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Unusual Low-Frequency Resonance Raman Spectra of Heme Observed for Hog Intestinal Peroxidase and Its Derivatives†

Shioko Kimura,‡ Isao Yamazaki, and Teizo Kitagawa*

ABSTRACT: The resonance Raman spectra of hog intestinal peroxidase (IPO) and its cyanide and fluoride complexes were observed for the first time. The Raman spectra in the 100-500-cm^{-1} region were quite dissimilar to those of the A-, B-, and C-type hemes so far observed, although the spectra in the 1200-1700-cm^{-1} region were similar to those of the B-type heme. The Raman spectral characteristics, including the presence of two anomalously polarized lines at 1344 and 1306 cm^{-1} and the absence of any Raman lines between 1650 and 1700 cm^{-1}, indicated the presence of a vinyl group at position 2 or 4, at least, and the absence of a C_β—C(=O)—R (R = H, alkyl, or *O*-alkyl) linkage in the heme periphery. The ν_4 line (oxidation-state marker) was observed at normal frequencies for both the ferrous and ferric states, indicating that the axial ligand is not of an unusual type. The ν_{10} frequency of ferri-IPO (1622 cm^{-1}) suggested coordination of a water molecule to the sixth coordination position at the heme iron of native ferri-IPO, in contrast with ferri-horseradish peroxidase. The Raman spectral characteristics of native ferri-IPO remained unaltered between pH 5.6 and 9.3. The cyanide complex of ferri-IPO gave rise to ν_{10} at 1638 cm^{-1}, implying the coordination of cyanide anion to the axial position of the heme iron. The cyanide and the fluoride complexes of ferri-IPO retained the unusual feature of the low-frequency Raman spectrum. On the other hand, for ferro-IPO, two kinds of Raman spectra were observed by varying the pH. One ($4.5 < pH < 6.5$) was of the low-spin type and the other ($7 < pH < 10$) of the high-spin type. The spin change from high to low spin was irreversible, and its rate was very slow between pH 6 and 7. The Raman spectra of the cyanide complex of ferro-IPO displayed a distinctly different feature from those of the cyanide complex of ferro-horseradish peroxidase. Both the Raman and absorption spectra of the cyanide complex of ferro-IPO exhibited a drastic change on further addition of a large amount of KCN.

The presence of a heme-containing peroxidase is a likely origin of the high peroxidase activity in animal tissues (Neufeld et al., 1958). So far, hog mucosa intestinal peroxidase has been purified, and its visible spectral characteristics were shown to have a close resemblance to those of cow lactoperoxidase among animal peroxidases (Stelmaszyńska & Zgliczyński, 1971). The intestinal peroxidase (IPO)[1] is a true peroxidase which reacts with H_2O_2 as a substrate similarly to horseradish peroxidase (HRP), but its spectral as well as biochemical properties have somewhat different aspects from those of HRP (Kimura & Yamazaki, 1978, 1979). Such properties substantially depend upon the nature of the heme itself and the heme environment. Although much information has been accumulated about HRP [Yamazaki et al. (1978) and references cited therein], little is known about IPO.

Resonance Raman scattering from hemoproteins has provided detailed structural information on the heme proximity through selective observation of the molecular vibrations of the heme group (Spiro, 1975; Felton & Yu, 1978; Kitagawa et al., 1978). The structural implication of the heme-linked ionization of HRP was analyzed recently by resonance Raman spectroscopy, and the importance of the hydrogen bonding of proximal histidine to protein was pointed out as the difference in the heme proximity between HRP and myoglobin (Mb) (Teraoka & Kitagawa, 1981). Application of this technique to IPO may bring about crucial information on the structure-function relationship of the peroxidase and may distinguish the specific property of IPO from common properties of hemoproteins. Accordingly, we have investigated the resonance Raman spectra of hog mucosa intestinal peroxidase.

Materials and Methods

The intestinal peroxidase was isolated from the mucosa membrane of hog intestine tissue by the method of Stelmaszyńska & Zgliczyński (1971) with a slight modification. As a molar absorbance of IPO is not known, an approximate concentration was inferred from the comparative experiments on lactoperoxidase for which the concentration could be determined spectrophotometrically on the basis of $\epsilon_{mM} = 114$ at 412 nm (Morrison et al., 1957). The buffer system used for the Raman experiments was 0.1 M sodium

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[1] Abbreviations used: IPO, intestinal peroxidase; HRP, horseradish peroxidase; IPO-CN, cyanide complex of intestinal peroxidase; HRP-CN, cyanide complex of horseradish peroxidase.

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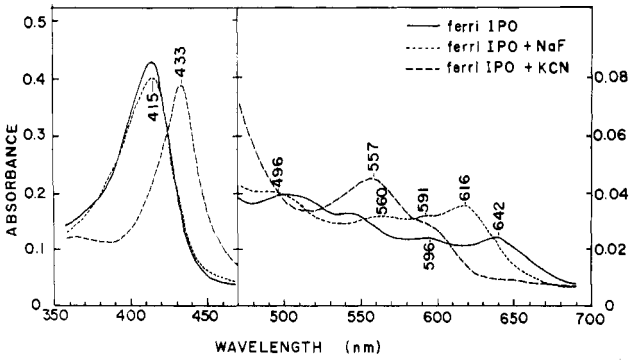

FIGURE 1: Absorption spectra of ferri-IPO (pH 5.05) and its fluoride and cyanide complexes (pH 5.40). The concentrations of IPO used are commonly 3.7 μM. The buffer consisted of 50 mM sodium acetate. Concentrations of NaF and KCN were about 100 and 10 mM, respectively.

phosphate unless otherwise noted, and its pH was adjusted with concentrated HCl or NaOH. The buffer systems used for the absorption spectra are specified in the individual figure captions. The pH value was determined at 15 °C for the solution in the Raman cell with a Radiometer PHM26 pH meter equipped with a long, thin Ingold combination electrode. The enzyme was reduced by a small amount of solid dithionite under an Ar atmosphere. The cyanide complex was formed by adding a small amount of solid KCN, and the pH of these preparations was determined after the Raman measurements.

Raman scattering was excited by an He–Cd laser (Kinmon Electrics, Model CDR30MGE) or an Ar laser (Spectra Physics, Model 164) and was recorded on a JEOL-400D Raman spectrometer equipped with a cooled HTV-R649 photomultiplier. The frequencies of the Raman spectrometer were calibrated with indene as a standard (Hendra & Loader, 1968) for individual measurements. For the Raman measurements, ca 0.1 mM enzyme solution was put in a 1-mm-thick air-tight cuvette, and the inside atmosphere was replaced by an Ar gas. The temperature of the cuvette was kept at 5 ± 1 °C during the measurements of Raman spectra, and after the Raman measurements, the absorption spectra were measured for all the preparations to confirm that the enzyme was not altered by laser illumination. The visible absorption spectra were measured with a Shimazu MPS-5000 spectrophotometer or a Hitachi-124 recording spectrophotometer.

Results

Figure 1 shows the absorption spectra of ferri-IPO and its cyanide and fluoride complexes. The former two spectra are in close agreement with those previously reported (Stelmaszyńska & Zgliczyński, 1971). Addition of fluoride to native IPO caused an appreciable spectral change in the Q region but little change in the Soret region, while addition of cyanide brought about drastic changes in both regions.

The resonance Raman spectra of these compounds excited at 441.6 nm are shown in Figure 2A (1200–1700 cm^{-1}) and Figure 2B (200–700 cm^{-1}). The native enzyme exhibited prominent Raman lines at 1622 (dp), 1586 (p), 1564 (ap), 1485 (p), and 1375 (p) cm^{-1} in the higher frequency region (p, polarized; dp, depolarized; ap, anomalously polarized), and these were assignable to ν_{10}, ν_2, ν_{19}, ν_3, and ν_4 of the porphyrin skeletal modes, respectively (Abe et al., 1978). The 1618-cm^{-1} line of ferri-IPO·CN was polarized, and therefore, it cannot be ν_{10}, but is presumably associated with the peripheral vinyl stretching vibration. The ν_{10} mode of ferri-IPO·CN was clearly observed at 1638 cm^{-1} upon excitation at 514.5 nm.

The ν_{10} frequency was recently shown to be sensitive to the coordination number of the heme iron and an identity of the

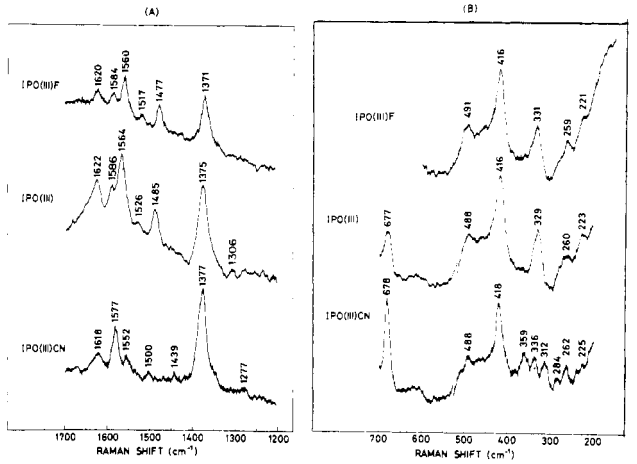

FIGURE 2: Resonance Raman spectra of ferri-IPO (pH 7.5) and its fluoride (pH 5.3) and cyanide (pH 8.0) complexes. Laser, 441.6 nm, 7 mW. Instrumental conditions: (A) sensitivity, 1000 pulses/s; time constant, 2 s; slit width, 200 μm; scan speed, 25 cm^{-1}/min; (B) sensitivity, 250 pulses/s; time constant, 16 s; slit width, 200 μm; scan speed, 10 cm^{-1}/min.

coordinated ligands, 1628–1630 cm^{-1} for the 5-coordinate ferric high-spin complexes and 1618–1625 cm^{-1} for the 6-coordinate ferric high-spin complexes (Teraoka & Kitagawa, 1980b). Accordingly, the ν_{10} frequency of native ferri-IPO (1622 cm^{-1}) indicated the 6-coordinate ferric high-spin structure. This contrasts well with ν_{10} = 1630 cm^{-1} of ferri-HRP (Rakshit & Spiro, 1974), which indicates the 5-coordinate ferric high-spin structure. The whole spectrum of ferri-IPO in the higher frequency region closely resembled those of aquomet-Mb and the benzhydroxamic acid complex of ferri-HRP (Teraoka & Kitagawa, 1981). Consequently, for ferri-IPO, coordination of the water molecule to the sixth coordination position of the heme iron is likely.

The Raman spectral feature was slightly altered in the fluoride complex. Such a small alteration seems reasonable when the sixth ligand is merely replaced by a weak ligand without a change of the spin state. On the other hand, when cyanide was bound to ferri-IPO, the apparent relative intensity of two lines at 1586 and 1564 cm^{-1} was reversed, and the ν_3 line was shifted to 1500 cm^{-1}. The relative intensity was previously pointed out as an indicator of the spin state of the heme iron (Yamamoto et al., 1973) and the ν_3 frequency as well (Spiro & Burke, 1976; Kitagawa et al., 1976). Therefore, the Raman spectral change upon addition of cyanide was in accord with what was expected for the change of spin state from the high- to the low-spin state. This implied that cyanide was bound to the sixth coordination position of the heme iron.

The ν_{10}, ν_{19}, and ν_3 frequencies are considered to serve as a porphyrin core size indicator (Spaulding et al., 1975; Spiro et al., 1979), and on the basis of the empirical relation given by Spiro et al. (1979), the Ct–N distance (d) of native ferri-IPO (Ct represents the center of the porphyrin in the average porphyrin plane) was calculated to be ~2.05 Å (2.055, 2.045, and 2.042 Å from ν_3, ν_{19}, and ν_{10}, respectively). A change of the Ct–N distance, Δd, upon coordination of fluoride or cyanide can be evaluated with the empirical equation $\Delta d = -\Delta\nu_i/K_i$ (Huong & Pommier, 1977) where $\Delta\nu_i$ is the frequency shift upon the coordination of the ligand and K_i is an empirical parameter determined for the individual band, 375.5, 555.6, and 423.7 for ν_3, ν_{19}, and ν_{10}, respectively (Spiro et al., 1979). The observed frequency shifts ($\Delta\nu_3$ = −8, $\Delta\nu_{19}$ = −4, and $\Delta\nu_{10}$ = −2 cm^{-1} for IPO·F and $\Delta\nu_3$ = 15, $\Delta\nu_{19}$ = 13, and $\Delta\nu_{10}$ = 16 cm^{-1} for IPO·CN) gave rise to Δd = 0.02, 0.007, and 0.004 Å for IPO·F and Δd = −0.039, −0.023, and −0.038 Å for

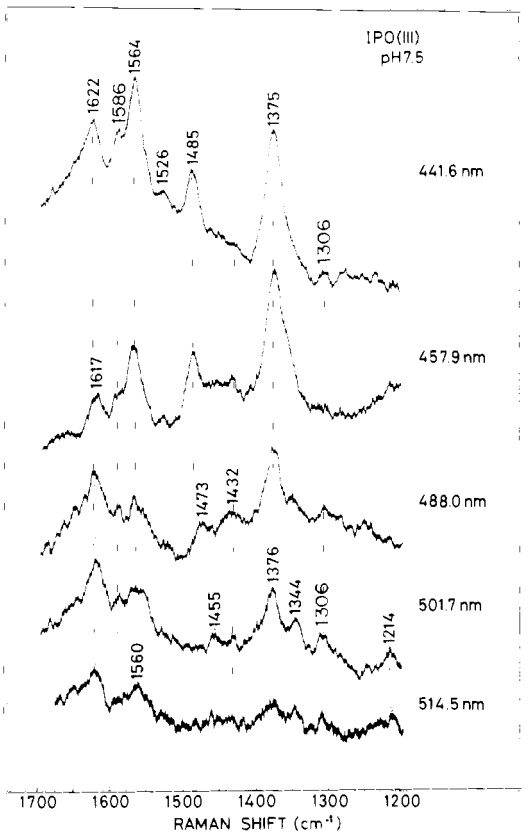

FIGURE 3: Resonance Raman spectra of ferri-IPO (pH 7.5) excited at 441.6 (7 mW), 457.9 (25 mW), 488.0 (18 mW), 501.7 (18 mW), and 514.5 nm (42 mW).

IPO·CN, respectively. Although the sizes of core expansion deduced from the three modes are not unique, probably due to errors involved in determination of Raman frequencies, it appeared that ca. 0.01-Å expansion or ca. 0.03-Å contraction took place upon the coordination of fluoride or cyanide, respectively.

The spectra of these compounds in the lower frequency region (Figure 2B) were obtained for the same preparation as for those in Figure 2A but with 4-fold higher gain. All the Raman lines in this region were polarized. These spectra are quite unusual compared to those of ferri-Mb derivatives (Desbois et al, 1979), ferri-Hb derivatives (Asher & Schuster, 1979; Nagai et al., 1980a), oxidized cytochrome P-450 (Champion et al., 1978), ferri-HRP (Teraoka & Kitagawa), and oxidized cytochrome oxidase (Babcock & Salmeen, 1979). For these hemoproteins, two intense lines, at least, are observed between 300 and 400 cm^{-1}, usually around 340 and 375 cm^{-1}. Nonetheless, the spectral pattern of native IPO was characterized by two intense lines at 416 and 329 cm^{-1} and the lack of any Raman lines between them. These Raman spectral characteristics were unaltered between pH 5.6 and 9.6 and by the addition of fluoride. As the origin of such unique spectra, two possibilities would be expected: (1) heme peripheral substituents differ from those of ordinary hemes, and (2) the fifth ligand is unusual. To determine the alternative, we examined the dependence of Raman intensity upon the excitation wavelengths and the Raman spectra of its pyridine ferrohemochrome.

Figure 3 shows the resonance Raman spectra of native ferri-IPO excited at 441.6, 457.9, 488.0, 501.7, and 514.5 nm. When excited at longer wavelengths, the signal to noise ratio became worse due to a decreased resonance effect, the Raman lines in the lower frequency region being too weak to be identified. However, it was repeatedly confirmed that there

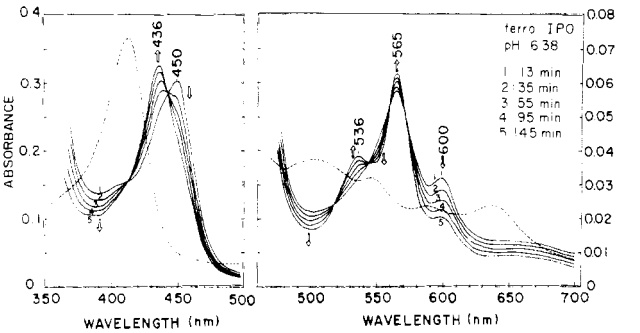

FIGURE 4: Time-dependent changes in the absorption spectrum of ferro-IPO. The lapsed time until the start of scanning after addition of sodium dithionite is specified in the spectra. A broken line designates the absorption spectra of ferri-IPO used for this measurement. Temperature, 20 °C; buffer, 50 mM potassium phosphate, pH 6.38; concentration of IPO, 3.2 μM.

was no sign of Raman lines between 1650 and 1700 cm^{-1} for any excitation wavelengths, although a prominent line should be present at 1650–1680 cm^{-1} if a carbonyl group were attached to the heme periphery in conjugation with the porphyrin π system (Babcock & Salmeen, 1979; Tsubaki et al., 1980). On the other hand, upon excitation at 501.7 nm, two Raman lines were evidently observed at 1344 and 1306 cm^{-1}. The two lines suggested the presence of a vinyl group at the heme periphery like protoporphyrin IX (Adar, 1975; Kitagawa et al., 1975). The dependence of whole Raman spectra of native ferri-IPO on the excitation wavelengths qualitatively agreed with that of met-Hb fluoride (Strekas et al., 1973).

The absorption spectrum of pyridine ferrohemochrome is reported to show a time-dependent change (Stelmaszyńska & Zgliczyński, 1971). Therefore, we determined its Raman spectrum 1 day after its formation. The marker lines were observed at 1361 cm^{-1} for ν_4 (oxidation-state marker), which is a typical frequency for ferrous low-spin hemoproteins, at 1617 cm^{-1} for ν_{10} (spin-state marker), and at 1575 cm^{-1} for ν_{19}, while other lines were considerably weak upon excitation at 441.6 nm. In the lower frequency region, four prominent Raman lines were observed at 494, 456, 414, and 271 cm^{-1}. The absence of any Raman lines between 300 and 400 cm^{-1} was unexpected from our present knowledge about the resonance Raman spectra of ferrous low-spin hemoproteins.

For a reduced form of native IPO, two kinds of absorption spectra were observed. One was favored at higher pH and the other at lower pH. At neutral pH, superposition of the two spectra was obtained, and relative intensities of the two sets of absorption bands were varied with time. Figure 4 illustrates the time-dependent change of the absorption spectrum in the Soret and Q band regions of ferro-IPO at pH 6.38, 20 °C. Immediately after the reduction, the absorption bands were observed at 450, 565, and 600 nm, but after a while, the Soret band was gradually shifted to 436 nm, and at the same time, a new band appeared at 536 nm. Simultaneously, the 565-nm absorption increased, and the 600-nm absorption decreased. As the isosbestic points were clearly observed, a structural change from one to the other must have taken place. Even after the measurement of the last spectrum in Figure 4, the spectrum still continued to change a little, but it was overlapped with autooxidation. The first spectrum in Figure 4 was very close to the stable one observed at pH 8–10.

The rate of the spectral change was faster at lower pH, although the solution became turbid below pH 4.5, and was sensitive to temperature, being slower at lower temperature. Since the change was irreversible, it cannot be a chemical equilibrium. In this pH region, it was practically impossible to define the absorption spectrum of ferro-IPO at a given pH

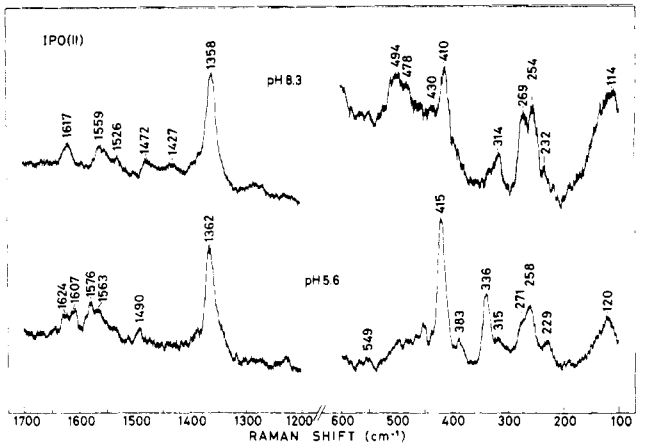

FIGURE 5: Resonance Raman spectra of ferro-IPO at pH 8.3 (upper spectra) and at pH 5.6 (lower spectra) excited at 441.6 nm. Instrumental conditions for the higher frequency region: sensitivity, 2500 pulses/s; time constant, 2 s; slit width, 200 μm; scan speed, 25 cm^{-1}/min. Instrumental conditions for the lower frequency region: sensitivity, 250 pulses/s; time constant, 8 s; slit width, 200 μm; scan speed, 10 cm^{-1}/min.

and thus to construct a pH titration curve.

Figure 5 shows the resonance Raman spectra of ferro-IPO at pH 8.3 and 5.6 excited at 441.6 nm. The spectrum at pH 5.6 was measured 2 h after the reduction. The Raman lines of the pH 8.3 preparation at 1472 (ν_3) and 1358 (ν_4) cm^{-1} were shifted to 1490 and 1362 cm^{-1} at pH 5.6, respectively. This spectral change was similar to what was observed for ferro-HRP upon the change from the 5-coordinate high-spin form (1473 and 1358 cm^{-1}) to the 6-coordinate low-spin form (1492 and 1360 cm^{-1}) (Teraoka & Kitagawa, 1980a). The change in relative intensity of the two Raman lines around 1580 and 1560 cm^{-1} was also consistent with the spectral change expected for the high-spin and low-spin interconversion (Yamamoto et al., 1973). Therefore, it is presumed that ferro-IPO adopts the 5-coordinate high-spin form at pH 8.3 and the 6-coordinate low-spin form at pH 5.6. Note that the interconversion from the low- to the high-spin form could not be observed by reversing the pH change.

The Raman spectra of the two forms of ferro-IPO in the low-frequency region were quite unusual compared with those of deoxy-Mb (Desbois et al., 1979; Kincaid et al., 1979; Kitagawa et al., 1979), deoxy-Hb (Nagai & Kitagawa, 1980; Nagai et al., 1980b), reduced cytochrome P-450 (Champion et al., 1978), ferro-HRP (Teraoka & Kitagawa, 1980a), and reduced cytochrome oxidase (Nafie et al., 1973; Salmeen et al., 1978; Adar & Erecińska, 1979). The spin interconversion of ferro-IPO upon the pH change was accompanied with pronounced spectral changes in the lower frequency region too. In the low-spin form, the intensity ratio, I_{335}/I_{315}, was markedly large, and I_{271}/I_{258} was lower than unity, and, in addition, the whole Raman spectrum was more than twice intense compared with that of the high-spin form.

Figure 6 illustrates the time-dependent change of the Raman spectrum of ferro-IPO at pH 6.6, 7 °C. As shown in the top trace, the Raman lines at 271 and 256 cm^{-1} were clearly resolved, and the line at 336 cm^{-1} was weak at 30 min after the reduction, but after 3 h, the 271-cm^{-1} line became a shoulder, and, conversely, the 336-cm^{-1} line became manifest. Since the sample was kept at a slightly higher pH and a lower temperature than in the experiment for absorption spectroscopy (Figure 4), the change was somewhat slower. At pH 7.5, the Raman spectrum remained similar to the upper trace of Figure 5 even at 3 h after the reduction. The critical pH for the change was inferred to be 6.9, although its precise value could not be determined from the present experiment. Consequently, we are now confident that there are two forms of ferro-IPO: one assumes the low-spin structure and is dominant between pH 4.5 and 6.5, and the other assumes the high-spin structure and is dominant between pH 7 and 10.

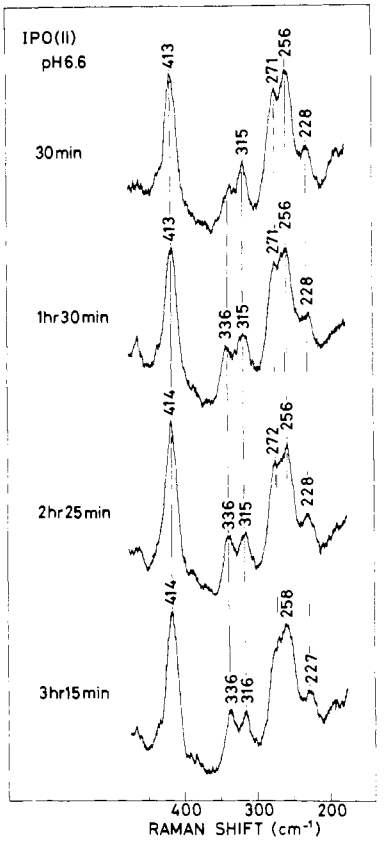

FIGURE 6: Time-dependent changes in the resonance Raman spectrum of ferro-IPO at pH 6.6. The lapsed time until the start of scanning is specified at the left side of the individual spectra. Temperature, 7 °C; laser 441.6 nm, 7 mW; sensitivity, 250 pulses/s; time constant, 16 s; slit width, 200 μm; scan speed, 10 cm^{-1}/min.

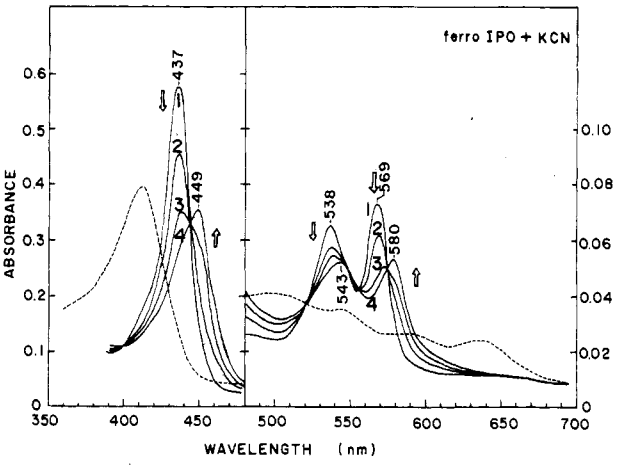

FIGURE 7: Change in the absorption spectrum of the cyanide complex of ferro-IPO induced by the further addition of a large amount of KCN. A broken line indicates the absorption spectrum of native ferri-IPO used for this measurement. Temperature, 20 °C; buffer, 50 mM potassium phosphate (pH 6.38); concentration of IPO, 3.5 μM. The concentration of KCN was 10 mM (pH 6.38) and 270 mM (pH 9.0) for spectrum 1 (identical), 380 mM (pH 9.5) for spectrum 2, and 540 mM (pH 9.8) for spectrum 3. Spectrum 4 was observed 1 h after the addition of 540 mM KCN.

Figure 7 shows a change of the absorption spectrum of the cyanide complex of ferro-IPO induced by further addition of KCN. Spectrum 1 was a typical spectrum obtained in the

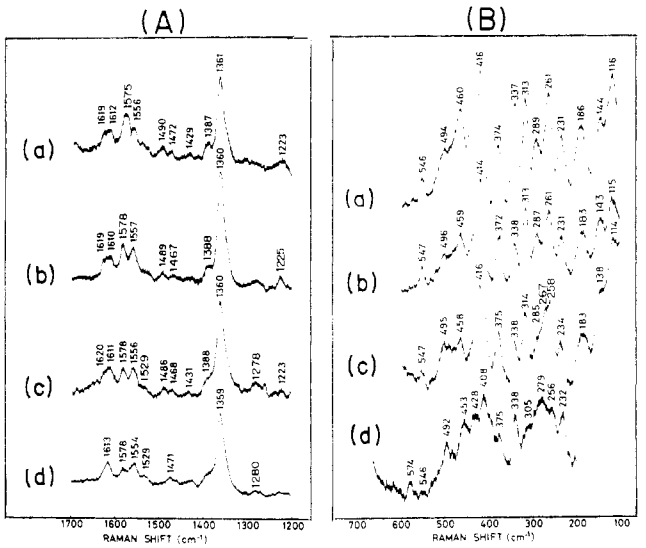

FIGURE 8: Resonance Raman spectra of the cyanide complex of ferro-IPO in the presence of a large amount of KCN. The concentrations of KCN were 0.23 M (pH 6.3) for (a), 0.57 M (pH 7.9) for (b), 1.0 M (pH 9.0) for (c), and 1.8 M (pH 9.8) for (d). The scanning of the spectrum was set about 1 h after the addition of KCN. Temperature, 5 °C; laser, 441.6 nm, 7 mW. Instrumental conditions: (A) sensitivity, 2500 pulses/s; time constant, 1 s; slit width, 200 μm; scan speed, 25 cm^{-1}/min; (B) sensitivity, 500 pulses/s (250 pulses/s for spectra c and d); time constant, 8 s; slit width, 200 μm; scan speed, 10 cm^{-1}/min.

presence of 10–200 mM KCN. This spectrum, having three absorption maxima at 437, 538, and 569 nm, was in agreement with the reported one (Stelmaszyńska & Zgliczyński, 1971). A sign of spectral change, however, appeared when the cyanide concentration was increased above 270 mM, and finally in spectrum 4, those three maxima were shifted to 449, 543, and 580 nm with the isosbestic points at 444, 519, 547, 556, and 575 nm. The rate of the spectral change was very slow in the presence of 270 mM KCN at pH 9. The spectral change did not take place when the pH was merely raised in the presence of 10 mM KCN or when the KCN concentration was increased at a fixed pH, although we failed to keep the pH below 9 with 1.8 M KCN. Therefore, the effects of the pH and the concentration of KCN both seemed essential for the spectral change.

Figure 8 illustrates the similar cyanide-induced Raman spectral change of the cyanide complex of ferro-IPO in the higher (A) and lower (B) frequency regions. In the spectrum of the typical cyanide complex (spectrum a), two Raman lines were recognizable at 1490 and 1472 cm^{-1}, which were noted as the indicator band (ν_3) of the low- and high-spin ferrous hemoproteins, respectively. A large value for the relative intensity of two indicator lines, I_{1575}/I_{1556} (Yamamoto et al., 1973), also indicated the presence of the low-spin form. As the KCN concentration and pH were raised simultaneously (spectra b–d), both the 1490- and 1575-cm^{-1} lines in addition to the 1619-cm^{-1} line were diminished. These spectral changes differed from those of the ferro-HRP·CN complex, in which the 1471-cm^{-1} line in the neutral solution was replaced by the 1491-cm^{-1} line in the alkaline solution (Teraoka & Kitagawa, 1980a). The Raman spectra shown in Figure 8B were measured with a 5-fold gain for the same preparation as described in Figure 8A. For the typical cyanide complex (spectrum a), prominent Raman lines were observed at 460, 374, 289, and 186 cm^{-1} which did not have a corresponding counterpart in the spectra of free ferro-IPO at both pH 5.6 and 8.3 (see Figure 5). The symmetric Raman line of the cyanide complex at 261 cm^{-1} (spectrum a) corresponded to doubletlike lines at 254 and 269 cm^{-1} for free ferro-IPO.

Ferro-IPO produced relatively stable compound III, the half-life time being on the order of 10 h at 2 °C (S. Kimura and I. Yamazaki, unpublished experiments). When we illuminated the compound with laser light at 441.6 nm, the sample on the light path was damaged, leaving a mark of the light path in the solution. The light-induced deterioration of the sample was reduced with reduced laser power but was not completely avoided with power of one-eighth of that used for other measurements. Thus, we failed to observe the Raman spectra of compound III with any excitation lines between 441.6 and 514.5 nm. We also failed to observe the Raman spectra of the carbon monoxide complex of ferro-IPO due to the occurrence of photodissociation.

Discussion

Structure of the Prosthetic Group. The type of heme present in native IPO has not been identified yet. The absorption maxima of its pyridine ferrohemochrome (423, 530, and 568 nm) do not match any of the established sets of absorption maxima: 418, 524, and 557 nm for protoheme IX; 405, 516, and 546 nm for hematoheme IX; 432, 537, and 583 nm for chlorocruoroheme; 430 and 587 nm for heme *a*; and 432 and 620 nm for heme *d*. The absorption maxima are relatively closer to those of cow lactoperoxidase (422, 525, and 563 nm), for which the absorption spectrum had been interpreted in terms of the heme covalently bound to the protein through an alkaline-labile chemical bond such as an ester or amido linkage (Morell & Clezy, 1963), or mesoheme IX, with two double bonds in conjugation with the porphyrin skeleton and hydroxyl groups attached to the side chains (Hultquist & Morrison, 1963). In recent years, however, the heme isolated from lactoperoxidase with Pronase was demonstrated to be a protoheme IX, and the curious set of absorption maxima of the pyridine ferrohemochrome was attributed to asymmetric coordination of the heme axial ligands, namely, a pyridine molecule and an amino acid residue (Carlström, 1969; Sievers, 1979). Although the Raman spectra of the pyridine ferrohemochrome of IPO were curious as stated above, their formation in an extreme alkaline solution may involve an unexpected modification of the heme periphery. Here, we discuss the nature of the heme on the basis of the spectra of native IPO at neutral pH.

The spectra shown in Figure 3 are negative to the presence of a carbonyl group conjugated with the porphyrin skeleton, which includes the C_β–C(=O)–R and C_β–C(=O)–OR linkages, although this is not always so for a nonconjugated ester linkage like C_β–O–C(=O)–R (R = H or an alkyl group). Other Raman features of ferri- and ferro-IPO in the higher frequency region (Figures 2, 3 and 5) suggest the presence of protoheme IX in IPO. If this is so, however, the structure at the heme proximity should be so unique as to make the low-frequency Raman spectra distinctly different from those of other protoheme-containing hemoproteins.

If the axial ligand of the heme iron were endowed with strong π basicity, the ν_4 frequency of ferro-IPO should be unusually low (below 1350 cm^{-1}) as found for various ferrocytochrome P-450's (Ozaki et al., 1976, 1978). On the basis of the ν_4 frequencies of ferro-IPO (1358 cm^{-1} at pH 8.3 and 1362 cm^{-1} at pH 5.6) and its cyanide complexes (1359–1361 cm^{-1}), coordination of such a strongly π-donating ligand as a sulfur anion to the heme iron seems less plausible.

If the unique environment around the heme of IPO were concerned with ionization of the coordinated histidylimidazole, the low-frequency Raman spectrum in the ferrous high-spin state (Figure 5, pH 8.3) should be between the spectra of

ferro-HRP and the iron protoporphyrin 2-methylimidazole complex placed in a micelle of detergent, because the former and the latter are supposed to have a very strongly and a very weakly hydrogen-bonded imidazole ligand, respectively (Teraoka & Kitagawa, 1981). Since the two different situations of the heme-coordinated ligand brought about almost identical Raman spectra in the 1200–1700-cm^{-1} region, the higher frequency spectrum of IPO may not be used for this diagnosis. The Raman line of ferro-IPO at 254 cm^{-1} is close to the Fe(II)–His stretching band of ferro-HRP-A (252 cm^{-1}) (Teraoka & Kitagawa, 1981), being suggestive of the presence of the very strongly hydrogen-bonded histidylimidazole as an axial ligand. However, other Raman spectral characteristics of ferro-IPO shown in Figure 5 (pH 8.3) are quite dissimilar to both of the two representative cases, and we could not succeed in reproducing the unique low-frequency spectrum in the model system by simply changing an axial ligand of the heme iron or hydrophobicity around the heme.

On the other hand, the low-frequency Raman spectrum of hemoproteins is expected to be particularly sensitive to the peripheral groups attached, because of strong vibrational coupling between the porphyrin deformation modes and the in-plane bending vibrations of peripheral groups (Abe et al., 1978). Indeed, the low-frequency Raman spectra of ferrocytochrome *c* excited at 413.1 nm (Champion & Albrecht, 1979) and ferrocytochrome b_5 excited at 441.6 nm (Kitagawa et al., 1979) are distinctly different, although their spectra are alike in the higher frequency region. Therefore, there is left a possibility that the unique low-frequency Raman spectrum results from the presence of a heme having one vinyl group and one covalent linkage to the protein moiety at positions 2 and 4 of the heme periphery.

Binding of Cyanide and Fluoride. Figures 1 and 2 demonstrated that fluoride anion binds to ferri-IPO, but the accompanied spectral change was relatively small. On the other hand, addition of cyanide to ferri-IPO caused much larger spectral changes, and the resultant Raman spectral characteristics suggested coordination of cyanide to the sixth coordination position without destruction of the unique property of heme.

The Raman spectrum of the typical cyanide complex of ferro-IPO (spectrum a in Figure 8A) contains additional Raman lines compared with the spectrum of free ferro-IPO at pH 5.6 (Figure 5), and the spectral features, including the appearance of two ν_3 lines at 1490 and 1472 cm^{-1} are most reasonably interpreted in terms of a mixture of the high-spin and low-spin species. The presence of two species cannot be ascribed to the chemical equilibrium of the cyanide-bound and free forms, because ferro-IPO is saturated spectrophotometrically with cyanide under the present experimental conditions. The cyanide anion bound to ferro-HRP was pointed out to be photodissociable by a Hg lamp (Keilin & Hartree, 1955), although it was not photodissociated upon illumination by the present excitation light (Teraoka & Kitagawa, 1980a). If ferro-IPO·CN is more photodissociable than ferro-HRP·CN, the high-spin species would be assigned to the photodissociated species in the same way as photodissociated (carbon monoxy)IPO.

The Raman spectrum of the cyanide complex of ferro-IPO observed in the presence of a large amount of KCN (spectrum d in Figure 8A) is characterized by ν_{10} = 1613 and ν_3 = 1471 cm^{-1}, and a small relative intensity of I_{1578}/I_{1554}. These features contrast well with those of ferro-HRP·CN (Teraoka & Kitagawa, 1980a), which satisfy the empirical rule derived for various ferrous low-spin hemoproteins (Spiro & Burke, 1976; Kitagawa et al., 1976). Presumably, a significant structural change of the heme must have taken place at a high concentration of KCN. Elucidation of the Raman intensity of the very intense 415-cm^{-1} line holds the key to the interpretation of the structural change.

Acid–Base Transition. The spectroscopic titration revealed the presence of a heme-linked ionization of ferri-IPO with pK_a = 4.75 (Kimura & Yamazaki, 1978). The higher frequency Raman spectra of ferri-IPO at pH 5.6–9.6 are close to those of aquomet-Mb, and accordingly are consistent with the water-coordinated model proposed previously (Kimura & Yamazaki, 1978).

With regard to ferro-IPO, the present study demonstrated the occurrence of an irreversible transition around pH 6.9, which was accompanied by a switch of the spin state. On the basis of the Raman spectra shown in Figure 5, we conclude that the 5-coordinate high-spin form is stable at alkaline pH and the 6-coordinate low-spin form at acidic pH. This had been rather unexpected because a strong-field ligand must be bound to the sixth coordination position of the heme iron to produce the low-spin state. Presumably, the time-dependent spin transition observed at acidic pH involves a large conformational alteration of the polypeptide chain, which may result in coordination of a methionine or histidine residue to the sixth coordination position of the heme iron of ferro-IPO in the low-spin form.

In conclusion, the Raman spectra of ferro- and ferri-IPO in the lower frequency region are very strange and probably specific to this enzyme in the native neutral form. The properties of ferro-IPO regarding the pH dependence as well as the binding of cyanide are also quite unusual compared with other hemoproteins. Further investigation is desired to explicate what causes such unusual properties specific to IPO and how they are reflected in its enzymatic catalysis.

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Solution Behavior of Proteins L7/L12 from the 50S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: The behavior of *Escherichia coli* 50S ribosomal subunit proteins L7/L12 has been investigated in ribosome reconstitution buffer, TMK buffer, by sedimentation equilibrium and analytical gel filtration. Contrary to previous reports that L7/L12 exists in solution solely as dimer species [Möller, W., Groene, A., Terhorst, C., & Amons, R. (1972) *Eur. J. Biochem. 25*, 5], results presented here indicate that L7/L12 undergoes a monomer–dimer–tetramer self-association, with equal equilibrium constants of 3.5×10^4 M^{-1} obtained for the monomer–dimer and dimer–tetramer steps. These results yield standard Gibbs' free energies of -6.1 ± 0.6 kcal/mol at 20 °C. The observed absence of temperature dependence of this interaction over the range 5–25 °C indicates a zero standard enthalpy of self-association. Gel filtration results are presented that confirm the highly elongated shape of the L7/L12 molecule. The data suggest the corresponding Stokes radii for the monomer, dimer, and tetramer are 21–23, 26–28, and 29–32 Å, respectively. The significance of these results is discussed.

In previous studies of the 50S ribosomal subunit of *Escherichia coli*, the proteins L12 and its N-acetylated modification, L7, have been shown to dramatically influence the functional capability of the ribosome (Hamel et al., 1972; Weissbach et al., 1972; Highland et al., 1973, 1974; Möller, 1974; Koteliansky et al., 1977; Glick, 1977; Kurland, 1977). The absence of the proteins L7/L12 from the ribosome greatly reduces the capacity of the ribosome to support elongation factor dependent translation of synthetic poly(U) messages in vitro. Readdition of L7/L12 to the L7/L12-deficient ribosomes completely restores the activity lost by its removal (Hamel et al., 1972; Möller, 1974; Koteliansky et al., 1977; Glick, 1977). It has been shown that labeling of 50S subunits with antibody directed against proteins L7/L12 abolished the binding of elongation factor G to the 50S subunit, while labeling with antibodies directed against the other 32 proteins had no effect (Highland et al., 1974). The apparent involvement of L7/L12 in partial reactions involving initiation factors and release factors has been reviewed in detail (Möller, 1974).

L7/L12 are the only proteins present in multiple copies in the ribosome (Subramanian, 1975; Brimacombe et al., 1978). Though originally thought to be present in two copies per ribosome (Möller et al., 1972), the number of copies of L7

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0006-2960/81/0420-4638$01.25/0