Resonance Raman Studies of Compounds I and II of Arthromyces ramosus Peroxidase: Close Similarities in Their Raman Spectra But Distinct Oxygen Exchangeability of the Fe=O Heme

DENIS A. PROSHLYAKOV,1,2 INSOOK R. PAENG,3 KY-JUNG PAENG,4 and TEIZO KITAGAWA1,*

1Institute for Molecular Science, Okazaki National Research Institutes, Okazaki 444, Japan; 2Chemistry Department and LASER Laboratory, Michigan State University, East Lansing, Michigan 48824, USA; 3Department of Chemistry, College of Natural Science, Seoul Women's University, Seoul, 139-774, Korea; and 4Department of Chemistry, College of Liberal Arts and Sciences, Yonsei University, Wonju 222-701, Korea

SYNOPSIS

Simultaneous measurements of resonance Raman and absorption spectra were performed for intermediates generated upon addition of hydrogen peroxide to ferric Arthromyces ramosus peroxidase (ARP) using the microcirculating system constructed in this laboratory, which enables generation of desirable intermediates under steady-state conditions. Compound I of ARP generated at neutral pH was stable over tens of minutes in the absence of laser illumination with this circulation system, but was gradually degraded under laser illumination, giving rise to a new irreversible species with an iron-oxo heme. Such photosensitivity was not observed for compound II in the steady state at alkaline pH. Surprisingly, the Raman spectrum of compound I of ARP in the high-frequency region, where characteristic frequency shifts are expected upon oxidation of the macrocycle, was quite close to that of compound II, despite the fact that the reduced Soret absorption indicated the formation of a π-cation radical. The Fe=O stretching ($\nu_{Fe=O}$) frequency of compound I was observed at 781 cm$^{-1}$ for the $^{16}$O derivative but appeared as a doublet at 744 and 731 cm$^{-1}$ for the $^{18}$O derivative. The isotope sensitivity of the $\nu_{Fe=O}$ mode of compound I was seen upon H$_2^{16}$O/H$_2^{18}$O solvent substitution but not upon H$_2^{16}$O$_2$/H$_2^{18}$O$_2$ peroxide substitution in H$_2^{16}$O at neutral pH. This directly indicates the occurrence of an oxygen atom exchange between the o xo-heme and bulk water, providing the first example of such exchange in compound I of peroxidases. The oxygen exchange was abolished for compound II at alkaline pH, for which the $\nu_{Fe=O}$ mode was seen at 787/749 cm$^{-1}$ only upon H$_2^{16}$O$_2$/H$_2^{18}$O$_2$ peroxide substitution. The oxygen exchangeability seems to depend on protonation of a nearby residue with $pK_a \sim 9$ and to correlate with stability of compound I.

INTRODUCTION

Arthromyces ramosus peroxidase (ARP) has attracted attention owing to its easier clinical appli-

cability, since it exhibits 100 times stronger chemiluminescence in the oxidation of luminol than does horseradish peroxidase (HRP).1 ARP is a single-polypeptide protein of 344 residues ($M_r = 38,000$) with an isoelectric point at pH 3.5, and has one Fe-protoporphyrin IX, two Ca ions, and carbohydrates attached to Asn-143 and Ser-339.2,3 It belongs to a class II peroxidase, like lignin and manganese peroxidases. X-ray crystallographic analysis has been completed for the resting high-spin,4 resting low-spin,5 and cyanide-bound forms of ARP,6 pointing out similarity of its overall tertiary structure to that of yeast cytochrome c peroxidase (CcP). It has
been demonstrated that ARP is oxidized by hydrogen peroxide first and performs stepwise one-electron reactions, giving compounds I and II as intermediates in its reaction cycle similar to HRP.\(^7\)

Compound I of peroxidases, which has the oxidation state higher than the ferric state by two oxidative equivalents,\(^8\) contains a ferroxyloporphyrin cation radical for HRP,\(^9,10\) but has a ferroxylo nonionized porphyrin with a tripyrrolic cation radical for CeP.\(^11,12\) Despite of extensive time-resolved studies exploring resonance Raman (RR) spectra of compound I of ARP,\(^13-16\) considerable disagreement exists in the observed spectra,\(^17\) partly due to its instability at neutral pH and partly to its high photosensitivity, while RR spectra of compound II are in agreement. On the other hand, Farhangrazi et al.\(^7\) demonstrated that ARP compound I is stable at neutral pH and reduced to the ferric state without apparent formation of compound II. The unusual behavior of ARP was ascribed to high reduction potential of compound II but not to an unusual structure of compound I.\(^7\) ARP compound I is optically similar to that of HRP: Prominently reduced absorbance of the Soret band compared with that of the ferric enzyme\(^7\) implies the formation of a porphyrin cation radical.

To obtain high-quality RR spectra for enzymatic reaction intermediates with short lifetimes, we have developed a novel microcirculating system, which enables us to generate desirable intermediates in a steady state, and successfully applied it to intermediates of cytochrome c oxidase in its reaction with hydrogen peroxide.\(^18-20\) In combination with high-performance Raman/absorption simultaneous determination technique,\(^19\) it enables us to correlate RR spectra of unstable and photolabile intermediates with known absorption forms.\(^21\) Accordingly, the application of these techniques to ARP may provide an opportunity to overcome difficulties associated with RR experiments on compound I of HRP. Here we report the Fe=O stretching (\(v_{\text{Fe=O}}\)) RR bands of compounds I and II of ARP in the pH range between 7.3 and 10.7 in addition to their high-frequency RR spectra. It will be shown that compounds I and II of ARP have unexpectedly similar vibrational properties of heme. We also report the first example of the oxygen exchange reaction for compound I of peroxidases.

### MATERIALS AND METHODS

Purified ARP was provided by Suntory Limited (Lots 93091311 and 93102011; Osaka, Japan). The 2 mM stock solution of ARP was made first and diluted to the final concentration of 50 \(\mu M\) with the \(\text{H}_2^{16}\text{O}\) or \(\text{H}_2^{18}\text{O}\) (95 atm %, Icon) buffer containing sodium phosphate (pH 7.3), sodium borate (pH 8.8 or 9.5), or sodium carbonate (pH 10.8). A certain amount of saturated ammonium sulfate solution was added where indicated. The final \(^18\text{O}\)-isotope enrichment for \(\text{H}_2^{16}\text{O}\) solutions was about 87%. All pH values were determined for the reaction medium after spectral measurements. \(\text{H}_2^{16}\text{O}_2\) (30%; Wako Chemicals, Osaka, Japan) was used as purchased, while \(\text{H}_2^{18}\text{O}_2\) was synthesized as described previously.\(^22\) Stock solutions of hydrogen peroxide were made to 10 or 50 \(\text{mM}\) and were stored frozen until use.

The Raman/absorption simultaneous measurements were carried out under steady-state conditions using the microcirculating system as described in detail elsewhere,\(^19\) except for the following modification; the total volume of the system was reduced to \(~\)0.6 mL by replacement of the tube in the peristaltic pump with a thinner one. For Raman measurements a monochromator with higher resolution (~0.4 cm\(^{-1}\)/channel, spectral slitwidth \(~4\) cm\(^{-1}\)) and a holographic cutoff filter for 406.7 nm were used. Raman scattering was excited by the 406.7 nm output of a Kr\(^+\) laser (Spectra Physics; model 2016) with a power of 1.0–2.5 mW at the sample point. The sample was flowed through a quartz flow cell (cross-section 0.6 \(\times\) 0.6 mm\(^2\)). To maintain a sufficiently high flow rate, the pump was operated at the maximal rate. The exposure time of an arbitrary molecule to the laser beam (diameter = 15–20 \(\mu\)m, \(l = 600 \mu\)m) was about 10 \(\mu\)s. To prevent the flowing sample from heating, all connecting tubes were enclosed into a jacket through which cooling water was circulated.

The reaction was initiated by rapid addition of hydrogen peroxide to the medium in the microcirculating system to make its final concentration 50–200 \(\mu M\), and at the same time the slow addition of hydrogen peroxide with the same isotope species into the circulating solution was initiated at a rate of 0.05–0.5 mol \(\text{H}_2\text{O}_2/mol \text{ARP}/\text{min}\) and continued over the whole period of spectral measurements. Every measurement was performed with a fresh sample and continued for 20–45 min. The measurements were repeated several times for each isotope in an alternating order. For measurements of absorption spectra without laser illumination (where indicated), a dichroic mirror in the light-mixing device\(^19\) was removed and a spectrum was measured in the 370–700 nm range, while all other conditions were kept the same.

The spectral data were integrated for every \(~1\)-
phase-shift noises of detector pixels were filtered out using the Galoy-Savitsky algorithm (pixel frame was set to about spectral slit width to preserve resolution).

In a series of preliminary experiments 50 μM solution of ARP at desired pH was titrated with subequimolar aliquots of H₂¹⁸O₂ solution until the maximal conversion to the desirable intermediate was achieved. This appeared to be in a range from an equimolar to 3-4-fold excess of hydrogen peroxide over ARP depending on the conditions. At the next stage a rate of the slow peroxide addition was increasingly adjusted until the decay of the objective compound to the ferric state was not seen. The spectral species thus obtained were stable in tens of minutes and presumably existed in an equilibrium between its formation by added peroxide and its decomposition in the peroxidase cycle.

RESULTS

The absorption spectrum of compound I of ARP thus produced at pH 7.3 is shown in Figure 1b, in comparison with that of the ferric enzyme (a). Since no significant spectral changes were detected over 40 min from the initial addition of peroxide, the spectrum was integrated over this period. Spectrum (b) is characterized by the reduced intensity (about half) of the Soret band, which was broadened and slightly (≈ 2 nm) shifted to a longer wavelength, and by broad bands at 535, 559, and 656 nm in the Q-band region. This spectrum qualitatively agrees with the characteristics reported for the formation of compound I of ARP at neutral pH, although the precise wavelengths of absorption maxima have not been reported.

When the flowing sample was oversupplied with H₂O₂ either in the fast or slow phase of addition, another form was observed as delineated by spectrum (c) in Figure 1. This has the Soret maximum at 415 nm and three characteristic bands at 550, 583, and 665 nm in the visible region, in agreement with the reported spectrum of compound III generated in the presence of ascorbate. Since ascorbate is absent in this experiment, presumably hydrogen peroxide also works as a reductant in the formation of compound III of ARP as reported earlier for lactoperoxidase. Compound III was unstable and converted back to compound I with time, but the addition of millimolar H₂O₂ produced compound III repeatedly, indicating that this process is reversible except for slow decomposition of ARP at high peroxide concentrations. This does not support the previous suggestion that compound III is unstable because of increased stability of compound I, but rather indicates the accelerated decomposition of peroxide in the reaction cycle.

The real-time Raman/absorption simultaneous measurements were carried out under the same conditions as those for the formation of compound I in Figure 1 except for the presence of 2.5-mW continuous-wave laser illumination; the results are shown in Figure 2. Panel C displays absorption spectra. Spectrum (a), observed over the first 5 min from the start of reaction, was similar to that seen without laser illumination [Fig. 1(b)]. However, in course of time the three characteristic bands of compound I at 535, 557, and 656 nm faded and a new band grew at 606 nm (trace b: 5-25 min; trace c: 25-45 min). This process was slower when either a larger volume of sample (1.1 mL) or a lower laser power was used, indicating that the spectral changes are primarily caused by laser illumination. The spectral change was irreversible, since the 606-nm band was stable in the absence of further laser illumination and without peroxide supply, while the remaining compound I was converted back to the original ferric state.

Panel A shows the corresponding changes in the medium-frequency RR spectrum. Spectrum (a) observed in the initial 0-5 min exhibited 2-3-fold weakening of all Raman bands compared with that of the ferric enzyme, in consonance with reduced absorbance at the excitation wavelength (406.7 nm). Spectra (b) and (c) reflect the photochanged states in the 5-25- and 25-45-min periods following the initial addition of H₂O₂, respectively, exhibiting the prominent enhancement of the bands at 1004, 952, and 748 cm⁻¹. Changes can also be seen in the overlapped bands around 933-926, 800-780, and ≈ 700 cm⁻¹.

For characterization of RR spectra the same measurements as those in panel A in Figure 2 were carried out with oxygen isotopes. When H₂¹⁸O₂ was used in H₂¹⁸O, there were no changes in RR spectra. However, when the H₂¹⁶O derivative was generated in H₂¹⁸O, a definite change was observed in the ≈ 750 cm⁻¹ region, where the Fe = O stretching (vFe=O) of an oxoiron intermediate is expected.
Figure 1. Absorption spectra of ARP at neutral pH without laser illumination: (a) ferric enzyme; (b) compound I under the steady state generated as described in Materials and Methods; (c) compound III generated by addition of 5 mM H₂O₂ to compound I. Instrumental conditions: 50 μM ARP in 20 mM sodium phosphate buffer, pH 7.3; accumulation time, 3 min for the ferric enzyme, 40 min for compound I, and 3 s for compound III; pathlength for absorption spectra, 0.6 mm.

Panel B in Figure 2 shows the corresponding isotope-difference spectra. In the initial 0–5 min following the addition of H₂O₂ (trace a), a broad difference pattern with major bands at 781 cm⁻¹ (H₂¹⁶O) and 731 cm⁻¹ (H₂¹⁸O) was observed. These bands became weaker with time and were replaced with sharper and stronger bands at 798/761 cm⁻¹ (trace c). Note that all these bands were seen upon water isotope substitution, but not with peroxide substitution, demonstrating the existence of an oxygen exchange reaction between intermediate and bulk water.

One may argue that if the white light and the laser beam were not strictly overlapped in the sample, an absorption spectrum would indicate compound I, while a Raman spectrum would probe largely a product of its photoreaction. To examine such a possibility, a control experiment was carried out. When the flow of medium was stopped, both Raman and absorption spectra exhibited large changes. If the volume examined by the absorption spectrum were different from the laser-illuminated volume from which Raman scattering is collected, complete conversion of compound I to other species would not be observed in the absorption spectrum. When the flow rate of the sample was gradually decreased while monitoring Raman and absorption spectra, appreciable changes in Raman and absorption spectra were detected only at considerably reduced rate. This indicates that ~10 μs exposure under conditions used for Figure 2 is much shorter than the time necessary for molecules in the laser beam to exhibit this photoinduced spectral changes (not be mixed with a photoproduc accumulate with time in a whole volume). Consequently, the possibility of the misoverlapping between the white light and the laser beam was ruled out from the observations mentioned above.

Figure 3 shows the results from similar real-time
Figure 2. The real-time Raman/absorption spectra of ARP compound I at neutral pH illuminated with laser light (2.5 mW, 406.7 nm). Panel A: Raman spectra of the H$_2^{16}$O$_2$ derivative in H$_2^{16}$O solution. Panel B: Solvent isotope difference Raman spectra of the H$_2^{16}$O$_2$ derivative in H$_2^{16}$O minus that in H$_2^{18}$O. Panel C: Absorption spectra of the enzyme observed simultaneously with Raman spectra (ordinate offset applied). Traces a, b, and c represent the spectra obtained by integration over the periods of 0–5, 5–25, and 25–45 min following the start of reaction, respectively. Experimental conditions were similar to that for compound I in Figure 1 except for laser illumination: reaction medium, 0.55 mL of 100 μM ARP in 5 mM sodium phosphate buffer, pH 7.3; initial concentration of H$_2$O$_2$, 180 μM; slow addition of H$_2$O$_2$, -0.5 [mol H$_2$O$_2$]/[mol ARP]• min$^{-1}$. Raman/absorption simultaneous measurements at pH 10.7, where compound II is more stable than compound I. Spectra (a), (b), and (c) were obtained for the periods of the initial 0–10, 10–20 and 20–30 min, respectively. No clear time dependence is recognized in either raw RR spectra (panel A) or absorption spectra (panel C). The characteristic band of compound I at 656 nm is absent and two Q bands are shifted to 530 and 553 nm in Figure 3(C), but no new band grew at 606 nm as was seen in Figure 2(C). These characteristics are in agreement with those of compound II of ARP. In the oxygen isotope difference RR spectra (panel B), a differential pattern was obtained at 788/749 cm$^{-1}$, but the difference peaks at 798/761 cm$^{-1}$ seen in Figure 2B are absent even in the last period of time. It is stressed that the results shown in Figure 3B were obtained with H$_2^{18}$O$_2$ and H$_2^{16}$O$_2$, both in the H$_2^{16}$O solutions, indicating that the oxygen exchange does not occur at pH 10.7. Consequently, the Fe$^{IV}$═O stretching mode of compound II, which is photostable unlike compound I, is assigned to the bands at 788 (18O) and 749 cm$^{-1}$ (16O).

Figure 4 illustrates pH dependencies of Raman/
Figure 3. The real-time Raman/absorption spectra of ARP compound II at alkaline pH illuminated by laser light (2.5 mW, 406.7 nm). Panel A: RR spectra of the H\textsubscript{2}\textsuperscript{16}O\textsubscript{2} derivative in H\textsubscript{2}\textsuperscript{18}O\textsubscript{2} solution. Panel B: Isotope-difference Raman spectra in the ~800-cm\textsuperscript{-1} region for H\textsubscript{2}\textsuperscript{16}O\textsubscript{2} derivative minus H\textsubscript{2}\textsuperscript{18}O\textsubscript{2} derivative in H\textsubscript{2}\textsuperscript{16}O. Panel C: Absorption spectra measured simultaneously with Raman spectra (ordinate offset applied). Spectra a, b, and c were obtained by integration over the periods of 0–10, 10–20, and 20–30 min following the start of reaction, respectively. Reaction medium, 50 \(\mu\)M enzyme in 20 mM sodium carbonate buffer, pH 10.7; Initial peroxide concentration, 100 \(\mu\)M; slow addition, ~0.1 [mol H\textsubscript{2}O\textsubscript{2}] * [mol ARP] \textsuperscript{-1} min\textsuperscript{-1}. Other conditions were the same as those in Figure 2.
RESONANCE RAMAN STUDIES OF *ARTHROMYCES Ramosus*

Figure 4. pH-dependence of Raman/absorption spectra of intermediates. Traces A and B: Raman spectra in the 600–1000 cm⁻¹ region of the H₂¹⁶O₂ derivatives in H₂¹⁶O at pH 9.5 and 8.8, respectively. Traces A' and B': absorption spectra measured simultaneously with spectra A and B, respectively. Trace C: difference spectrum of H₂¹⁶O₂ derivative in H₂¹⁶O minus that in H₂¹⁸O at pH 9.5. Trace D: Difference spectrum of H₂¹⁶O₂ derivative minus H₂¹⁸O₂ derivative in H₂¹⁶O at pH 9.5. Trace E: Difference spectrum of H₂¹⁶O₂ derivative minus H₂¹⁸O₂ derivative in H₂¹⁶O at pH 9.5. All spectra were obtained in 30 mM sodium borate buffer; 1.8% (v/v) saturated ammonium sulfate solution was added for traces B/B' and E; integration period, 2–22 min from the start of reaction; ordinate scale is doubly expanded for traces C–E; ordinate offset applied for absorption spectra; all other conditions were the same as those in Figure 3.

However, the absorption and Raman spectra of intermediates were little influenced by the presence of ammonium sulfate. The H₂¹⁶O versus H₂¹⁸O difference RR spectrum is delineated by trace (E) in Figure 4, in which the positive band is shifted down to 780–781 cm⁻¹, the frequency identical to that at pH 7.3. The negative band is shifted to ~ 744 cm⁻¹ and another band appeared at 731 cm⁻¹. In the peroxide-isotope difference spectrum there was no difference peak, indicating rapid exchange of the oxo-oxygen with bulk water.

Below pH 8.8 the absorption spectra were dominated by that of compound I and Raman spectra...
Figure 5. The high-frequency RR (left) and simultaneously measured absorption (right) spectra of ARP compounds probed with 1 mW laser power. Traces A/A', ferric enzyme before addition of H$_2$O$_2$; traces B/B' and C/C' were obtained for compound I at pH 7.3 by integration over the periods of 0–15 and 15–25 min following the start of reaction, respectively; traces D/D' were obtained for compound II at pH 10.7 by integration over a period of 0–15 min from the start of reaction. All other conditions were the same as those in Figures 2 and 3 for pH values 7.3 and 10.7, respectively, except for laser power. The effect of concentration difference (50 vs. 100 µM) on the Raman intensity was compensated by scaling of the ordinate of spectrum. Traces A and D were scaled by a factor of 0.5.

were increasingly disturbed by photosensitivity along with the growing population of compound I. To confirm that the RR spectra obtained in Figure 2 arose from compound I, similar experiments were carried out with reduced laser power (1 mW) at pH 7.3 and the results are shown in Figure 5. The absorption spectrum (B') was virtually identical to the spectrum measured without laser illumination [Fig. 1(b)]. Spectra were also measured over next 10 min (traces C') as a control. Even in the second period of time the amount of generated photoprod-uct was small judging from the low intensity of the 606-nm absorption band (compare it with trace C in Fig. 2). These absorption spectra are distinct from those of the ferric state (A') and compound II at pH 10.7 (D'), whose simultaneously observed RR spectra in the high-frequency region are displayed as spectra A and D in Figure 5, respectively.

Upon the formation of compound I, overall Raman intensity decreased to nearly one half. The $v_3$ and $v_4$ modes of the ferric state (1494 and 1372 cm$^{-1}$ in spectrum A) are shifted to higher frequencies in compound I (1506 and 1376 cm$^{-1}$ in spectrum B). The band at 1626 cm$^{-1}$ for the ferric en-zyme is apparently split into three bands at 1636, 1626, and 1619 cm$^{-1}$ for compound I. The $v_2$ mode is shifted from 1566 to 1579 cm$^{-1}$ and is practically the only band exhibiting some changes with time;
in comparison of traces B with C in Figure 5, the band first appeared at 1579 cm\(^{-1}\) and then was replaced by two bands at 1589 and 1576 cm\(^{-1}\). On this basis the 1579-cm\(^{-1}\) frequency is considered to be the correct position of the \(v_2\) mode of compound I, while the 1589-cm\(^{-1}\) band is assignable to a photoproduct. Although polarization measurements are necessary for definite assignment of Raman bands in the \(v_{16} (1600-1650\text{ cm}^{-1})\) and \(v_{11} (\sim 1550\text{ cm}^{-1})\) regions, it is surprising that the RR spectrum of compound I in the 1300–1700-cm\(^{-1}\) region is very close to that of compound II as illustrated by spectrum D despite the significant difference between absorption spectra B’ and D’.

The RR spectra in the midfrequency region were remeasured with reduced laser power (1 mW) and the observed spectra for different time periods are shown in Figure 6 (left). Spectra (B) and (C) were measured for the periods of the initial 0–15 and 15–25 min following the start of reaction, respectively. They are distinct from the spectrum of the ferric state shown by spectrum (A) as well as that of photoproduct [Fig. 2 (A–C)]. The intensity ratio of the 688–674-cm\(^{-1}\) bands is reversed from that of compound I shown in Figure 3. Accordingly, spectrum (B) is considered to reflect compound I of ARP before photoreactions. The spectral differences between the H\(_2\)\(^{16}\)O and H\(_2\)\(^{18}\)O solutions were calculated for periods of the initial 0–15 and subsequent 15–25 min and are shown by traces (D) and (E) respectively, in Figure 6. The isotopic difference peaks at 781/731 cm\(^{-1}\) are dominant and the 798/761-cm\(^{-1}\) peaks seen in Figure 2 (B) are absent in trace D. The negative peak at 744 cm\(^{-1}\) seems to be slightly more intensified in spectrum (E) than in spectrum (D), but this intensity change can be attributed to the distortion of the baseline due to the appearance of weak bands at 798/761 cm\(^{-1}\) bands in the second period.

**DISCUSSION**

**Oxygen Exchange in Compound I**

Substitution of the oxygen isotope in compound I yielded an asymmetrical shift pattern in the corresponding difference spectrum. For most pure compound I [Fig. 6 (D)], a single band was observed at 781 cm\(^{-1}\) for \(^{16}\)O but a doublet was observed at 731 and 744 cm\(^{-1}\) for \(^{18}\)O. As mentioned above, the 781/731–744-cm\(^{-1}\) bands could be seen only upon oxygen isotope substitution of water, but no such oxygen isotope–sensitive bands were seen upon peroxide isotope substitution carried out in H\(_2\)\(^{16}\)O at neutral pH. This fact definitely indicates the occurrence of an oxygen exchange between the oxo-intermediate and bulk water.

So far there have been no reports on the oxygen exchange in compound I of peroxidases. Positive evidences for the oxygen exchange have been reported for compounds II of various peroxidases.\(^{34-38}\) The oxygen exchange reaction of compound II does not take place when the distal histidine was deprotonated at alkaline pH, exhibiting close correlation with the heme-linked ionization.\(^{29-34}\) Occurrence of the oxygen exchange in compound I of ARP below pH 9 strongly suggests that a distal residue (probably histidine) is involved and only its protonated state allows the oxygen exchange reaction with bulk water. Judging from the pH dependence, the stability of ARP compound I seems to correlate with the oxygen exchangeability, and thus to depend on the protonation of the distal residue.

Diatomic approximation for the Fe=O oscillator predicts a downshift of its frequency by approximately 4.6% (36 cm\(^{-1}\) for 781 cm\(^{-1}\)) for the \(^{16}\)O to \(^{18}\)O substitution. The 781/744-cm\(^{-1}\) pair of Raman bands of compound I [Fig. 6 (D)] gives a reasonable value of shift (37 cm\(^{-1}\)), although the 744-cm\(^{-1}\) band is appreciably weaker than the 781-cm\(^{-1}\) band. However, the 731-cm\(^{-1}\) band gives a 48-cm\(^{-1}\) downshift from 781 cm\(^{-1}\) upon \(^{16}\)O/\(^{18}\)O substitution. Although the O—O stretching of a peroxo species is also expected in this frequency region, a covalent O—O bond is not expected to undergo the oxygen exchange with water molecules.

There would be two possible explanations. One is to assume the presence of two \(v_{16}=\) cm\(^{-1}\) bands, which have not been resolved for the \(^{16}\)O species in Figure 6 (D), but are resolved for the \(^{18}\)O species. In this case the individual higher- and lower-frequency components reflect the nonhydrogen-bonded and hydrogen-bonded Fe=O bonds, respectively. The other is to assume that the Fe=\(^{16}\)O species yields a single band, but the Fe=\(^{18}\)O species exhibits splitting owing to interactions with a porphyrin mode. In this case the intrinsic \(v_{16}=\) cm\(^{-1}\) mode of the Fe=\(^{18}\)O species is located around 738 cm\(^{-1}\) and interacts with a porphyrin vibration at 737 cm\(^{-1}\). Although precise frequencies of these bands in the raw spectra are difficult to determine, owing to the broad and weak features, even in the difference spectra, the former alternative appears more likely since the isotopic frequency shift is still too large for the latter alternative.

It is noted that the oxygen-isotope frequency shift of the photoproduc of compound I [\(\Delta \nu = 37\) cm\(^{-1}\) for the 798/761-cm\(^{-1}\) species in Fig. 2 (B)] is remarkably close to that expected for the Fe=O
The observed difference in $v_{\text{Fe}=0}$ frequencies of compounds I and II of ARP reported here is 5–6 cm$^{-1}$, which is within this range. Although to dis-

oscillator, suggesting the formation of an oxoheme compound. If laser illumination results in simple photoreduction of compound I, the photoproduct should be a protonated form of compound II, but its stability is inconsistent with the reported character of compound II$^7$ and the apparent upshift of $v_{\text{Fe}=0}$ band upon protonation (by 10 cm$^{-1}$) does not agree with the expectations (see below).

The $v_{\text{Fe}=0}$ Mode of Compound II

Compound II of ARP, which is stable at alkaline pH, does not exhibit the oxygen exchange, since the $v_{\text{Fe}=0}$ mode at 788/749 cm$^{-1}$ [Fig. 3 (B)] is stable and can be clearly seen upon isotope substitution of peroxide. By analogy with other peroxidases it means that the distal residue in this compound is deprotonated, and therefore the hydrogen bond to the oxo atom is absent. It has been demonstrated for peroxidases compound II that the $v_{\text{Fe}=0}$ shifts up by 9–18 cm$^{-1}$ when the hydrogen bond to the oxo atom is broken owing to deprotonation of the distal residue.$^{22,24-26}$ The amount of the upshift would be smaller when the hydrogen bond is weaker.

Figure 6. The Raman/absorption spectra of compound I of ARP probed with 1 mW laser power (406.7 nm). Left: RR spectra in the 600–1000-cm$^{-1}$ region. Right: simultaneously observed absorption spectra. Traces A/A', ferrie ARP (Raman spectrum scaled). Traces B/B' and C/C', the $H_2^{16}O_2$ derivative in $H_2^{16}O$ by integration over the periods of 1–15 and 15–25 min, respectively, following the start of reaction. Traces D and E, doubly expanded Raman isotope-difference spectra of $H_2^{16}O_2$ derivative in $H_2^{16}O$ minus that in $H_2^{18}O$ obtained over the same periods as traces B and C, respectively. All conditions are the same as for pH 7.3 in Figure 5.
cuss fine differences in the \( v_{Fe=O} \) frequencies, the \( v_{Fe=O} \) frequencies of compounds I and II in an identical protonation state should be compared, it is practically difficult because protonated form of compound II and deprotonated form of compound I are very unstable.\(^7\) A sole possibility to overcome this problem is to compare the Raman/absorption spectra at pH 8.8 and 7.3. As described above, both pH values are lower than the \( pK_a \) of the distal residue as seen from the rapid oxygen exchange reaction. Protonation of the distal residue at pH 8.8 is also supported by the downshift of the \( v_{Fe=O} \) band from that at pH 9.5, while absorption spectra indicate that this is not the result of an increased population of compound I. Presumably, the samples at pH 7.3 and 8.8 exist in two different forms, namely, compounds I and II, respectively. The differences are reflected in their absorption and Raman spectra (Fig. 4) and different photosensitivity. Nevertheless, the Raman isotope-difference spectra give the \( v_{Fe=O} \) modes at the same frequencies with slightly different relative intensities of two bands for \(^{16}O \) derivative. This observation suggests that the Fe=O stretching mode of ARP is scarcely affected by the oxidation state of the enzyme.

**RR Spectral Characteristics of Compounds I and II**

It has been a matter of controversy whether the oxidation of porphyrin ring affects the \( v_{Fe=O} \) frequency, owing to the difficulties associated with photolability of the compound I of HRP. Some groups \(^{35,36} \) reported the \( v_{Fe=O} \) mode at substantially lower frequency for a porphyrin \( \pi \)-cation radical than for neutral porphyrin, while in other cases it appeared at the same frequency \(^{15} \) or could not be detected.\(^{21}\) In the \( \pi \)-cation radical of the macrocycle, an electron is removed from HOMO, which is either of \( a_{1u} \) or \( a_{2u} \) symmetry. The two types of orbitals have different nodal patterns and thus different bonding, antibonding and nonbonding characters for a given bond. Accordingly, appreciable up- or downshifts of stretching frequencies have been observed for metalloporphyrins upon oxidation of the macrocycle.\(^{37-39}\)

As to the porphyrin vibrations, RR spectra of compounds I and II of ARP are remarkably alike as shown here upon simultaneous observations of their absorption spectra [see Fig. 5 (B) and 5 (D)]. Furthermore, the reduction of relative intensity of the \( v_4 \) band reported for compounds I of chloroperoxidase,\(^{40} \) of HRP upon ultraviolet excitation,\(^{16} \) and of HRP upon Soret excitation\(^{35,36} \) was not recognized for ARP compound I, although whole Raman intensity was more reduced for compound I than for the ferric state. In respect of similarities between compounds I and II, the present results on ARP are closer to some earlier reports on HRP\(^{21,36} \) but clearly different from others.\(^{16,35} \) One of the possible explanations for slight difference between compounds I and II is to ascribe to delocalization of the hole of the macrocycle to the axial ligand(s) as proposed earlier for HRP\(^{36,41,42} \) and cytochrome P-450.\(^{43} \) It is still unclear, however, why the Soret band becomes twice weaker if the radical does not reside at the macrocycle. Although Chuang and Van Wart\(^{36} \) reported that \( v_{Fe=O} \) frequency is reduced by as much as 55 cm\(^{-1} \) upon oxidation of the macrocycle, the present observation of this mode for ARP compounds I and II did not exhibit a significant difference.

**Applicability of the Present Technique to Photolabile Intermediates**

Figure 2 revealed temporal changes of Raman and absorption spectra upon laser illumination. The spectral dependencies on a laser power and sample volume mentioned before mean that generation of the new compound is proportional to integral photon numbers per unit volume of the circulating solution, but not a consequence of an immediate process within the laser beam. In other words, only a small fraction of molecules exposed to the laser beam undergoes photoconversion in each moment, but such a fraction increases with time as the sample is repeatedly flowed through the beam. In fact, the Raman spectra in Figure 2 (A) observed in the first period of time is clearly different from that observed in the last period of time.

In the resonance Raman studies on the photosensitive compounds, the interference from the fast-reversible photoreactions in the laser beam are often considered. The present results emphasize a possibility of the slow integral changes, though prominent, in the sample under the influence of light. Application of the real-time Raman/absorption spectroscopy to potentially unstable samples allows to distinguish the two kinds of side reactions and perform unambiguous assignments of Raman and absorption forms.

In respect of ARP, the present study reveals an intriguing discrepancy between radical characters of compound I, which have been discussed from the viewpoints of its photosensitivity and optical absorption spectra from one side, and unexpectedly small changes in the vibrational character of the heme from the other. This calls for further investigation of \( \pi \)-cation radical model systems and their applicability to heme proteins.
The authors express their gratitude to Suntory Limited for the courtesy of providing purified ARP. This study was supported by a grant-in-aid for scientific research on priority areas (Molecular Biometallics) from the Ministry of Education, Science, Culture, and Sports, Japan, to T.K. (08249106).

REFERENCES


24. S. Hashimoto, R. Nakajima, I. Yamazaki, Y. Tat-suno, and T. Kitagawa, “Oxygen exchange between the Fe(IV) = O heme and bulk water for the A3 iso-


Received May 1, 1996
Revised and accepted July 1, 1996