Proton NMR Study of Methemoglobin and Its Isolated Chains

EFFECT OF THE SUBUNIT ASSOCIATION ON THE STRUCTURE OF THE SUBUNITS*

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Saburo Neya and Isao Morishima‡
From the Department of Hydrocarbon Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606, Japan

In order to investigate the effect of the αβ subunit contacts on the subunit structure of human adult methemoglobin, the hyperfine shifted proton NMR spectra of several high spin complexes (water, cyanate, thiocyanate, formate, fluoride, and nitrite) and low spin complexes (imidazole, azide, and cyanide) of methemoglobin and its isolated subunits were characterized at 220 MHz and 22°C.

The spectra of ferric low spin derivatives of the isolated subunits were approximately superimposable on the corresponding hemoglobin spectra. On the other hand, the high spin spectra of the isolated subunits were greatly different from each other. The spectral anomaly in the ferric high spin complexes of the isolated β subunit were interpreted to indicate other structural change than the hemichrome formation in the β heme pocket. Difference in the subunit association effect between the high and low spin complexes of the isolated β subunit was interpreted on the basis of a conformational change of the apoprotein dependent on the spin state of the β heme iron.

The subunit interaction has been recognized to be important in the allosteric ligand binding process of hemoglobin. The isolated α and β subunits of hemoglobin have higher oxygen affinity similar to that of myoglobin with decreased cooperativity, while the affinity is much decreased and the cooperativity is increased upon the formation of the heterogeneous tetramer with partner subunit chains (1). The theoretical model for the allosteric process proposed by Monod et al. (2) explained the sigmoidal curve in ligand binding in terms of the subunit interaction, and the structural model from the x-ray analysis of hemoglobin by Perutz (3) also emphasized the importance of the subunit interaction produced by the distal histidyl contacts on the subunit structure of human adult methemoglobin, the hyperfine shifted proton NMR spectra of several high spin complexes of hemoglobin in ferric high spin state have not been extensively investigated except the precedent works by Kurland et al. (11, 12), possibly because of some difficulties in the observation of the much broadened signals with large chemical shifts. We have recently obtained the proton NMR spectra of ferric high spin hemoproteins by Fourier transform method with quadrature phase detection technique. Using this technique on hemoglobin and its isolated subunits, especially those in ferric high spin state, we examined whether and how the effect of the subunit association is exerted on the heme electronic structure of the constituent subunits. Here the hyperfine shifted heme methyl proton resonances are monitored because they are known to be excellent probes for the changes in the heme electronic structure which ultimately reflect the structural perturbations in the polypeptide skeleton (13).

MATERIALS AND METHODS

Human adult blood was obtained from a local blood bank and was washed several times with isotonic saline (0.9% NaCl). After addition of an equal volume of distilled water to the red cells, the hemolysate was centrifuged to remove cell debris, and dialyzed against several changes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl to remove organic phosphate (14). Methemoglobin was prepared by adding a 5-fold molar excess of potassium ferricyanide. Methemoglobin solution was dialyzed against several changes of 0.1 M deuterated phosphate buffer, pH 7.0, exhaustively dialyzed against cold distilled water to remove salt, and was lyophilized. The methemoglobin powder thus obtained was dissolved in 10 mM Tris-HCl buffer or 0.1 M deuterated phosphate buffer, pH 7.0. Visible absorption and proton NMR spectra of various methemoglobin derivatives exhibited the same feature before and after lyophilization. The isolated subunits in carbonmonoxy form were prepared by p-hydroxymercuribenzoate treatment (15) and separated by subsequent chromatography on a DEAE-cellulose column.

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‡ To whom inquiries should be addressed.
Separation of the subunits was verified by proton NMR spectra of the subunit chains in ferric azide form, where the heme methyl proton resonances only from the isolated subunits were observed. After regeneration of the sulphydryl groups by mercaptoethanol treatment (16), the isolated subunits were concentrated and the solvent was exchanged from H_2O to D_2O by repeated ultrafiltration through a membrane filter (Amicon, model 12). Ferric subunits in 1 M glycine in D_2O were prepared according to Banerjee and Cassoly (17). The methemoglobin complexes were prepared by adding a 50-fold excess of ligand to reagent grade aquomethemoglobin. The NMR samples were generally about 0.3 ml with heme concentration of 2 to 4 mM. The pD value was the direct reading of a pH meter, Radiometer, model B 200. Ferric Low Spin Derivatives—Addition of the external ligands to methemoglobin significantly altered the hyperfine shifted resonances from those of acid methemoglobin to intermediate and low spin (imidazole and met) complexes. The magnitude of the hyperfine shifts is dependent on the nature of ligands. The variation of the hyperfine shifts is in accordance with the ligand affinity to methemoglobin, i.e., imidazole < azide < cyanide (1). The resonances whose chemical shifts are given in parts per million units have been assigned to the heme methyl proton resonances for the cyanide (6) and azide (18, 19) complexes. In the cyanide spectrum, those at 22.7, 16.6 ppm and 22.7, 15.7 ppm are assigned to the \( a \) and \( \beta \) heme methyl protons, respectively (6). In the azide spectrum, the heme methyl resonances at 27.9, 22.0 ppm and 26.7, 21.1 ppm are assigned to the \( a \) and \( \beta \) subunits, respectively (18, 19). The nonequivalence of the heme methyl shifts of the \( a \) and \( \beta \) subunits indicates that the \( a \) and \( \beta \) heme environments are not equivalent as already pointed out by Ogawa et al. (6) and by Davis et al. (19). The imidazole complex, in contrast, did not exhibit the apparent \( a \beta \) heme methyl doublet. In order to resolve and assign the heme methyl from the constituent subunits in imidazole methemoglobin, the isolated chains were prepared and their proton NMR spectra were recorded. Figs. 2 and 3 show the NMR spectra of the low spin derivatives of the isolated subunits in 0.1 M phosphate at 22°C. The resonances labeled with their chemical shifts show the heme methyl resonances. The resonance at 22.8 ppm of the cyanide spectrum is known to be composed of the two heme methyl resonances from the \( a \) and \( \beta \) subunits (6, 19). Each of those at 32.2 and 24.6 ppm in the azide (Im) spectrum was identified as two heme methyl resonances from the \( a \) and \( \beta \) subunits. The heme concentration was about 2 mM for each sample.

**RESULTS**

**Ferric Low Spin Derivatives**—Addition of the external ligands to methemoglobin significantly altered the hyperfine shifted resonances from those of acid methemoglobin to intermediate and low spin (imidazole and met) complexes. The magnitude of the hyperfine shifts is dependent on the nature of ligands. The variation of the hyperfine shifts is in accordance with the ligand affinity to methemoglobin, i.e., imidazole < azide < cyanide (1). The resonances whose chemical shifts are given in parts per million units have been assigned to the heme methyl proton resonances for the cyanide (6) and azide (18, 19) complexes. In the cyanide spectrum, those at 22.7, 16.6 ppm and 22.7, 15.7 ppm are assigned to the \( a \) and \( \beta \) heme methyl protons, respectively (6). In the azide spectrum, the heme methyl resonances at 27.9, 22.0 ppm and 26.7, 21.1 ppm are assigned to the \( a \) and \( \beta \) subunits, respectively (18, 19). The nonequivalence of the heme methyl shifts of the \( a \) and \( \beta \) subunits indicates that the \( a \) and \( \beta \) heme environments are not equivalent as already pointed out by Ogawa et al. (6) and by Davis et al. (19). The imidazole complex, in contrast, did not exhibit the apparent \( a \beta \) heme methyl doublet. In order to resolve and assign the heme methyl from the constituent subunits in imidazole methemoglobin, the isolated chains were prepared and their proton NMR spectra were recorded. Figs. 2 and 3 show the NMR spectra of the low spin derivatives of the isolated subunits in 0.1 M phosphate at 22°C. The resonances labeled with their chemical shifts show the heme methyl resonances. The resonance at 22.8 ppm of the cyanide spectrum is known to be composed of the two heme methyl resonances from the \( a \) and \( \beta \) subunits (6, 19). Each of those at 32.2 and 24.6 ppm in the azide (Im) spectrum was identified as two heme methyl resonances from the \( a \) and \( \beta \) subunits. The heme concentration was about 2 mM for each sample.
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FIG. 3. The proton NMR spectra of several low spin derivatives of the isolated $\beta$ subunit in 0.1 M phosphate at 22°C. The resonances labeled with their chemical shifts show the heme methyl peaks. Heme concentration was about 2 mM for each sample. Im, imidazole.

FIG. 4. The proton NMR spectra of several high spin derivatives of the isolated $\alpha$ subunit in 1 M glycine at 22°C. The spectra are similar to those corresponding hemoglobin derivatives and greatly different from those of the corresponding $\beta$ high spin derivatives.

Thus it is evident that the nonequivalence of the $\alpha$ and $\beta$ heme methyl resonances is generally observed in ferric low spin complexes of hemoglobin as pointed out by Ogawa et al. (6, 7) for cyano hemoglobin and by Davis et al. (19) for azide hemoglobin. This nonequivalence may be interpreted in terms of a difference in the interaction between heme iron and proximal histidine as suggested by the proton NMR studies for the model system, protohemin complex with substituted pyridine derivatives, by Hill and Morallee (20), and for the protein system by Morishima et al. (18).

Ferric High Spin Derivatives—Water, fluoride, and some other ligands are known to form ferric high spin complexes with methemoglobin (1). Fig. 4 shows the hyperfine shifted proton NMR spectra of various methemoglobin complexes in purely high spin state (fluoride) and in predominantly high spin state (water, formate, thiocyanate, cyanate, and nitrite). The hyperfine shifts of the ferric high spin complexes observed about 30 to 90 ppm are significantly larger than those of ferric low spin complexes, because of the larger scaling factor $S (S + 1)$ in the NMR hyperfine shift formalism (13), where the total electron spin $S$ is 5/2 for high spin hemes, and because of the structural characteristic in high spin heme environment (3, 11, 12). The magnitude of the hyperfine shifts is in the order of the ligand affinity as in the case for the ferric low spin spectra in Fig. 1. For water, fluoride, formate, and cyanate complexes the resonances labeled with their chemical shifts...
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were assigned to the heme methyl proton resonances from their integrated intensities and line shapes. Especially those of acid methemoglobin were assigned from the comparison with acid metmyoglobin spectrum where the four heme methyl resonances were observed at 91.1, 85.0, 72.2, and 51.6 ppm in 0.5 M phosphate at pD 7.5 and 22°C (18). The line widths of the resonances in the fluoride spectrum are considerably larger than those of other high spin complexes with comparable hyperfine shifts. These large line widths of the heme methyl resonances in fluoro hemoglobin spectrum are due to longer electron spin relaxation time of the heme iron, which predominantly determines the line width of the proton resonance (21), and the spectrum of the fluoride complex is not clearly resolved. As pointed out previously for formate-methemoglobin complex (22), the spectrum of formate hemoglobin also exhibits similar feature to that of an abnormal hemoglobin, hemoglobin M Milwaukee. Hemoglobin M Milwaukee is a naturally occurring valency hybrid containing two permanently oxidized hemes in the ß subunits (ß67 E11 Val → Ghu). Fung et al. (23) reported that the hyperfine shifted resonances of this hemoglobin are observed in the range between 50 and 65 ppm from DSS. This hyperfine shift range is in good agreement with that in formate hemoglobin spectrum of 40 to 60 ppm as in Fig. 4. For thiocyanate and nitrite derivatives the two clearly resolved resonances that would be assigned to the heme methyl resonances were observed at 55.0, 46.8 ppm and 40.6, 35.3 ppm at 22°C, respectively. Each of the resonances is thought to be composed of two heme methyl proton resonances, judging from the spectra of thiocyanate and nitrite myoglobin (horse heart) where they were observed at 70.1, 64.2, 56.9, 50.5 ppm and 58.1, 55.2, 47.7, 44.1 ppm in 0.1 M phosphate at pD 7.0 and 22°C, respectively.

Another interesting feature of the ferric high spin spectra in Fig. 4 is that they do not exhibit ß ß heme methyl doubling despite the environmental difference in the ß and ß hemes. Such ß ß heme methyl doublings are generally seen in ferric low spin spectra in Figs. 1 and 3 as reported by Ogawa et al. for valency hybrid hemoglobins (6, 7) and by Davis et al. for azide hemoglobin (19). It is also possible that other proton resonances are hidden under the heme methyl resonances to make the spectra more ambiguously resolved. In order to solve the spectral contributions from the ß ß subunits in ferric high spin spectra in Fig. 4, the isolated subunits were prepared and their proton NMR spectra were recorded. Figs. 5 and 6 show the NMR spectra of ferric high spin complexes of the isolated subunits in 1 M glycine at 22°C, where the heme methyl resonances are labeled with their chemical shifts in parts per million units from DSS. The spectral features of the ß ß subunit complexes in Fig. 5 are similar to those of the corresponding hemoglobin complexes in Fig. 4. On the other hand, as shown in Fig. 6, the high spin spectra of the isolated ß subunit are distinctly different from those of methemoglobin and the ß subunit except for the fluoride spectrum. Since the high spin spectra of the ß subunit are not clearly resolved, the heme methyl proton resonances were not unambiguously assigned in Fig. 6. Resonances that would be assigned to the ß heme methyl groups were labeled with their chemical shifts.

DISCUSSION

Ferric Low Spin Derivatives—From the present results on the proton NMR spectra of the ferric low spin complexes of methemoglobin, it was generally shown that the chemical shifts of the ß ß and ß ß heme methyl resonances are not identical, indicating the structural nonequivalence of the ß ß and ß ß heme environments in ferric low spin state. The structural nonequivalence of the ß ß and ß ß subunits in hemoglobin was already pointed out by kinetic (24) and equilibrium (25) lidand binding studies, and by proton NMR observations on cyanogen (6, 7) and azide (18, 19) hemoglobins. Comparison of the low spin spectra of methemoglobin and its isolated subunits in Figs. 1 to 3 reveals that the heme methyl shifts of each isolated subunit are approximately superimposable on those of the corresponding tetrameric hemoglobin and that the ß heme methyl resonances are usually observed at higher field side of the ß ß heme methyl doublings. This general feature in ferric low spin complexes is probably due to the different mode of the heme iron-proximal histidine interactions of the subunits in hemoglobin (8, 18, 26). The effectiveness of transmission of electron spin densities from the paramagnetic heme iron to the peripheral heme methyl proton is expected to depend on the bonding interaction between the heme iron and proximal histidine, which in turn is reflected as the difference in the magnitude of the heme methyl shifts (13). Thus, the appearance of the

The chemical shift in the original report (23) is the reading from HDO. The chemical shift from HDO was reduced to those from DSS by adding 4.9 ppm to the reading in parts per million from HDO in order to compare the current value.

The proton NMR spectral comparison in Figs. 4 to 6 also suggests that when the β subunit is integrated into the intact hemoglobin a large conformational change in the surrounding of the β heme occurs. This is in contrast with the result for the ferric low spin complexes where the subunit association does not affect the β heme crevice structure. The difference of the effect of the subunit association between the high and low spin complexes of the isolated β chain may be explained by the ligand-dependent conformational difference of the apoprotein moiety. Steinhardt et al. (29) studied the acid denaturation of methemoglobin derivatives with various high and low spin ligands. They showed that the stability of methemoglobin is much increased upon complex formation with low spin ligand such as cyanide and azide. High spin ligand such as fluoride and thiocyanate stabilized methemoglobin to a lesser extent if at all. These observations were explained in terms of the difference in the apoprotein conformation and these authors suggested that the heme plays an essential part in stabilizing the hemoglobin molecule. In the light of their observation it is reasonable to assume that the protein conformation of the isolated β subunit is much dependent on the nature of the external ligands and hence the spin state of the β heme iron. Thus this may explain why the effect of the β subunit integration to tetrameric hemoglobin is different between the high and low spin complexes.

As pointed out by Dr. L. W. Fung, the spectral anomaly of the isolated high spin β subunit might not be surprising if the oligomeric states of the α and β chains in tetrameric hemoglobin and in the isolated state are compared. The isolated α and β chains self-associate to form dimers, α₅, or tetramers, β₅, and they associate into heterogeneous tetramers, α₅β₅, to form normal hemoglobin. When the oligomeric states were compared for each subunit, the α subunit is dimeric in both states, while the β subunit is dimeric in native hemoglobin and tetrameric in the isolated state. Thus the changes in the oligomeric states may in part be responsible for the abnormal features of the ferric β high spin spectra in Fig. 6.

This observation in ferric high spin β chain parallels the results for ferrous high spin β chain. Electronic absorption study by Antonini et al. (31) or Banerjee et al. (8) and CD spectral study by Nagai et al. (32) show that the spectral feature of isolated β deoxy chain is different from that of deoxyhemoglobin and that the spectrum of deoxyhemoglobin is not represented as a simple sum of those of deoxy isolated chains. Perutz et al. (33) also reported that the superimposed proton NMR spectra of deoxy subunits do not give the spectrum of deoxyhemoglobin, the deoxy β spectrum being ex-

1 L. W.-M. Fung, private communication.
hibiting an abnormal feature.

From the present data alone, it is not possible to discuss the spectral anomaly of the isolated high spin \( \beta \) subunit on the structural basis such as a conformational change of proximal histidine relative to the heme iron (8) or the heme distortion caused by the changes in heme-globin contacts. Yet the spectral anomaly of ferric high spin \( \beta \) chain apparently dependent on the ligand size and the observation that the isolated ferric high spin \( \beta \) chain is structurally more flexible than the high spin \( \alpha \) chain may allow us to expect that the heme pocket is easily perturbed by subunit dissociation from hemoglobin to the isolated \( \alpha \) chain, reflecting more seriously the steric effect of bulky high spin ligands.

In summary the effect of the subunit association on the heme crevice structure is different between the high and low spin complexes of the isolated \( \beta \) subunit. The observed spectral anomaly in the isolated high spin \( \beta \) subunit show that the \( \beta \) heme pocket is easily perturbed by subunit dissociation from tetrameric hemoglobin. Although extrapolation of the present result to ferrous hemoglobin is not directly possible, the flexibility of the \( \beta \) polypeptide and its influence to the heme electronic structure may suggest that the \( \alpha \beta \) nonequivalence is to be considered in the cooperative ligand binding process of ferrous hemoglobin as has been extensively investigated by a number of investigators (5, 34, 35).

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S Neya and I Morishima


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