tectable spectrum (Figure 3). Radical **4** is persistent under these conditions for a few days. The temperature was monitored by inserting a thermocouple in the place of the sample before or after each spectral determination.

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X-ray Absorption Spectral Study of Ferric High-Spin Hemoproteins: XANES Evidences for Coordination Structure of the Heme Iron

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Abstract: The Fe XANES (X-ray absorption near-edge structure) spectra were measured at 80 K for hemoproteins in a ferric high-spin state. As ferric hemoproteins, we chose sperm whale myoglobin (Mb), its derivative modified at the distal histidine (His) by cyanogen bromide (BrCN-Mb), *Aplysia* Mb, and horseradish peroxidase (HRP), which have different structures at the heme distal side. In comparison of the spectra among the three Mbs, we found that the spectral features, in particular the intensities of the preedge P peak and the fine structure C_1 at the K-edge absorption, serve as a sensitive probe for the iron six-coordination structure; intense P and weak C_1 correspond to five-coordinated iron, while weak P and intense C_1 to six-coordination. Comparing the XANES spectrum of HRP with those of three Mbs, we suggested that the water molecule is absent at the sixth site of HRP, with pentacoordination of the heme iron.

X-ray diffraction has long been applied to a variety of protein single crystals to determine their three-dimensional structures.[1-3] Most attempts to explain protein or enzyme function have been based on the static structure obtained by these studies.[4] However, it is now recognized that conformation of the protein in a solution state is dynamically fluctuating and, as a consequence, it can readily associate or dissociate substrates and/or ligands.

X-ray absorption spectroscopy, with synchrotron radiation, is a powerful emerging technique for high-resolution studies of metal binding sites in proteins because a specific metal serves as a probe in a large molecular system.[5] The spectroscopy gives information on the local structure around the metal binding site, being sensitive to short-range order in atomic arrangements rather than to long-range order. Recently, this technique has been used for the study of hemoproteins in the solution state, especially for determining the local structure at the active site of such compounds as iron porphyrin.[6] In particular, the spectrum in the region of X-ray absorptin near-edge structure (XANES) contains information on the coordination geometry of the heme iron such as the Fe–ligand bond angle and on the distortion of symmetry due to small atomic displacement around the iron.[7,8]

We report here the XANES spectra of four hemoproteins in a ferric high-spin state: sperm whale myoglobin (Mb), its derivative chemically modified at its distal histidyl imidazole by cyanogen bromide (BrCN-Mb),[9] *Aplysia* Mb, and horseradish peroxidase (HRP). These hemoproteins have different types of structure at the heme distal side, although the heme iron of these four proteins has protoporphyrin IX as a planar ligand and a histidyl imidazole as the fifth ligand. (Detailed structures will

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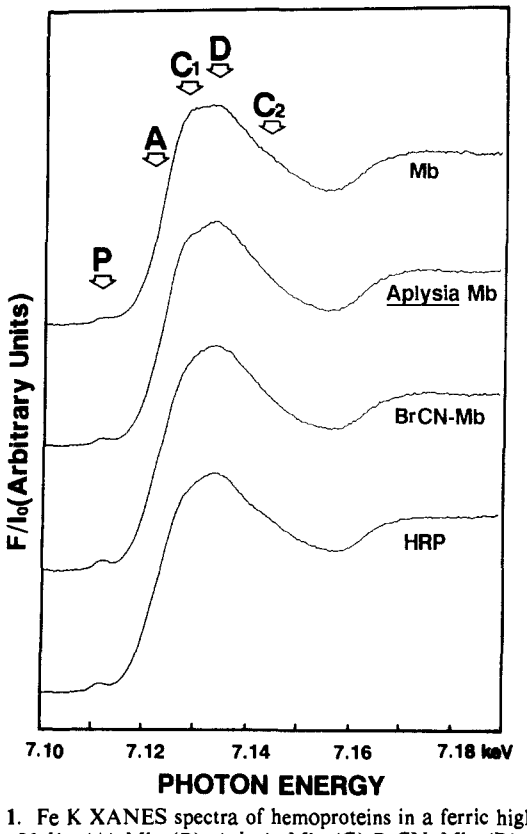

Figure 1. Fe K XANES spectra of hemoproteins in a ferric high-spin state at 80 K: (A) Mb, (B) *Aplysia* Mb, (C) BrCN-Mb, (D) HRP. Sample conditions were shown in Materials and Methods. The spectral characteristics (P, A, C_1, D, and C_2) were denoted according to Bianconi et al.[7f] All of the spectra were normalized on the basis of integration of the absorption from 7100 to 7190 eV.

be cited in the Discussion.) It has been pointed out that several amino acid residues at the heme distal side control the reactivity of the heme iron, i.e., the binding of an external ligand, such as H_2O, CN^-, CO, and O_2, through hydrophobic interactions or hydrogen bonds.[10] Thus, it is important to characterize the coordination structure of the sixth ligand to the iron, which is closely relevant to the interaction of the distal group with the ligand. In the present study, we compare first the XANES spectra of the three Mbs and delineate the relationships of the spectral feature with the coordination structures of the ferric heme iron. On the basis of the results, we discuss the iron six-coordination structure of HRP, whose X-ray crystallographic analysis is not yet available.[11]

Materials and Methods

Sperm whale Mb was purchased from Sigma Chemical Co. and used after further purification by CM-cellulose (Whatman) column chromatography, BrCN-Mb was prepared by the reaction of Mb with an excess amount (1.5 times) of BrCN at pH 7.[9a] *Aplysia* Mb was isolated from the radular muscle of *Aplysia kurodai* according to the method of Rossi-Fanelli and Antonini[12] and was further purified by DEAE-cellulose (Whatman) chromatography.[13] Horseradish peroxidase (Type III) was purchased from Toyobo Co. Ltd. (Osaka) and used after purification by CM-cellulose chromatography.[14]

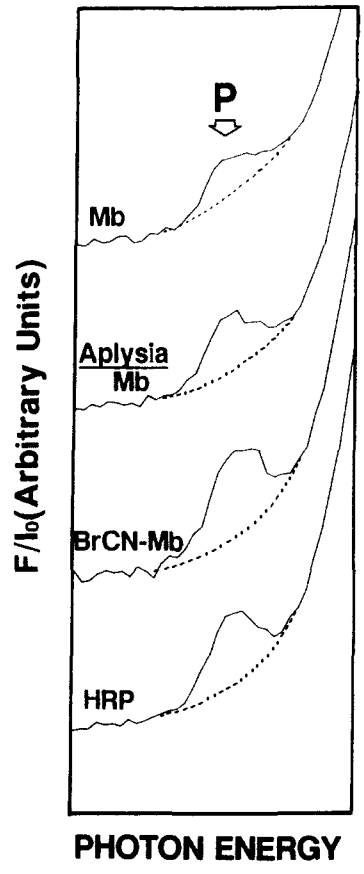

Figure 2. P-peak region of the XANES spectra in Figure 1 expanded. Although energy positions at the P peak are essentially identical, their intensities vary among four ferric hemoproteins in a high-spin state. The base line (shown by the dotted line) was drawn on the basis of Spline function, which fits well the XANES curve above and below the P-peak region.

Protein concentrations for the XANES measurements were all adjusted to be 5 mM. Potassium phosphate buffer (0.1 M) at pH 7.0 was employed for Mb, BrCN-Mb, and HRP and at pH 6.4 for *Aplysia* Mb. Each sample, in an aluminum cell with a Kapton window, was rapidly frozen by immersion into liquid nitrogen and was immediately transferred to a cryostat that had been cooled to 80 K with a closed-cycle helium refrigerator.

The XANES spectra were obtained by a fluorescent detection spectrometer at the Photon Factory (Tsukuba) with employment of sagittally bent crystal monochromator. Details for the measurements have been already reported elsewhere.[8] The time to obtain the final spectrum was about 4 h. The obtained spectra were normalized on the basis of the spectral integrations between 7100 and 7190 eV.

Results

In Figure 1, XANES spectra are compared for the three Mbs and HRP at 80 K. The spectrum of Mb (Figure 1A) is almost identical with that measured at room temperature: a weak absorption at 7112 eV (preedge, P) corresponds to the quadrupole-allowed transition from Fe 1s to empty 3d states and the intense one around 7127 eV to the Fe 1s-4p transition.[7] The fine structures at this absorption, which have been originally denoted A, C_1, C_2, and D by Bianconi and his co-workers,[7f] represent scattering of the photoelectron by atoms around the heme iron. The modification of the distal His of Mb by BrCN gave a spectrum (Figure 1C) different from that of native Mb (Figure 1A); the shoulder absorption of C_1 disappeared, in association with an increase in the intensity of the preedge P absorption. On the other hand, the XANES spectral characteristics of *Aplysia* Mb (Figure 1B) were intermediate between those of Mb and BrCN-Mb, while that of HRP (Figure 1D) bore a good resemblance to that of BrCN-Mb.

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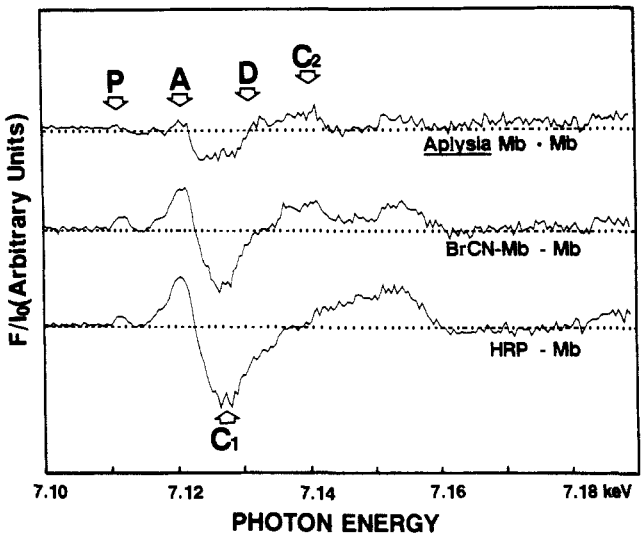

Figure 3. Difference XANES spectra of ferric hemoproteins on the basis of Mb, *Aplysia* Mb–Mb, BrCN–Mb–Mb, and HRP–Mb. A decrease in the C_1 peak concomitantly accompanies increases in the P and the A peaks.

To clarify the subtle differences in the XANES spectra among these hemoproteins, the expanded spectra in the P peak region and the difference spectra of *Aplysia* Mb, BrCN–Mb, and HRP minus Mb were illustrated in Figures 2 and 3, respectively. The P peak intensity (Figure 2) is in the order Mb < *Aplysia* Mb < HRP ≤ BrCN–Mb, indicating that the site symmetry around the heme iron is reduced in this order (discussed below). As seen in all the difference spectra (Figure 3), the increase in P peak intensity accompanies proportionally the decrease in the C_1 peak and the increase in the A peak. The difference spectra also show that the spectral patterns of *Aplysia* Mb to Mb and BrCN–Mb to Mb are similar but they are slightly different from that of HRP. For example, the D position is a node or an isosbestic point among the spectra of Mb, *Aplysia* Mb, and BrCN–Mb, while the absorption of HRP at this position is smaller than that of the Mbs. The spectral shapes in the C_2 region from 7140 to 7160 eV are also slightly different among HRP and the Mbs.

After X-ray irradiation, optical absorption spectra of these samples were taken, but they showed no indication of radiation damage. At the cryogenetic temperature, the samples were extremely stable and neither decyanation reaction at the modified site nor precipitation was observed, even for BrCN–Mb.[9a]

Discussion

Iron Six-Coordination Structure of Metmyoglobins. The peak denoted P was observed at 7112 eV in all of the XANES spectra of the Mbs (Figure 1), but its intensity is varied in these protein species (Figure 2). Since this peak has been ascribed to a forbidden transition, its intensity is sensitive to the site symmetry of the heme iron and, thus, serves as a marker of the iron coordination structure;[7] an increase in its intensity indicates a decrease in the molecular symmetry around the iron. Thereby, an intense P peak of BrCN–Mb, compared with that of Mb, is indicative of the reduction of the molecular symmetry around the heme iron upon the BrCN modification of the distal histidyl imidazole of Mb. Both Mb and BrCN–Mb have protoporphyrin as an iron planar ligand and a histidyl imidazole as the fifth axial ligand. Their bond nature, in particular that of the iron imidazole, is supposed to be also identical between these two species, on the basis of our previous NMR studies.[9b] Furthermore, our recent EPR and X-ray diffraction studies on the azide complex of BrCN–Mb crystal also show no alteration of the protein structure of Mb upon the distal His modification except for the distal structure.[9c,15] (The ferric high-spin form of BrCN–Mb is somewhat unstable at room temperature to form a single crystal.) Thus, we can conclude that the XANES spectral change on going from Mb to BrCN–Mb, e.g., the increase in the P peak intensity, is accounted for by the structural change at the sixth coordination site of the heme iron.

The iron sixth site of Mb is occupied by a water molecule, as was manifested by the X-ray crystallographic analysis.[2a] Many spectroscopic studies proved that water directly binds to the heme iron as a sixth ligand to form a six-coordination structure even in the solution state. It is thus readily concluded that the XANES spectrum of Mb, its weak P peak, comes from typical hexacoordinated ferric iron in hemoproteins. On the other hand, it is likely that the intense P peak in the XANES spectrum of BrCN–Mb, which reflects the reduced iron symmetry, corresponds to five-coordination of the heme iron. This finding is consistent with our previous result obtained by several spectroscopic measurements.[9a,b] Upon BrCN modification of the distal His, the Soret absorption in the visible spectrum blue shifted by 10 nm with a decrease in its intensity and the porphyrin *meso*-H NMR signals moved from the downfield to the upfield side. We have explained these spectral changes in terms of the conformational changes induced by the distal His modification, which expels the water molecule from the iron sixth site.

Corresponding to the increase in the P peak intensity upon BrCN modification, the fine structure C_1 disappeared (Figure 1). Peak C_1 has been assigned to the scattering of the photoelectron from the axial ligands along the heme normal direction, on the basis of the angular-dependent XANES observations of an MbCO single crystal.[7f] So, it may be reasonable that the C_1 peak diminishes in the spectrum of the five-coordinated iron of BrCN–Mb, while it is clearly observed at 7130 eV in that of the six-coordinated Mb.

Here, we can propose the criterion for the iron six-coordination structure of hemoproteins in the ferric high-spin state by utilizing the XANES spectral features such as the P and the C_1 peak intensities; a weak P and an intense C_1 indicate a six-coordinated ferric high-spin iron, while an intense P and a weak C_1 a five-coordination. In contrast to these features, peak D, which is due to scattering in the in-plane direction of the porphyrin,[7f] is not affected by the coordination of the water ligand to the iron sixth site. This feature is in good agreement with our previous results that the modification of the distal His causes no alteration of the porphyrin conformation of Mb.[9]

In comparison of the XANES spectra among Mbs, it is worth noting that the spectral feature of *Aplysia* Mb (Figure 1B) is intermediate between those of Mb (Figure 1A) and BrCN–Mb (Figure 1C). Judging from the P and the C_1 intensities, it looks as if half of a water molecule is present at the iron sixth site. Surprisingly, this finding does not parallel previous spectroscopic results. The NMR and absorption spectral features of *Aplysia* Mb are closely similar to those of BrCN–Mb rather than those of Mb, the upfield resonance of the porphyrin *meso*-H NMR signal[16] and the reduced absorption intensity of the Soret region[17] suggesting that the heme iron of *Aplysia* Mb is exactly five-coordinated. However, the absorption and NMR spectral features predominantly depend on the location of the iron toward the porphyrin plane, whether it locates in-plane or out-of-plane of the porphyrin. In contrast, the XANES spectrum is more informative on the presence or absence of the ligand atom near the iron, because it reflects the scattering of the photoelectron by the neighboring atoms. Therefore, the XANES results of *Aplysia* Mb suggest that the water molecule is present at its iron sixth site, presumably with a longer interatomic distance from the iron than that of Mb. Indeed, the recent X-ray crystallographic study of *Aplysia limacina* Mb by Bolognesi et al.[3] showed that there is an isolated electron density in the neighborhood of the distal residue (Val E7) 4.6 Å from the iron, which is assignable to the

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water molecule. However, the distance is too long to form a strong chemical bond with the heme iron, compared with Mb (2.0 Å of Fe–OH_2).[2a] Hereby, it is likely that the water ligand for *Aplysia* Mb exchanges with the bulk water more rapidly than that for Mb, consistent with the ^1H NMR relaxation measurements of the bulk water by Giacometti and his co-workers.[17]

The accessibility of the water molecule to the heme iron is of importance in relation to characteristic properties of hemoproteins. For example, in the case of *Aplysia* Mb, its oxy complex is much more susceptible to the autoxidation than that of oxyMb.[13] This is most probably because easier attack on the Fe–O_2 bond by the water molecule promotes the formation of ferric iron from oxyferrous iron in *Aplysia* Mb.[18] By contrast, the oxy complex of BrCN–Mb is extremely stabilized[19] because the bulky substituent, such as imidazole–CN, sterically hinders the access of water to the active site.

Iron Six-Coordination Structure of Horseradish Peroxidase. We demonstrate hereafter that the XANES method is feasible for the detailed characterization of the local structure at the ferric iron sixth site, even for hemoproteins whose crystallographic analyses are not available. In this context, it is interesting to characterize the iron coordination structure of HRP in a ferric high-spin state. In its catalytic cycle, hydrogen peroxide is decomposed to a water molecule at the iron sixth site and the water is released to the bulk solution. The visible absorption spectral feature of HRP was similar to those of BrCN–Mb and *Aplysia* Mb, rather than that of Mb,[17] indicative of no coordination of the water to the iron. Resonance Raman[20] and ^1H NMR relaxation[21] measurements of ferric HRP also suggested the five-coordinated heme iron.

In the XANES spectrum of HRP (Figures 1D and 2D), we can see an intense P peak and no fine structure of C_1. Comparison of this spectrum with those of the three Mbs (Figures 1 and 2) showed that it is similar to that of BrCN–Mb rather than those of *Aplysia* Mb and Mb. Along with our criterion, this observation indicates that the water molecule is more than 4.6 Å distant from the heme iron or absent at the sixth site of HRP, which is consistent with optical, NMR, and Raman results. However, the present result for HRP is sharply contrasted with the X-ray crystallographic data of cytochrome *c* peroxidase (CCP), in which the water molecule is present at the iron sixth site with an interatomic distance of 2.4 Å between Fe and OH_2.[1a,b] This may reflect the difference of hydrophobicity in the heme cavity between HRP and CCP. A recent study by Yonetani and Anni[22] showed that iron coordination of CCP at the sixth site is altered, depending on whether the enzyme is fresh or aged and on solvent condition. It is not the case for HRP. Therefore, the structure at the heme vicinity may not be necessarily the same between HRP and CCP, in spite of the similar enzymatic properties.

Inspection of Figure 3 shows that the spectral pattern of HRP minus Mb at the D and the C_2 regions is slightly different from those of BrCN–Mb and of *Aplysia* Mb. Since the D peak has been ascribed to the in-plane scattering of the photoelectron from porphyrin moiety, the difference in the D region is responsible for the subtle difference in porphyrin structure. Indeed, the porphyrin plane of Mb is slightly distorted into a bowl shape, while that of HRP is considered to be saddle shaped.[1a] Moreover, the C_2 peak has been shown to alter in the change of the spin state of the ferric iron, as was manifested by the temperature-dependent change of the XANES spectrum of MbOH.[8] These facts led us to think that either the coordination structure of the porphyrin and the fifth ligand or the electronic structure of the heme iron is subtly different between HRP and Mbs. The nature of the bond between iron and the proximal histidyl imidazole of HRP is different from that of Mb, since the proximal His of HRP has more anionic character caused by a strong hydrogen bond of its imidazolyl NH with the surrounding base.[1a,20] These structural effects may be discerned in the D and the C_2 regions of the XANES spectra.

In summary, we showd that the XANES spectra of hemoproteins provide useful information about the six-coordination structure of the ferric heme iron. This technique will make it possible to characterize the heme environment of some hemoproteins in a solution state, whose crystallographic analysis is not available.

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