the Supporting Ligand

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[*Angew. Chem., Int. Ed.* in press]

Functionalization of aliphatic C–H bond mediated by copper complexes with O_2 and/or H_2O_2 is of great interest in biological significance and industrial applicability. In this communication we report the formation, structure, and reactivity of a novel ligand-based alkylperoxo copper(II) complex, $[Cu(Me\text{-tpa-}CH_2OO)]^+$, which is produced by the reaction of $[Cu(Me_2\text{-tpa})]^+$ with excess of H_2O_2. The complex is the first example of a structurally characterized ligand-based alkylperoxo-copper(II) complex isolated as a reaction intermediate, which is further converted into ligand-based alkoxo, carboxylato complexes, and some other species upon decomposition. We believe that the findings in this study provide fundamental basis for functionalization of aliphatic C–H bond by Cu–O_n species and are of great interest to wide audience.

X-E-28 Synthesis, Characterization, and Reactivities of Manganese(V)-Oxo Porphyrin Complexes

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[*J. Am. Chem. Soc.* in press]

The reactions of manganese(III) porphyrin complexes with terminal oxidants, such as *m*-chloroperbenzoic acid, iodosylarenes, and H_2O_2, produced high-valent manganese(V)-oxo porphyrins in the presence of base in organic solvents at room temperature. The manganese(V)-oxo porphyrins have been characterized with various spectroscopic techniques, including UV-vis, EPR, ^1H and ^{19}F NMR, resonance Raman, and X-ray absorption spectroscopy. The combined spectroscopic results indicate that the manganese(V)-oxo porphyrins are diamagnetic low-spin ($S = 0$) species with a longer, weaker Mn–O bond than in previously reported Mn(V)-oxo complexes of non-porphyrin ligands. This is indicative of double bond character between the manganese(V) ion and the oxygen atom. The $[(Porp)Mn^V{=}O]^+$ species are stable in the presence of base at room temperature. The stability of the intermediates is dependent on base concentration. In the absence of base, $(Porp)Mn^{IV}{=}O$ is generated instead of the $[(Porp)Mn^V{=}O]^+$ species. The stability of the $[(Porp)Mn^V{=}O]^+$ species also depends on the electronic nature of porphyrin ligands; $[(Porp)Mn^V{=}O]^+$ complexes bearing electron-deficient porphyrin ligands are more stable than those bearing electron-rich porphyrins. Reactivity studies of manganese(V)-oxo porphyrins revealed that the intermediates are capable of oxygenating PPh_3 and thioanisoles, but not olefins and alkanes at room temperature. These results indicate that the oxidizing power of $[(Porp)Mn^V{=}O]^+$ is low in the presence of base. However, when the $[(Porp)Mn^V{=}O]^+$ complexes were associated with iodosylbenzene in the presence of olefins and alkanes, high yields of oxygenated products were obtained in the catalytic olefin epoxidation and alkane hydroxylation reactions. Mechanistic aspects, such as oxygen exchange between $[(Porp)Mn^V{=}^{16}O]^+$ and $H_2{}^{18}O$, are also discussed.