IX-H Biomolecular Science

Elucidation of a structure-function relationship of metalloproteins is a current subject of this group. The primary technique used for this project is the stationary and time-resolved resonance Raman spectroscopy excited by visible and UV lasers. The main themes that we want to explore are (1) mechanism of oxygen activation by enzymes, (2) mechanism of active proton translocation and its coupling with electron transfer, (3) coupling mechanism of proton- and electron transfers by quinones in photosynthetic reaction center, (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, cytochrome P450s, and peroxidases, and also treated their enzymatic 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 and we are going to apply it to a giant protein like cytochrme c oxidase. More recently, we succeeded in pursuing protein folding of apomyoglobin by combining UV time-resolved Raman and rapid mixing device. In (3) we succeeded in observing RR spectra of quinones A and B in bacterial photosynthetic reaction centers for the first time, but we have focused our attention on detecting tyrosine radical for the P intermediate of terminal oxidases. Some positive evidence was obtained for cytochrome *bo*. 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 nanosecond resolution to discuss protein dynamics. With the newly developed third generation UV Raman spectrometer, 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. As a model of Tyr244, an imidazole-bound *para*-cresol was synthesized and its UV resonance Raman was investigated. For (5) we purified soluble guanylate cyclase from bovine lung and observed its RR spectra. To further investigate it, we are developing an expression system of this protein.

IX-H-1 Stationary and Time-Resolved Resonance Raman Spectra of His77 and Met95 Mutants of the Isolated Heme Domain of a Direct Oxygen Sensor from *E. coli*

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[*J. Biol. Chem.* **277**, 32650 (2002)]

The heme environments of Met95 and His77 mutants of the isolated heme-bound PAS domain (*Ec* DOS PAS) of a direct oxygen sensing protein from *E. coli* (*Ec* Dos) were investigated with resonance Raman (RR) spectroscopy and compared to the wild type enzyme (WT). The RR spectra of both the reduced and oxidised WT enzyme were characteristic of six-coordinated low-spin heme complexes from pH 4 to 10. The time-resolved RR spectra of the photo-dissociated CO-WT complex had an Fe–His stretching band ($\nu_{Fe–His}$) at 214 cm^{-1}, and the $\nu_{Fe–CO}$ *vs* ν_{CO} plot of CO-WT *Ec* DOS PAS fell on the line of His-coordinated heme proteins. The photo-dissociated CO-His77Ala mutant complex did not yield the $\nu_{Fe–His}$ band but gave a $\nu_{Fe–Im}$ band in the presence of imidazole. The RR spectrum of the oxidised Met95Ala mutant was that of a six-coordinated low-spin complex, *i.e.* the same as that of the WT enzyme, whereas the reduced mutant appeared to contain a five-coordinated heme complex. Taken together, we suggest that the heme of the reduced WT enzyme is coordinated by His77 and Met95, and that Met95 is displaced by CO and O_2. Presumably, the protein conformational change that occurs on exchange of an unknown ligand for Met95 following heme reduction, may lead to activation of the phosphodiesterase domain of *Ec* Dos.

IX-H-2 Resonance Raman Studies on Xanthine Oxidase: Observation of the Mo^{VI}-Ligand Vibration

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[*J. Biol. Inorg. Chem.* in press]

Resonance Raman spectra were investigated for the sulfo- and desulfo-forms of cow's milk xanthine oxidase with various visible excitation lines between 400 and 650 nm, and the Mo^{VI}-ligand vibrations were observed for the first time. The Mo^{VI}=S stretch was identified at 474 and 462 cm^{-1} for the ^{32}S and ^{34}S-sulfo-forms, respectively, but was absent in the reduced state and in the desulfo form. The Mo^{VI}=O stretch was weakly observed at 899 cm^{-1} for the sulfo-form and shifted to 892 cm^{-1} with very weak intensity for the di-oxo desulfo-form. In measurements of an excitation profile, the two bands at 474 and 899 cm^{-1} showed maximum intensity at similar excitation wavelengths, suggesting that the Raman intensity of the metal-ligand modes is owed to the $Mo^{VI} \leftarrow S$ CT transition, and that this is the origin of the intrinsically weak features of the Mo^{VI}-ligand Raman bands. When the sulfo-form was regenerated from the desulfo-form, the 899 cm^{-1} band reappeared. However, the band at 899 cm^{-1} showed no frequency shift when regeneration was conducted in $H_2{}^{18}O$, or after several turnovers in the presence of xanthine in $H_2{}^{18}O$. When the sulfo-form was reduced and reoxidized in $H_2{}^{18}O$ buffer, the 899 cm^{-1} band reappeared without any frequency shift. These observa-

tions suggest that the oxo oxygen in the Mo center of xanthine oxidase is not labile. Low-frequency vibrations of the Mo-center were observed together with those of the Fe_2S_2 center with some overlaps, while FAD modes were observed clearly. The absence of dithiolene modes in XO is in contrast to the Mo^{VI}-centers of DMSO reductase and sulfite oxidase.

IX-H-3 Changes in the Abnormal α-Subunit upon CO-Binding to the Normal β-Subunit of Hb M Boston: Resonance Raman, EPR, and CD Study

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[*Biophys. Chem.* **98**, 217 (2002)]

Heme-heme interaction in Hb M Boston (Hisα58 → Tyr) was investigated with visible and UV resonance Raman (RR), EPR, and CD spectroscopies. Although Hb M Boston has been believed to be frozen in the *T* quaternary state, oxygen binding exhibited appreciable cooperativity (n = 1.4) and the near-UV CD spectrum indicated weakening of the *T* marker at pH 9.0. Binding of CO to the normal β subunit gave no change in the EPR and visible Raman spectra of the abnormal α subunit at pH 7.5, but it caused an increase of EPR rhombicity and significant changes in the Raman coordination markers as well as the Fe(III)-tyrosine related bands of the α subunit at pH 9.0. The UVRR spectra indicated appreciable changes of Trp but not of Tyr upon CO binding to the β subunit at pH 9.0. Therefore, we conclude that the ligand binding to the β heme induces quaternary structure change at pH 9.0 and is communicated to the α heme presumably through Hisβ92 → Trpβ37 → Hisα87.

IX-H-4 Coordination Geometry of Cu-Porphyrin in Cu(II)-Fe(II) Hybrid Hemoglobins Studied by Q-Band EPR and Resonance Raman Spectroscopies

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[*J. Inorg. Biochem.* **88**, 310 (2002)]

Cu(II)-Fe(II) hybrid hemoglobins were investigated by UV-vis, Q-band (35 GHz) EPR and resonance Raman spectroscopies. EPR results indicated that Cu-porphyrin in α-subunit within hybrid hemoglobin had either 5- or 4-coordination geometry depending on the pH conditions, while Cu-porphyrin in β-subunit had only 5-coordination geometry at high and low pH values. These results were consistent with UV-vis absorption results. A new resonance Raman band appeared around 190 cm^{-1}, which was present whenever 5-coordinated Cu-porphyrin existed in Cu(II)–Fe(II) hybrid hemoglobins irrespective of the coordination number in Fe(II) subunit. This Raman band might be assigned to Cu–His stretching mode. These results are direct demonstration of the existence of coordination changes of Cu-porphyrin in α-subunit within hybrid hemoglobin by shifting the molecular conformation from fully unliganded state to intermediately liganded state.

IX-H-5 Fine-Tuning of Copper(I)-Dioxygen Reactivity by 2-(2-Pyridyl)ethylamine Bidentate Ligands

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[*J. Am. Chem. Soc.* **124**, 6367 (2002)]

Copper(I)-dioxygen reactivity has been examined using a series of 2-(2-pyridyl)ethylamine bidentate ligands $^{R1}Py1^{R2,R3}$. The bidentate ligand with the methyl substituent on the pyridine nucleus $^{Me}Py1^{Et,Bz}$ (*N*-benzyl-*N*-ethyl-2-(6-methylpyridin-2-yl)ethylamine) predominantly provided a (μ-η^2:η^2-peroxo)dicopper(II) complex, while the bidentate ligand without the 6-methyl group $^{H}Py1^{Et,Bz}$ (*N*-benzyl-*N*-ethyl-2-(2-pyridyl)ethylamine) afforded a bis(μ-oxo)dicopper(III) complex under the same experimental conditions. Both Cu_2O_2 complexes gradually decompose, leading to oxidative N-dealkylation reaction of the benzyl group. Detailed kinetic analysis has revealed that the bis(μ-oxo)dicopper(III) complex is the common reactive intermediate in both cases and that O–O bond homolysis of the peroxo complex is the rate-determining step in the former case with $^{Me}Py1^{Et,Bz}$. On the other hand, the copper(I) complex supported by the bidentate ligand with the smallest *N*-alkyl group ($^{H}Py1^{Me,Me}$, *N*,*N*-dimethyl-2-(2-pyridyl)ethylamine) reacts with molecular oxygen in a 3:1 ratio in acetone at a low temperature to give a mixed-valence trinuclear copper(II, II, III) complex with two μ_3-oxo bridges, the UV-vis spectrum of which is very close to that of an active oxygen intermediate of lacase. Detailed spectroscopic analysis on the oxygenation reaction at different concentrations has indicated that a bis(μ-oxo)dicopper(III) complex is the precursor for the formation of trinuclear copper complex. In the reaction with 2,4-di-*tert*-butylphenol (DBP), the trinuclear copper(II, II, III) complex acts as a two-electron oxidant to produce an equimolar amount of the C–C coupling dimer of DBP (3,5,3',5'-tetra-*tert*-butyl-biphenyl-2,2'-diol) and a bis(μ-hydroxo)dicopper(II) complex. Kinetic analysis has shown that the reaction consists of two distinct steps, where the first step involves a binding of DBP to the trinuclear complex to give a certain intermediate that further reacts with the second molecule of DBP to give another intermediate, from which the final products are released. Steric and/or electronic effects of the 6-methyl group and the *N*-alkyl substituents of the bidentate ligands on the copper(I)-dioxygen reactivity have been discussed.

IX-H-6 Modulation of the Copper-Dioxygen Reactivity by Stereochemical Effect of Tetradentate Tripodal Ligands

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[*Chem. Lett.* 416 (2002)]

Dioxygen reactivity of a copper(I) complex having a sterically hindered Me-3-tpa and monooxygenase activity of its oxygenated species toward the ligand were significantly modulated by the presence of the 6-methyl group onto pyridyl group.

IX-H-7 Reactivity of Hydroperoxide Bound to a Mononuclear Non-Heme Iron Site

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[*Inorg. Chem.* **41**, 616 (2002)]

The first isolation and spectroscopic characterization of the mononuclear hydroperoxo-iron(III) complex $[Fe(H_2bppa)(OOH)]^{2+}$ (**2**) and the stoichiometric oxidation of substrates by the mononuclear iron-oxo intermediate generated by its decomposition have been described. The purple species **2** obtained from reaction of $[Fe(H_2bppa)(HCOO)](ClO_4)_2$ with H_2O_2 in acetone at –50 °C gave characteristic UV-vis (λ_{max} = 568 nm, ε = 1200 $M^{-1}cm^{-1}$), ESR (g = 7.54, 5.78, and 4.25, S = 5/2), and ESI mass spectra (m/z 288.5 corresponding to the ion, $[Fe(bppa)(OOH)]^{2+}$, which revealed that **2** is a high-spin mononuclear iron(III) complex with a hydroperoxide in an end-on fashion. The resonance Raman spectrum of **2** in d_6-acetone revealed two intense bands at 621 and 830 cm^{-1}, which shifted to 599 and 813 cm^{-1}, respectively, when reacted with ^{18}O-labeled H_2O_2. Reactions of the isolated $(bppa)Fe^{III}$-OOH (**2**) with various substrates (single turnover oxidations) exhibited that the iron-oxo intermediate generated by decomposition of **2** is a nucleophilic species formulated as $[(H_2bppa)Fe^{III}\text{-}O^{\bullet}]$.

IX-H-8 A New Mononuclear Iron(III) Complex Containing a Peroxocarbonate Ligand

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[*Angew. Chem. Int. Ed. Engl.* **41**, 1205 (2002)]

Stabilization of a peroxocarbonate ligand by formation of a five-membered chelate ring. The mononuclear peroxocarbonate complex **1** was prepared by the reaction of a bis(μ-hydroxo)diiron(III) complex with H_2O_2 and CO_2. Compound **1** is the first crystallography characterized transition metal complex with a peroxocarbonate ligand. Formation of the peroxocarbonate moiety in **1** proceeds by a nulceophilic addition of a peroxide anion to CO_2. Hqn = quinaldic acid.

IX-H-9 Formation, Characterization, and Reactivity of Bis(μ-oxo)dinickel(III) Complexes Supported by a Series of Bis[2-(2-pyridyl)ethyl]amine Ligands

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[*J. Am. Chem. Soc.* **123**, 11168 (2001)]

Bis(μ-oxo)dinickel(III) complexes supported by a series of bis[2-(2-pyridyl)ethyl]amine ligands have been successfully generated by treating the corresponding bis(μ-hydroxo)dinickel(II) complexes or bis(μ-methoxo)dinickel(II) complex with an equimolar amount of H_2O_2 in acetone at low temperature. The bis(μ-oxo)dinickel(III) complexes exhibit a characteristic UV-vis absorption band at ~ 410 nm and a resonance Raman band at 600–610 cm^{-1} that shifted to 570–580 cm^{-1} upon ^{18}O-substitution. Kinetic studies and isotope labeling experiments using $^{18}O_2$ imply the existence of intermediate(s) such as peroxo dinickel(II) in the course of formation of the bis(μ-oxo)dinickel(III) complex. The bis(μ-oxo)dinickel(III) complexes supported by the mononucleating ligands ($\mathbf{L1^X}$ = para-substituted *N*,*N*-bis[2-(2-pyridyl)ethyl]-2-phenylethylamine; X = OMe, Me, H, Cl) gradually decompose, leading to benzylic hydroxylation of the ligand side arm (phenethyl group). The kinetics of the ligand hydroxylation process including kinetic deuterium isotope effects (KIE), *p*-substituent effects (Hammett plot), and activation parameters ($\Delta H_H^{\ddagger}$ and $\Delta S_H^{\ddagger}$) indicate that the bis(μ-oxo)dinickel (III) complex exhibits an ability of hydrogen atom abstraction from the substrate moiety as in the case of the bis(μ-oxo)dicopper(III) complex. Such a reactivity of bis(μ-oxo)dinickel(III) complexes has also been suggested by the observed reactivity toward external substrates such as phenol derivatives and 1,4-cyclohexadiene. The thermal stability of the bis(μ-oxo)-dinickel(III) complex is significantly enhanced when the dinucleating ligand with a longer alkyl strap is adopted instead of the mononucleating ligand. In the *m*-xylyl ligand system, no aromatic ligand hydroxylation occurred, showing a sharp contrast with the reactivity of the (μ-η^2:η^2-peroxo)dicopper(II) complex with the same ligand which induces aromatic ligand hydroxylation *via* an electrophilic aromatic substitution mechanism. Differences in the structure and reactivity of the active oxygen complexes between the nickel and the copper systems are discussed on the basis of the detailed

comparison of these two systems with the same ligand.

IX-H-10 UV Resonance Raman and NMR Spectroscopic Studies on the pH Dependent Metal Ion Release from Pseudoazurin

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[*Inorg. Chim. Acta* in press]

UV resonance Raman (UVRR) and ^{1}H NMR spectra are measured for native Cu(I)- and Cu(II)-pseudoazurin, its apo-protein, and a few metal-substituted derivatives. The pH titration experiments of ^{1}H NMR enabled us to determine the pK_a* values of three His residues (His6, His40, and His81). The UVRR band characteristic of a metal coordinated histidyl imidazole was observed at 1385 cm^{-1} for Cu(II)-pseudoazurin in D_2Obut not for Cu(I)-pseudoazurin. This frequency is consistent with the N_δ coordination of His. For the Cu(I)–pseudoazurin a characteristic band of histidyl imidazolium was detected at 1408 cm^{-1} at acidic pH. This is assigned to protonated His81, which is deligated from Cu(I) at low pH values. The imidazolium Raman band at 1408 cm^{-1} was also detected in the UVRR spectrum of apo-pseudoazurin at pH* = 3.9, and the acidification was accompanied by a significant change in the X-Pro bands at 1467 cm^{-1}. Pseudoazurin substituted with Zn^{2+} and Cd^{2+} gave the characteristic Raman bands of the metal coordinated imidazole at 1388 and 1384 cm^{-1}, respectively, at neutral pH, but its intensity diminished upon lowering the pH, and instead the imidazolium band at 1408 cm^{-1} grew. The X-Pro bands of the pseudoazurins substituted with Zn^{2+} and Cd^{2+} exhibited the pH dependence very similar to that observed for apo-pseudoazurin. These findings indicate that the Zn^{2+} and Cd^{2+} ions are released from the active site at acidic pH and it is accompanied by a change in hydrogen bonding state of Pro80. This behavior is clearly absent in both the Cu(I) and Cu(II) proteins, meaning that pseudoazurin discriminates between copper and the other metal ions.

IX-I Fast Dynamics of Photoproducts in Solution Phases

Picosecond time-resolved resonance Raman (ps-TR^3) spectroscopy is a promising technique to investigate ultrafast structural changes of molecules. However, this technique has not been used as widely as nanosecond TR^3 spectroscopy, mainly due to the lack of light source which has suitable repetition rates of pulses and wavelength tunability. In order to obtain qualified TR^3 spectra, first we need two independently tunable light sources for pump and probe pulses. Second, the repetition rate should be higher than kiloHertz to keep a moderate average laser power without making the photon density of probe pulse too high. We succeeded in developing light sources for ps-TR^3 spectroscopy having wide tunability and kHz repetition, and applied them to study fast dynamics of photo-excited molecules. For carbonmonoxy myoglobin (MbCO), vibrational relaxation with the time constant of 1.9 ps was observed for CO-photodissociated heme. For Ni–octaethylporphyrin in benzene, differeces in rise times of population in vibrationally excited levels among various modes were observed in the anti-Stokes spectra for the first time. This technique has been applied to identify the trans ligand of CO in the CO-bound transcriptional factor, Coo A.

On the other hand, we have constructed a nanosecond temperature-jump apparatus using a water absorption in near infrared. The new apparatus based on a Nd:YAG laser was combined with a time-resolved Raman measurement system and applied successfully to explore thermal unfolding of ribonucelase A.

IX-I-1 Time-Resolved Resonance Raman Study on Ultrafast Structural Relaxation and Vibrational Cooling of Photodissociated Carbonmonoxy Myoglobin

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[*Biopolymers (Biospectroscopy)* **67**, 207 (2002)]

A localized small structural change is converted to a higher order conformational change of protein and extends to a mesoscopic scale to induce a physiological function. To understand such features of protein, ultrafast dynamics of myoglobin (Mb) following CO photolysis have been investigated in this laboratory. Recent results are summarized here with a stress on structural and vibrational energy relaxation. The core expansion of heme takes place within 2 ps but the out-of-plane displacement of the heme iron and the accompanied protein conformational change occur in ~10 and ~ 100 picosecond regimes, respectively. It was found from UV resonance Raman spectra that Trp7 in the N-terminal region and Tyr151 in the C-terminal region undergo appreciable structural changes upon ligand binding/dissociation and as a result, the rate of spectral change of iron-histidine (Fe–His) stretching band is influenced by viscosity of solvent. Temporal changes of the anti-Stokes Raman intensity demonstrated immediate generation of vibrationally excited heme upon photodissociation and its decay with a time constant of 1.1 ps.

IX-I-2 Vibrational Energy Relaxation of Metalloporphyrins in a Condensed Phase Probed by Time-Resolved Resonance Raman Spectroscopy

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[*Bull. Chem. Soc. Jpn.* **75**, 623 (2002)]

Recent experimental work on vibrational energy relaxation of metalloporphyrins in a condensed phase carried out in this laboratory is summarized. The formation of a vibrationally excited photoproduct of metalloporphyrins upon (π, π^*) excitation and its subsequent vibrational energy relaxation were monitored by picosecond time-resolved resonance Raman spectroscopy. Results related to intramolecular relaxation of octaethylporphyrinato nickel (NiOEP) are described. Stokes Raman bands due to a photoproduct of NiOEP instantaneously appeared upon the photoexcitation. Their intensities decayed with a time constant of ~300 ps, which indicates an electronic relaxation from the (*d*, *d*) excited state (B_{1g}) to the ground state (A_{1g}), being consistent with the results of transient absorption measurements. Anti-Stokes ν_4 and ν_7 bands for vibrationally excited (*d*, *d*) state of NiOEP decayed with time constants of ~ 10 and ~ 300 ps. The former is ascribed to vibrational relaxation, while the latter corresponds to the electronic relaxation from the (*d*, *d*) excited state to the electronic ground state. While the rise of anti-Stokes ν_4 intensity was instrument-limited, the rise of anti-Stokes ν_7 intensity was delayed by 2.0 ± 0.4 ps, which indicates that intramolecular vibrational energy redistribution has not been completed in the subpicosecond time regime. To study the mechanism of intermolecular energy transfer, solvent dependence of the time constants of anti-Stokes kinetics was investigated using various solvents. No significant solvent dependence of the rise and decay constants was observed for NiOEP. For an iron porphyrin, we observed two phases in intermolecular energy transfer. The fast phase was insensitive to solvent and the slow phase depended on solvents. A model of classical thermal diffusion qualitatively reproduced this behavior. For myoglobin, temporal changes of the anti-Stokes Raman intensity of the ν_4 and ν_7 bands demonstrated immediate generation of a vibrationally excited heme upon photodissociation and subsequent decays of the excited populations, whose time constants were 1.1 ± 0.6 and 1.9 ± 0.6 ps, respectively. This direct monitoring of the cooling dynamics of the heme cofactor within the protein matrix allows the characterization of the vibrational energy flow through the protein moiety and to the water bath. For solute-solvent energy transfer process, low-frequency modes of proteins seem to be less important.

IX-I-3 Mode Dependence of Vibrational Energy Redistribution in Nickel Tetraphenylporphyrin Probed by Picosecond Time-Resolved Resonance Raman Spectroscopy: Slow IVR to Phenyl Peripherals

MIZUTANI, Yasuhisa; KITAGAWA, Teizo

[*Bull. Chem. Soc. Jpn.* **75**, 965 (2002)]

The formation of the (*d*, *d*) excited state of (*meso*-tetraphenylporphyrinato)nickel (II) ([Ni(tpp)]) upon (π, π^*) excitation, and its vibrational energy relaxation were monitored by picosecond time-resolved resonance Raman spectroscopy. Stokes resonance Raman bands due the (*d*, *d*) excited state instantaneously appeared upon the photoexcitation into the (π, π^*) excited state. Their intensities decayed with a time constant of about 250 ps, which corresponds to electronic relaxation from the (*d*, *d*) excited state to the electronic ground state. This is consistent with the results of ultrafast absorption measurements reported by Eom *et al.* [H. S. Eom, S. C. Jeoung, D. Kim, J. H. Ha and Y. R. Kim, *J. Phys. Chem. A* **101**, 3661 (1997)]. Anti-Stokes ν_4 (macrocycle in-plane mode) intensities of [Ni(tpp)] in the (*d*, *d*) excited state appeared promptly and decayed with a time constant of 3.6 ± 0.6 ps. The rise and decay of anti-Stokes intensity are interpreted as vibrational excitation due to the excess energy and intermolecular vibrational energy transfer to the surrounding solvent molecules, respectively. The ϕ_4 mode. which is mainly ν(CC) of the peripheral phenyl groups, gave no detectable anti-stokes intensity although the mode gave appreciable Stokes intensity. This means that the ϕ_4 mode is left vibrationally less excited than the ν_4 mode in the process of vibrational energy relaxation and that intramolecular vibrational energy redistribution is not completed in a subpicosecond time regime. These results for [Ni(tpp)] demonstrate that the vibrational modes of peripheral groups are vibrationally less excited shortly after the formation of the (*d*, *d*) excited state and that energy redistribution in the peripheral groups takes place in picoseconds, such a short time is competitive with vibrational energy transfer to the surrounding solvent molecules.