# Modification of the Heme Distal Side in Myoglobin by Cyanogen Bromide. Heme Environmental Structures and Ligand Binding Properties of the Modified Myoglobin†

Yoshitsugu Shiro and Isao Morishima*

ABSTRACT: Met, deoxy, and CO forms of myoglobin (Mb) react with a stoichiometric amount of cyanogen bromide (BrCN) to cause substantial changes in the $^1H$ NMR, optical absorption, and infrared spectra. These spectral changes were interpreted as arising from the substantial alterations in the heme environments, most probably due to the modification of the histidine residue at the heme distal side. It is also revealed that the modified Mb does not combine with some exogenous ligands such as $CN^-$, $CH_3NH_2$, and $O_2$, although it does with $N_3^-$ or CO. These unique ligand binding properties are also discussed with relevance to a role of the distal histidine in stabilizing the coordinated ligand through a hydrogen bond and to a steric constraint.

Myoglobin (Mb) and hemoglobin (Hb) reversibly combine with molecular oxygen by virtue of containing ferrous iron protoporphyrin IX as a prosthetic group. Crystallographic studies have shown that the oxygen molecule bound to the heme iron lies in a tight pocket surrounded by two hydrophobic groups (Phe CD1 and Val E11) and the imidazole group of His E7 (Antonini & Brunori, 1970, pp 13–97; Takano, 1977; Phillips, 1978). Great importance for the formation of the stable iron–oxygen complex in Mb and Hb has been ascribed to His E7, which is called the "distal" His and located closely enough to interact with the oxygen molecule. In fact, the neutron diffraction study on oxyMb (Phillips & Schoenborn, 1981) afforded evidence for the hydrogen bonding between the distal His and the coordinated oxygen. Further support for this view seems to be provided by the behavior of *Aplysia* Mb, in which the distal His is replaced by other amino acid residue (Tentori et al., 1971), and the oxygen binding affinity and its dissociation rate constant are significantly different from those for normal Mb and Hb (Antonini & Brunori, 1970, pp 219–234). These findings have frequently allowed one to suggest that the distal His may play a crucial role in the binding of the oxygen molecule to the heme iron. By contrast, the fact that Hb Zürich or *Chironomus thumi* Hb, in which the distal His is displaced by other amino acid residues (Winterhalter et al., 1969; Huber et al., 1970), forms a stable and reversible complex with oxygen has led to a controversy concerning the specific role of the distal imidazole in the formation of an iron–oxygen complex (Antonini & Brunori, 1970, pp 13–39). The X-ray crystallographic study of Hb Zürich (Tucker et al., 1978), however, indicated that the substitution of the distal His not only leaves an empty space in the heme pocket but also renders the entrance to the pocket widely open. Furthermore, the X-ray study by Steigemann & Weber (1979) suggested that the stereochemistry of ligand binding to the prosthetic group in *C. thumi* Hb differs in several respects from that in Mb and Hb A. So the studies on the ligand binding in these abnormal Hb's do not seem to sufficiently clarify the role of the distal His.

One of the approaches to evaluate the role of the distal His could be the chemical modification of its imidazole group and the examination of the physicochemical properties of the modified Mb. Several approaches along this line have been tried to specifically modify either of histidine residues in the heme vicinity of myoglobin. The use of some modification reagents resulted in the reaction at not only His but also other amino acid residues like Cys, Met, Trp, Lys, Tyr, and so on (Hirs, 1967; Ray & Koshland, 1962). Gurd and co-workers (Banaszak et al., 1963; Hartzell et al., 1968) have also attempted to modify the imidazole groups of His in Mb by certain alkylating reagents, such as bromoacetate and iodoacetamide, but they were unfortunately unsuccessful in modifying the distal His without causing the protein denaturation.

On the other hand, Jajczay and co-workers examined the use of cyanogen bromide (BrCN) to modify the distal His of Mb and showed that the reaction of BrCN with Mb occurs with a 1:1 stoichiometry (Jajczay, 1970; Schonbaum & Chance, 1976). They also pointed out that this reaction could involve the modification of the imidazole group of His E7 with cyanation at the imidazolyl NH site and is suitable especially for Mb, because Mb contains no Cys residue, which is more reactive than His toward BrCN. However, the instability of this modified Mb prevented its isolation, and direct demonstration of the modified site and the structural characterization of the heme environments have remained open to further studies.

We have attempted here to directly characterize the heme environmental structures and ligand binding properties of BrCN-modified Mb by NMR, IR, and absorption spectroscopic measurements and to delineate the conformational changes in the heme vicinity of its met, deoxy, and CO derivatives accompanied by the BrCN modification. The relevance of the present results to the ligand binding properties of Mb is also discussed in order to explore the structural relation of the distal His with the reactivity at the heme sixth coordination site.

## Materials and Methods

Sperm whale myoglobin (Type II) was obtained from Sigma and cyanogen bromide from Wako Pure Chemical, Ltd. The reaction of Mb with BrCN was proceeded by the method described previously (Jajczay, 1970), that is, simply addition of a stoichiometric amount of BrCN to metMb solution at pH 7. All chemicals were used without further purification.

Proton NMR spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. For recording of the paramagnetically shifted proton

† From the Division of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606, Japan. *Received February 28, 1984.* This work is supported by a grant from Ministry of Education, Japan (57470119).

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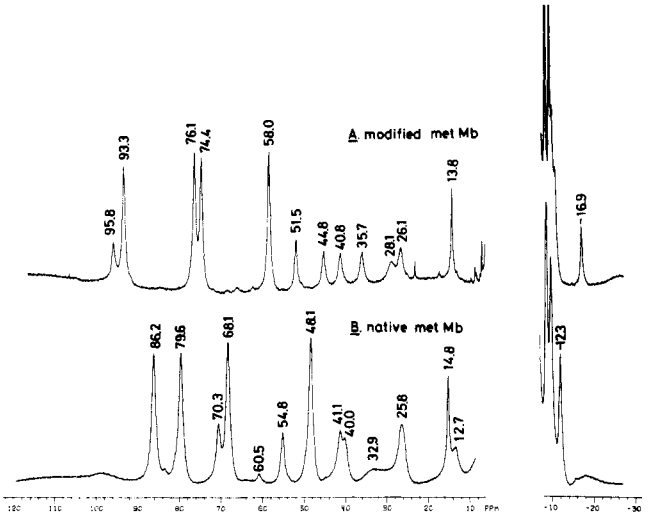


FIGURE 1: Proton NMR spectra of (A) BrCN-modified and (B) native metmyoglobins at pH 7 and 23 °C.

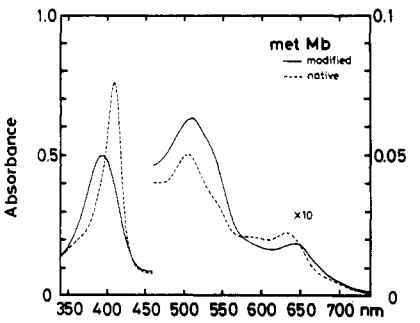


FIGURE 2: Optical absorption spectra of BrCN-modified and native metmyoglobins at pH 7 and 23 °C.

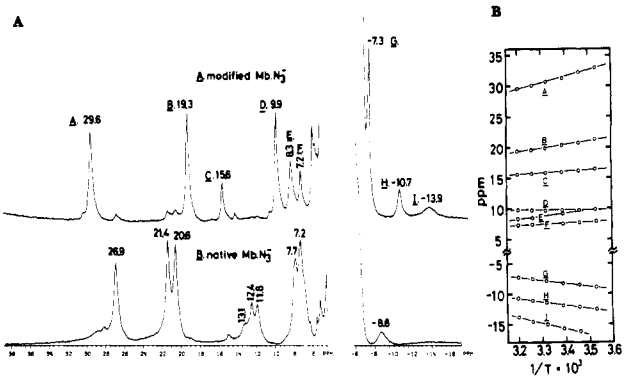


FIGURE 3: (A) Proton NMR spectra of the azide complexes of BrCN-modified and native myoglobins at pH 7 and 40 °C. (B) Temperature dependence (10–40 °C) of the proton resonances of the BrCN-modified Mb·$N_3^-$ at pH 7. The chemical shifts are plotted against $1/T$ (Curie's plot). The designations of the signals correspond with that in (A).

NMR spectra, typically 10–30K transients were accumulated to obtain the Fourier transformed spectra with 8K data points and a 5.7-$\mu$s 90° pulse. For the measurement of diamagnetic species such as Mb·CO, 1000 transients were accumulated with 32K data points and 1-s delay time. Proton chemical shifts are referenced with respect to the proton signal of the residual $H_2O$ in the protein solution, assigning a positive value for the low-field resonance.

The visible absorption spectra were recorded with a Union-Giken SM-401 spectrometer by using a cell with a 1-cm path length.

The infrared spectra of Mb·CO were recorded on a Nicolet Model 6000 FT-IR spectrometer with indium antimonite detector. A total of 512 transients with a resolution of 4 $cm^{-1}$ was accumulated to yield the resultant spectra. The $CaF_2$ IR cell with a 0.025-mm path length was used.

All the spectral measurements were carried out within 10 min after sample preparation, because of instability of BrCN-modified Mb. All the sample solutions for the spectral measurements were prepared in 0.1 M phosphate buffer, pH 7.

## Results

Figure 1 illustrates the hyperfine-shifted proton NMR spectra of native and BrCN-treated metMb's. For native metMb, the hyperfine-shifted four heme peripheral methyl peaks are observed at 86.2, 79.6, 68.1, and 48.1 ppm far downfield from the HDO resonance, while the signals for BrCN-treated metMb are located at 93.3, 76.1, 74.4, and 58.0 ppm. Corresponding absorption spectral changes are shown in Figure 2. Upon addition of BrCN, the Soret band drastically decreases in its intensity. By use of the value of the maximum absorptivity for native metMb ($\epsilon_{409nm}$ = 164 $mM^{-1}$ $cm^{-1}$) (Hardman et al., 1966), the one for the BrCN-treated metMb was determined as 103 $mM^{-1}$ $cm^{-1}$ at 397 nm.

The titration of BrCN to metMb was followed by the $^1H$ NMR spectra. The proton resonances for the native metMb are decreased in their intensities, and a new set of signals grows up in proportion to the concentration of added BrCN. This spectral change in the slow NMR exchange limit is possibly due to the cyanation reaction at the modified site accompanied by a conformational change in the heme vicinity. The effect of BrCN modification on the spectrum was saturated upon addition of almost 1 molar equivalent of BrCN. With addition of more BrCN, no further changes of the spectrum were encountered. Under conditions employed here (pH 7), metMb was stable in its BrCN-modified form and its NMR spectrum remained unchanged for about 1 h. After that, the spectrum gradually converted to the native one, indicating that the native Mb is regenerated in time. This may be due to a decyanation reaction involving the hydrolysis of the modified group. With an excess of BrCN, the protein was finally denatured to form the precipitate. Schonbaum and Jajczay investigated extensively the regeneration rate of the modified site in the pH range from 4.5 to 9 (Jajczay, 1970) and showed that the BrCN-treated Mb was relatively stable in a neutral region (pH 6–7) examined in the present study and the regeneration toward the native form is accelerated in the acid and alkaline pH region.

The BrCN-modified metMb exhibited no spectral changes when external ligands such as $CN^-$ and $CH_3NH_2$ were added, showing that it does not bind such ligands at the heme sixth coordination site, in sharp contrast to the normal Mb. However, azide ion ($N_3^-$) does bind to the heme iron of the modified metMb to afford a characteristic NMR spectrum that is quite different from that of native Mb·$N_3^-$ (Figure 3A). The unusual temperature dependence of the hyperfine-shifted proton signal designated D (Figure 3B) suggests that the heme iron of the BrCN-treated Mb·$N_3^-$ is also presumably in the thermal spin equilibrium state as the case for native Mb·$N_3^-$ (Morishima & Iizuka, 1974; Iizuka & Morishima, 1974).

The BrCN-modified metMb was readily reduced with dithionite to form comparably stable BrCN-modified deoxyMb. Its $^1H$ NMR and absorption spectra are compared with those of native deoxyMb in Figures 4 and 5, respectively. In the $^1H$ NMR spectra, the heme peripheral proton signals are observed at 11.7, 9.2, and 6.5 ppm for native deoxyMb, whereas those of BrCN-treated deoxyMb are located at 9.7,

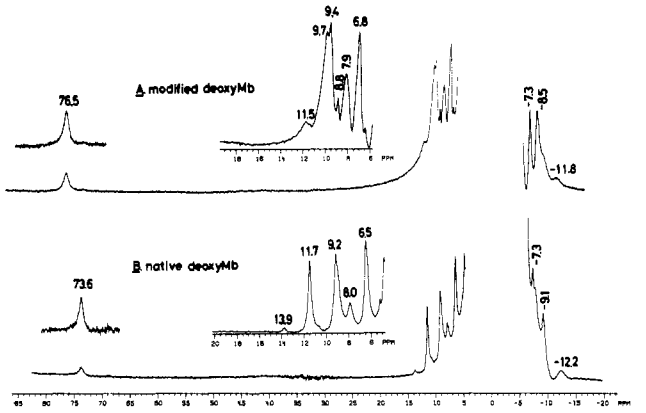


FIGURE 4: Proton NMR spectra of (A) BrCN-modified and (B) native deoxymyoglobins at pH 7 and 23 °C.

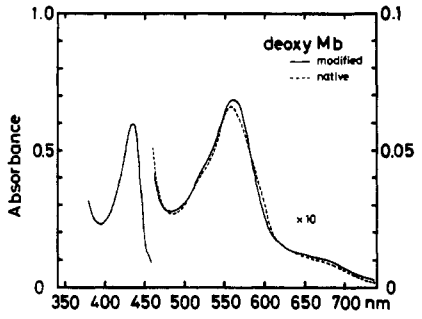


FIGURE 5: Optical absorption spectra of BrCN-modified and native deoxymyoglobins at pH 7 and 23 °C.

9.4, 7.9 and 6.8 ppm from the $H_2O$ resonance. The broad and single proton peak at 73.6 ppm, which has been assigned to the proximal histidyl NH, is shifted to 76.5 ppm upon the BrCN modification.

As to the reactivity of the heme iron in the BrCN-modified deoxyMb, it must be noted that the absorption and NMR spectra of the modified deoxyMb were not changed immediately after gel filtration with Sephadex G-25 to remove excess dithionite and to form the $O_2$ complex. After several minutes, they gradually changed toward those of the BrCN-modified metMb, indicating that the BrCN-modified deoxyMb does not bind with $O_2$ or the oxy form is too unstable to be detected but is autoxidized to a ferric form.

The BrCN-modified deoxyMb reacted with CO to afford a corresponding CO form and exhibited characteristic NMR and absorption spectra that were changed in time. Figure 6 shows the time course of $^1H$ NMR spectra of modified Mb·CO. The ring current shifted Val E11 methyl proton peak was observed at −6.81 ppm immediately after CO is bound to the modified deoxyMb. After several minutes, the signal at −6.81 ppm decreased in its intensity with the concomitant appearance and increase in the intensity of the peak at −7.17 ppm. After 2 h, the spectrum was indistinguishable from that of native Mb·CO (Shulman et al., 1970; Lindstorm et al., 1972) as shown at the upper line in Figure 6. These time-dependent spectral changes indicate that the CO complex of BrCN-modified Mb is unstable and reverts in time to native Mb·CO with the regeneration of the native site. The modified Mb·CO form appears to be more susceptible to this regeneration reaction than other forms. In Figure 7, the absorption spectra for CO complexes are also compared between native and BrCN-treated Mb's. The regeneration of the modified site was also recognized in the absorption spectral changes.

We then tried to estimate the affinity of CO for the BrCN-treated Mb to be compared with that of native Mb. Because of the instability of modified Mb·CO as stated above,

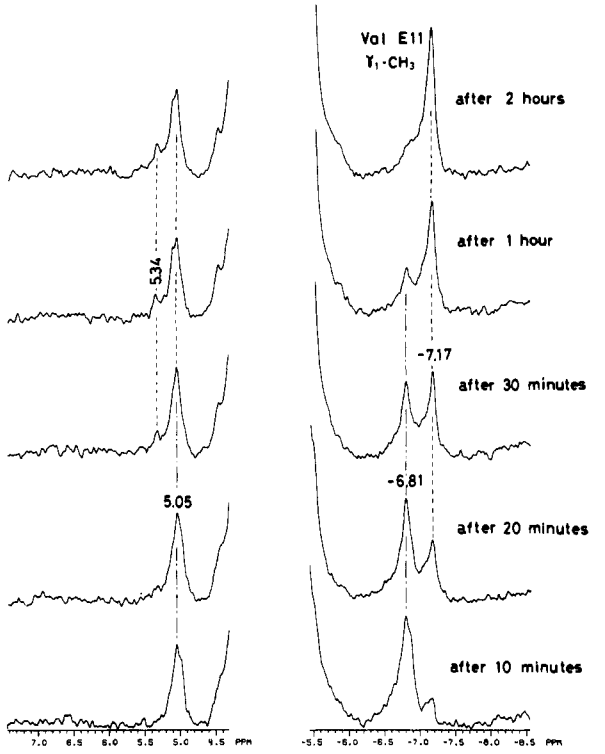


FIGURE 6: Time course of proton NMR spectra of the carbon monoxide complex of BrCN-modified myoglobin at pH 7 and 23 °C.

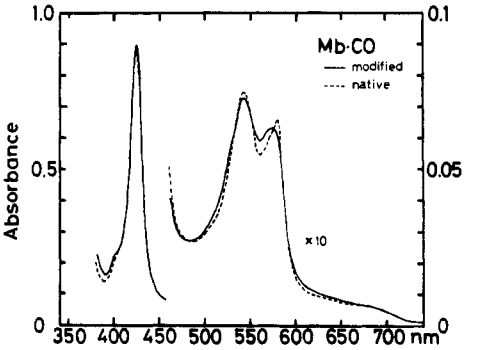


FIGURE 7: Optical absorption spectra of modified and native Mb·CO's at pH 7 and 23 °C.

it was not possible to obtain the precise value of the CO binding constant by a usual method. So we roughly estimated the affinity of CO by titrating the CO-saturated water to the native or the BrCN-treated deoxyMb's solutions and by following the absorption spectral changes under the same conditions (data are not shown). The spectral response for the BrCN-treated Mb was about 5 times less than that for the native Mb, showing that the affinity of CO to BrCN-treated Mb was reduced to about one-fifth of that of native Mb.

The IR absorption spectra in the iron-ligated CO stretching region, 1900–2000 $cm^{-1}$, of native and BrCN-treated Mb·CO's in solution are illustrated in Figure 8. The spectrum of native Mb·CO consists of three distinguishable components for the iron-bound CO stretching frequencies and is identical with that described by Makinen et al. (1979), in which the most prominent absorption at 1944 $cm^{-1}$ is characteristically observed (Figure 8A). On the other hand, the treatment by BrCN resulted in a relative increase in the fraction of the conformer giving rise to the absorption at 1967 $cm^{-1}$ (Figure 8B). Absorption near 1967 $cm^{-1}$ has frequently been assigned to a denatured form of the hemoproteins because acid denaturation of Mb·CO and Hb·CO affords a broad absorption band in this region (Alben, 1978). However, the IR spectrum reverted to

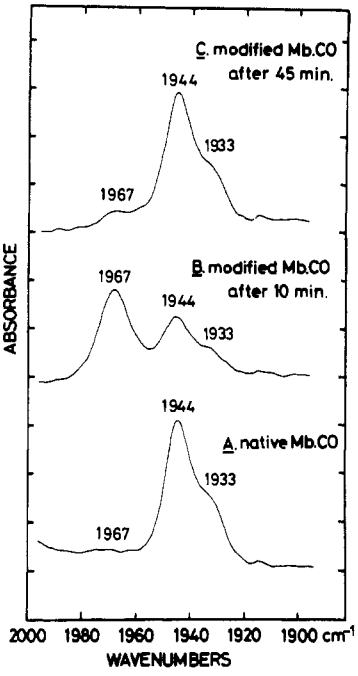


FIGURE 8: IR spectra in the C–O stretch regions (2000–1900 $cm^{-1}$) of myoglobin. (A) Native Mb·CO, (B) BrCN-modified Mb·CO, 10 min after CO bound, and (C) BrCN-modified Mb·CO, 45 min after CO bound.

that of native Mb·CO (Figure 8C) after 45 min, showing that the original site is regenerated in time and is consistent with the results from absorption and NMR measurements. This indicates that the absorption band at 1967 $cm^{-1}$ does not arise from the denatured species but arises from the modified species.

## Discussion

Cyanogen bromide, which serves as a protein modification reagent, has been found to react with the imidazole group of His (cyanation of imidazole) as well as Met peptide bond, with sulfydryl group of Cys, and with $\epsilon$-amino group of Lys in a polypeptide (Gross & Witkop, 1962). Recently Schonbaum and co-workers applied this reagent to modify catalase and showed that the modification elicits the heme-linked perturbation correlated with the loss of the activity and ligand binding characteristics of catalase (Jajczay, 1970; Schonbaum & Chance, 1976). They also extended this modification reaction to Mb and found that 1 mol of BrCN reacts with 1 mol of protein to modify a single amino acid residue with cyanation at, most probably, the imidazole group of the distal His. However, the BrCN-modified Mb was not stable enough to be isolated for the analysis of the modified residue, and the characterization of the heme environmental structures of the BrCN-modified Mb has remained open to further studies. Thus, the spectroscopic measurements employed here appear to make the present system suitable for its structural characterization in the heme vicinity. The present NMR spectral changes for met-, deoxy- and CO-Mb derivatives induced by the reaction of 1 mol of BrCN with 1 mol of protein and the substantial changes in the ligand binding characteristics appear to support the view that the modification of a single amino acid residue takes place in the immediate vicinity of the heme, most probably at the imidazole group of the distal His (Jajczay, 1970), and consequently induces the structural changes in the heme environment. This will be discussed in more detail in the following paragraphs.

The structural changes in the heme vicinity upon the modification are sensitively manifested in the $^1H$ NMR spectra as the spectral shifts of the ring current shifted Val E11 $\gamma_1$-methyl resonance for a CO form and of the hyperfine-shifted proximal histidyl NH resonance for a deoxy form. It has been well established that these two resonances serve as sensitive probes to monitor directly the conformational changes in the heme pocket. The Val E11 residue is located in the heme distal side situated next to the distal His as was visualized by X-ray crystallographic analysis (Antonini & Brunori, 1970; Takano, 1977), and its $^1H$ NMR resonance position sensitively reflects the geometric relationship between the heme group and Val E11 methyl group (Lindstorm et al., 1972; Lindstorm & Ho, 1973; Mims et al., 1983). The spectral shift of this Val E11 methyl signal as shown in Figure 6 indicates the structural changes in the heme distal side upon the BrCN modification. According to the calculation made by Shulman et al. (1970), the downfield shift by 0.3 ppm of its signal implies that the methyl group of Val E11 moves up to 0.3 Å farther away from the heme plane under the perturbation.

As shown in Figure 4, the BrCN-induced shift by 3 ppm for the proximal histidyl NH for deoxyMb also significantly reflects the subtle perturbation at the heme proximity (Nagai et al., 1982; La Mar & de Ropp, 1982). Since the heme iron of deoxyMb has no sixth coordinated ligand (Takano, 1977) and eventually has no direct interaction with the distal His, the spectral change in the NH signal may result from the changes in the binding profiles of the proximal imidazole such as bond compression or tilting of the iron–imidazole bond or modulation of the NH hydrogen bonding. This suggests that the conformational changes associated with the BrCN modification could occur in the heme distal side and to some extent in the proximal side as well.

Further insight into the conformational changes in the heme vicinity was gained on the basis of the IR spectra of Mb·CO's as shown in Figure 8. It is well-known that in sperm whale Mb·CO three structurally distinguishable components are involved in solution with the IR absorptions at 1933, 1944, and 1967 $cm^{-1}$ for the iron-bound CO stretching, and the configuration of the iron–carbonyl with respect to the immediate coordination environment of the heme iron has been assigned for each $\nu$(CO) stretching frequency. According to Makinen et al. (1979), the differences in the $\nu$(CO) frequency between the 1944- and the 1967-$cm^{-1}$ components were interpreted in terms of the structural difference in the two comformers where the relative position of the N atom of the distal imidazole with respect to the ligand and the deviation of the Fe–N (proximal imidazole) bond from the heme normal are substantially different. The distance of the distal imidazole N atom from the N atom of pyrrole ring A for the 1967-$cm^{-1}$ conformer is larger than that for 1944-$cm^{-1}$ one. The deviation of the Fe–N bond from the heme normal axis is also different between the 1967- and the 1944-$cm^{-1}$ conformers. As mentioned under Results, the treatment of Mb with BrCN increased the relative proportion of the 1967-$cm^{-1}$ component (Figure 8B). It is then reasonable to see that the proportion of the 1967-$cm^{-1}$ conformer is more favored than the 1944-$cm^{-1}$ one if the distal His is displaced farther away from the porphyrin ring by the BrCN treatment. All the present spectroscopic measurements suggest that the treatment of Mb with BrCN induces the structural changes in the heme vicinity; the amino acid residues in the heme distal side are forced to move farther away from the heme plane and also to change the binding nature of the proximal imidazole to the heme iron.

We then discuss the modification-induced structural changes at the heme sixth coordination site. The NMR and absorption spectral changes of Mb in the met form upon BrCN modi-

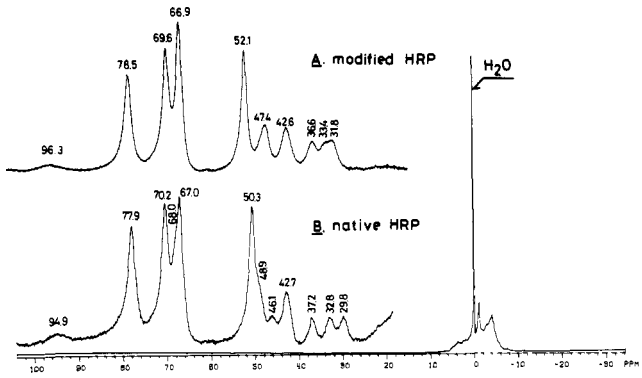


FIGURE 9: Proton NMR spectra of (A) *p*-CBC-modified and (B) native HRP's at pH 7 and 23 °C.

fication are very prominent, in comparison with those in a ferrous state, indicating some marked structural changes in the heme pocket in the ferric state. The significant difference in the Soret region between native and modified metMb's (Figure 2) is noticeable. The spectrum of the BrCN-modified metMb is almost superimposable to that of horseradish peroxidase (HRP) or *Aplysia* Mb (Giacometti et al., 1981), and its absorptivity at maximum absorption ($\epsilon_{397nm}$ = 103 $mM^{-1}$ $cm^{-1}$) is comparable with that of HRP ($\epsilon_{402nm}$ = 102 $mM^{-1}$ $cm^{-1}$) (Schonbaum & Lo, 1972), which is significantly different from those of hemoproteins having a bound water as a sixth ligand (for aquometMb $\epsilon_{409nm}$ = 164 $mM^{-1}$ $cm^{-1}$) (Hardman et al., 1966). Relevant to this point is the suggestion (Giacometti et al., 1981) that pentacoordinated ferric heme iron affords the Soret absorption with low absorptivity. In fact, HRP and *Aplysia* Mb have been suggested to have no water molecule at the heme sixth coordination positions as evidenced by several physical measurements (Bolognesi et al., 1982; Kobayashi et al., 1980; Gupta et al., 1980). On the basis of these results, the heme environmental structure of the BrCN-modified metMb would demand the absence of a coordinated water molecule. Thus, the modification of the heme distal side in aquometMb releases the water molecule from the iron sixth coordination site, which may account for the substantial spectral changes stated above.

To further confirm this view, we tried to see the effect of the modification at the heme distal side of HRP on the NMR spectra. Since the ferric heme of HRP is pentacoordinated in its native form, the modification at the heme distal site, if any, is expected to exert no significant effect on the heme electronic structures and eventually affect only slightly the hyperfine-shifted NMR spectra. We used *p*-chlorobenzoyl chloride (*p*-CBC) as the modification reagent of His by following the reported method (Schonbaum et al., 1979). The modification reaction occured with 1:1 stoichiometry, which was followed by the hyperfine-shifted proton spectral measurements. Figure 9 illustrates the hyperfine-shifted proton NMR spectra of ferric HRP's in its native and modified forms. The two spectra are distinguishable, but their difference is expectedly quite small, compared with the case for metMb. The corresponding absorption spectral change was reported to be small (Schonbaum et al., 1979). In the resting state of HRP, the lack of the iron-bound water could be responsible for the small spectral responses to the modification at the heme distal side. These differences in the modification-induced spectral responses, depending on whether the ferric heme iron is penta- or hexacoordinated, may allow one to expect that the modification site in the heme proximity of aquometMb could play an important role in stabilizing the iron-bound water by hydrogen bonding. Therefore, it seems reasonable to identify the modification site of Mb as the heme distal His, as suggested by previous workers (Jajczay, 1970; Schonbaum & Chance, 1976). The cyanation of the distal histidyl NH group could be involved in the BrCN-modification reaction of Mb, although the detailed mechanism of this reaction can hardly be disclosed in the present system.[1] The *N*-cyano group is unstable and the decyanation reaction could easily occur to regenerate the native form. This reverse reaction is promoted for the Mb·CO form where a steric interaction between the iron-bound CO and the distal His is involved.

We next discuss the structural changes in the heme environment so far mentioned with relevance to the unique ligand binding properties of the ferrous modified Mb; $O_2$ did not bind to the modified Mb, while CO did bind although its affinity was much weaker than that of native Mb. One of the prevalent arguments on this point is the situation of Val E11 methyl groups with respect to the heme, which has been shown to be directly related to the ligand affinity of Hb (Lindstorm & Ho, 1973). It has been shown that the location of the Val E11 methyl group closer to the heme plane is correlated with the lower oxygen affinity of Hb in the presence of anions or organic phosphates. Along with this line, the movement of the distal residues farther away from the heme plane upon the modification allows us to expect an increase in the ligand affinity of Mb. However, it was not the case in the present study, indicating that the distal His modification of Mb may affect its ligand binding properties in the different way from what anions or organic phosphates do to Hb.

One of the most important structural factors to control the ligand binding to the heme iron has been suggested to be the interaction of the distal His with the coordinated ligand, such as hydrogen bond (Yonetani et al., 1974; Ikeda-Saito et al., 1977, 1981) and van der Waals contact. Neutron diffraction studies of Mb·$O_2$ and Mb·CO indicated that the distal His interacts with the ligated $O_2$ molecule through a hydrogen bond, while the iron-bound CO molecule is in van der Waals contact with its surroundings without any hydrogen bond (Phillips & Schoenborn, 1981; Norvell et al., 1975; Hanson & Schoenborn, 1981). As mentioned in the former section, BrCN treatment altered the structure of Mb at the heme distal side and resulted in the inability of hydrogen bonding of the distal His with the ligand. The resultant effects may be manifested as the unique behaviors of the $O_2$ or CO binding by the modified Mb. The lack of the hydrogen-bond ability of the distal His in the modified Mb could be responsible for the present findings that ferrous modified Mb is readily oxidized rather than forming a stable oxy complex. This suggestion could be further supported by the behavior of the abnormal Hb or Mb, where the distal His is replaced by other amino acid residues. The oxygen affinity of *Aplysia* Mb is much lower than that of native Mb (Antonini & Brunori, 1970, pp 219–234), and Hb M Boston, which has the "distal" Tyr instead of His, is stable in a ferric state rather than in a ferrous-oxygenated form (Antonini & Brunori, 1970, pp 55–97).

Other structural factors may account for the specific ligand binding properties of the modified Mb. One is the BrCN-induced change in the binding nature of the proximal His to the heme iron, although it is structurally subtle. Another is the change of the polarity in the heme cavity, which was

[1] The direct evidence for the formation of an unstable N–CN bond was not obtained in the present study. An IR spectral measurement is expected to give such evidence. However, it does not appear feasible because the N–CN IR band could be mixed with a complex spectrum arising from the protein.

pointed out as an important factor by Sharma et al. on the basis of the kinetics of the reactions of $O_2$ and CO with opossum Hb (Sharma et al., 1982). These possibilities could not be necessarily ruled out at the present stage.

It is also of interest to note that cyanide is not accommodated at the heme sixth coordination position, while azide is bound to the ferric heme iron in the modified Mb. Keeping in mind that most of hemoproteins lacking the distal His can bind $CN^-$ and $N_3^-$ at the heme coordination site, the above findings may suggest that some steric constraints could be exerted in the heme distal site so that the entry of $CN^-$ at the sixth coordination position is blocked while the azide binding is free from this steric restraint. It is therefore likely that the azide binding at the heme coordination site of Mb could occur regardless of the ligand–His hydrogen bonding. This appears to be consistent with the suggestion made on the basis of the pH-dependent features of the azide IR bands that the hydrogen bonding is not present between iron-bound $N_3^-$ and distal His in Mb·$N_3^-$ (Maxwell & Caughey, 1976; McCoy & Caughey, 1970).

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**Registry No.** HRP, 9003-99-0; *p*-CBC, 122-01-0; BrCN, 506-68-3; $N_3$, 14343-69-2; CO, 630-08-0; His, 71-00-1; heme, 14875-96-8.

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