Detection of UV Resonance Raman Bands of the Distal Histidine in Cyanide-Bound Horseradish Peroxidase: Evidence for Two Hydrogen Bonding States of the Imidazolium Side Chain

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Horseradish peroxidase (HRP) catalyzes the decomposition of H_2O_2 to H_2O and concomitant oxidation of a variety of aromatic molecules. The catalytic active center of HRP contains a single iron protoporphyrin IX, whose center iron Fe(III) is coordinated by a histidine residue (His170) from the proximal side.[1] On the other side of the heme are located a distal histidine residue (His42) and an arginine residue (Arg38). According to the stereochemical model proposed for heme peroxidases including HRP,[2] H_2O_2 binds to the heme iron in the HOO^- form after transferring a proton to the imidazole ring of distal His42. In the next step, the protonated imidazole (imidazolium) ring of His42 and the guanidinium group of Arg38 cooperatively attract the HO^- part of the iron-bound HOO^-, forming a transition state for O−O cleavage. The imidazolium proton of His42 is then back-transferred to the HO^- fragment, which leaves the active site as a water molecule. After the decomposition of H_2O_2, HRP becomes compound I, a two-electron oxidized intermediate with ferryl iron [Fe(IV)═O]. Although the distal His plays a key role in the formation of compound I, its structure and environments during the catalytic cycle are not fully elucidated. Ultraviolet resonance Raman (UVRR) spectroscopy is a possible probe to obtain structural information on the distal His in HRP. However, His is a weak Raman scatterer compared to other aromatic amino acid residues[3,4] and His UVRR bands have not been detected except for those of a few proteins.[5−7] In this study, we have successfully detected the UVRR bands of His42 in HRP complexed with an inhibitor, cyanide, by using a high-performance UV Raman spectrometer.[8] The observed Raman bands provide evidence that the protonated His42 side chain forms two types of hydrogen bond, strong and weak, in cyanide-bound HRP.

Figure 1A shows a 244-nm-excited UVRR spectrum of native HRP isozyme C. Most of the prominent bands in the spectrum are assigned to 5 Tyr, 1 Trp, and 20 Phe residues in the protein[9] as indicated with labels Y (Tyr), W (Trp), and F (Phe) followed by their mode numbers.[10] The 1455 cm^{-1} band is ascribed to the imide II modes of 17 X-Pro linkages.[11] UVRR bands of 3 His residues (proximal His170, distal His42, and additional His40) are too weak to be identified in spectrum A.

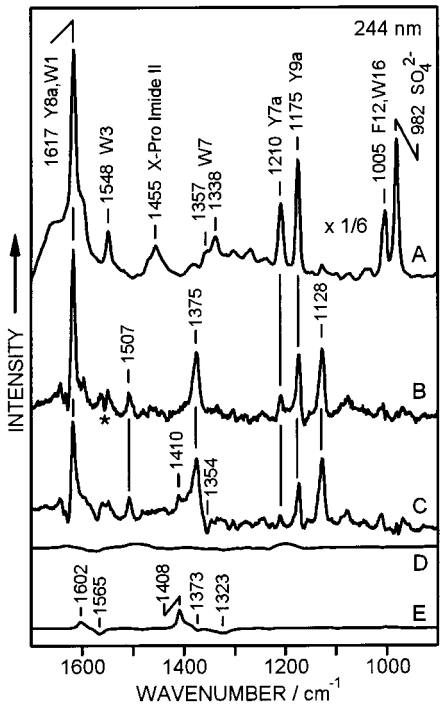

Figure 1. UV (244 nm) resonance Raman spectra of HRP and amino acid histidine. (A) Native HRP isozyme C in 20 mM sodium phosphate buffer (pH 7.0) containing 100 mM Na_2SO_4 as a Raman intensity standard. (B, C) Difference spectra computed by subtracting the spectrum of native HRP from that of cyanide-bound HRP in H_2O (B) and D_2O (C). (D, E) Difference spectra, histidinium (pH 3.0) − histidine (pH 9.0), in H_2O (D) and D_2O (E). The Raman spectrometer and the continuous-wave UV laser used were described in refs 8 and 24. Samples were circulated through a quartz capillary tube, and Raman scattered light was collected with a UV achromatic lense. Lyophilized powder of HRP (TOYOBO Co., type IC, Rz 3.5) was dissolved in the phosphate buffer at a protein concentration of 160 μM. The solution was divided into two equal portions and a small amount of concentrated solution of potassium cyanide (pH or pD 7.0) was added to one of them. The binding of cyanide was confirmed by a shift of Soret absorption. A difference spectrum was computed for each pair of cyanide-free and cyanide-added samples. The difference spectra in B−E are averages of two or three difference spectra obtained for independent sample preparations and are expanded by an intensity scale factor of 6. A dip marked with * in B is due to the overlapping Raman band of atmospheric oxygen.

Cyanide binds to the heme iron of HRP from the distal side. Concomitantly, the His42 imidazole ring is protonated as is proposed for the binding of H_2O_2.[12] To detect possible spectral changes associated with the conversion from imidazole to imidazolium of the His42 side chain, we have computed a difference spectrum by subtracting the spectrum of native HRP from that of the cyanide adduct (HRP−CN^-). Figure 1B shows the result of subtraction for the spectra recorded in H_2O solution. Three positive peaks at 1507, 1375, and 1128 cm^{-1} are assigned, consecutively, to the ν_3 and ν_4 modes of the heme ring[13] and to the heme C_b-vinyl stretch mode[14] of HRP−CN^-. Positive peaks

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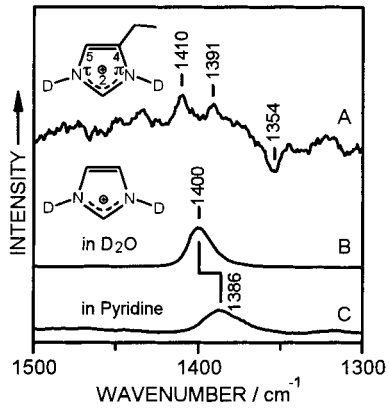

Figure 2. (A) Double difference spectrum obtained by subtracting the difference spectrum in Figure 1B (H_2O solution) from that in Figure 1C (D_2O solution). Two positive peaks at 1410 and 1391 cm^{-1} and a negative peak at 1354 cm^{-1} represent deuteration-sensitive components of the spectral changes induced by the cyanide binding. (B, C) Visible (488 nm) Raman spectra of N-deuterated imidazolium dissolved in D_2O (B) and pyridine (C). Solvent Raman bands were subtracted.

around 1550 cm^{-1} may be due to an overlap of the heme ν_{38}[14] and Trp W3[10] modes. The frequencies of the heme ring modes are consistent with those reported for HRP−CN^- in a previous Raman study employing visible−laser excitation resonant with the heme absorption.[15] Other positive peaks at 1617, 1210, and 1175 cm^{-1}, indicating intensity increases of the Tyr bands, are ascribed to an increase of the strength of hydrogen bonding at the phenolic oxygen of a Tyr residue.[16] This Tyr residue is likely to be Tyr233 whose phenolic oxygen is within hydrogen-bonding distance of the carboxylate of Asp247, which is in turn hydrogen bonded with His170.[1] In contrast to the clear peaks due to the heme and Tyr, His does not give any signals in the difference spectrum in H_2O solution. This is because the UV resonance Raman intensity is distributed over many vibrational modes and each Raman band is weak for the N−H forms of imidazole and imidazolium,[17] as demonstrated by the difference spectrum, histidinium − histidine in H_2O, in Figure 1D. N-deuteration, however, changes the vibrational modes of the His side chain, and only a few bands of the N-deuterated imidazolium ring gain UVRR intensity.[17]

Figure 1C shows the HRP−CN^- − HRP difference spectrum obtained for D_2O solution. Upon deuteration, additional positive and negative peaks appear at 1410 and 1354 cm^{-1}, respectively. The 1410 cm^{-1} positive peak is assigned to the N−C−N symmetric stretch of the N-deuterated imidazolium ring[18] of His42 in HRP−CN^-. This assignment is supported by the appearance of a positive peak at 1408 cm^{-1} in the difference spectrum, histidinium − histidine in D_2O (Figure 1D). The negative peak at 1354 cm^{-1} may be ascribed to the N-deuterated imidazole ring of His42 in cyanide-free HRP or to an intensity decrease of a His170 band upon cyanide binding. It is known that N_π-deuterated, N_τ-metal-ligated His, such as His170 of HRP, usually gives a strong Raman band around 1350 cm^{-1}.[7,19]

In addition to the 1410 cm^{-1} band mentioned above, a weak positive shoulder is seen on the high-frequency side of the 1375 cm^{-1} heme ν_4 band in Figure 1C. We have computed a double difference spectrum, trace C − trace B in Figure 1, to precisely locate the weak band. Figure 2A shows the double difference spectrum where the deuteration-insensitive heme ν_4 band is removed and a positive peak is clearly seen at 1391 cm^{-1}. To elucidate the origin of the 1391 cm^{-1} band, we have examined the effect of hydrogen bonding on the N−C−N stretch frequency of the N-deuterated imidazolium ring. Traces B and C in Figure 2 show 488-nm-excited Raman spectra of N-deuterated imidazolium in D_2O and pyridine solutions. The 1400 cm^{-1} band in D_2O solution is assigned to the N−C−N stretch band of imidazolium, and this band downshifts by 14 cm^{-1} in pyridine solution. Since pyridine is a much stronger proton acceptor than water (basicity: H_2O, 0.18; pyridine, 0.64),[20] the frequency downshift is ascribed to a strong hydrogen bonding. In light of this model compound study, the 1391 cm^{-1} band is assigned to a strongly hydrogen-bonded state of the His42 imidazolium ring. The appearance of two N−C−N stretch bands indicates that His42 of HRP assumes two hydrogen bonding states, weak (1410 cm^{-1}) and strong (1391 cm^{-1}), when cyanide binds to the heme iron. These two bands disappear at pD 12.5 (data not shown), being consistent with the reported p*K* value (∼11) of His42 deprotonation.[12]

A visible resonance Raman study has shown that cyanide binds to the heme iron of HRP in two distinct forms.[21] In the major form, the Fe−C−N linkage is linear, whereas it is bent in the minor form. We have built a model for cyanide-bound HRP by using the recently solved X-ray structure of HRP.[1] The model shows that the nitrogen atom of cyanide would not be very appropriately positioned to accept a proton from His42 when cyanide binds to the heme iron in the linear form although His42 was supposed to be the proton donor in the visible resonance Raman study. Instead, Arg38 is a good hydrogen bond donor to the cyanide. In order for His42 to form a strong hydrogen bond with the Fe-bound cyanide, the Fe−C−N linkage must be bent. Accordingly, the more intense 1410 cm^{-1} band of His42 is reasonably ascribed to the major linear form, and the less intense 1391 cm^{-1} band to the minor bent form. The two hydrogen bonding states of His42 in HRP−CN^- may have relevance to the role of the distal His in the heterolytic O−O cleavage of H_2O_2, which is accomplished in collaboration with Arg38. Recent studies on HRP mutants have confirmed the direct involvement of His42 in the catalytic mechanism.[22,23]

Visible and UV resonance Raman spectroscopy has played an important part in providing information on the structure and dynamics of heme proteins including HRP. However, direct structural information on distal amino acid residues has scarcely been obtained. Ligands and/or substrates bind to the heme iron from the distal side, and structural information on distal amino acid residues such as His42 of HRP are highly demanded to fully understand the mechanisms of heme protein functions. Here, we have demonstrated, for the first time, that UVRR spectroscopy in combination with hydrogen−deuterium exchange is a useful tool for studying the hydrogen bonding state of catalytically important distal His.

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