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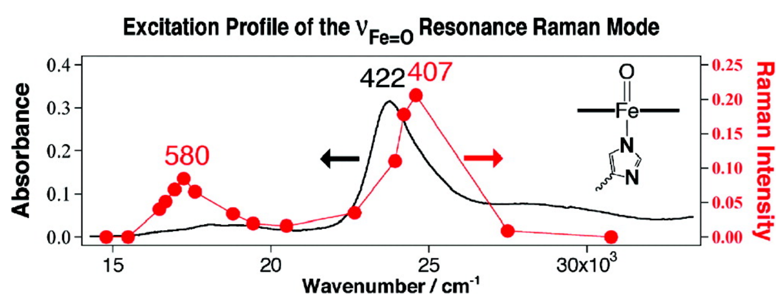

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Red-Excitation Resonance Raman Analysis of the $\nu_{Fe=O}$ Mode of Ferryl-Oxo Hemoproteins

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Heme enzymes, such as peroxidases, catalases, and oxidases, which catalyze oxidation−reduction reactions of hydrogen peroxide and dioxygen, produce high-valent reaction intermediates with an Fe═O heme moiety. Resonance Raman (RR) spectroscopy has been recognized as a powerful technique[1] for elucidation of the reaction mechanisms of such enzymes because it reveals the detailed coordination and electronic structures of the heme which functions as the catalytic site of the reaction intermediates.

The RR spectra of the Fe═O intermediates of heme enzymes have so far been obtained using Soret excitation between 400 and 450 nm.[2] A maximum at ∼410 nm in a Raman excitation profile (REP) has been observed for the $\nu_{Fe=O}$ mode of HRP-II and was attributed to an oxygen-to-iron charge-transfer transition lying on the higher energy side of the Soret absorption band of porphyrin.[3]

We have identified a new REP maximum of an oxygen-to-iron charge-transfer transition for the $\nu_{Fe=O}$ mode at 580 nm. This finding has suggested that red-excited RR spectroscopy could be used in new investigations which produce much less photochemical damage than blue-excited RR. This would represent a new and powerful tool for identification of the sensitive photolabile $\nu_{Fe=O}$ Raman band of heme enzymes and their model compounds.

Figure 1 depicts RR spectra of HRP-II excited at 580 nm. Spectra A and B are for ^{16}O- and ^{18}O-adducts, and spectrum C is a Raman difference spectrum obtained by subtracting spectrum B from spectrum A. The bands at 829, 808, and 756 cm^{-1} in spectrum A are attributable to porphyrin vibrations. The band at 787 cm^{-1} (A) shows a downshift to 756 cm^{-1} upon ^{18}O-substitution (B). The latter is overlapped by a porphyrin mode at a very close frequency as seen in spectrum A. The isotopic shift is more clearly seen in the Raman difference spectra (^{16}O minus ^{18}O) shown in spectrum C. Figure 2 shows the REP of the $\nu_{Fe=O}$ mode (upper panel) and the absorption spectrum of HRP-II (lower panel). Details of the procedure used to determine the REP are given in the Supporting Information.

In addition, we have identified the $\nu_{Fe=O}$ mode of compound II of ARP (*Arthromyces ramosus* peroxidase, a protoheme containing peroxidase, Figure SI3) and ferryl myoglobin (a protoheme containing oxygen binding protein, Figure SI4) excited at 590 nm. We have also identified the mode of the P intermediate of CcO (a heme A containing oxidase) from bovine heart (Figure SI5) and CcO of *Paracoccus denitrificans* (Figure SI6). Upon excitation of these enzyme species at 590 nm, the intensity of the $\nu_{Fe=O}$ Raman band

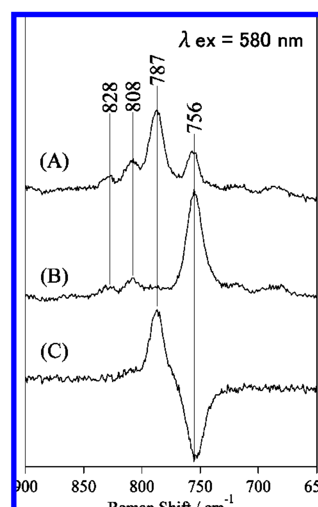

Figure 1. RR Spectra of ^{16}O-HRP-II (A) and ^{18}O-HRP-II (B). Spectrum C is an isotopic difference spectrum. The spectral slit width was 6 cm^{-1}, and the laser power at the sample was 35 mW. Accumulation time was 40 min. Spectra A and B are the sum of 4 spectra obtained in 10 min accumulations.

observed relative to porphyrin bands is also significantly large with respect to the intensity of this same band observed with Soret excitation as shown in Figure 1.

The REP of the ν_{Fe-O2} mode of oxy hemoglobin is similar to that of the porphyrin $\pi-\pi^*$ transitions.[4] In this case, dioxygen, a π-acid ligand, competes effectively with the porphyrin π^* orbitals for back-donation from the $Fe^{II}d_\pi$ orbitals, and as a result, the ν_{Fe-O2} mode has a substantial effect on the porphyrin π^* orbital energies.[4] Thus, the excitation profile of the ν_{Fe-O2} mode coincides with the absorption spectrum. On the other hand, the REP of the Fe═O stretching mode for HRP-II was previously reported to be located on the higher energy side of the Soret transition of HRP-II at 422 nm.[3] Hence, the resonance enhancement of the $\nu_{Fe=O}$ mode was thought to be not through the $\pi-\pi^*$ resonance of the heme, but through the charge-transfer electronic transition from O to Fe. A new maximum was identified in the REP at ∼580 nm (Figure 2, upper panel), which is a longer wavelength than that of the α-band. This indicates that a charge-transfer electronic transition mechanism plays a role in resonance enhancement. Theoretical calculations based on time-dependent density functional theory (TD-DFT) indicated the presence of two electronic excitations to the Fe(IV)═O molecular orbitals in the visible region in addition to the Soret- and α-bands.[5] The results of these calculations might explain the present results.

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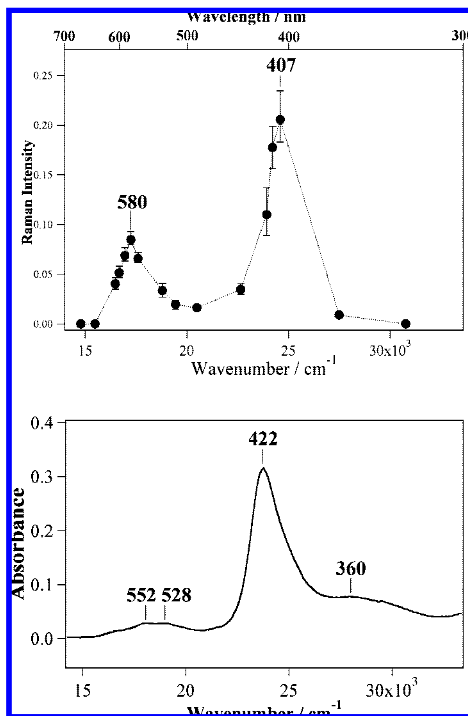

Figure 2. REP of $\nu_{Fe=O}$ band of HRP-II (upper panel) and absorption spectrum of HRP-II (lower panel). The data points are connected by a thin dotted line in REP. The numbers on the peaks in both REP and absorption spectrum indicate their wavelengths in nm.

The RR spectra of the $\nu_{Fe=O}$ Raman band of HRP-II measured upon 580 nm excitation shown in Figure 1 have a remarkably high quality relative to typical RR spectra of reaction intermediates of a heme enzyme.[3,6,7] See Figure SI2-(L) and Figure SI2-(D) for comparison. The intensity of the band at 787 cm^{-1} relative to the porphyrin modes at 829, 808, and 756 cm^{-1} in Figure 1A is sufficiently high to enable precise determinations of the frequencies. Since the lower photon energy of red light produces much less sample damage, a much higher laser power of 35 mW could be applied to the sample. In contrast, existing RR techniques that employ Soret excitation require use of laser power $<$10 mW. In certain cases where sensitive samples are investigated, even $<$1 mW laser power is necessary to avoid sample damage.

The $\nu_{Fe=O}$ modes of ARP-II, ferryl myoglobin, and of the P intermediate of CcO identified using 590 nm excitation are strongly suggestive of the presence of a charge-transfer electronic transition from O-to-Fe that is similar to that of HRP-II. However, the possibility exists that the $\nu_{Fe=O}$ mode of the P intermediates of CcO observed in the visible Raman spectrum could be due to resonance enhancement through a $\pi-\pi^*$ transition.[8] In any case, these results indicate the applicability of the red-excited RR technique to investigations of most hemoprotein Fe=O intermediates.

It is expected to be worthwhile to compare red-excited RR measurements with Soret-excited measurements. The relative intensity of the REP obtained with excitation at 407 nm is ~2-fold higher than that of the REP obtained with excitation at 580 nm (Figure 2, upper panel). In contrast, the absorbance intensity at 407 nm is 10-fold greater than the 580-nm absorbance (Figure 2, lower panel). The spectral profiles strongly suggest the following advantages in using the red-excitation light in the RR measurement of the Fe=O mode: (1) the extent of overlap of the porphyrin modes which are mainly due to their $\pi-\pi^*$ transitions is much lower in the red-excitation RR measurement than that in the Soret-excitation RR measurement; and (2) since laser irradiation damage is directly related to the intensity of the absorption at the wavelength of the laser, the ratio of the RR intensity to the extent of the photochemical damage in a Raman measurement is much higher with red-excitation than with Soret excitation.

Furthermore, the lower energy red light provides less photochemical damage than the higher energy wavelengths in the Soret region. Another important advantage in using red excitation wavelengths is that the red excitation measurement provides higher accuracy in the spectral measurement for resolution of single spectral bands overlapping with each other.

These advantages are obvious as evidenced by the ferryl myoglobin results as given in Figure SI3. The $\nu_{Fe=O}$ frequency of an ^{18}O-adduct was obtained at 760 cm^{-1} with the red-excitation RR measurement described herein. In contrast, Soret excitation RR measurements produced a value of 771 cm^{-1}.[6] The discrepancy is most likely due to the higher extent of the porphyrin band overlap and to the lower resolution for the spectral measurement using the Soret excitation. In fact, a weaker band near 760 cm^{-1} is detectable as a shoulder in the reported spectra.

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Supporting Information Available: RR measurements procedures and RR spectra of four Fe=O enzyme species. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Abbreviations: ARP: *Arthromyces ramosus* peroxidase; ARP-II: Compound II of ARP; Compound II: a reaction intermediate of peroxidases that has one oxidative equivalent higher than the ferric state; CcO: Cytochrome *c* oxidase; HRP: Horseradish peroxidase; HRP-II: Compound II of HRP; Mb: myoglobin; P intermediate: a reaction intermediate of cytochrome *c* oxidase that has two oxidative equivalents higher than the ferric state produced on heme a_3 and having an Fe=O fragment like Compound II with the location of one oxidative equivalent being uncertain; REP: Raman excitation profile; RR: resonance Raman.

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