Humason, G. L. (1972) in *Animal Tissue Techniques* (Freeman, W. H., Ed.) pp 1-68, Academic Press, San Francisco, CA.
Ivanova, E., Pironcheva, G., & Djondjurov, L. (1981) *Eur. J. Biochem. 113*, 569-573.
Jackson, V., Shires, A., Tanphaichitr, N., & Chalkley, R. (1976) *J. Mol. Biol. 104*, 471-483.
Jahn, C., & Litman, G. (1979) *Biochemistry 18*, 1442-1449.
Kaneko, M., & Gerutti, P. A. (1980) *Cancer Res. 40*, 4313-4319.
Kelley, R. I. (1973) *Biochem. Biophys. Res. Commun. 54*, 1588-1594.
Kornberg, R. D., & Thomas, J. O. (1974) *Science (Washington, D.C.) 184*, 865-869.
Lieberman, M. W., & Forbes, P. D. (1973) *Nature (London), New Biol. 241*, 199-201.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. Y. (1951) *J. Biol. Chem. 193*, 265-275.
Melli, M., Spinelly, G., & Arnold, E. (1977) *Cell (Cambridge, Mass.) 12*, 167-174.
Ohba, Y., Hayashi, K., Nakagawa, Y., & Yamaguchi, Z. (1975) *Eur. J. Biochem. 56*, 343-352.
Oliver, D., Granner, D., & Chalkley, R. (1974) *Biochemistry 13*, 746-749.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337-346.
Pegg, A. (1977) *Adv. Cancer Res. 25*, 195-269.
Pehrson, J. R., & Cole, R. D. (1982) *Biochemistry 21*, 456-460.
Piha, R. S., Guenod, M., & Waelsch, H. (1966) *J. Biol. Chem. 241*, 2397-2404.
Price, G. B., Modak, S. P., & Makinodan, T. (1971) *Science (Washington, D.C.) 171*, 917-920.
Sanders, M. M. (1981) *J. Cell Biol. 91*, 579-583.
Schimke, R. T. (1975) in *Intracellular Protein Turnover* (Schimke, R. T., & Katunuma, N., Eds.) pp 173-186, Academic Press, New York, San Francisco, and London.
Sperling, R., & Bustin, M. (1975) *Biochemistry 14*, 3322-3331.
Sperling, R., & Wachtel, E. (1981) *Adv. Protein Chem. 34*, 1-52.
Stegeman, H. (1960) *Hoppe-Seyler's Z. Physiol. Chem. 319*, 102-109.
Stein, G., Park, W. D., Stein, J. L., & Lieberman, M. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1466-1470.
Stocker, E., Schultze, B., Heine, W.-D., & Liebscher, H. (1972) *Cell Tissue Res. 125*, 306-331.
Tarnowka, M. A., Baglioni, C., & Basilico, C. (1978) *Cell (Cambridge, Mass.) 15*, 163-171.
Tsanev, R. (1981) in *Eurkaryotic Gene Regulation* (Kolodny, G. M., Ed.) Vol. II, pp 57-112, CRC Press, Boca Raton, FL.
Van Lente, F., Jackson, J. F., & Weintraub, H. (1975) *Cell (Cambridge, Mass.) 5*, 45-50.
Westra, J., Kriek, E., & Hittelhausen, H. (1976) *Chem.-Biol. Interact. 15*, 149-164.
Wu, R., & Bonner, W. M. (1981) *Cell (Cambridge, Mass.) 27*, 321-330.
Yancheva, N., & Djondjurov, L. (1982) *Eur. J. Biochem. 121*, 309-316.

High-Pressure Nuclear Magnetic Resonance Studies of Hemoproteins. Pressure-Induced Structural Changes in the Heme Environments of Ferric Low-Spin Metmyoglobin Complexes†

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ABSTRACT: In order to gain an insight into nonbonded interactions in the heme microenvironments of hemoproteins, proton NMR spectra of the cyanide and methylamine complexes of metmyoglobin and its derivatives reconstituted with deutero- and *meso*-hemins in H_2O were studied under high pressures. The exchangeable NH proton of distal histidyl imidazole exhibits substantial pressure-induced shift while the proximal histidyl NH proton shows no pressure effect for the cyanide complexes. The heme peripheral proton signals, especially 5- and 8-methyl and vinyl $C_\alpha H$ resonances, were also affected by pressure. These observations are interpreted as arising from pressure-induced structural changes in the heme crevice in which the pressure effects are localized to the distal side rather than the proximal side and from possible changes in the van der Waals contacts at the heme periphery with nearby amino acid residues.

The heme group in hemoprotein is buried in the heme crevice made up by the three-dimensional arrangements of the polypeptide chain by covalent or coordinative bonds and by a multitude of weak bonds, i.e., hydrogen bonds, ionic bonds, and van der Waals interactions. The structure of polypeptide chain and the heme group in hemoprotein molecules are closely related to their functions. The importance of the heme–apoprotein interactions is underscored by their proposed focal role in modulating the reactivity of the iron center. Of particular interest are the roles of the heme proximal ligand and the heme distal amino acid residues at the heme proximal, distal, and peripheral sides in modulating the heme electronic structures and ligand exchange phenomena. These primary

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roles played by covalent and noncovalent interactions in the heme vicinity make them attractive for structural studies of hemoproteins by NMR[1] under high pressures.

The effect of pressure on the hemoprotein structures has been the subject of considerable investigations in which drastic structural alterations such as pressure–denaturation (Zipp & Kauzmann, 1973) and the open to close heme crevice structural changes (Ogunmola et al., 1977) are detected by the use of UV–visible spectral studies under pressure. However, we have demonstrated that high-pressure NMR provides a more powerful tool to delineate pressure-induced subtle structural changes of hemoproteins (Morishima et al., 1979, 1980; Morishima & Hara, 1982).

We wish to report here some details of the direct evidence for pressure-induced subtle structural changes of the protein in heme environments of ferric low-spin metmyoglobin complexes. We followed the proton NMR spectrum of horse and sperm whale cyanometmyoglobin (MbCN) as well as the methylamine complex of myoglobin in H_2O at various pHs and pressures with attention to the hyperfine-shifted proton resonances of heme peripheral groups and exchangeable NH signals of distal and proximal histidyl residues which are sensitive probes for minute structural variation in the heme environments. The present high-pressure NMR study could serve as a quite powerful method in the study of structure–function relationships of hemoproteins. The preliminary study has been published (Morishima & Hara, 1982).

Experimental Procedures

Materials. Horse heart myoglobin (Sigma type III) and sperm whale myoglobin (Sigma type II) were dissolved in 0.1 M Tris-HCl buffer. The pH of Tris-HCl buffer has been shown to be independent of pressure up to 4000 atm (Newmann et al., 1973). Therefore, we used Tris-HCl buffer throughout the present high-pressure NMR study. Myoglobins reconstituted with deutero- and *meso*-hemins were prepared by following the reported procedures (Antonini et al., 1964). The solution was centrifuged to remove any precipitate, and a 9-fold excess of NaCN or methylamine hydrochloride was added. All chemicals were reagent grade and were used without further purification. The protein concentration was about 7 mM in each of the samples. The pH value was adjusted by careful addition of 0.1 M NaOH or HCl successively to the solution, and the pH value was found by direct reading of the pH meter (radiomater) equipped with a microelectrode (Ingold).

Methods. We used the high-pressure NMR glass cell (capiallary) filled with the pressure indicator (a mixture of 99 mol % of phenylacetylene and 1 mol % of cyclohexane) in the safty jacket which can be spun. The details of the use of this simple device are noted in our previous paper (Morishima et al., 1980). Proton NMR spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Typical spectra of cyanometmyoglobin consisted of 40000 transients with 8K data points and 5.8-μs 90° pulse after the strong solvent resonance in H_2O solution was suppressed by a 500-μs low-power 180° pulse. Redfield 2-1-4 spectra were recorded by an 8K data point transform of the 5K bandwidth after 2000 pulses with a pulse width of 21.5 μs. The spectra at 1 atm were obtained with the usual 5-mm sample tube with spinning. Since we used a glass capillary (1 mm i.d.) for the high-pressure NMR cell, the effect of spinning of the high-pressure cell on the hyperfine-shifted signal line width was not noticeable. Therefore, we did not spin the high-pressure NMR cell in the present study. Proton shifts were referenced with respect to the water proton signal. Estimation of the inner pressures was achieved by measurement of the chemical shift of the ethynyl proton of phenylacetylene with respect to the internal reference of cyclohexane, which was precalibrated (Yamada et al., 1981).

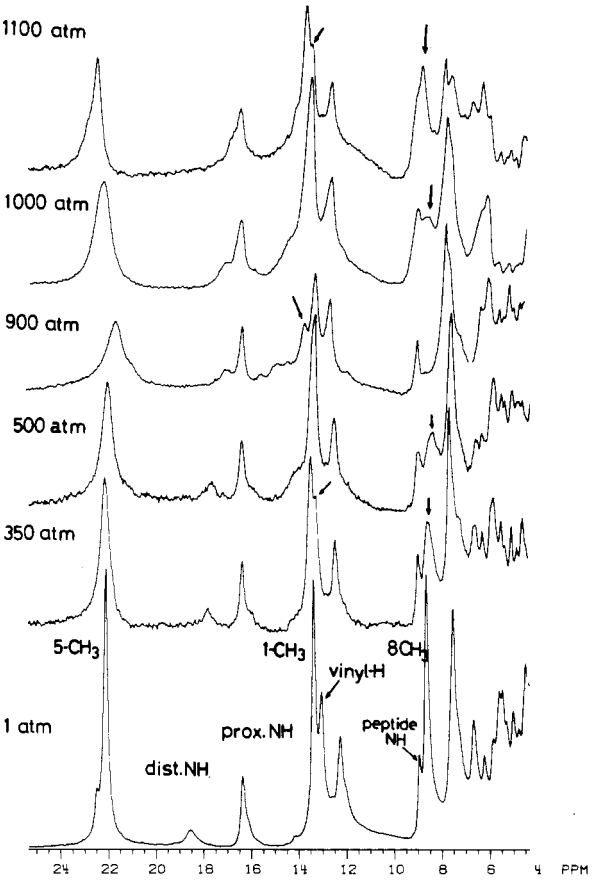

FIGURE 1: Pressure dependence of proton NMR spectra for horse MbCN at 30 °C, pH 7.8, in 0.1 M Tris-HCl. The numbering of the heme methyl groups is shown in Figure 5.

Results

Figure 1 illustrates the hyperfine shifted portions of the proton NMR spectra of horse MbCN in Tris-HCl buffer, pH 7.8, in H_2O at various pressures. The previously determined heme methyl assignments (Mayer et al., 1974) are included in Figure 1. There appear three paramagnetically shifted exchangeable peaks at 18.6, 16.4, and 8.9 ppm, which have been assigned to the distal histidyl (E7) NH proton, proximal histidyl (F8) NH proton, and proximal histidine (F8) peptide NH proton, respectively (Sheard et al., 1970; La Mar et al., 1981). As the pressure is increased from 1 to 1100 atm, the distal histidyl NH resonance exhibits progressively substantial upfield shift from 18.6 to 16.4 ppm while the proximal histidyl NH and peptide NH resonances are insensitive to pressure. Another interesting feature in Figure 1 is the specific shift accompanied by broadening of the heme methyl resonances, especially the 8-methyl signal at 8.7 ppm, upon pressurization. With increasing pressure from 1 to 900 atm, the 8- and 5-methyl peaks exhibit sizable upfield shift accompanied by broadening. When pressure is further increased above 1000 atm, these spectral changes for the two methyl resonances appear to return to those for lower pressures, with the distal histidyl NH resonance exhibiting a progressive upfield shift.

[1] Abbreviations: NMR, nuclear magnetic resonance; MbCN, cyanometmyoglobin; Tris, tris(hydroxymethyl)aminomethane.

Table I: Pressure-Dependent Shifts of the Hyperfine-Shifted Proton Resonances for Cyanide Complex of Horse Heart Ferric Myoglobin in Tris Buffer Solution at pH 7.8 and 30 °C

peak assignment	pressure (atm)						
	1	350	500	800	900	1000	1100
5-CH_3	22.2	22.1	22.0	21.9	21.7	22.3	22.5
His E7 N_3H (distal)	18.6	17.8	17.5	17.2	16.9	16.8	16.4
His F8 N_1H (proximal)	16.4	16.3	16.3	16.2	16.3	16.4	16.4
1-CH_3	13.4	13.4	13.3	13.3	13.2	13.6	13.7
vinyl $C_\alpha H$	13.1	13.2			13.7		13.4
His F8 peptide NH	8.9	8.9	8.9	8.9	8.9	9.0	
8-CH_3	8.7	8.5	8.3			8.6	8.8

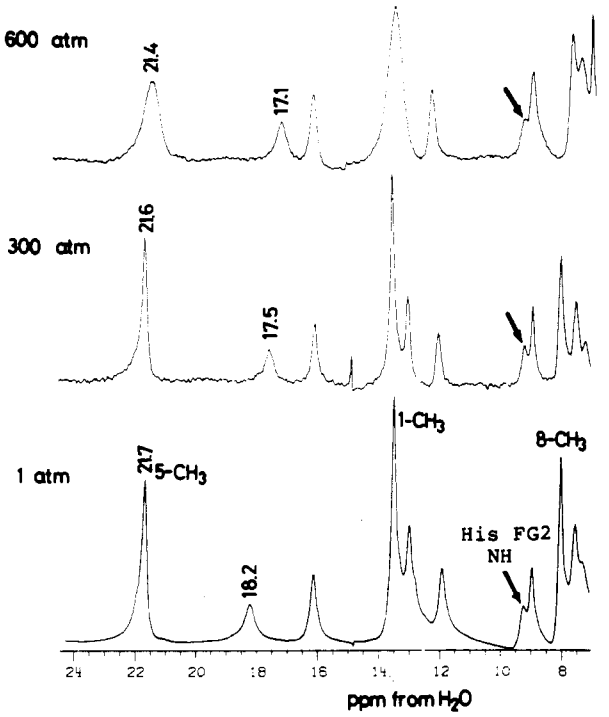

FIGURE 2: Pressure dependence of Redfield 2-1-4 spectra at 300 MHz for sperm whale MbCN at 35 °C, pH 7.8, in 0.1 M Tris-HCl.

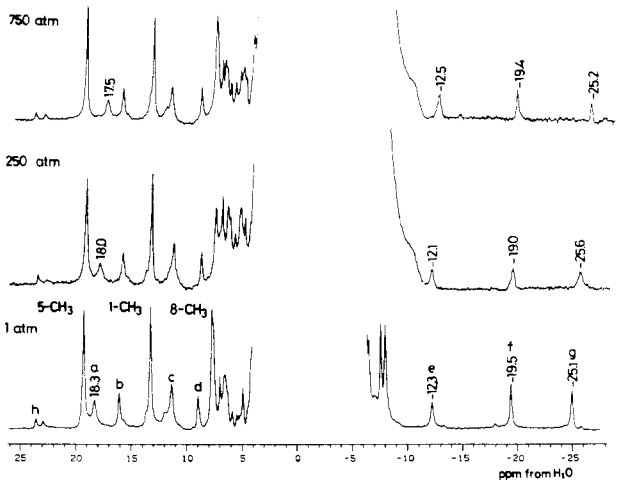

FIGURE 3: Pressure dependence of proton NMR spectra for sperm whale deutero-MbCN at 30 °C, pH 7.8, in 0.1 M Tris-HCl.

The single proton peak at 13.1 ppm, previously assigned to the vinyl $C_\alpha H$ (Shulman et al., 1969), shows noticeable downfield shift upon pressurization up to 900 atm and beyond this pressure seems to move upfield toward the signal position at 1 atm. Pressure-dependent shifts of the hyperfine-shifted resonances for horse MbCN at pH 7.8 are summarized in Table I.

We have also examined the pressure dependence of the proton NMR spectra for sperm whale MbCN at pH 7.8 and 30 °C (the result is not shown). Pressure-induced spectra for sperm whale MbCN are almost the same as those of horse MbCN except that the 5-methyl resonance did not experience pressure-induced broadening, in contrast to horse MbCN exhibiting signal broadening. At a somewhat higher temperature of 35 °C, however, the 5-methyl resonance for sperm whale MbCN exhibited broadening upon pressurization, as shown in Figure 2. The peak at 9.3 ppm, which was assigned to the His FG2 N_3H proton (La Mar et al., 1981), is insensitive to pressure in the pH range 7.8–8.2.

NMR spectra for the cyanide complex of sperm whale deuteromyoglobin (the vinyl groups at the 2- and 4-positions of the heme are replaced by a proton) at pH 7.8 are presented in Figure 3. Pressure-induced spectral changes for deutero-MbCN is almost the same as that of native sperm whale MbCN. The distal histidyl N_3H signal a shifts upfield at elevated pressures, while the proximal histidyl N_1H peak b remains unchanged. Peaks f and g, which were assigned to 2,4-protons (Shulman et al., 1969), experience noticeable pressure-induced shifts. In the case of deuteromyoglobin, rotation of the low symmetry perturbation about the α–γ meso axis of deuterohemin results in interchange of environments of 1-methyl with 3-methyl and 5-methyl with 8-methyl (La Mar et al., 1978). The two sets of proton signals are therefore attributed to two forms of deutero-MbCN in the different orientations of the deuterohemin that are interconvertible. Peak h in Figure 3, which was assigned to the 8-methyl of the reversed orientation with the prophyrin rotated 180° in the heme pocket about the α–γ meso axis, remains unchanged upon pressurization. This observation appears to correspond with the pressure-independent behavior of the 5-methyl signal in the normal orientation as found in the native protein. Peak i, which was also assigned to the 3-methyl or the reversed orientation of deuterohemin, is insensitive to pressure, as expected from the absence of the pressure effects on the 1-methyl resonance for the normal orientation.

We have also examined high-pressure NMR for the cyanide complex of sperm whale *meso*-myoglobin (the vinyl group at the 2- and 4-positions of the heme are replaced by ethyl groups) at pH 7.8 in Tris-HCl (the result is not shown). The entire hyperfine-shifted spectrum was changed for *meso*-MbCN upon pressurization, suggesting that replacement of a vinyl group by a bulkier ethyl group significantly affects the heme environmental structure at high pressure. The details of the effects of 2,4-substitution of the heme on the pressure-dependent structural changes of the heme environments are under investigation and will be reported afterward.

Figure 4 illustrates the pressure dependence of the proton NMR spectra for horse MbCN at pH 8.2 in H_2O. The 5- and 8-methyl resonances exhibit progressive broadening with increasing pressure. With reference to the integrated intensity

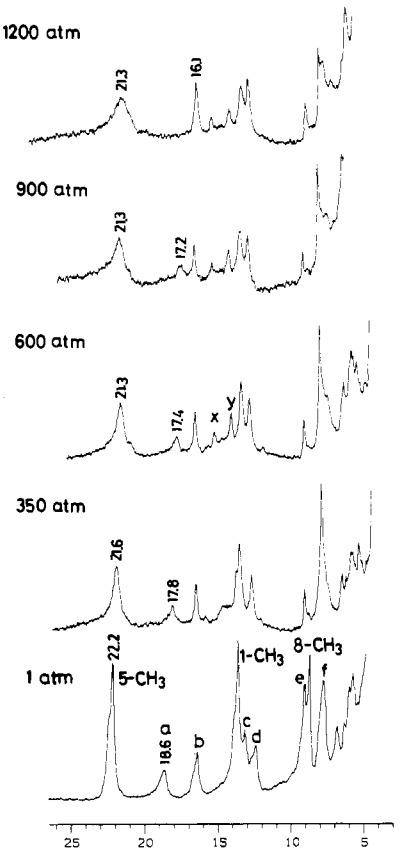

FIGURE 4: Pressure dependence of proton NMR spectra for horse MbCN at 30 °C, pH 8.2, in 0.1 M Tris-HCl.

of the two-proton peak at 16.1 ppm and 1200 atm in Figure 4, the 5-methyl peak intensity at 1200 atm amounts to more than three protons, suggesting that it consists of an unresolved pair of the 5-methyl peak and another peak. This unresolved peak exhibits clearly doubling after 3 months under high pressure. It is of particular interest to note in Figure 4 that hyperfine-shifted new peaks marked x and y appear at 600 atm. With increased pressure, these two peaks exhibited a slight downfield shift. The apparent absence of these two peaks in D_2O solution suggests that these peaks originate from exchangeable protons. These new peaks x and y were not observed for sperm whale. However, after 3 months under high pressure, such new peaks appeared.

The pressure-dependent shifts of the hyperfine-shifted proton resonances for methylamine complex of sperm whale ferrimyoglobin at pH 8.0 are summarized in Table II. On the basis of the similarity of the heme methyl shift pattern for the low-spin cyanide and methylamine complexes of myoglobin, the assignment of the heme methyl resonances for the latter complex is made possible as shown in Table II. There appear three paramagnetically shifted exchangeable protons. The spectral characteristics of these proton resonances were similar to those of the exchangeable peaks in MbCN, allowing us to assign them to the distal histidyl N_3H proton, proximal histidyl N_1H proton, and proximal histidine peptide NH proton, respectively. It is to be noted that these three exchangeable resonances are insensitive to pressure, in contrast to MbCN which exhibits upfield shifts for the distal histidyl NH signal. With increasing pressure, the 1-methyl resonance shifts slightly upfield, the 5-methyl resonance also exhibits a slight upfield shift accompanied by slight broadening, and the 8-methyl peak experiences a slight upfield shift with sizable broadening. The vinyl $C_\alpha H$ proton resonance shows small but appreciable downfield shifts.

Table II: Pressure-Dependent Shifts of the Hyperfine-Shifted Proton Resonances for Methylamine Complex of Sperm Whale Ferrimyoglobin in Tris Buffer Solution at pH 8.0 and 30 °C

peak assignment	pressure (atm) 1	200	400
5-CH_3	30.6	29.8	29.5
vinyl $C_\alpha H$	15.9	16.1	16.2
1-CH_3	15.3	14.9	14.8
His E7 N_3H (distal)	9.3	9.3	9.2
His F8 N_1H (proximal)	8.9	8.8	8.8
8-CH_3	5.8		
His F8 peptide NH	5.7	5.7	5.7

Discussion

Inspection of the pressure-dependent feature of exchangeable NH signals in the paramagnetically shifted region for MbCN suggests that the binding feature of the proximal histidyl imidazole to the heme iron is not altered, while a local structural change is induced at the distal side at elevated pressures.

The distal histidyl N_3H proton is located near the heme iron closely enough to experience the substantial pseudocontact shift due to the anisotropic paramagnetic center of the heme iron (Takano, 1977; Sheard et al., 1970). Decrease in the downfield paramagnetic shift for this resonance upon pressurization could be attributed to dislocation of this histidyl imidazole group in a way that the iron–N_3H distance increases and/or the N_3H moves far off the heme pseudo 4-fold axis. This structural change could be induced by a swinging away of the imidazole group of the distal histidine upon pressure-induced structural change. According to an X-ray analysis for sperm whale MbCN (Brethscher, 1968), the distal histidyl N_3H proton forms a hydrogen bond with the nitrogen atom of the iron-bound cyanide ion. Some changes in this hydrogen bond interaction between the N_3H proton and the iron-bound CN^- and in the tautomeric equilibrium of the imidazole ring could not be ruled out as causes for the pressure-induced shift of the NH resonance.[2] Such a marked pressure-induced spectral shift was not encountered for the methylamine complex of myoglobin, probably because there could be no possible hydrogen bond between the distal histidyl imidazole and the iron-bound methylamine.

It is well established that the spin state of the hemoprotein in the purely low-spin state does not change at high pressure (Ogunmola et al., 1977; Morishima et al., 1979, 1980). Since the hyperfine shifts of the heme peripheral proton groups are modulated predominantly by the iron-bound ligands, our present finding of the pressure-independent behaviors of both proximal histidyl NH and heme peripheral 1-methyl resonances also indicates that the binding mode of the fifth and sixth ligands to the heme iron is not altered at high pressures. It therefore follows that the pressure-dependent behavior of the specific heme methyl and the vinyl $C_\alpha H$ signals appears to be associated with local structural alterations of the heme periphery resulting from steric interactions with nearby amino acid residues. Nonbonded interactions, which are presumably responsible for signal broadening and shift for these proton resonances, may be modulated by pressure-induced structural changes. The steric constraint exerted on these methyl groups may be released at specific pressure, say 1000 atm, because

[2] We have also examined the effect of pressure on the exchange behavior of the distal histidyl imidazole exchangeable N_3H proton for MbCN (La Mar et al., 1981). The degree of saturation transfer from bulk water to distal histidyl NH resonance was unchanged upon pressurization.

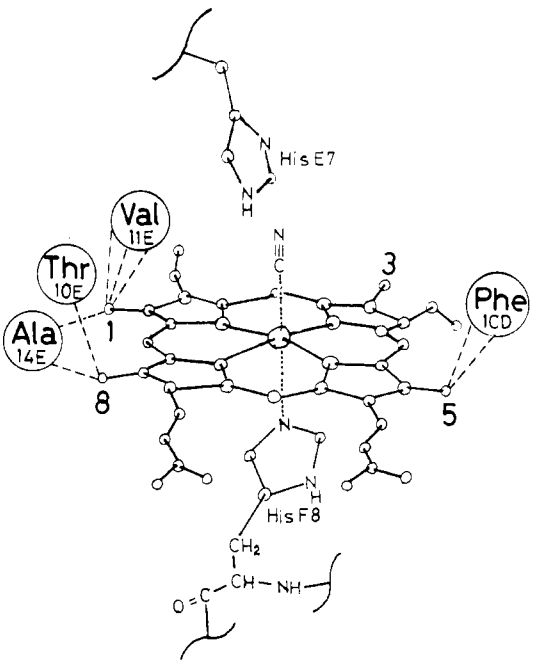

FIGURE 5: Heme environmental structure of MbCN based on the X-ray structural analysis (Takano, 1977). Open circles represent the residues which are in van der Waals contact with 1-, 5-, and 8-methyl groups. The dotted lines stand for the interatomic contacts with 3.9 Å.

the spectral features of these resonance at 1 atm appear to be reproduced at a pressure above 1000 atm (Figure 1). This local structural alteration could be exerted discontinuously on the peripheral proton groups, while pressure-induced structural change may occur in proportion to applied pressure, as is manifested as a progressive shift of the distal histidyl N_3H peak.

On the basis of X-ray structure analysis of sperm whale metmyoglobin (Takano, 1977), the 8-methyl group is in van der Waals contact with alanine 14E and threonine 10E[3] at the distal histidine side, while the 5-methyl is in contact with phenylalanine 1CD and 1-methyl with alanine 14E and valine 11E at the same side (Figure 5). As figure 2 shows, the NH proton of histidine FG2, which is in close contact with the 5-methyl group at the proximal histidine side, is insensitive to pressure. This observation suggests that a slight shift and broadening for the 5-methyl resonance at high pressure is probably due to the change in contact with phenylalanine 1CD at the distal histidine side. It is also likely that the pressure-induced structural change occurs in such a way that the contact at the 8-methyl group is preferentially strengthened, leading to restricted rotation of the methyl group so as to broaden the methyl proton resonance. We have measured the spin–spin relaxation time, T_2, for the 8-methyl resonances and revealed that it is insensitive to pressure. This may allow us to suggest that pressure-induced structural change could result in restricting methyl rotation so as to differentiate chemical shifts among three methyl protons, which is manifested as a broadening of the methyl resonance at high pressure owing to an insufficient resolution. We have also examined high-pressure NMR of other hemoproteins in low-spin state, such as cyano horseradish peroxidase and cyanomethemoglobin. The specific broadening of the heme methyl signal was quite characteristic of MbCN and not prominent for other hemoproteins.

[3] This 67th amino acid residue is changed from threonine for sperm whale Mb to valine for horse Mb (Dayhoff & Eck, 1972).

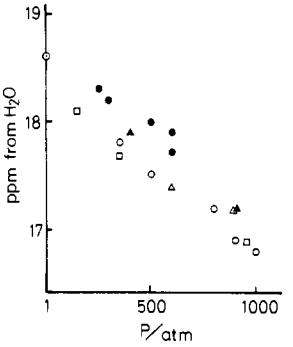

FIGURE 6: Plots of the chemical shift of distal histidyl NH resonances for horse and sperm whale MbCN at 30 °C as a function of applied pressure. Open symbols represent the data for horse MbCN and closed symbols for sperm whale MbCN. The data were obtained at pH 7.5 (□), 7.8 (○, ●), and 8.2 (△, ▲).

It is also worth noting that there is a slight difference in the pressure-induced spectral change between the cyanide complexes of horse and sperm whale myoglobins. At pH 7.8, the 5-methyl resonance for sperm whale MbCN did not experience pressure-induced shift and broadening, in contrast to horse MbCN which exhibited signal broadening, as shown in Figure 1. At pH 8.2 for horse MbCN, there appear two unassignable exchangeable proton resonances marked x and y with a concomitant appearance of the peak which is located near the 5-methyl resonance as shown in Figure 4. Such a spectral change is not obtained for sperm whale MbCN at pH 8.2. However, these spectral changes occur after 3 months at high pressure. These observations suggest that homologous protein can differ appreciably in their conformational stability. We further characterize the difference in conformational stability between horse and sperm whale MbCN by comparing pressure-dependent features of the distal histidyl N_3H proton signal for horse MbCN with that for sperm whale MbCN as shown in Figure 6. The distal histidyl N_3H proton signals for horse MbCN are more susceptible to pressure effects than those for sperm whale MbCN, suggesting that sperm whale MbCN is more stable than horse MbCN under pressure. It was reported that the free energy for total protein unfolding by urea and guanidine hydrochloride for sperm whale myoglobin is larger than that for horse myoglobin (Puett, 1973; McLendon, 1977). The present NMR study also supports the greater conformational stability for sperm whale MbCN that that for horse MbCN at least in the heme vicinity.

In summary, the present high-pressure NMR study of MbCN revealed that the pressure affects the heme environmental structure at the distal side. The present study also showed that sperm whale MbCN is more resistant to pressure than horse MbCN.

Acknowledgments

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References

Antonini, E., Brunori, M., Caputo, A., Chiacone, E., Rossi-Fanelli, A., & Wyman, J. (1964) *Biochim. Biophys. Acta 79*, 248.

Brethscher, P. A. (1968) Ph.D. Thesis, Cambridge University, Cambridge, England.

Dayhoff, M. O., & Eck, R. V. (1972) *Atlas of Protein Sequence and Structure*, National Biochemical Research Foundation, Silver Spring, MD.

La Mar, G. N., Budd, D. L., Visco, D. B., Smith, K. M., & Langry, K. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5755–5759.
La Mar, G. N., Cutnnel, J. D., & Kong, S. B. (1981) *J. Am. Chem. Soc. 103*, 3567–3572.
Mayer, A., Ogawa, S., Shulman, R. G., & Yamane, T. (1974) *J. Mol. Biol. 86*, 749–756.
McLendon, G. (1977) *Biochem. Biophys. Res. Commun. 77*, 959–966.
Morishima, I., & Hara, M. (1982) *J. Am. Chem. Soc. 104*, 6833–6834.
Morishima, I., Ogawa, S., & Yamada, H. (1979) *J. Am. Chem. Soc. 101*, 7074–7076.
Morishima, I., Ogawa, S., & Yamada, H. (1980) *Biochemistry 19*, 1569–1575.
Newmann, R. C., Jr., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.
Ogunmola, G. B., Zipp, A., Chen, F., & Kauzmann, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1.
Puett, D. (1973) *J. Biol. Chem. 248*, 4623–4634.
Sheard, B., Yamane, T., & Shulman, R. G. (1970) *J. Mol. Biol. 53*, 35–48.
Shulman, R. G., Wüthrich, K., Yamane, T., Antonini, E., & Brunori, M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 623–628.
Takano, T. (1977) *J. Mol. Biol. 110*, 537–568.
Yamada, H., Nishitani, S., & Sera, A. (1981) *Chem. Lett.*, 791–792.
Zipp, A., & Kauzmann, W. (1973) *Biochemistry 12*, 4217–4228.

Subunit Structure and Dissociation of *Homarus americanus* Hemocyanin. Effects of Salts and Ureas on the Acetylated and Unmodified Hexamers†

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ABSTRACT: At neutral pH and in the presence of divalent cations the hemocyanin of the lobster *Homarus americanus* exists largely as a dodecamer with a molecular weight of 940 000. Light-scattering investigation has shown that the dodecamers dissociate to hexamers followed by dissociation of the hexamers to monomers. In the absence of calcium ion, both the acetylated hemocyanin at pH 7.8 and the unmodified protein at pH 8.8 were found to be largely in the hexameric state, with molecular weights close to 450 000. The effects of various salts of the Hofmeister series and the urea series on the subunit organization and dissociation of the basic hexameric unit were investigated and analyzed. The salts as dissociating agents are found to be fairly effective, while the ureas are rather ineffective. The effects of these two groups of reagents on the dissociation of the hexameric structure to form monomers closely parallel their effects on the parent dodecamers, dissociating to hexamers [Herskovits, T. T., San George, R. C., & Erhunmwunsee, L. J. (1981) *Biochemistry 20*, 2580–2587]. This suggests that polar and ionic interactions, rather than hydrophobic forces, are the dominant forces that stabilize both the basic hexameric unit and the dodecamers in solution. The analysis of our data obtained with the acetylated hexamers gave apparent estimates of amino acids at the contact areas of the monomers that are nearly the same as the number of groups estimated previously for the contact areas of the hexamers. This suggests that the contact areas of the hexamers and monomers forming the dodecamer are comparable in size. Comparable surface areas of contact are also suggested by the recent models of arthropod hemocyanin dodecamers built by Lamy et al. [Lamy, J., Bijlholt, M. M. C., Sizaret, P.-Y., Lamy, J., & van Bruggen, E. F. J. (1981) *Biochemistry 20*, 1849–1856] and Markl et al. [Markl, J., Kempter, B., Linzen, B., Bijlholt, M. M. C., & van Bruggen, E. F. J. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1631–1641] on the basis of the X-ray crystallographic structure of the hexamer of *Panulirus interruptus* hemocyanin [Van Schaick, E. J. M., Schutter, W. G., Gaykema, W. P. J., van Bruggen, E. F. J., & Holt, W. G. J. (1981) in *Invertebrate Oxygen-Binding Protens: Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 353–362, Marcel Dekker, New York].

The hemocyanins found in the hemolymph of many invertebrates are copper-containing, multisubunit proteins of varying complexity charged with the transport of oxygen. The hemocyanins of the lobsters and other arthropods are assemblies consisting of one to eight basic hexameric units, ranging from approximately 0.45×10^6 to 3.3×10^6 daltons (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977). The subunit structure and the interactions among the various subunits related to the stability and the oxygen binding of the various hemocyanins have been the topic of interest of a number of recent studies [for recent reviews, see Van Holde & van Bruggen (1971), Antonini & Chiancone (1977), and Bonaventura et al. (1977)]. Most hemocyanins exhibit subunit heterogeneity (Di Giamberardino, 1967; Konings et al., 1969; Van Holde et al., 1977), which alters their association–dissociation properties. This has been a complicating factor in the description and analysis of the fundamental monomer to hexamer step of assembly of the hemocyanins of the arthropod species. The few hemocyanins that have been found that appear to be homogeneous or show only moderate effects of subunit heterogeneity are those of the isopods *Ligia exotica* and *Bathynomus giganteus* (Terwilliger et al., 1979; Van Holde & Brenowitz, 1981) and the lobster *Homarus americanus* (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). The association–dissociation behavior of the latter dodecameric

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