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Interaction of Fully Liganded Valency Hybrid Hemoglobin with Inositol Hexaphosphate. Implication of the IHP-Induced T State of Human Adult Methemoglobin in the Low-Spin State†

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ABSTRACT: To gain further insight into the quaternary structures of methemoglobin derivatives in the low-spin state, the interaction of fully liganded valency hybrid human hemoglobins with IHP was studied by proton NMR spectroscopy. Upon addition of IHP to $(\alpha_{CO}\beta^{+}_{N_3^-})_2$, the same resonances as the previously reported IHP-induced NMR peaks for azidomethemoglobin $(\alpha^{+}_{N_3^-}\beta^{+}_{N_3^-})_2$ appeared, whereas the binding of IHP did not significantly affect the NMR spectra for $(\alpha^{+}_{N_3^-}\beta_{CO})_2$. The binding of IHP also brought about more pronounced spectral changes for $(\alpha_{CO}\beta^{+}_{Im})_2$ and $(\alpha_{CO}\beta^{+}_{H_2O})_2$ than for $(\alpha^{+}_{Im}\beta_{CO})_2$ and $(\alpha^{+}_{H_2O}\beta_{CO})_2$. Therefore, the IHP-induced NMR peaks for azidomethemoglobin are attributed to the β heme methyl group. Such IHP-induced β heme methyl resonances were also observed for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$, which undergoes quaternary structural change, analogously to the R–T transition by the binding of IHP. From the above results, it was suggested that the IHP-induced heme methyl resonances for azidomethemoglobin and $(\alpha_{CO}\beta^{+}_{N_3^-})_2$ may also be associated with the quaternary structure of these Hbs, implying the presence of the IHP-induced "T-like" state in low-spin metHb A.

A variety of physicochemical properties of hemoglobin tetramer have been often understood on the basis of two quaternary conformational states: the T and R states, normally associated with deoxy- and oxyhemoglobin structures and also characterized by low and high oxygen ligand binding affinity, respectively (Monod et al., 1965). These two quaternary states have been defined in terms of some detailed protein and heme environmental structures (Shulman et al., 1975; Baldwin, 1975). The X-ray structural analysis revealed that the iron is out of the heme plane in deoxyhemoglobin (Fermi, 1975) but planar in oxyhemoglobin (Shaanan, 1982). On the basis of these structural differences, Perutz (1970) has proposed that this movement of iron displacement into or out of the heme plane, linked to the spin state of heme iron, exerts an influence on the globin structures of the protein that promote the T to R structural transition upon ligand binding. In support of such

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relationship between the quaternary structural change and the spin state of the heme iron, Perutz and co-workers (1974a–c) extended their spectral studies to high- and low-spin derivatives of methemoglobin and found that the allosteric effector IHP,[1] which binds preferentially to the T state, converts the high-spin methemoglobin derivatives with the iron located out of the heme plane from the R to T state (Perutz et al., 1974a,b; Massana et al., 1978; Fermi & Perutz, 1977; Hensley et al., 1975a,b; Edelstein & Gibson, 1975). For low-spin methemoglobin derivatives, however, Perutz et al. (1974b,c) showed that the IHP binding does not change their quaternary structures, although their tertiary structure is perturbed.

We have previously studied the effect of IHP on the tertiary and quaternary structures of methemoglobin derivatives by the use of proton NMR spectroscopy and found that the IHP-induced hyperfine-shifted proton peaks which are distinguishable from those observed in the absence of IHP appeared for the azide and imidazole complexes in the low-spin state where the heme iron is in the heme plane (Neya & Morishima, 1980, 1981). These IHP-induced NMR peaks for the low-spin azidomethemoglobin (HbN_3^-) were not observed when azide was titrated to stripped aquomethemoglobin, indicating that IHP-induced peaks are not due to the dissociation of azide. Azide titration to aquomethemoglobin in the presence of IHP further revealed that the IHP-induced peaks are not due to the localized structural change but rather to the presence of an IHP-induced new conformer (Neya & Morishima, 1981). The pD dependence of the intensities of these peaks was found to be closely similar to that of the exchangeable proton NMR peak that has been used as a marker of the T quaternary conformation (Perutz et al., 1978; Huang, 1979). It was further suggested that the IHP-induced quaternary transition is accompanied by the tertiary structural alterations in both of the α and β subunits (Neya & Morishima, 1981). Above previous findings of the IHP-induced NMR peaks for those low-spin methemoglobin derivatives led us to suggest that the low-spin methemoglobin can switch from the R to T quaternary structure by the binding of IHP. Although these IHP-induced NMR peaks were attributed to a new conformer in low-spin methemoglobin, an unequivocal support for the presence of the T quaternary structure was incomplete, because these IHP-induced heme methyl resonances have not so far been observed in the hemoglobin derivative that is definitely known to be in the T quaternary state.

In order to complement the results obtained previously and structural changes in the ferric low-spin methemoglobin, we examined here the effect of IHP binding on the NMR spectra of human adult fully liganded valency hybrid Hbs, which may serve to assign the IHP-induced NMR peaks to α and/or β subunits and also to delineate their quaternary structures in the presence or absence of IHP.

Materials and Methods

Human adult hemoglobin was prepared from whole blood as reported previously (Morishima & Hara, 1983). The isolated α and β chains were prepared by the method of Ikeda-Saito et al. (1981).

The azide–carboxy hybrids were prepared by addition of the metazide derivatives of one chain with the carboxyl form of the other with a slight excess of the β chain. Then the mixture was passed through Sephadex G-25 equilibrated with 10 mM phosphate buffer, pH 6.5, and applied to CM-52 (Whatman) equilibrated with the same buffer. The excess chain was washed off by the same buffer, and the hybrid hemoglobin tetramer was eluted with 0.1 M phosphate buffer, pH 7.0.

The azide–nitrosyl hybrids were obtained following the procedure described by Henry and Banerjee (1973) with a slight modification. The oxygenated chain in 0.1 M phosphate buffer, pH 7.0, was deoxygenated by flushing the dry nitrogen gas. Nitric oxide was then allowed to react by the successive addition of sodium nitrite and dithionite. Excess NO and dithionite were removed by filtering rapidly in a Sephadex G-25 column equilibrated with 0.05 M deoxygenated Bis-Tris buffer, pH 6.5, under completely anaerobic conditions. Then, an equivalent amount of metazide chain was mixed immediately.

The aquomet–carboxy hybrids were prepared as follows: The oxygenated chain in 0.1 M phosphate buffer, pH 7.0, was oxidized by adding a 1.2 equiv of ferricyanide. Excess ferri- and ferrocyanides were removed by passing through a Sephadex G-25 column equilibrated with the same buffer and then the carboxy chains injected immediately with a slight excess of β chain. The hybrids thus obtained were purified according to the same procedures as described for azide–carboxy hybrids. The purity of the samples was checked by calculating the fraction of carboxyferroheme (Banerjee & Cassoly, 1969).

The imidazole–carboxy hybrids were prepared by adding the imidazole to aquomet–carboxy hybrids. All of the operations were carried out at 4 °C.

Proton NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Typical NMR spectra consisted of 5000 pulses, and hyperfine-shifted NMR spectra were obtained by a 4K data transform of 30-kHz (high-spin complexes) and 12-kHz (low-spin complexes) spectral width and 5.5-μs 90° pulse after the strong solvent resonance in H_2O solution was suppressed by a 500-μs low-power pulse. We used Redfield 2-1-X pulse sequence with 22.9-μs pulse and 8K data points over an 8-kHz spectral width for recording the exchangeable proton resonances in the subunit interface of Hb. Probe temperature was determined by the temperature control unit of the spectrometer, accurate to ±0.5 °C. The volume of the NMR sample was 0.3 mL, and heme concentration was about 2 mM. Proton shifts were referenced with respect to the water proton signal, which is 4.8 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 23 °C.

Results

IHP-Induced Tertiary Structural Changes for Valency Hybrid Hemoglobins. The hyperfine-shifted proton NMR spectra of HbN_3^- in the presence and absence of IHP have been described before (Neya & Morishima, 1980, 1981). In the absence of IHP at pH 6.5 and 23 °C, the α heme methyl resonances were observed at 23.0, 17.1, and 9.8 ppm and β heme methyl resonances at 21.8, 16.1, and 9.0 ppm (Davis et al., 1969). Upon addition of IHP, new peaks appeared at 26.3, 20.0, and 15.4 ppm. The contributions of the α and β subunits to these IHP-induced resonances in HbN_3^- could be evaluated by studying the valency hybrids $(\alpha^+_{N_3}\beta_{CO})_2$ and $(\alpha_{CO}\beta^+_{N_3})_2$, where only the ferri–azide chains give paramagnetically shifted resonances.

Figure 1 shows the proton NMR spectra of $(\alpha^+_{N_3}\beta_{CO})_2$ and $(\alpha_{CO}\beta^+_{N_3})_2$ in the presence and absence of IHP in 0.05 M Bis-Tris buffer at pH 6.5. Without IHP, the hyperfine-shifted α and β heme methyl resonances of HbN_3^- appear to be a composite of those of valency hybrids, indicating that these fully liganded hemoglobins are more or less the same in their

[1] Abbreviations: IHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane.

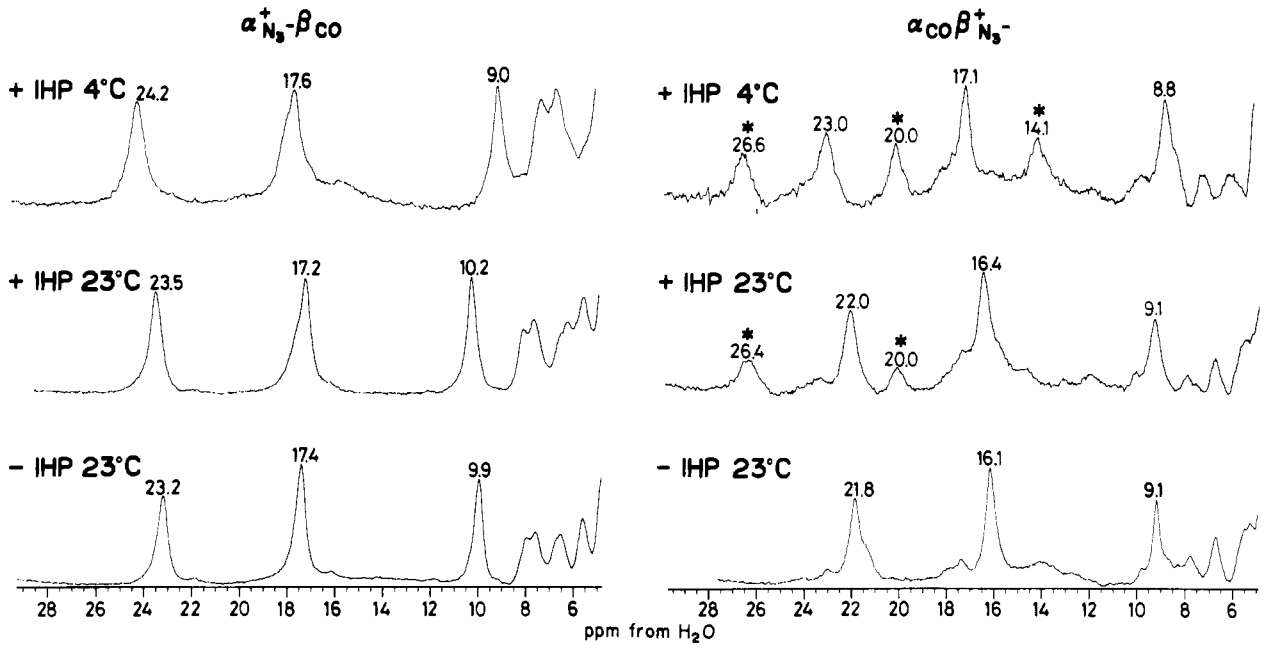

FIGURE 1: Proton NMR spectra of the azide–carboxy valency hybrids, with and without IHP, in 0.05 M Bis-Tris at pH 6.5 and 4 and 23 °C. Five moles of IHP/mol of tetramer was added. Only the ferri–azide chains give the paramagnetically shifted resonances. Asterisks show the IHP-induced heme methyl resonances.

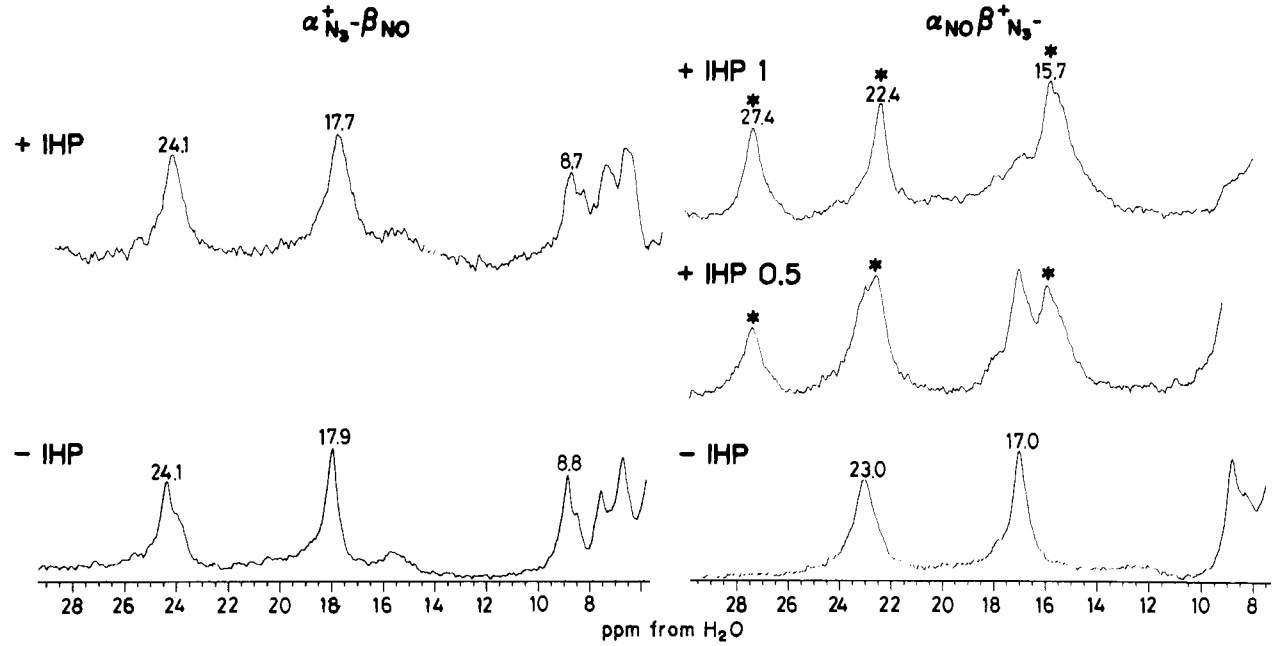

FIGURE 2: Proton NMR spectra of azide–nitrosyl valency hybrids, with and without IHP, in 0.05 M Bis-Tris at pH 6.5 and 4 °C. Five moles of IHP/tetramer was added to $(\alpha^{+}_{N_3}\text{-}\beta_{NO})_2$. For $(\alpha_{NO}\beta^{+}_{N_3})_2$, the IHP/tetramer ratio was included in the figure. Asterisks show the IHP-induced heme methyl resonances.

heme environmental conformations. The NMR spectra for $(\alpha^{+}_{N_3}\text{-}\beta_{CO})_2$ are not changed by the addition of IHP except for the slight IHP-induced increase in the line width of three heme methyl resonances, while those for $(\alpha_{CO}\beta^{+}_{N_3})_2$ are much influenced by IHP. Upon addition of IHP to $(\alpha_{CO}\beta^{+}_{N_3})_2$, there appear new resonances at 26.4 and 20.0 ppm at 23 °C. The intensities of these resonances gradually increased with lowering the temperature from 23 to 4 °C. It is to be noted that those IHP-induced peaks for $(\alpha_{CO}\beta^{+}_{N_3})_2$ are identical with those for HbN_3^-. The IHP-induced NMR peaks for HbN_3^- could therefore be attributed to the β subunit.

The effect of IHP binding on the NMR spectra of $(\alpha^{+}_{N_3}\text{-}\beta_{NO})_2$ and $(\alpha_{NO}\beta^{+}_{N_3})_2$ was also examined (Figure 2). The IHP-induced spectral changes for nitrosyl–azide valency hybrids are almost the same as those for carboxy–azide valency hybrids. Upon addition of IHP, the NMR spectrum of $(\alpha^{+}_{N_3}\text{-}\beta_{NO})_2$ remains unchanged, while for $(\alpha_{NO}\beta^{+}_{N_3})_2$ the intensities of the heme methyl resonances around 23.0 and 17.0 ppm gradually decrease with a concomitant appearance of the characteristic resonances at 27.4, 22.4, and 15.7 ppm. The 23.0 and 17.0 ppm peaks, which are observed in the absence of IHP, completely disappear to give only a set of IHP-induced new peaks when a saturating amount of IHP is added. These IHP-induced resonances for $(\alpha_{NO}\beta^{+}_{N_3})_2$ are placed at slightly lower field by 1–2 ppm, compared with those for $(\alpha_{CO}\beta^{+}_{N_3})_2$ and HbN_3^-.

In Figure 3 are shown the proton NMR spectra of $(\alpha_{NO}\beta^{+}_{N_3})_2$ with and without IHP in 0.05 M Bis-Tris buffer at pH 6.5 and 23 °C. There appears a broad signal at 27.2 ppm in the absence of IHP. This peak could be attributed to

Table I: Resonance Positions of Heme Methyl Groups for Azide and Imidazole Derivatives

Hb		resonance position (ppm)						
HbN_3[a]	−IHP		23.0	21.8		17.1	16.1	
	+IHP	26.3*[b]	23.0	21.8	20.0*	17.1	16.1	15.4*
$(\alpha^{+}_{N_3}\text{-}\beta_{CO})_2$[a]	−IHP		23.2			17.4		
	+IHP		23.5			17.2		
$(\alpha_{CO}\beta^{+}_{N_3})_2$[a]	−IHP			21.8			16.1	
	+IHP	26.4*		22.0	20.0*		16.4	14.1*[c]
$(\alpha^{+}_{N_3}\text{-}\beta_{NO})_2$[c]	−IHP		24.1			17.9		
	+IHP		24.1			17.7		
$(\alpha_{NO}\beta^{+}_{N_3})_2$[c]	−IHP			23.0			17.0	
	+IHP	27.4*			22.4*			15.7*
HbIm[c]	−IHP		28.6	22.5	20.0			14.0
	+IHP	31.2*[b]	28.6	22.5	20.1			14.8
$(\alpha^{+}_{Im}\beta_{CO})_2$[c]	−IHP		28.7	23.2				14.3
	+IHP		28.7	23.1				14.1
$(\alpha_{CO}\beta^{+}_{Im})_2$[c]	−IHP		28.7		20.6			14.5
	+IHP	31.2*	28.8	21.0			15.2	

[a] Measured at 23 °C. [b] Asterisks show the IHP-induced heme methyl resonances. [c] Measured at 4 °C.

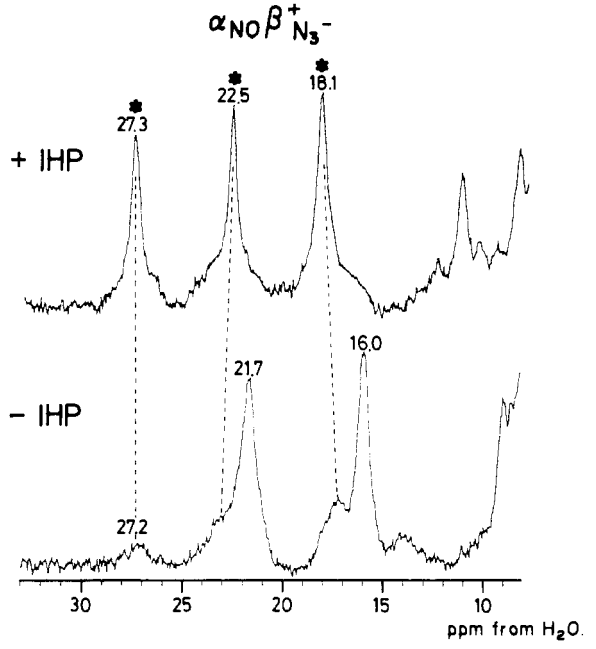

FIGURE 3: Proton NMR spectra of $(\alpha_{NO}\beta^{+}_{N_3})_2$, with and without IHP, in 0.05 M Bis-Tris at pH 6.5 and 23 °C. One mole of IHP/tetramer was added. Asterisks show the IHP-induced heme methyl resonances.

the same resonance as the IHP-induced one since an appreciable increase in intensity is encountered upon addition of IHP. As shown in Figure 2, this broad signal is not observed in the absence of IHP at 4 °C, probably because it is too broadened to be detectable at this temperature. The appearance of the same resonance as the IHP-induced one even in the absence of IHP indicates that the IHP-induced peaks for $(\alpha_{NO}\beta^{+}_{N_3})_2$ are not due to the IHP-induced localized structural change within $(\alpha_{NO}\beta^{+}_{N_3})_2$, but rather to the presence of the new conformer to which IHP preferentially binds and which could stabilize its conformation.

We also examined the effect of IHP binding on the NMR spectra of $(\alpha^{+}_{Im}\beta_{CO})_2$ and $(\alpha_{CO}\beta^{+}_{Im})_2$ at pH 6.5 and 4 °C in 0.05 M Bis-Tris buffer, as shown in Figure 4. For $(\alpha^{+}_{Im}\beta_{CO})_2$, the NMR spectrum in the presence of IHP is identical with that in the absence of IHP, while that of $(\alpha_{CO}\beta^{+}_{Im})_2$ exhibits a marked spectral change upon addition of IHP. This observation may also allow us to describe the IHP-induced NMR peaks for HbIm, which was previously reported (Neya & Morishima, 1980, 1981), to the β subunit. All the resonance positions of azide and imidazole derivatives are assembled in Table I.

The left side of Figure 5 illustrates the NMR spectra of $(\alpha^{+}_{H_2O}\beta_{CO})_2$ and $(\alpha_{CO}\beta^{+}_{H_2O})_2$ with and without IHP. The resonances that appear over the spectral region from 30 to 90 ppm come from the heme peripheral proton groups and/or the protons of the amino acid residues in the immediate surroundings of the heme group. These resonances for $(\alpha^{+}_{H_2O}\beta_{CO})_2$ exhibit minor spectral changes upon addition of IHP, as shown in Figure 5. The exchangeable resonance at

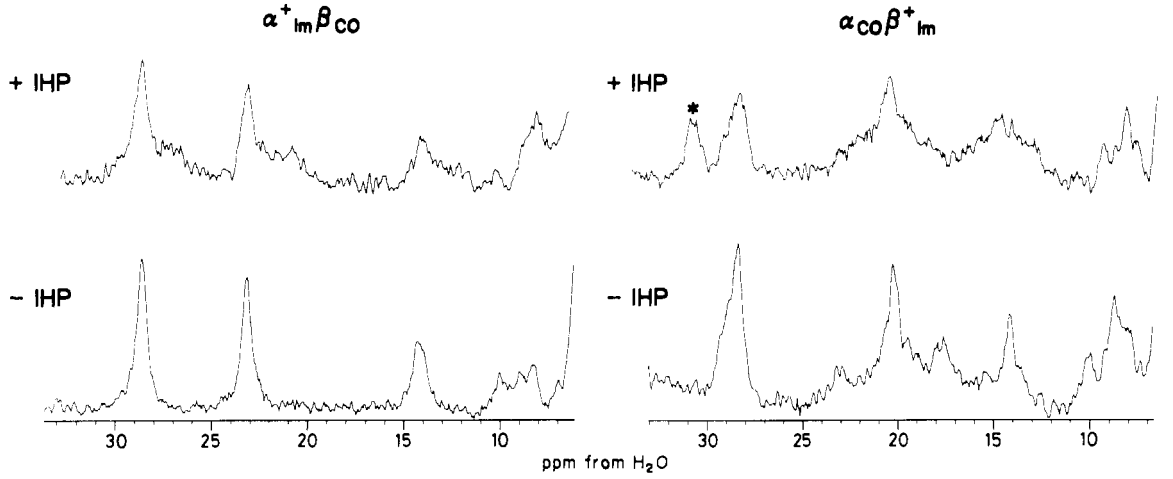

FIGURE 4: Proton NMR spectra of imidazole–carboxy valency hybrids, with and without IHP, in 0.05 M Bis-Tris at pH 6.5 and 4 °C. Five moles of IHP/tetramer was added. The asterisk shows the IHP-induced resonance.

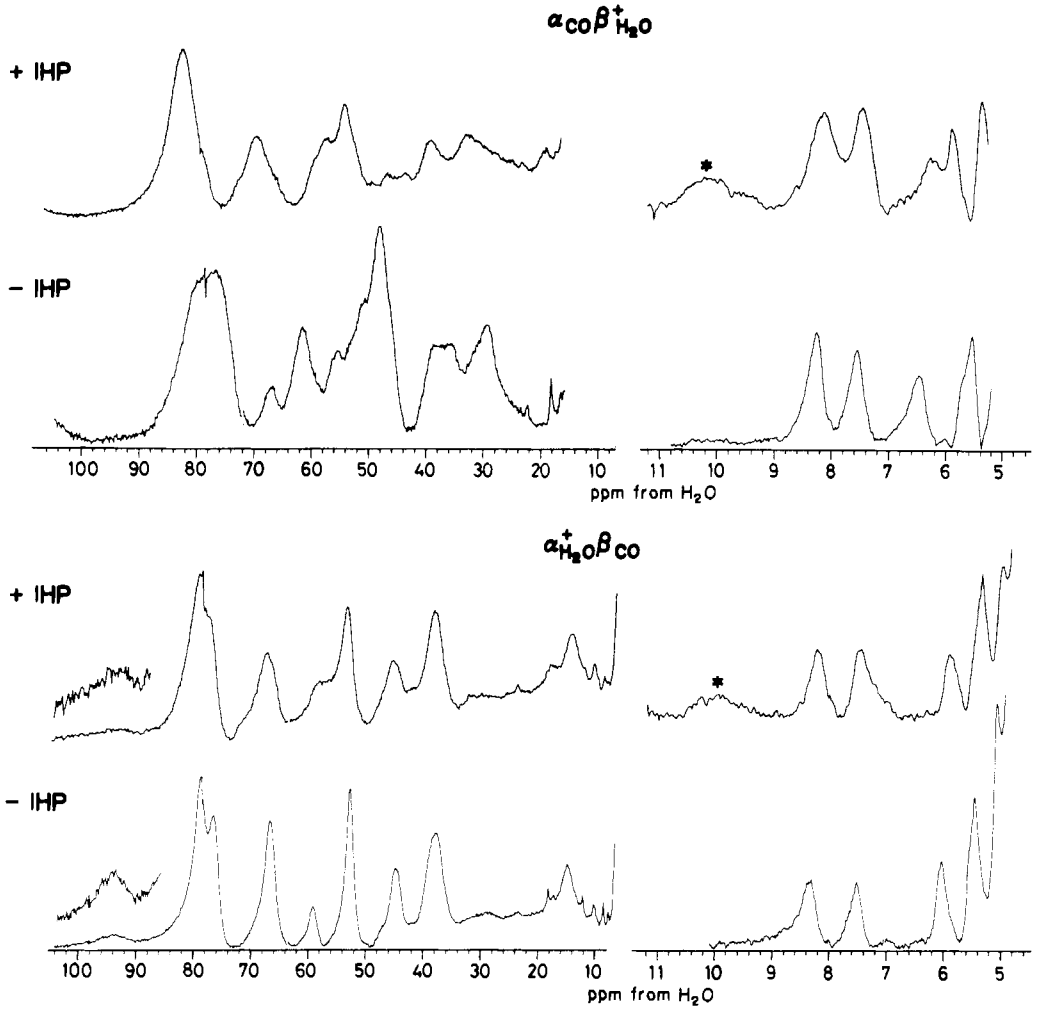

FIGURE 5: Hyperfine-shifted proton resonances (left side) and exchangeable resonances (right side) for aquomet–carboxy valency hybrids, with and without IHP, in 0.05 M Bis-Tris at pH 6.0 and 23 °C. Five moles of IHP/tetramer was added. Asterisks show the IHP-induced resonances.

93.9 ppm, which is most probably assigned to the proximal histidyl (E7) NH proton in the ferric α subunit (La Mar & de Ropp, 1979), also remains unchanged. For $(\alpha_{CO}\beta^{+}_{H_2O})_2$, the more obvious spectral change was induced as shown in Figure 5. The binding of IHP produces more pronounced spectral changes for $(\alpha_{CO}\beta^{+}_{H_2O})_2$ over the region from 40 to 90 ppm, in contrast to $(\alpha^{+}_{H_2O}\beta_{CO})_2$, which experiences no significant spectral changes.

IHP-Induced Quaternary Structural Changes for Valency Hybrid Hemoglobins. Figure 6 shows the exchangeable proton resonances for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ in the presence and absence of IHP at 4 °C. Of particular interest is the appearance of the exchangeable proton resonances at 9.3 and 6.2 ppm by addition of IHP. The former resonance has been assigned to the *inter*subunit hydrogen bond between tyrosine-α42(C7) and aspartic acid-β99(G1), characteristic of the deoxy quaternary structure (Fung & Ho, 1975), and the latter to the *intra*subunit hydrogen bond between valine-β98(FG5) and tyrosine-β145(HC2), which is characteristic of the deoxy tertiary structure (Viggiano et al., 1978). It also worthy to note that the intensity of the resonances at 9.3 and 6.2 ppm for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ with IHP appears to bear half of the signal intensity of the 9.4 and 6.4 ppm peaks for deoxyhemoglobin (Perutz et al., 1974). This suggests that IHP can switch the quaternary structure for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$, although the quaternary structure of the hybrid Hb in the presence of IHP is somewhat different from the usual T quaternary structure. An attempt to look for the exchangeable proton resonances associated with the hydrogen

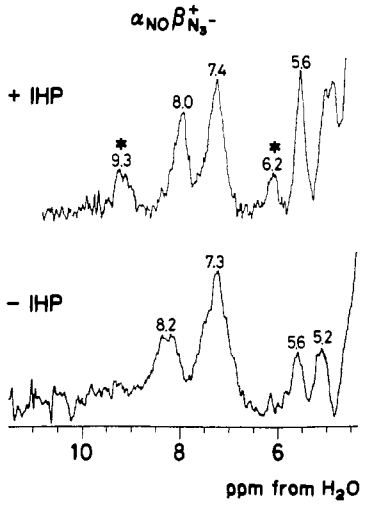

FIGURE 6: Exchangeable proton resonances of $(\alpha_{NO}\beta^{+}_{N_3^-})_2$, with and without IHP, in 0.05 M Bis-Tris at pH 6.5 and 4 °C. One mole of IHP/tetramer was added. Asterisks show the IHP-induced resonances.

bond at the subunit interface in HbN_3^-, carboxy–azide hybrid Hbs, and $(\alpha^{+}_{N_3^-}\beta_{NO})_2$ was not successful because of the spectral overlaps in this region with the paramagnetically shifted resonances.

In the right side of Figure 5, the exchangeable proton resonances of carboxy–aquomet hybrid hemoglobin with and without IHP are shown. Although Fung et al. (1976, 1977)

Table II: Resonance Positions of Hydrogen-Bonded Protons for Valency Hybrid Hemoglobins[a]

Hb		resonance position (ppm)				
HbH_2O	−IHP		8.1	7.4		
	+IHP	10.1*[b]	8.1	7.4	6.4	
$(\alpha^{+}_{H_2O}\beta_{CO})_2$	−IHP		8.4	7.5		6.0
	+IHP	10.1*	8.2	7.5		5.9
$(\alpha_{CO}\beta^{+}_{H_2O})_2$	−IHP		8.3	7.6		6.5
	+IHP	10.2*	8.3	7.5		6.3
$(\alpha_{NO}\beta^{+}_{N_3^-})_2$[c]	−IHP		8.1	7.2		
	+IHP	9.3*	8.0	7.4	6.3*	

[a] Measured at 23 °C. [b] Asterisks show the IHP-induced resonances. [c] Measured at 4 °C.

reported the NMR spectra of hemoglobin M Milwaukee, which contains the mutant aquomet β heme, the quaternary structures of the aquomet–carboxy hybrid Hbs have not been reported. In the spectrum of $(\alpha^{+}_{H_2O}\beta_{CO})_2$, a resonance at 6.0 ppm, which has been utilized as an indicator of the R quaternary structure (Fung & Ho, 1975), loses its signal intensity and shifts to 5.9 ppm upon addition of IHP. The most obvious change is the appearance of the broad resonance at 10.1 ppm, which has been established as a reliable T state marker in aquomethemoglobin (Perutz et al., 1978; Huang, 1979). $(\alpha_{CO}\beta^{+}_{H_2O})_2$ also exhibits the T marker signal at 10.1 ppm upon addition of IHP, indicating that IHP switches the quaternary structure of this hybrid Hb from the R to T state, although its paramagnetic resonances experience no significant changes as shown in Figure 5. These observations show that some of the hybrid Hb derivatives undergo the R to T quaternary structural transition by the binding of IHP. It is also noteworthy that the resonance at 6.5 ppm which has been assigned to the *intra*subunit hydrogen bond, characteristic of the tertiary structure within the T state, is observed for $(\alpha_{CO}\beta^{+}_{H_2O})_2$. Upon addition of IHP, a 6.5 ppm peak experienced a slight upfield shift to 5.9 ppm. All the resonance positions of the hydrogen-bonded protons for the aquomet derivatives and $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ are summarized in Table II.

Discussion

Tertiary Structural Changes Induced by Addition of IHP for Low-Spin Methemoglobin Derivatives. The present NMR results on carboxy–azide and nitrosyl–azide valency hybrids have served to identify the IHP-induced NMR peaks in the hyperfine-shifted region for HbN_3^- and give additional support for the presence of the new conformer which is induced by the addition of IHP to low-spin methemoglobin as was proposed in our previous report (Neya & Morishima, 1980, 1981).

In the NMR spectrum of HbN_3^- with IHP, contributions from the α and β subunits to IHP-induced NMR peaks are easily distinguished when the spectrum of $(\alpha^{+}_{N_3^-}\beta_{CO})_2$ or $(\alpha^{+}_{N_3^-}\beta_{NO})_2$ with IHP is compared with that of $(\alpha_{CO}\beta^{+}_{N_3^-})_2$ or $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ without IHP. As shown in Table I, it is thus confirmed that the IHP-induced NMR peaks in HbN_3^- are originated from the β subunit. Accordingly, we now correct our previous suggestion that both α and β subunits for HbN_3^- are equally perturbed by the binding of IHP and the IHP-induced NMR peaks arise from the structural alteration in both subunits (Neya & Morishima, 1981). In our previous report, we have performed the azide titration to IHP-bound aquomethemoglobin and found that the IHP-induced NMR peaks appeared first and then the α,β heme methyl groups grow up with increasing amount of azide. This observation could also be reinterpreted as follows: the heme methyl peaks characteristic of the IHP-induced conformer appeared first, then followed by the appearance of the native conformer peaks. This seems to be in good agreement with the result obtained by Olson (1976) that the azide binding rate at the β subunit is 50-fold larger than that at the α subunit in the presence of IHP.

Although the structural origin of the IHP-induced resonances for $(\alpha_{CO}\beta^{+}_{Im})_2$ is not certain at the present stage, IHP gives rise to more pronounced NMR spectral changes for $(\alpha_{CO}\beta^{+}_{Im})_2$ than for $(\alpha^{+}_{Im}\beta_{CO})_2$ as shown in Table I. In the case of aquomet–carboxy hybrids (Figure 5), the β heme moiety of $(\alpha_{CO}\beta^{+}_{H_2O})_2$ also undertakes more pronounced structural change than the α heme moiety of $(\alpha^{+}_{H_2O}\beta_{CO})_2$.

Such a different behavior of responses toward organic phosphate between the complementary pairs of valency hybrids seems to be a general phenomenon. Cassoly and Gibson (1972) have shown that addition of IHP to $(\alpha_{deoxy}\beta^{+}_{CN^-})_2$ produces the marked absorption difference spectrum in the Soret region, while the spectrum of $(\alpha^{+}_{CN^-}\beta_{deoxy})_2$ remains almost unchanged. They also studied the effect of DPG binding on the kinetics of carbon monoxide binding to cyanide–deoxy valency hybrids which exhibits a strong heterogeneous character in the absence of organic phosphate. Upon addition of DPG, only $(\alpha_{deoxy}\beta^{+}_{CN^-})_2$ showed a decrease in the fraction of fast-phase component although DPG is more strongly bound to $(\alpha^{+}_{CN^-}\beta_{deoxy})_2$ than to $(\alpha_{deoxy}\beta^{+}_{CN^-})_2$. Perutz et al. (1976) have reported that addition of IHP to $(\alpha_{NO}\beta^{+}_{CN^-})_2$ causes a substantial change of the absorption spectra in either the UV or visible region, but $(\alpha^{+}_{CN^-}\beta_{NO})_2$ experiences no significant spectral changes. This was also encountered for the ESR spectra (Rein et al., 1972; Henry & Banerjee, 1973; Miura & Morimoto, 1980; Nagai et al., 1978). The ESR spectra of $(\alpha_{NO}\beta^{+}_{CN^-})_2$, $(\alpha_{NO}\beta^{+}_{H_2O})_2$, and $(\alpha_{NO}\beta^{+}_{F^-})_2$ exhibited three distinct hyperfine structures upon addition of IHP, whereas those of their complementary hybrids were insensitive to the addition of IHP. We have no available data to show the difference in the affinity of IHP toward both complementary hybrids. However, it is unlikely that the apparent affinity of IHP to these hybrids can differ remarkably, since IHP is a strong allosteric effector which binds, for example, to oxy- and azidomethemoglobin with the large binding constants, 1.4 and 1.88×10^4 M^{-1}, respectively (Neya et al., 1983; Jonig et al., 1971). Therefore, we suggest that the α and β subunits could have different sensitivity to organic phosphate.

Quaternary Structural Changes Induced by Addition of IHP—Evidence for the T-like Structure in Low-Spin Methemoglobin. In the quaternary structural transition from the R to T state in hemoglobins, Perutz et al. (1974a,b) previously found the characteristic changes in ultraviolet absorption and circular dichroism. In ferric high-spin methemoglobin, aquomethemoglobin, or fluoromethemoglobin, exactly the same changes as those for ferrous hemoglobin can be produced by the addition of IHP. NMR spectra (Perutz et al., 1978) also showed that the addition of IHP to human fluoro- and aquomethemoglobin leads to the slowly exchanging proton resonance at about 10 ppm diagnostic of the T structure. Thus, Perutz et al. (1978) concluded that IHP is capable of switching the quaternary structure of certain high-spin ferric hemoglobins from the R to T state. As to ferric low-spin methemoglobin, however, Perutz et al. (1974a) also investigated azido- and cyanomethemoglobin and concluded that IHP appears to modify the structure of these low-spin derivatives without changing their quaternary structure to the T state.

On the other hand, Neya and Morishima (1980, 1981) found an IHP-induced new conformer in low-spin azidomethemoglobin by utilizing NMR spectra and suggested that human low-spin methemoglobin can be switched into the T

Table III: Resonance Positions of Hydrogen-Bonded Protons for Native and Hybrid Hemoglobins[a]

Hb		resonance position (ppm)				state
oxyHb			8.3	7.4		R
deoxyHb		9.4*	8.3	7.6	6.4*	T
metHbH$_2$O	−IHP		8.1	7.4		R
	+IHP	10.1*	8.1	7.4	6.4*	T
$(\alpha_{NO}\beta^{+}_{N_3^-})_2$[c]	−IHP		8.1	7.2		R
	+IHP	9.3*	8.0	7.4	6.3*	T-like

[a] Measured at 23 °C. [b] Asterisks show the IHP-induced resonances. [c] Measured at 4 °C.

quaternary structure by the binding of IHP. Neya et al. (1983) also reported the UV-spectral evidence for the IHP-induced quaternary structural alteration in HbN_3^-; IHP induced the changes in the UV absorption spectra of Tyr-α42(C7) and Trp-β37(C3), corresponding to the perturbation of the subunit boundary structure.

The resonances of the hydrogen-bonded proton located at the *inter*- and *intra*subunits are expected to further clarify the quaternary structure of $(\alpha_{NO}\beta^{+}_{N_3^-})_2$. As shown in Table III, the quaternary structure of the new conformer is analogous to the T-state structure in ferrous hemoglobin. Since the T marker signal at 9.4 ppm for native Hb is shifted to 9.3 ppm for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ and the intensity of the T marker for the hybrid hemoglobin is half of the one for deoxyhemoglobin, the quaternary structure of the new conformer could be described as the "T-like" state rather than the usual T state. The T marker signal at 6.4 ppm for native Hb is also shifted to 6.3 ppm for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$. It is apparent from Figure 3 that the "T-like" structural marker signals not only are induced by addition of IHP but also exist even in the absence of IHP. This may suggest that IHP changes the R–T conformational equilibrium inherent in $(\alpha_{NO}\beta^{+}_{N_3^-})_2$. Huang (1979) reported that stripped nitrosylhemoglobin is 70% in the T state below pH 6.4 and is in the R state at pH above 6.4 and that IHP raises this transition point from pH 6.4 to 7.5. These findings indicate that the IHP can shift the equilibrium from the R to T quaternary state for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ and the nitrosylhemoglobin as well. It is thus likely that IHP induces the quaternary structural change of the valence hybrid hemoglobin containing ferric low-spin iron, $(\alpha_{NO}\beta^{+}_{N_3^-})_2$, which is analogous to the R–T transition as was found for ferric high-spin Hbs. On the basis of the close similarity of the IHP-induced heme methyl resonances among HbN_3^-, $(\alpha_{CO}\beta^{+}_{N_3^-})_2$, and $(\alpha_{NO}\beta^{+}_{N_3^-})_2$ as shown in Table I, the IHP-induced resonances for HbN_3^- and $(\alpha_{CO}\beta^{+}_{N_3^-})_2$ could also be attributed to the T-like conformer of these hemoglobins. Accordingly, we can conclude that IHP induces the quaternary structure not only in ferric high-spin hemoglobins but also in low-spin hemoglobins, and the quaternary structural change by addition of IHP is similar to the R-T transition, although quaternary structure of ferric low-spin hemoglobin with IHP is somewhat different from that of ferric high-spin of ferrous hemoglobins.

Here one may ask why the NMR spectra of the complementary pairs $(\alpha^{+},\beta)_2$ are little affected by addition of IHP as shown in Figures 1, 2, and 4. It is also to be noted that Cassoly and Gibson (1972) and Perutz et al. (1976) suggested on the basis of no optical spectrum responses to the addition of organic phosphate that organic phosphate does not change the quaternary structure of valency hybrid hemoglobin containing ferric heme in the α subunit. Therefore, the energy to change the quaternary structure for $(\alpha,\beta^{+})_2$ seems to be smaller than that for $(\alpha^{+},\beta)_2$, since the binding energy of IHP would suffice to change quaternary structure of the protein to the T-like state for $(\alpha,\beta^{+})_2$ but not for $(\alpha^{+},\beta)_2$. In other words, the T-like quaternary structure is more favored in $(\alpha,\beta^{+})_2$ compared with $(\alpha^{+},\beta)_2$. This is further confirmed by the present observation that there appears the T-state marker signal at 6.5 ppm for stripped $(\alpha_{CO}\beta^{+}_{H_2O})_2$ but not for $(\alpha^{+}_{H_2O}\beta_{CO})_2$ as shown in Figure 5. The same result was also obtained here for carboxy fluoride valency hybrids (the result is not shown). The higher oxygen affinity for $(\alpha^{+}_{N_3^-}\beta_{deoxy})_2$, $(\alpha^{+}_{H_2O}\beta_{deoxy})_2$, and $(\alpha^{+}_{F^-}\beta_{deoxy})_2$ than for their complementary hybrids (Banerjee & Cassoly, 1969) may also be interpreted along these lines. However, Ogawa and Shulman (1972) and Ogawa et al. (1972) reported that oxy quaternary structure is much more favored in $(\alpha_{deoxy}\beta^{+}_{CN^-})_2$ than the deoxyhemoglobin-like structure because an extremely strong allosteric effector like IHP is required to switch its quaternary structure, whereas in $(\alpha^{+}_{CN^-}\beta_{deoxy})_2$, the energy to change its quaternary structure is rather small and can be switched by DPG or inorganic phosphate. Therefore, it is premature to conclude that there is an unequivocal correlation between those different physicochemical properties and quaternary conformations in the complementary pairs of hybrids. It may be more safe to say that the different spectral responses between the complementary pairs of valency hybrids to organic phosphate could be due to a difference in their quaternary conformations. Possible presence of so many different quaternary structures also suggests that the two structural models are not always adequate to describe the quaternary structures of Hb, as mentioned for the case of the asymmetric hybrid Hbs (Miura & Ho, 1982).

In summary, the present NMR results have served to elucidate the different sensitivities to IHP between α and β subunits and gave additional support for the presence of the "T-like" state in ferric low-spin methemoglobins, which means that the quaternary structure of hemoglobins does not always link to the movement of iron displacement into or out of the heme plane.

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