High-Pressure Proton Nuclear Magnetic Resonance Studies of Hemoproteins. Pressure-Induced Structural Change in Heme Environments of Myoglobin, Hemoglobin, and Horseradish Peroxidase†

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ABSTRACT: Hyperfine shifted proton NMR spectra of metmyoglobin, methemoglobin, and their complexes with azide, imidazole, and cyanide as well as the spectrum of native horseradish peroxidase were obtained at high pressures up to 2000 atm with a specially designed high-pressure cell for 220-MHz superconducting NMR spectrometer. For the azide complexes of metmyoglobin and methemoglobin and the imidazole complex of metmyoglobin, in all of which the iron atoms are in thermal spin equilibrium between high- and low-spin states, the increased pressure shifted their heme methyl proton signals to the upfield side. For the cyanide complexes of metmyoglobin and methemoglobin and for the fluoride complex of metmyoglobin, which are in purely low- and high-spin states, respectively, the spectra were almost insensitive to changes in pressure up to 2000 atm. The heme methyl proton signals of aquometmyoglobin, its formate complex, and horseradish peroxidase showed appreciable upfield shifts upon pressurization. These results were interpreted to indicate that the primary effect of pressure on the hemoprotein structure is to shift the spin equilibrium in favor of the low-spin form. Hemichrome formation of methemoglobin at high pressures was also observed, and the effect of pressure on the heme environmental structure of deoxyhemoglobin and deoxymyoglobin was also discussed.

The effect of pressure on the physicochemical properties of hemoproteins has attracted considerable attention over the last decade. These studies have been concerned with the denaturation of protein (Zipp & Kauzmann, 1973) and changes in the functional properties and thermodynamic properties accompanying the ligand binding (Ogunmola et al., 1976). Electronic spectra were used to follow the changes of the heme environmental structures of various hemoproteins such as myoglobin, hemoglobin, cytochrome *c*, and horseradish peroxidase when these molecules are subjected to high hydrostatic pressures (Ogunmola et al., 1977; Zipp et al., 1972; Fabry & Hunt, 1968; Gibson & Carey, 1975). The visible and Soret spectral changes upon pressurization have been examined to show the drastic structural changes from native high-spin form (open crevice) to hemichromogen (closed crevice) in the low-spin state. However, these pressure-induced structural changes were concerned with the replacement of the ligand in the sixth coordination position with the distal histidyl imidazole, and the pressure-induced structural perturbations such as subtle changes in the profile of the iron–ligand bond have been missed by the UV[1] and visible spectroscopic method.

We have initiated the high-pressure and high-resolution NMR studies of hemoproteins to gain an insight into the structural changes in their heme environments (Morishima et al., 1979), in the hope that the pressure-induced subtle structural changes can be monitored by the shift of the hyperfine shifted proton resonances of the heme peripheral groups which have been proved to be a very sensitive probe for minute structural variation in the heme environments (Wüthrich, 1970; Morishima et al., 1978a). In the present report we wish to show that the primary effect of pressure on hemoprotein structure is to shift their spin equilibrium in favor of the low-spin form. Application of pressure up to 2000 atm to ferric hemoproteins in purely high- and low-spin states as well as to ferrous deoxy forms of myoglobin and hemoglobin also perturbs their heme environmental structures.

Materials and Methods

Human hemoglobin was prepared by the usual method (Beetlestone & Irvine, 1964) and oxidized to methemoglobin with twofold excess potassium ferricyanide. Ferrocyanide and excess ferricyanide were removed by dialysis against 0.05 M sodium chloride at pH 6.0. Metmyoglobin from sperm whale purchased from Sigma Chemical Co. (type II) was used without further purification. Deoxyhemoglobin and deoxymyoglobin were prepared by the action of dithionite. Horseradish peroxidase purchased from Toyobo (Tokyo) (type G-C-I) was used without further purification. All the protein samples for the NMR measurements were prepared in 50 mM phosphate–^2H_2O buffer at p^{2}H 7.5, and their concentrations were about 5 mM. We examined the sample quality of hemoproteins, particularly aquomethemoglobin and deoxyhemoglobin, before and after the NMR measurements at high pressures with the use of an optical absorption spectrometer.

We used a simple device designed by one of the authors (H.Y.) for high-pressure and high-resolution NMR performance which allows sample tube spinning and temperature variation (Yamada, 1974; Yamada et al., 1977, 1979). The high-pressure glass NMR cell (capillary) filled with the pressure indicator (a mixture of 95 mol % of phenylacetylene and 5 mol % of cyclohexane), purified mercury, and a sample solution is put in a safety jacket fitted with a 5-mm o.d. spinner. The glass cell with 1.6-mm i.d. and 3–3.3-mm o.d. is made of a usual laboratory glass tube or a Pyrex tube and is etched inside with a 5% aqueous hydrogen fluoride solution. Melting and thermal expansion of the pressure indicator sufficed to generate an inner pressure up to 2500 bars. The pressure indicator was frozen by immersing the glass cell in a liquid nitrogen bath, while an additional portion of the

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[1] Abbreviations used: NMR, nuclear magnetic resonance; IR, infrared; UV, ultraviolet; metHb, aquomethemoglobin; metMb, aquometmyoglobin.

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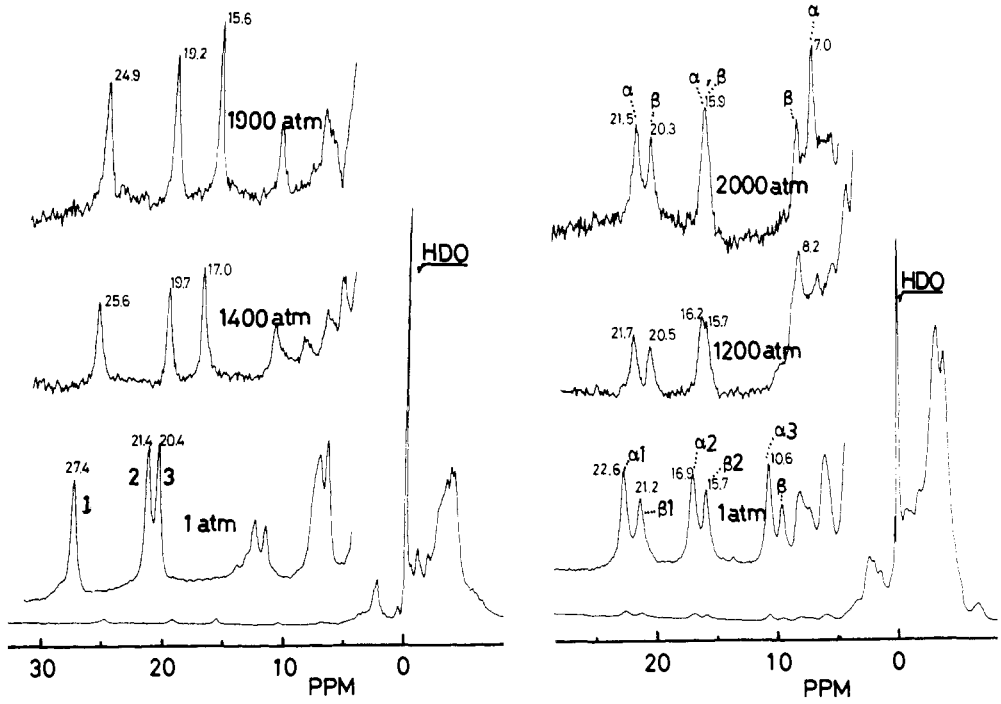

FIGURE 1: Hyperfine shifted proton NMR spectra of azide complexes of sperm whale metmyoglobin (left) and human methemoglobin (right) at high pressures. The spectra were obtained at pH 7.5 in 50 mM phosphate-^2H_2O buffer and 32 °C.

sample solution was sucked into the glass cell through an open-ended capillary, which was sealed by a microburner. By controlling the extent of freezing of the indicator, one can nearly generate the desired pressure in the cell. Estimation of the inner pressure was achieved by measurement of the chemical shift of the ethynyl proton of phenylacetylene whose pressure dependence was thoroughly investigated (Yamada et al., 1979). For this purpose, its chemical shift was measured in situ at an upside down position in the safety jacket.

Proton NMR spectra at 220 MHz were recorded at 32 °C with a Varian HR-220/Nicolet TT-100 spectrometer in a pulsed Fourier transform mode. The spectra were obtained by a 4K data point transform of 40-KHz spectral width after 16 000–64 000 pulses with a pulse repetition time of 0.051 s. The spectra at 1 atm were obtained with the usual 5-mm sample tube with spinning. The high-pressure spectra were recorded without sample spinning in the present study. Proton chemical shift is referenced with respect to the residual water proton signal in the sample solution.

The optical spectra at high pressures were obtained with a Union (Osaka) Model RA pressure cell with a range up to 3000 atm.

Results

Figure 1 illustrates the hyperfine shifted proton resonances of azide complexes of metmyoglobin (metMb·N_3^-) and methemoglobin (metHb·N_3^-) obtained at 220 MHz under several pressures. Upon pressurization, three of the four heme methyl proton signals of metMb·N_3^- at 27.4, 21.4, and 20.4 ppm at 1 atm shifted upfield in proportion to the applied pressures. This phenomenon was reversible with the application and the release of the pressure. A similar spectral change of metHb·N_3^- upon pressurization is seen in the Figure 1. Three heme methyl resonances of metHb·N_3^- appear in the downfield spectral region, accompanied by doubling for each signal due to nonequivalence of α and β subunits. Identification of the signals from the α and β subunits has been established (Davis et al., 1969; Morishima et al., 1978b) as depicted in the figure. The methyl proton signals of the α subunits preferentially

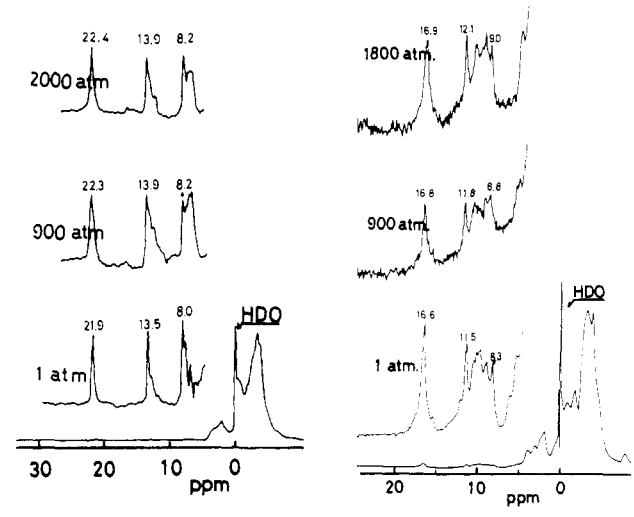

FIGURE 2: Proton NMR spectra of cyanide complexes of metmyoglobin (left) and methemoglobin (right) at high pressures at 32 °C and pH 7.5. The hyperfine shifted spectral regions are illustrated.

shifted upfield upon pressurization, the signals at 16.9 and 10.6 ppm exhibiting the most prominent effect.

Figure 2 shows the pressure effect on the spectra of cyanide complexes of metmyoglobin (metMb·CN^-) and methemoglobin (metHb·CN^-). No drastic shifts are seen for both cyanide complexes, although small but appreciable downfield shifts are observed for the hyperfine shifted signals upon pressurization.

In Figure 3 are demonstrated the pressure-induced spectral shifts for imidazole (metMb·Im) and pyrazole (metMb·pyrazole) complexes of metmyoglobin. For metMb·Im, the heme methyl proton signal at 11.6 ppm preferentially shifted upfield. A similar spectral feature is seen for the pyrazole complex upon pressurization. We have also examined the effect of pressure on the NMR spectrum of the imidazole complex of methemoglobin (metHb·Im) and found that there was no spectral shift upon pressurization up to 1000 atm, accompanied by a gradual decrease in the signal intensities

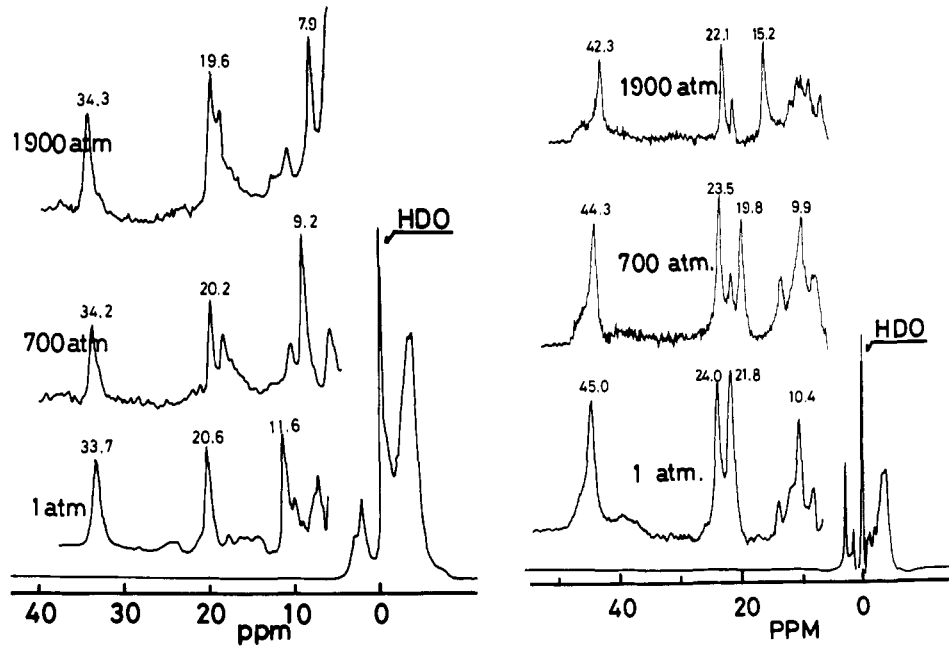

FIGURE 3: Proton NMR spectra of the imidazole complex of metmyoglobin (left) and the metmyoglobin pyrazole complex (right) at high pressures at 32 °C and pH 7.5.

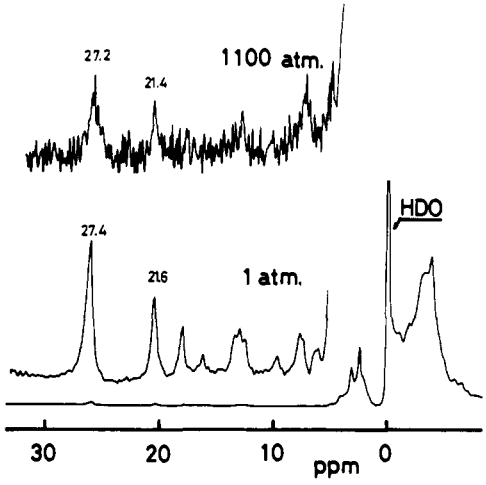

FIGURE 4: Proton NMR spectra of the imidazole complex of methemoglobin at 1 and 1100 atm at pH 7.5 and 35 °C. Upon pressurization above 1000 atm, the hyperfine shifted signals gradually lose their signal intensities, and at 2000 atm these signals disappeared from the hyperfine shifted spectral region.

(Figure 4). At 2000 atm, the hyperfine shifted signals of metHb·Im disappeared, suggesting that denaturation of the protein occurred at 2000 atm.

Figure 5 shows the proton spectra of metmyoglobin derivatives of fluoride (metMb·F^-) and formate (metMb·$HCOO^-$) in the ferric high-spin states at different pressures. The broadened four heme methyl signals for the fluoride complex are observed in the range 30–60 ppm. Upon pressurization up to 2000 atm, each methyl signal of the fluoride complex tends to shift downfield, while the heme methyl signals of the formate complex experience slight upfield shifts.

In Figure 6 is shown the effect of pressure on the NMR spectra of aquometmyoglobin (metMb) and aquomethemoglobin (metHb). With increasing pressure, the hyperfine shifted heme methyl resonances at 48.0, 67.4, 79.0, and 85.7 ppm for metMb at 1 atm appear to shift upfield, while methyl signals for metHb disappeared from the paramagnetically shifted spectral region at 2000 atm. In the intermediate pressure region, the hyperfine shifted signals of metHb decreased in their signal intensities compared to those of the 1-atm spectrum. The effect of pressure on the spectrum of horseradish peroxidase (HRP) was similar to that for metMb as shown in Figure 7. The heme methyl proton signals of horseradish peroxidase shifted slightly upfield upon pressurization.

We have also studied at high pressures the NMR spectra of deoxyhemoglobin and deoxymyoglobin as shown in Figure 8. Increased pressure up to 2000 atm shifted the heme methyl signals of deoxyhemoglobin to the upfield side, while a small upfield shift of a heme methyl signal of deoxymyoglobin was seen upon pressurization. At 2000 atm deoxyhemoglobin exhibited no signals in the hyperfine shifted spectral region, suggesting denaturation of the protein at this pressure. It is also noticeable that the hyperfine shifts of the heme methyl resonances at high pressure of deoxyhemoglobin are quite similar to those of deoxymyoglobin, although they are very much different at 1 atm. The signals at 10.7, 8.9, and 5.9 ppm for deoxyhemoglobin at 1100 atm correspond to those at 17.6, 12.1, and 7.6 ppm at 1 atm, respectively, which have been assigned to the heme methyl resonances for β, α, α subunits (Johnson & Ho, 1974). The behavior of deoxyhemoglobin under pressure differs from that of deoxymyoglobin in the way that pressure needed to effect the NMR spectral change is much less for deoxyhemoglobin than is required for deoxymyoglobin, as is the case for metMb and metHb.

Discussion

Pressure-Induced Displacement of the High-Spin–Low-Spin Equilibrium. metMb·N_3^-, metHb·N_3^-, and metMb·Im are in thermal equilibrium between high- and low-spin ferric states, while metMb·CN^-, metHb·CN^-, and metHb·Im are in purely low-spin states and fluoride and formate complexes of these hemoproteins are in ferric high-spin states (Smith & Williams, 1968; Beetlestone & George, 1964; Iizuka & Kotani, 1969). Present NMR observations that only metMb·N_3^-, metHb·N_3^-, and metMb·Im showed a striking spectral shift upon pressurization and others did not indicate that the pressure-induced spectral shift is most probably due to displacement of the high-spin and low-spin equilibrium toward the low-spin state. The upfield bias of the hyperfine shifted heme methyl reso-

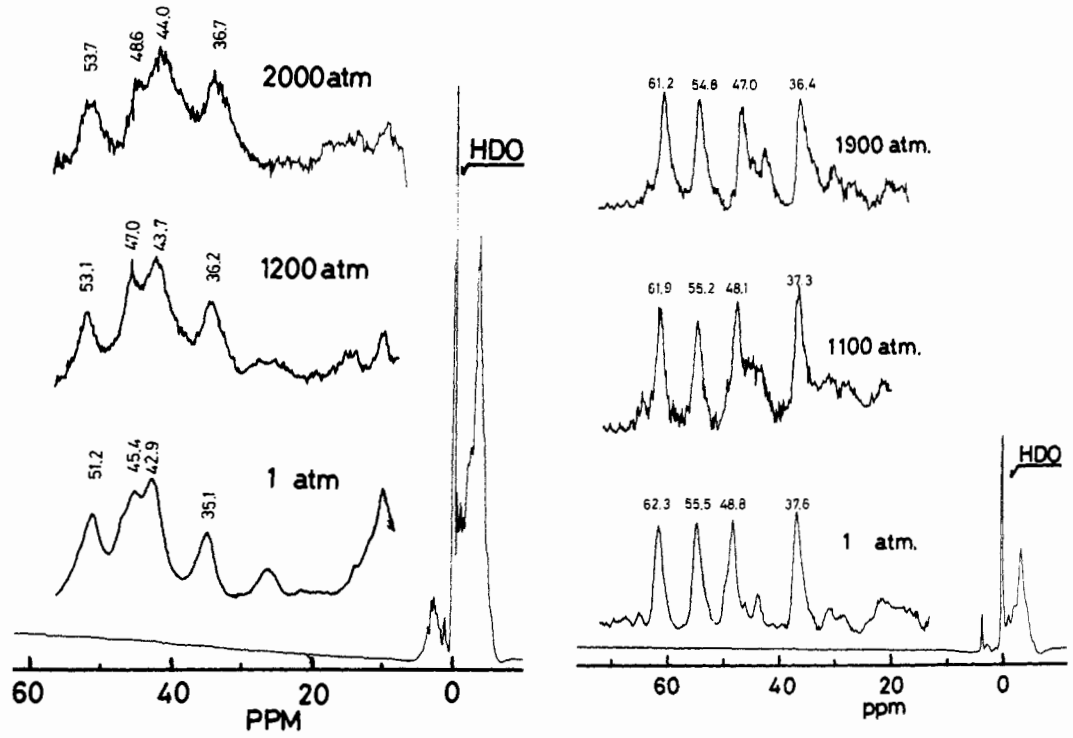

FIGURE 5: High-pressure proton NMR spectra of fluoride (left) and formate (right) complexes of metmyoglobin at 32 °C and pH 7.5.

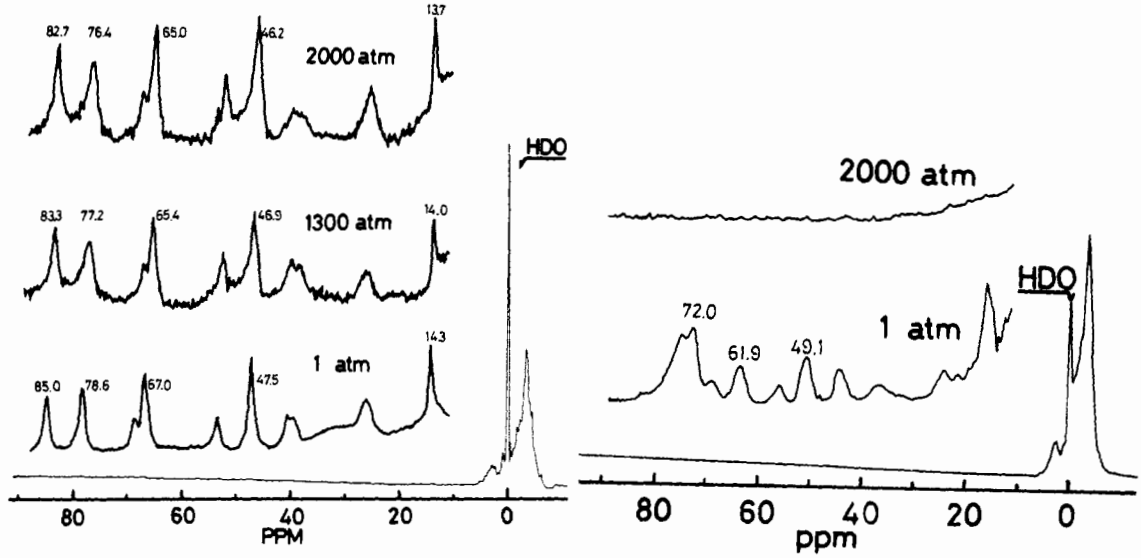

FIGURE 6: High-pressure proton NMR spectra of aquometmyoglobin (left) and aquomethemoglobin (right) at 32 °C and pH 7.5. The hyperfine shifted spectral regions are illustrated. At 2000 atm, the signals of methemoglobin disappeared from the spectral region.

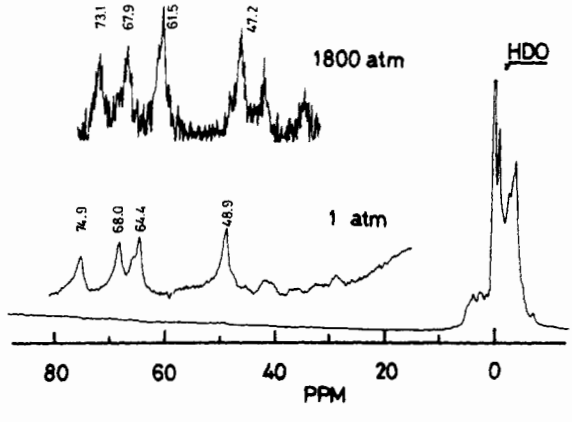

FIGURE 7: Proton NMR spectra of horseradish peroxidase at 1 and 1800 atm at pH 7.5 and 32 °C.

nances for metMb·N_3^-, metHb·N_3^-, and metHb·Im at high pressures is believed to reflect an increase in the amount of low-spin content of their iron atoms. This was also confirmed by the optical absorption spectra at high pressures. The high-pressure optical spectra up to 3000 atm were obtained for various methemoglobin and metmyoglobin derivatives at 23 °C and pH 7.0. With increasing pressure, the low-spin bands at 540 and 580 nm for metMb·N_3^- increased in their intensities with concomitant decrease in the intensity of a high-spin band at 635 nm. The optical spectral change of the azide complex with increasing pressure from 1 to 2000 atm corresponded to an increase in the low-spin content of the heme iron atom from ~80 to ~90%.

The spin equilibrium of hemoproteins is modulated by temperature variation as studied by magnetic susceptibility (Iizuka & Kotani, 1969), IR (Alben & Fager, 1972), optical

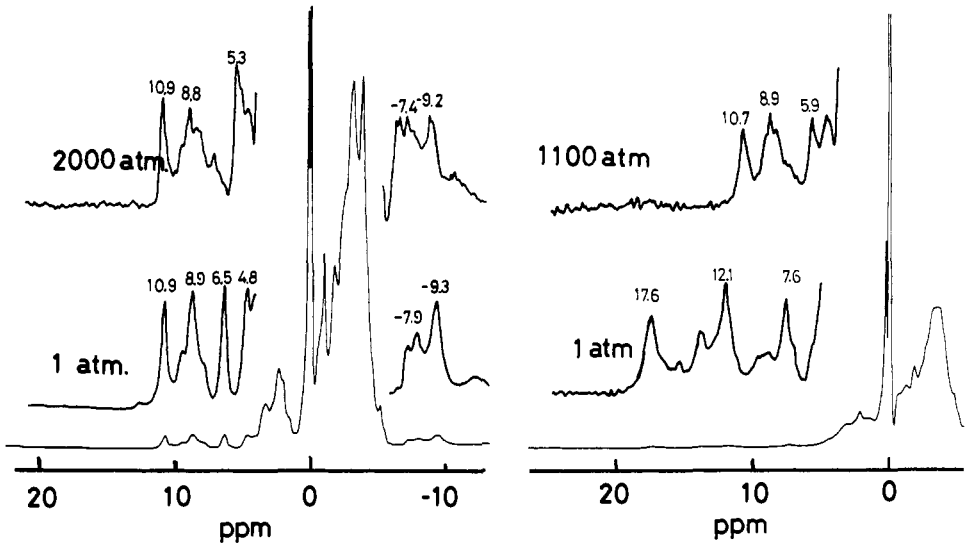

FIGURE 8: High-pressure proton NMR spectra of deoxymyoglobin (left) and deoxyhemoglobin (right) at pH 7.5 and 32 °C. The hyperfine shifted spectral regions are illustrated. The increased pressure shifted the proton signals of deoxyhemoglobin to the upfield side, and at above 2000 atm these signals disappeared from the hyperfine shifted spectral region.

Table I: Low-Spin and High-Spin Contents and Observed and Simulated Hyperfine Shifts (ppm) of the Heme Methyl Signals for Azide Complexes of Metmyoglobin (metMb·N_3^-) and Methemoglobin (metHb·N_3^-) at 1 atm and High Pressures at 32 °C

hemoprotein	pressure (atm)	spin content (%) low spin	spin content (%) high spin	obsd chemical shift (ppm) 1	2	3	simulated chemical shift[a] (ppm) 1	2	3
metMb·N_3^-	1	80	20	27.4	21.4	20.4	27.3	22.4	20.6
	2000	93	7	24.9	19.2	15.6	24.3	19.9	15.7

hemoprotein	pressure (atm)	spin content (%) low spin	spin content (%) high spin	obsd chemical shift (ppm) α1	β1	α2	β2	α3	simulated chemical shift[a] (ppm) α1	β1	α2	β2	α3
metHb·N_3^-	1	82	18	22.6	21.2	16.9	15.7	10.6	22.5	21.0	16.7	15.7	10.3
	1900	86	14	21.5	20.3	15.9	15.9	7.0	21.7	20.5	16.1	15.3	8.8

[a] Calculated with the use of the relation: $(\text{shift})_{\text{spin equil}}$ = (low-spin limiting shift) × (low-spin content) + (high-spin limiting shift) × (high-spin content).

absorption spectra (Smith & Williams, 1968; Beetlestone & George, 1964), NMR (Iizuka & Morishima, 1974), and many other methods. However, the effect of pressure on the spin equilibrium has not been studied except for the optical absorption studies of aquometmyoglobin at alkaline and intermediate pH region under high pressure (Ogunmola et al., 1977). The NMR hyperfine shifts are sensitive to follow the displacement of the spin equilibrium in hemoproteins, since the heme methyl hyperfine shifts of the low-spin hemoprotein are small compared with those of the high-spin one. In our earlier work, we separated the observed hyperfine shifts of each heme methyl proton resonance for metMb·N_3^-, metMb·Im, and metHb·N_3^- into the limiting shifts in purely high- and low-spin states by analyzing the temperature-dependent hyperfine shifts (Iizuka & Morishima, 1974; Morishima et al., 1978b). With these limiting shifts at 33 °C (Table I), the three methyl resonance positions for metMb·N_3^- are plausibly reproduced when we assume that the low-spin content is increased with pressure from 80% at 1 atm to 93% at 2000 atm (Table II). This low-spin content of the azide complex obtained by the simulation of the NMR hyperfine shifts appears to agree with the results by the present optical absorption measurement at high pressures.

The shift of the spin equilibrium with pressure in favor of the low-spin state for metMb·N_3^- may be caused either by a conformational change accompanying a compression of the ligand–iron bond or by replacement of the iron sixth ligand by a distal base such as the E-7 histidyl imidazole. The NMR spectral method may be more suitable than the optical absorption method to distinguish these causes, because these structural changes at the heme crevice site would be reflected in the spectrum as the shift of the heme methyl resonances (fast transition on NMR time scale) or as the entire spectral change (slow transition on NMR time scale), respectively. The slow transition would be associated with the ligand exchange process at the sixth coordination site. Thus, the NMR spectral changes in Figures 1 and 3 suggest that what we observed for metMb·N_3^-, metHb·N_3^-, metMb·Im, and the pyrazole complex of metMb with varying pressure may be due to the compression effect on the ligand–iron binding, which is responsible

Table II: Effect of High Pressure on the High-Spin–Low-Spin Equilibrium

hemoprotein	pressure (atm)	low-spin content (%)	high-spin content (%)	ΔV (mL/mol) at 32 °C
MB·N_3^-	1	80	20	−15
	2000	93	7	
HB·N_3^-	1	82	18	−5
	1900	86	14	

for the shift of the high- and low-spin equilibrium in favor of the low-spin form.

It is of interest to note that the pressure-induced shift of the heme methyl resonances for metHb·N_3^- is different between α and β subunits, as is shown in Figure 1. The proton signals of the α subunit shifted upfield upon pressurization, whereas the signals of the β subunit are almost insensitive to the changes in pressure up to 2000 atm. It is also worth noting that the pressure-induced shift for metHb·N_3^- is much less than that for metMb·N_3^-. The question then may arise whether these different behaviors of the pressure-induced spectral shifts between α and β subunits of methemoglobin azide and between metMb·N_3^- and metHb·N_3^- are due to the difference in the extent of the pressure-induced increase of their low-spin contents or to their different limiting shifts for the heme methyl proton signals in purely high- and low-spin states or to a combination of both. In order to answer this question, we tried to analyze the pressure-induced NMR shifts for metHb·N_3^- as the case for metMb·N_3^- with recourse to the limiting shifts of the heme methyl resonances in purely high- and low-spin states obtained in our earlier work (Morishima et al., 1978b). The methyl hyperfine shifts both for α and β subunits of metHb·N_3^- were well simulated if the low-spin contents of the α and β heme are assumed to be 82% at 1 atm and 86% at 1900 atm (Tables I and II). In the above analysis for the metmyoglobin azide, increased pressure from 1 to 2000 atm shifted its low-spin content from 80 to 93%. Thus, the different behavior of the pressure-induced spectral shifts between metMb·N_3^- and metHb·N_3^- may predominantly result from the difference in the extent of the pressure-induced increase of their low-spin contents. On the other hand, the difference in the methyl proton-limiting shifts of the purely high- and low-spin states of the α and β subunits of metHb·N_3^- appears to be responsible for their different pressure-induced shifts of the proton NMR signals. The above analysis for metHb·N_3^-, however, is based on the assumption that the heme iron atoms in the α and β subunits have the same spin content, which would not necessarily be the case. Therefore, we could not exclude the possibility that the different pressure-induced shifts between the subunits of metHb·N_3^- may result from the different pressure dependence of their spin equilibrium.

We have also analyzed the pressure-induced proton shifts for metMb·Im in terms of a shift of the spin equilibrium. However, the chemical shifts of the methyl signals at high pressures were not reproduced by assuming the appropriate amount of spin mixing. This indicates that pressure-induced shifts for the methyl signals of metMb·Im are caused by the shift of the spin equilibrium and by some other structural perturbations in the heme environment, probably by a steric hindrance of the iron-bound imidazole by pressure. The heme environmental structures of metMb·Im in purely high- and low-spin states at 1 atm may be different from those in purely high- and low-spin states at high pressures.

The volume change associated with the pressure-induced high-spin and low-spin conversion in the spin equilibrium of the hemoproteins may be calculated by the relation

$$\partial \ln K/\partial p = -\Delta V/(RT)$$

where T is the absolute temperature, R is the gas constant, and K is the equilibrium constant for the spin equilibrium system. The calculated ΔV values are given in Table II. The minus sign indicates that contraction of the volume of the hemeproteins occurs upon pressurization.

The volume change obtained here for metMb·N_3^- is comparable with the one (−11 mL/mol) for the azide binding reactions by metHb and metMb obtained by Ogunmola et al. (1977), where the high-spin and low-spin conversion occurs in the azide exchange reaction at the sixth coordination site of these hemoproteins. The volume change of the order of −10 mL/mol is much larger than that associated with the simple movement of the ferric iron into the heme plane when it changes from the high-spin to the low-spin state. The observed volume change reflects changes on the protein molecule other than that in the heme group. It is therefore likely that the conversion of the high-spin heme into the low-spin heme is accompanied by an overall contraction of the protein.

Pressure-Induced Structural Perturbation in the Heme Environments of Low-Spin and High-Spin Ferric Complexes of Metmyoglobin, Methemoglobin, and Horseradish Peroxidase. It is revealed in Figures 2, 4–6, and 7 that metMb and metHb and their complexes with low-spin and high-spin ligands such as CN^-, H_2O, F^-, and $HCOO^-$ experience a small amount of the pressure-induced shifts for the hyperfine shifted heme methyl resonances. This is in sharp contrast with the results for their azide and imidazole complexes in the spin equilibrium states. Aquometmyoglobin, its formate ($HCOO^-$) complex, and horseradish peroxidase tend to exhibit a slight upfield shift upon pressurization, while small downfield shifts were observed for the cyanide complexes and fluorometmyoglobin. It is to be noted here that cyanide and fluoride complexes of metMb and metHb are in purely low- and high-spin states, respectively, while metMb and its formate complex are in spin equilibrium with a large high-spin content (89% for metMb and 86% for the formate complex) at room temperature (Smith & Williams, 1968). It is then likely that pressure-induced small upfield shifts of the heme methyl proton signals for metMb and the formate complex are caused by the shifts of their spin equilibrium by pressure in favor of the low-spin form. Like the case of these metmyoglobins in nearly high-spin states, the increased pressure up to 2000 atm shifted slightly the heme methyl signals of horseradish peroxidase to the upfield side. This suggests that horseradish peroxidase may be in the thermal spin equilibrium between high- and low-spin states with a large high-spin content at room temperature or in a quantum spin mixed state between $S = 5/2$ and $S = 3/2$ as pointed out by Maltempo et al. (1979). However, higher pressures than those employed here may be needed to induce the spin-state change of horseradish peroxidase.

Although the high-pressure optical spectroscopic study has indicated that cyanide ion prevents pressure-induced spectral change in metMb up to 8000 atm (Ogunmola et al., 1977), the present NMR study at high pressures up to 2000 atm clearly showed pressure-induced subtle structural changes of the heme vicinity of the cyanide complex of metMb. The increased pressure appreciably shifted the heme methyl signals of the cyanide complexes of metMb and metHb and of the fluoride complex of metMb to the downfield side. Changes in the microenvironmental structure at the heme coordination site such as the changes in the orientation of the proximal base to the heme plane, the strength of the iron–proximal histidine binding, and the interaction between the distal histidine and the coordinated ligand may be responsible for the pressure-induced downfield shifts of the heme methyl signals of these metHb and metMb derivatives in purely low-spin or purely high-spin states.

Hemichrome Formation of Methemoglobin at High Pressure. Our observation of the drastic spectral change of metHb upon pressurization may be associated with a slow ligand exchange process at the heme sixth coordination position accompanied by high-spin to low-spin conversion. This finding

appears to be in accord with the results of the visible spectroscopic study of metHb at high pressures that metHb is converted from the ferric high-spin form to the ferric low-spin form in which the distal histidyl imidazole displaces the H_2O ligand to form the so-called hemichrome. The hyperfine shifted resonances of the heme methyl protons of the hemichrome in the ferric low-spin state might be hidden under the envelope of the protein resonances in the diamagnetic region. This is, however, to be compared with the hyperfine shifted spectrum of metHb·Im in the ferric low-spin state (Figure 4). The heme methyl proton signals of the imidazole complex are seen at 27.4 and 21.6 ppm. Although the structural feature in the heme site is similar between hemoglobin hemichrome and metHb·Im, the structure of the heme coordination site being imidazole–iron(III)–proximal histidine for both hemoglobin derivatives, different proton NMR spectra between hemichrome and the imidazole complex may indicate that the structure of the iron coordination site is quite different between them. The orientation of the proximal and distal histidines to the heme plane or the coordination strength of the distal and proximal histidines to the ferric iron appears to be different in these two hemoglobin derivatives.

Effect of Pressure on the NMR Spectra of Deoxyhemoglobin and Deoxymyoglobin. For deoxyhemoglobin and deoxymyoglobin, whose ferrous iron atoms are in purely high-spin ($S = 2$) states, the heme methyl signals shifted upfield upon pressurization, suggesting that conformational lability is different between the ferric and ferrous hemoproteins. For deoxymyoglobin, the signal at 6.5 ppm at 1 atm preferentially shifted upfield upon pressurization, whereas, for deoxyhemoglobin, all the signals shifted upfield. This indicates that the pressure needed to effect the spectral change is much less for deoxyhemoglobin than for deoxymyoglobin. These changes in the pressure-induced shifts of the heme methyl signals of deoxyhemoglobin and deoxymyoglobin may be caused by a structural change in the five-coordinated heme, possibly by the change in the displacement of the iron atom from the heme plane. It is of interest to note that the hyperfine shifted spectrum of deoxyhemoglobin at 1100 atm is similar to that of deoxymyoglobin at 1 atm, indicating that the heme environmental structure of deoxyhemoglobin in its contracted form under high pressure resembles that of deoxymyoglobin. It is also noted in the spectra of deoxyhemoglobin that the heme methyl signals of β subunit is much more sensitive to the change in pressure than that of the α subunit. This suggests that the β subunit in the tetrameric hemoglobin is more susceptible for the structural changes in the heme coordination site.

In summary, the present high-pressure NMR study firmly established that the primary effect of pressure on hemoprotein structure is to shift their spin equilibrium between high- and low-spin states in favor of the low-spin state. The present study also showed that the heme environmental structure of hemoglobin hemichrome is much different from that of metHb·Im. The heme environmental structure of deoxyhemoglobin is also sensitive to the change in pressure.

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