Nitta, K., Takayanagi, G., & Kawauchi, H. (1983) *Chem. Pharm. Bull. 31*, 315.
Noguchi, H. (1903) *Zentralbl. Bakteriol., Parasitenkd., Infektionskrankh. Hyg., Abt. 1: Orig. 133*, 362.
Omenn, G. S., Fontana, A., & Anfinsen, C. B. (1970) *J. Biol. Chem. 245*, 1895.
Pauley, G. B., Granger, G. A., & Krassner, S. M. (1971) *J. Invertebr. Pathol. 18*, 207.
Ravindranath, M. H., Higa, H. H., Cooper, E. L., & Paulson, J. C. (1985) *J. Biol. Chem. 50*, 8850.
Roche, A.-C., Schauer, R., & Mosigny, M. (1975) *FEBS Lett. 57*, 245.
Sakakibara, F., Takayanagi, G., Ise, H., & Kawauchi, H. (1977) *Yakugaku Zasshi 97*, 855.
Sakakibara, F., Kawauchi, H., Takayanagi, G., & Ise, H. (1979) *Cancer Res. 39*, 1347.

Proton Nuclear Magnetic Resonance and Spectrophotometric Studies of Nickel(II)–Iron(II) Hybrid Hemoglobins†

Naoya Shibayama,*,‡ Toshiro Inubushi,‡ Hideki Morimoto,§ and Takashi Yonetani‡

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6089, and Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

Received September 19, 1986; Revised Manuscript Received December 12, 1986

ABSTRACT: Ni(II)–Fe(II) hybrid hemoglobins, $\alpha(Fe)_2\beta(Ni)_2$ and $\alpha(Ni)_2\beta(Fe)_2$, have been characterized by proton nuclear magnetic resonance with Ni(II) protoporphyrin IX (Ni-PP) incorporated in apoprotein, which serves as a permanent deoxyheme. $\alpha(Fe)_2\beta(Ni)_2$, $\alpha(Ni)_2\beta(Fe)_2$, and NiHb commonly show exchangeable proton resonances at 11 and 14 ppm, due to hydrogen-bonded protons in a deoxy-like structure. Upon binding of carbon monoxide (CO) to $\alpha(Fe)_2\beta(Ni)_2$, these resonances disappear at pH 6.5 to pH 8.5. On the other hand, the complementary hybrid $\alpha(Ni)_2\beta(Fe\text{–}CO)_2$ showed the 11 and 14 ppm resonances at low pH. Upon raising pH, the intensities of both resonances are reduced, although these changes are not synchronized. Electronic absorption spectra and hyperfine-shifted proton resonances indicate that the ligation of CO in the β(Fe) subunits induced changes in the coordination and spin states of Ni-PP in the α subunits. In a deoxy-like structure, the coordination of Ni-PP in the α subunits is predominantly in a low-spin ($S = 0$) four-coordination state, whereas in an oxy-like structure the contribution of a high-spin ($S = 1$) five-coordination state markedly increased. Ni-PP in the β subunits always takes a high-spin five-coordination state regardless of solution conditions and the state of ligation in the partner α(Fe) subunits. In the β(Ni) subunits, a significant downfield shift of the proximal histidyl $N_\delta H$ resonance and a change in the absorption spectrum of Ni-PP were detected, upon changing the quaternary structure of the hybrid. Furthermore, the proximal histidyl resonance of $\alpha(Fe\text{–}CO)_2\beta(Ni)_2$ undergoes a slight upfield shift upon raising the pH from 6.5 to 8.5, while the 14 ppm resonance, the marker signal of a deoxy quaternary structure, hardly changes in this pH region. In addition, ring current shifted resonances in both hybrid Hbs were examined. The chemical shifts were analyzed in terms of the E11-Val methyls vs. the porphyrin rings in hybrid Hbs.

Even though Hb[1] is a well-investigated allosteric protein, the mechanism of cooperative oxygenation is not fully understood. This is caused by the difficulty in characterizing the intermediate species directly in the course of oxygenation, since the intermediate species of ligation are present in low concentrations in a cooperative system. Thus, many kinds of hybrid Hbs, which were regarded as models for the intermediate species, have been artificially prepared and examined (Banerjee & Cassoly, 1969; Ikeda-Saito et al., 1977; Blough & Hoffman, 1984; Simolo et al., 1985).

We have surveyed metal-substituted hybrid Hbs, $\alpha(Fe)_2\beta(M)_2$ and $\alpha(M)_2\beta(Fe)_2$, in which the hemes in either α or β subunits are substituted with porphyrins containing iron-series transition metal ions (M). The oxygen equilibrium properties of these hybrid Hbs indicated that Ni(II) protoporphyrin IX, Cu(II) protoporphyrin IX, and Zn(II) protoporphyrin IX behave like a permanent deoxyheme when the first oxygen molecule binds to these hybrid Hbs (Shibayama et al., 1986a; N. Shibayama et al., unpublished results; Miyazaki et al., unpublished results). We further carried out several chemical modifications such as des-Arg(α141), *N*-ethylsuccinimide–β93-Cys [NES(β93-Cys)], and NES(β93-Cys)-des-Arg(α141) on Ni–Fe hybrid Hbs (Shibayama et al., 1986b). By comparison with the oxygen equilibrium data between modified

†This work was supported by a grant from the NIH (HL 14508 to T.Y.). All NMR spectra were taken in the medical school NMR facility at the University of Pennsylvania, which is partially supported by Grants SO7-RR-05415 and SO7-RR-07083 from the NIH.

‡University of Pennsylvania.
§Osaka University.

[1] Abbreviations: Hb, hemoglobin; M–Fe hybrid Hb, hybrid hemoglobin in which hemes in either the α or β subunit are substituted with an iron-series transition metal ion (M); $\alpha(Fe)_2\beta(Ni)_2$, hybrid hemoglobin containing ferrous protoporphyrin IX in the α subunits and nickel(II) protoporphyrin IX in the β subunits; $\alpha(Ni)_2\beta(Fe)_2$, hybrid hemoglobin complementary with $\alpha(Fe)_2\beta(Ni)_2$; NiHb, hemoglobin containing nickel(II) protoporphyrin IX in both the α and β subunits; Ni-PP, nickel(II) protoporphyrin IX; NMR, nuclear magnetic resonance; CO, carbon monoxide; Bistris, 2-[bis(2-hydroxymethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; FeHb, hemoglobin containing ferrous protoporphyrin IX in both the α and β subunits; IHP, inositol hexaphosphate; ZnHb, hemoglobin containing zinc(II) protoporphyrin IX in both the α and β subunits; SH, sulfhydryl.

0006-2960/87/0426-2194$01.50/0

Ni–Fe hybrid Hbs and the corresponding FeHbs, it was confirmed that Ni-PP acts like a deoxyheme even in modified apoprotein. Thus it may be reasonable to use Ni-PP as a model for a permanent deoxyheme.

The coordination of Ni-PP in $\alpha(Ni)_2\beta(Fe)_2$ was very sensitive to the structure of Hb, while that in $\alpha(Fe)_2\beta(Ni)_2$ was hardly affected (Shibayama et al., 1986a). Therefore, we could detect quite large spectrophotometric changes in the α(Ni) subunits in relation to a structural change of hybrid Hb; namely, four-coordinated Ni-PP was more stable than five-coordinated Ni-PP under the conditions that stabilize a deoxy-like structure.

Recently, proton NMR spectroscopy has given a great deal of information regarding the detailed structure of Hb in solution. Some H–D exchangeable protons have been assigned to particular hydrogen bonds associated with the quaternary structure of Hb and can be used as marker signals to characterize the quaternary structure of Hb. The exchangeable proton resonance at about 14 ppm downfield from sodium 3-(trimethylsilyl)propionate has been assigned to the intersubunit hydrogen bond between $\alpha_1$42 Tyr and $\beta_2$99 Asp (Fung & Ho, 1975), which stabilizes a deoxy quaternary structure. The exchangeable proton resonance at about 11 ppm downfield from sodium 3-(trimethylsilyl)propionate has been believed to originate from the intrasubunit hydrogen bond (β145 Tyr–β98 Val) in a deoxy-like structure (Viggiano et al., 1978). These exchangeable proton resonances have been used to characterize the structures of Ni–Fe hybrid Hbs in various conditions.

The presence or absence of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances as well as electronic absorption spectra provides information on the coordination and spin states of Ni-PP in each subunit. Especially, the magnitude of the hyperfine shift can be correlated to the bond strength between the proximal histidine and the porphyrin metal (La Mar et al., 1977, 1980; Takahashi et al., 1980; Inubushi et al., 1983).

Ring current shifted resonances of E11-Val methyl groups, which arise from local magnetic fields produced by the delocalized π electrons in the porphyrin plane, are sensitive to the geometrical relationship between the methyl groups and the porphyrin plane (Shulman et al., 1969, 1970). Therefore, we can survey the movements of E11-Val methyl groups of CO-bound Fe subunits in relation to the quaternary structure of Hb.

In this paper, the NMR results on deoxy Ni–Fe hybrid Hbs are used to check the validity of using Ni-PP as a model for a deoxyheme. Then, the ligand-induced structural changes of Hb will be discussed by use of the NMR results on CO-bound hybrid Hbs. Finally, we will point out a discrepancy between the two-state allosteric model (Monod et al., 1965) and our results.

Experimental Procedures

NiHb and Ni(II)–Fe(II) hybrid hemoglobins were prepared as previously described (Shibayama et al., 1986a). Proton NMR spectra were obtained by a Bruker WH-360 spectrometer equipped with an ASPECT 2000A computer system operating at 360.04 MHz. Hydrogen-bonded proton resonances and ring current shifted resonances were measured by a modified Redfield 2-1-4 pulse sequence to minimize the water signal. For each spectrum, four to eight blocks of 256 Fourier-transformed free induction decays (FIDs) were accumulated with a 3-Hz line broadening applied. Hyperfine-shifted signals were obtained by super WEFT (Inubushi & Becker, 1983) with an 80-kHz spectral window and a 0.1-s repetition of pulse sequence. In order to obtain a reasonable signal to noise ratio, 32–128 blocks of 1024 Fourier-transformed FIDs were accumulated with a 20-Hz line broadening. Measurements were carried out at 20 °C. All samples were prepared in approximately 5% D_2O (v/v) and 50 mM Bistris or Tris buffer with 100 mM chloride. The concentration of samples was 0.5–1.0 mM for tetrameric Hb. Chemical shifts were measured from the resonance position of the internal standard *p*-dioxane [approximately 0.05% (v/v)] and converted to the shifts from 3-trimethylsilylpropionate (TSP), by using a 3.53 ppm offset and assigning positive values to downfield shifts.

Electronic absorption spectra were recorded on a Hitachi 557 spectrophotometer, which was interfaced to an IBM personal computer for storing the data and calculating the difference spectra. Measurements were carried out at 20 °C in 50 mM Bistris or Tris buffer with 100 mM chloride. The concentration of samples was 7.4 μM for tetrameric Hb. The pass length of the detection light is 5 mm.

Results

Hydrogen-Bonded Proton Resonances. The proton NMR spectra of NiHb, deoxy Ni–Fe hybrid Hbs, and deoxy-FeHb are illustrated in Figure 1B. These Hbs show exchangeable proton resonances at about 14 and 11 ppm, which have been assigned to the hydrogen bonds stabilizing a deoxy-like structure (Fung & Ho, 1975; Viggiano et al., 1978). These resonances of NiHb, deoxy Ni–Fe hybrid Hbs, and deoxy-FeHb are hardly affected by pH variation (pH 6.5–8.5) and the presence or absence of IHP. NiHb in D_2O solution exhibits broad nonexchangeable proton resonances around 12.6 and 10.9 ppm and sharper resonances at 10.3 and 9.8 ppm. We found that the two broad nonexchangeable resonances are due to the β(Ni) subunits and other sharper resonances are due to the α(Ni) subunits, by comparison with the spectra of Ni–Fe hybrid Hbs in D_2O solution (spectra are not shown). It should be noted that the chemical shifts of these broader nonexchangeable proton resonances are temperature-dependent, suggesting the presence of a paramagnetic center within the β(Ni) subunits.

Half-liganded hybrid Hb, $\alpha(Fe–CO)_2\beta(Ni)_2$, exhibits none of the exchangeable proton resonances at 14 and at 11 ppm (pH 6.5–8.5). However, it exhibits another exchangeable proton resonance at 10.8 ppm (Figure 2B), which has been assigned to the hydrogen bond in an oxy-like structure (Fung & Ho, 1975). A broad resonance observed at around 11 ppm is due to a nonexchangeable proton as mentioned above. In the presence of IHP, the 14 and 11 ppm resonances appear, while the 10.8 ppm resonance disappears at pH 6.5 to pH 7.4 (Figure 2B). A 10-fold excess of IHP showed no appreciable effect on the NMR spectrum of $\alpha(Fe–CO)_2\beta(Ni)_2$ at pH 8.5.

In the case of the complementary hybrid Hb, $\alpha(Ni)_2\beta(Fe–CO)_2$, the 14 and 11 ppm resonances are observed in full intensities only at low pH as shown in Figure 3A. Upon raising the pH, the 14 ppm resonance first decreases in its intensity, followed by line broadening above pH 7.4. At pH 7.9 the 14 ppm resonance has become much broader and the intensity of the 11 ppm resonance is decreased, and concomitantly, the 12.7 ppm signal is split into two peaks. Above pH 7.9, a small resonance at 10.6 ppm is detected. In the presence of IHP, the intensities of the 14 and 11 ppm resonances increased significantly (spectra are not shown).

Absorption Spectra of Ni Subunits in Ni–Fe Hybrid Hbs. Coordination states of Ni-PP in the α subunits were affected by pH, IHP, and the ligation of the partner β(Fe) subunits, if the structure is biased too much to neither a deoxy-like

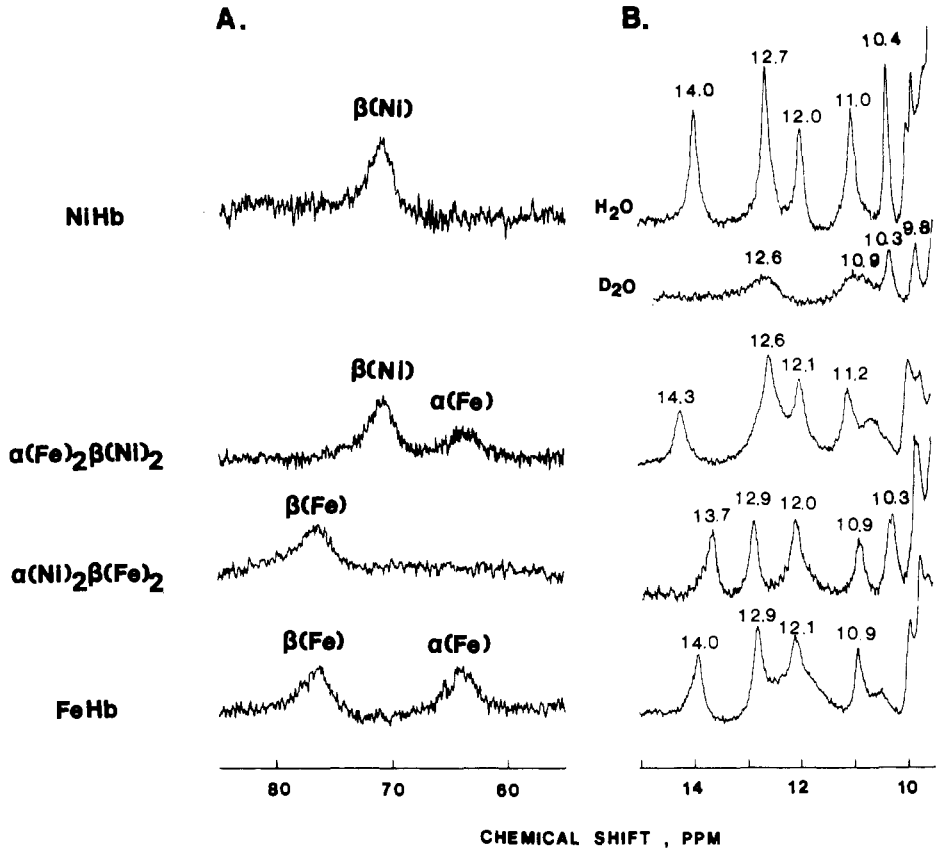

FIGURE 1: Proton NMR spectra (360.04 MHz) of NiHb, deoxy $\alpha(Fe)_2\beta(Ni)_2$, deoxy $\alpha(Ni)_2\beta(Fe)_2$, and deoxy-FeHb in 50 mM Bistris with 100 mM chloride buffer at pH 7.4 at 20 °C: (A) hyperfine-shifted proximal histidyl $N_\delta H$ proton resonances; (B) hydrogen-bonded proton resonances.

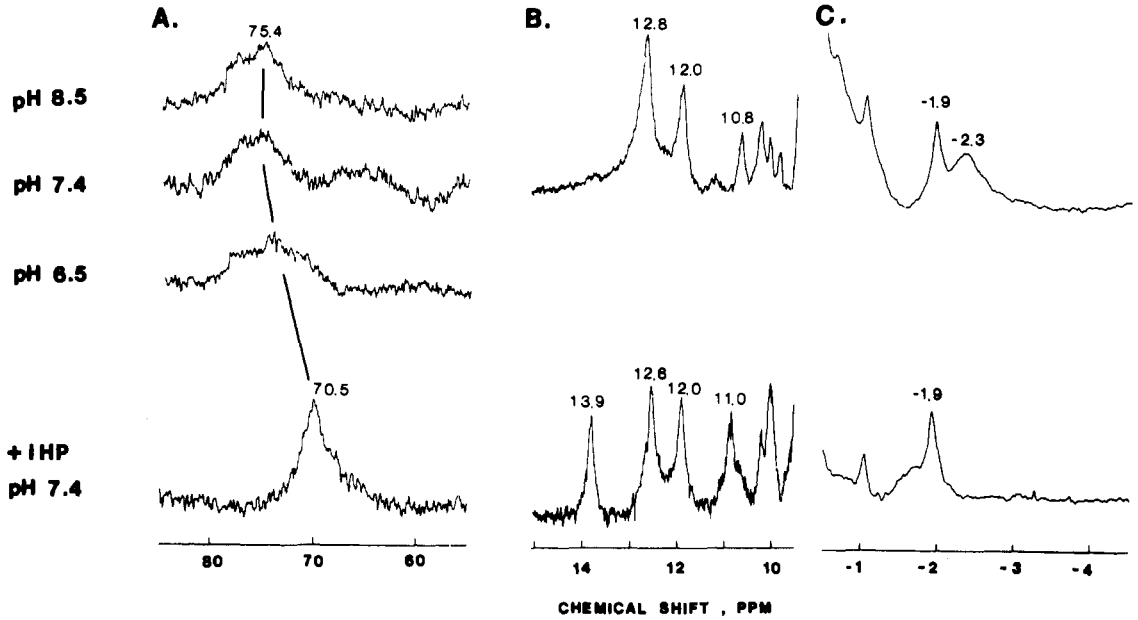

FIGURE 2: Proton NMR spectra (360.04 MHz) of $\alpha(Fe{-}CO)_2\beta(Ni)_2$ in 50 mM Bistris or Tris buffer with 100 mM chloride at 20 °C: (A) hyperfine-shifted proximal histidyl resonances; (B) hydrogen-bonded proton resonances; (C) ring current shifted resonances.

structure nor an oxy-like structure (Shibayama et al., 1986a). In deoxy $\alpha(Ni)_2\beta(Fe)_2$, Ni-PP always takes a four-coordination state regardless of solution conditions (Shibayama et al., 1986). However, in $\alpha(Ni)_2\beta(Fe{-}CO)_2$, the coordination states of Ni-PP are pH-dependent. Figure 4A shows the difference spectra of $\alpha(Ni)_2\beta(Fe{-}CO)_2$ at pH 6.5, 6.9, 7.4, and 7.9 taken against a reference spectrum at pH 8.5. These difference spectra are due to the change of the coordination state in the $\alpha(Ni)$ subunits. A peak at 398 nm and a trough at 422 nm have been assigned to four-coordination and five-coordination states of Ni-PP, respectively (McLees & Caughey, 1968; Alston et al., 1984; Shelnutt et al., 1986; Shibayama et al., 1986a). At pH 6.5, the $\alpha(Ni)$ subunits are dominantly in the four-coordination state. Upon raising the pH, the five-coordinated Ni-PP is markedly increased in $\alpha(Ni)_2\beta(Fe{-}CO)_2$. According to our preliminary estimations, about half of the Ni-PPs remain in the four-coordination state even at pH 8.5 in the absence of IHP.

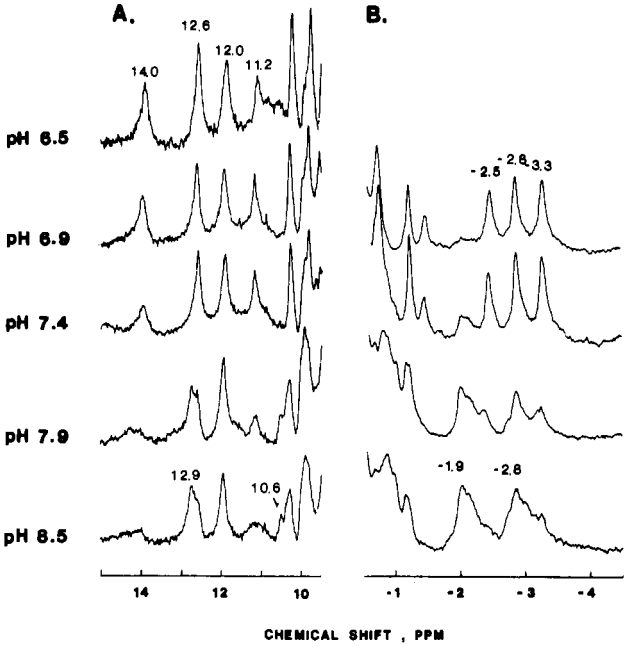

FIGURE 3: Proton NMR spectra (360.04 MHz) of $\alpha(Ni)_2\beta(Fe–CO)_2$ in 50 mM Bistris or Tris buffer with 100 mM chloride at 20 °C: (A) hydrogen-bonded proton resonances; (B) ring current shifted resonances.

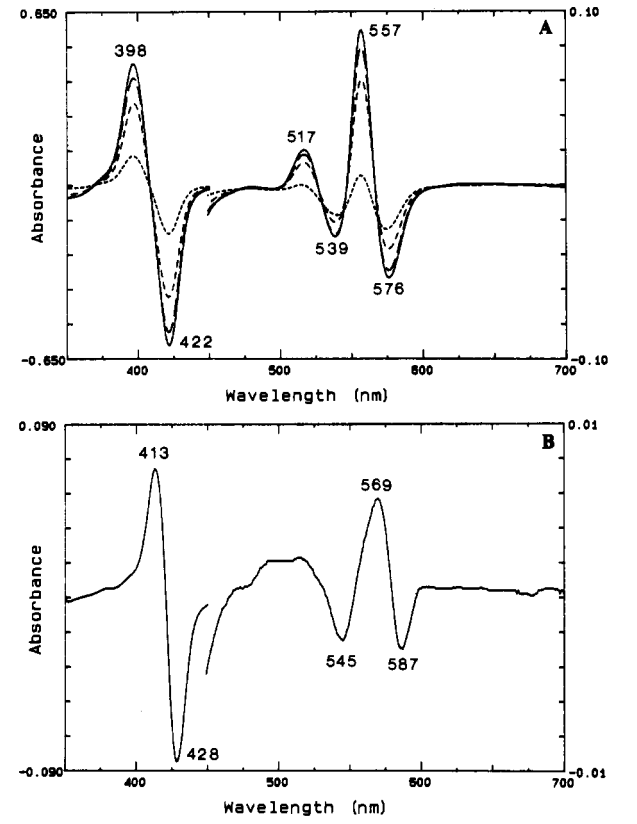

FIGURE 4: (A) Difference absorption spectra for $\alpha(Ni)_2\beta(Fe–CO)_2$ at pH 6.5 (—), pH 6.9 (– –), pH 7.4 (- - -), and pH 7.9 (···) in 50 mM Bistris or Tris buffer with 100 mM chloride at 20 °C, using the spectrum at pH 8.5 in 50 mM Tris buffer with 100 mM chloride as a reference. The protein concentration is 7.4 μM for tetrameric Hb, and the pass length of light is 5 mm. (B) Difference absorption spectrum for $\alpha(Fe–CO)_2\beta(Ni)_2$ at pH 7.4 in 50 mM Bistris buffer with 100 mM chloride in the presence and absence (reference) of 330 μM IHP. The protein concentration is 7.4 μM for tetrameric Hb, and the pass length of light is 5 mm.

In the case of the β(Ni) subunits, only the five-coordination state of Ni-PP is observed regardless of solution conditions.

Table I: Resonance Position of the Proximal Histidyl NH for NiHb, Ni–Fe Hybrid Hbs, and FeHb[a]

	pH	α(Ni)[b]	β(Ni)[b]	α(Fe)[b]	β(Fe)[b]
NiHb	7.4		70.8		
$\alpha(Fe)_2\beta(Ni)_2$	7.4		70.5	63.6	
$\alpha(Fe–CO)_2\beta(Ni)_2$	6.5		73.0		
	7.4		75.5		
	8.5		75.4		
	7.4 (+IHP)[c]		70.5		
$\alpha(Ni)_2\beta(Fe)_2$	7.4				76.1
$\alpha(Ni)_2\beta(Fe–CO)_2$	7.4	77.0[d]			
	8.5	77.3			
	7.4 (+IHP)				
FeHb	7.4			64.0	76.4

[a] Experimental conditions are as follows: temperature, 20 °C; buffer, 50 mM Bistris or Tris buffer with 100 mM chloride; sample concentration, approximately 1 mM for tetrameric Hb. [b] Chemical shifts from sodium 3-(trimethylsilyl)propionate in ppm. [c] In the presence of IHP (9 mM). [d] The resonance shows apparently reduced intensity.

However, the Soret absorbance increases slightly upon addition of IHP (Shibayama et al., 1986a). Figure 4B shows a difference spectrum of $\alpha(Fe–CO)_2\beta(Ni)_2$ at pH 7.4 in the presence and absence (reference) of IHP. This difference spectrum corresponds to a blue shift of the absorbance maximum and an increase of intensity upon addition of IHP. The intensity of this spectral change is about 10 times smaller than that observed in the α(Ni) subunits.

Hyperfine-Shifted Proton Resonances. Deoxy-FeHb shows two exchangeable proton resonances at 64.0 and 76.4 ppm, which have been assigned to the proximal histidyl $N_\delta H$ protons in deoxy α(Fe) and deoxy β(Fe) subunits, respectively (La Mar et al., 1977; Takahashi et al., 1980). The proton NMR spectra of NiHb, deoxy $\alpha(Fe)_2\beta(Ni)_2$, deoxy $\alpha(Ni)_2\beta(Fe)_2$, and deoxy-FeHb in the proximal His regions, from 55 to 85 ppm, are shown in Figure 1A. By comparison of the spectra between NiHb and deoxy $\alpha(Fe)_2\beta(Ni)_2$, the resonance at about 70.5 ppm, which is observed in both NiHb and deoxy $\alpha(Fe)_2\beta(Ni)_2$ but not in deoxy $\alpha(Ni)_2\beta(Fe)_2$ and deoxy-FeHb, is assigned to the hyperfine-shifted proximal histidyl $N_\delta H$ resonance in the β(Ni) subunits. The resonance position is comparable to those of hyperfine-shifted proximal histidyl $N_\delta Hs$ bound to Fe porphyrin in deoxy-Hbs. This assignment is further supported by the absence of this peak in D_2O (spectra are not shown). It should be noted that the α(Ni) subunits in NiHb and deoxy $\alpha(Ni)_2\beta(Fe)_2$ do not show such a hyperfine-shifted exchangeable proton resonance in the region 25–95 ppm.

Deoxy α(Fe) subunits in $\alpha(Fe)_2\beta(Ni)_2$ and deoxy β(Fe) subunits in $\alpha(Ni)_2\beta(Fe)_2$ exhibit hyperfine-shifted proximal histidyl $N_\delta H$ proton resonances at 63.6 and 76.1 ppm, respectively. These chemical shifts are identical with those for deoxy-FeHb (Figure 1A and Table I).

Similar measurements were carried out for the half-liganded Hbs, $\alpha(Fe–CO)_2\beta(Ni)_2$ and $\alpha(Ni)_2\beta(Fe–CO)_2$, as summarized in Table I. The CO-bound Fe subunits do not show hyperfine-shifted resonances because of diamagnetism of porphyrin iron. The $N_\delta H$ resonances of the β(Ni) subunits are shifted downfield by approximately 5 ppm upon CO binding to the partner α(Fe) subunits at pH 7.4 and pH 8.5, and then, the resonances are somewhat broader than those of deoxy $\alpha(Fe)_2\beta(Ni)_2$. In the low-pH region, a much broader signal is obtained around 73 ppm. With the presence of IHP at pH 7.4, the $N_\delta H$ signal is observed at the same position as that for deoxy $\alpha(Fe)_2\beta(Ni)_2$ and recovers its narrower line width.

In the case of $\alpha(Ni)_2\beta(Fe–CO)_2$, the $N_\delta H$ signal of the α(Ni) subunits is detected at 77 ppm only at high pHs (Table I). At pH 7.4 the $N_\delta H$ resonance markedly reduced its intensity, and at lower pH or in the presence of IHP the reso-

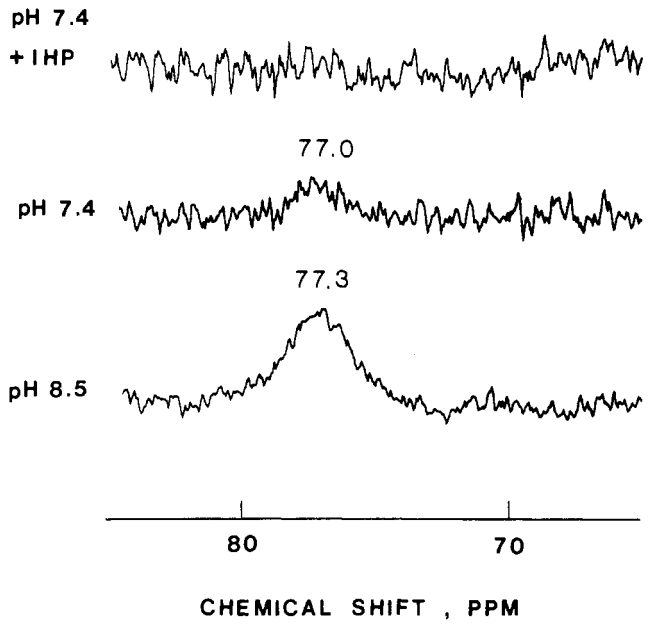

FIGURE 5: Hyperfine-shifted proximal histidyl $N_\delta H$ proton NMR spectra (360.04 MHz) of $\alpha(Ni)_2\beta(Fe–CO)_2$ at pH 8.5, pH 7.4, and pH 7.4 in the presence of IHP (9 mM) at 20 °C. Buffers consist of 50 mM Tris (pH 8.5) or Bistris (pH 7.4) and 100 mM chloride.

nance became beyond detection as shown in Figure 5.

Ring Current Shifted Resonances. In carbonmonoxy-FeHb (HbCO), the upfield resonance at about −1.8 ppm has been assigned to the γ_2-methyl of E11-Val in both α(Fe–CO) and β(Fe–CO) subunits (Lindstrom et al., 1972; Lindstrom & Ho, 1973; Dalvit & Ho, 1985), since the γ_2-methyl of E11-Val is the closest methyl group to the heme plane in both subunits, as indicated by the X-ray studies of HbCO (Baldwin & Chothia, 1979; Baldwin, 1980). Figure 6 shows the NMR spectra of NiHb, deoxy $\alpha(Fe)_2\beta(Ni)_2$, deoxy $\alpha(Ni)_2\beta(Fe)_2$, and HbCO in the upfield region. In this region, deoxy Fe subunits do not show the resonances of the E11-Val because the paramagnetic iron ion exists in the porphyrin center. Thus, two sharp resonances around −3 ppm, which are observed in both NiHb (−2.7 and −2.9 ppm) and deoxy $\alpha(Ni)_2\beta(Fe)_2$ (−2.8 and −3.1 ppm), can be considered as the ring current shifted resonances in the α(Ni) subunits. Similarly, the broader resonances at around −2 ppm, which are observed in both NiHb and deoxy $\alpha(Fe)_2\beta(Ni)_2$, can be derived from the β(Ni) subunits. At this time, it is rather difficult to assign these upfield resonances in both Ni subunits; particularly in the case of β(Ni), it should be necessary to estimate the paramagnetic effect on these peaks. However, these NMR spectra are very useful for assigning the ring current shifted resonances of CO-bound Fe subunits in $\alpha(Fe–CO)_2\beta(Ni)_2$ and $\alpha(Ni)_2\beta(Fe–CO)_2$.

In $\alpha(Fe–CO)_2\beta(Ni)_2$, the α(Fe–CO) subunits exhibit a resonance of the E11-Val γ_2-methyl at about −1.9 ppm, and the β(Ni) subunits show a broad resonance around −2.3 ppm (Figure 2C). In the presence of IHP, the β(Ni) signal shifts to −2 ppm, although the resonance position of the α(Fe–CO) signal is not changed significantly. The resonances of $\alpha(Ni)_2\beta(Fe–CO)_2$ are more complicated as shown in Figure 3B. At low pH or in the presence of IHP, the α(Ni) subunits show two resonances at −2.8 and −3.3 ppm, and the β(Fe–CO) subunits show a resonance of the E11-Val γ_2-methyl at −2.5 ppm. Assignment of these signals was carried out by comparison with the spectra between deoxy $\alpha(Ni)_2\beta(Fe)_2$ and $\alpha(Ni)_2\beta(Fe–CO)_2$ at low pH. It should be mentioned that the three resonances of $\alpha(Ni)_2\beta(Fe–CO)_2$ in a deoxy-like structure

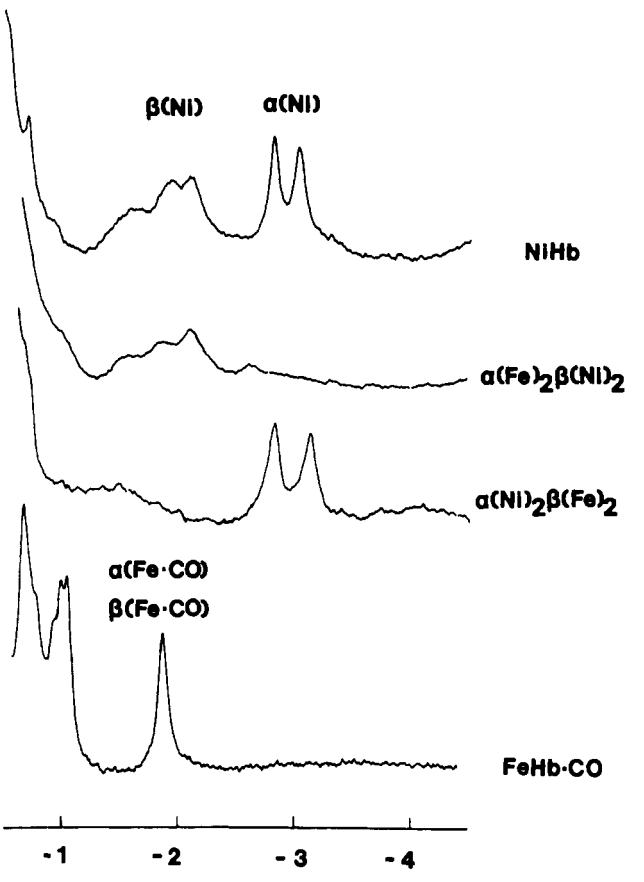

FIGURE 6: Ring current shifted proton NMR spectra (360.04 MHz) of NiHb, deoxy $\alpha(Fe)_2\beta(Ni)_2$, deoxy $\alpha(Ni)_2\beta(Fe)_2$, and HbCO in 50 mM Bistris buffer with 100 mM chloride at pH 7.4 at 20 °C.

show approximately equal integrated intensities. Upon raising the pH, the −2.8 ppm resonance, which could be due to the α(Ni) subunits in an oxy-like structure, and the −1.9 ppm resonance of the E11-Val γ_2-methyl in the β(Fe–CO) subunits are intensified. The position of the latter resonance is almost identical with that of HbCO.

Discussion

Features of NiHb. Recently, NiHb has been characterized by various physicochemical measurements such as the β93 Cys-SH reactivity, tetramer–dimer equilibrium, circular dichroic spectra, and X-ray crystallographic structural data (Alston et al., 1984). These data supported a conclusion that the structure of NiHb is very similar to that of deoxy-FeHb. The present NMR results of NiHb reveal the existence of hydrogen bonds that stabilize a deoxy-like quaternary structure of Hb (Figure 1).

Electronic absorption and resonance Raman spectra of NiHb distinguished two types of porphyrin environments in NiHb (Alston et al., 1984; Shelnutt et al., 1986). One is a four-coordinated Ni(II) site, and the other is a five-coordinated Ni(II) site. Our previous results suggested that the four-coordinated and five-coordinated Ni(II) ions are localized in the α and β subunits in NiHb, respectively, by use of Ni–Fe hybrid Hbs (Shibayama et al., 1986a). Further, the present NMR data on hyperfine-shifted resonances clearly show that the proximal His (F8) serves as an axial ligand only in the paramagnetic β(Ni) subunits of NiHb (Figure 1).

Spin States and Coordination States of Ni-PP in Hybrid Hbs. The spin states of four-coordinated (square-planar) and

six-coordinated (square-bipyramidal) Ni porphyrin derivatives have been reported to be low-spin Ni(II) ($3d_8$, $S = 0$) and high-spin Ni(II) ($3d_8$, $S = 1$) species, respectively (McLees & Caughey, 1968). Furthermore, the existence of both high- and low-spin five-coordinated Ni porphyrin derivatives has been indicated by NMR studies (Abraham & Swinton, 1969; La Mar & Walker, 1979). As shown in Table I, in Ni–Fe hybrid Hbs, only five-coordinated Ni-PPs can show its paramagnetism, which resulted in the observed hyperfine-shifted resonances.[2] Thus, one can conclude that the four-coordinated Ni-PP in the α subunits is in a low-spin state and the five-coordinated Ni-PP in both subunits contains a high-spin Ni(II) ion. In deoxy $\alpha(Fe)_2\beta(Ni)_2$, the β(Ni) subunits show the proximal histidyl $N_\delta H$ resonance with full intensity corresponding to two protons per tetrameric Hb by comparison with the $N_\delta H$ resonance of the deoxy α(Fe) subunits. This finding indicates that only high-spin Ni(II) ion exists in the five-coordinated Ni(II) site, and thus, a low-spin five-coordinated Ni(II) has not been observed in Ni–Fe hybrid Hbs.

It should be mentioned that the $N_\delta H$ resonance of $\alpha(Ni)_2\beta(Fe–CO)_2$ is observed at about 77 ppm in a high-pH region (Figure 5), although the α(Ni) subunits contain both low-spin (four-coordinated) and high-spin (five-coordinated) Ni(II) ions, suggesting the slow conversion of protein structure on an NMR time scale, which controls Ni-PP environments.

Ring Current Shifted Resonances. In the upfield region, NiHb shows sharp peaks at −2.7 and −2.9 ppm for the α(Ni) subunits ($S = 0$) and broad peaks around −2 ppm for the paramagnetic β(Ni) subunits. This broadening observed in only β(Ni) subunits could be due to the presence of paramagnetic Ni-PP.

Previously, two sets of upfield resonances have been reported in Zn^{II}Hb ($S = 0$), which exhibits characteristics of deoxy-Hb. The authors mentioned that one of the resonances in each subunit was not observed in the CO-bound Zn(II)–Fe(II) hybrid Hbs (Simolo et al., 1985). In this study, we found that the two resonances in the α(Ni) subunits are due to the two methyl groups in a deoxy-like structure, since each resonance has the same integrated intensity corresponding to one methyl group by comparison with the resonance of the E11-Val γ_2-methyl in the β(Fe–CO) subunits (Figure 3B, pH 6.9). In the case of deoxy-FeHb, the E11-Val γ_2-methyl is located closest to the heme plane in both subunits, as indicated by X-ray studies (Fermi, 1975). If it is assumed that the structure of NiHb is identical with that of deoxy-FeHb, the most likely candidate for one of two upfield resonances in the α(Ni) subunit could be the E11-Val γ_2-methyl.[3]

In an oxy-like structure, one of the methyl groups, which may be probably the E11-Val γ_2-methyl, resonates at −2.8 ppm in the α(Ni) subunits. While the other methyl resonance is missing in the region −4 to −1 ppm (Figure 3B, pH 8.5), suggesting that the position of this group relative to the heme plane is very sensitive to the change of the quaternary structure. This result is consistent with the data of ring current shifted resonances for Zn(II)–Fe(II) hybrid Hbs (Simolo et al., 1986).

Ni-PP in both α and β subunits in Ni–Fe hybrid Hbs in an oxy-like structure bears paramagnetism as discussed above. In these cases, not only a ring current effect but also a paramagnetic effect (pseudocontact shift) could contribute to the chemical shifts, if five-coordinated Ni shows nonnegligible magnetic anisotropy. In order to detail the geometry of the porphyrin-surrounding residues in the deoxy subunits with Ni subunits as models, it is necessary to estimate the contribution of the paramagnetic effects and ring current effects, respectively.

Ring current shifted resonances of the CO-bound Fe subunits in Ni–Fe hybrid Hbs show significant subunit inequivalence upon changing the quaternary structure. The α(Fe–CO) subunits exhibit the ring current shifted resonance at −1.9 ppm regardless of the quaternary structure (Figure 2). The β(Fe–CO) subunits within an oxy-like structure show the resonance at −1.9 ppm, whereas in a deoxy-like structure the resonance is shifted upfield by approximately 0.5 ppm (Figure 3). This finding, i.e., a larger shift of the β(Fe–CO) subunits upon changing the quaternary structure, is consistent with the previous measurements of Hb Kansas (β102 Asn → Thr) and Hb M Iwate (α87 His → Tyr) reported by Ogawa and coworker (Ogawa et al., 1972; Mayer et al., 1973). These subunit inequivalences are derived from a larger movement of E11-Val relative to the heme plane in the β(Fe–CO) subunits compared with that of the α(Fe–CO) subunits, upon the quaternary structural change.

Quaternary Structure of Ni–Fe Hybrid Hbs. We have reported that Ni-PP is a good model for a permanent deoxyheme judging from its effect on the heme in the partner Fe subunits in Ni–Fe hybrid Hbs (Shibayama et al., 1986). Hyperfine-shifted proximal histidyl $N_\delta H$ resonances of the Fe subunit in deoxy Ni–Fe hybrid Hbs show the same chemical shifts as those for deoxy-FeHb (Table I and Figure 1A), and hydrogen-bonded proton resonances of deoxy hybrid Hbs are similar to those of deoxy-FeHb resonances (Figure 1B). These spectral similarities also support our prognostication that Ni-PP acts like a deoxyheme in the Hb molecule. If Ni-PP can stay as a permanent deoxyheme even in diliganded hybrid Hbs, one may conclude that the ligation of the α subunits sensitively affects the quaternary structure of Hb compared with the ligation of the β subunits.

We previously reported that K_1 values (the equilibrium constants for the first oxygen molecule) of Ni–Fe hybrid Hbs agreed well with those of native FeHb in a variety of environmental conditions, whereas two types of Ni–Fe hybrid Hbs showed different degrees of cooperativity upon oxygenation (Shibayama et al., 1986). $\alpha(Ni)_2\beta(Fe)_2$ showed a Hill coefficient of near unity, while $\alpha(Fe)_2\beta(Ni)_2$ exhibited a Hill coefficient significantly higher than unity at high pH. At high pH oxygenation of one β(Fe) subunit could affect the coordination states of the α(Ni) subunit[4] without affecting the oxygen affinity of the other β(Fe) subunit in $\alpha(Ni)_2\beta(Fe)_2$. This observation indicated that the ligand-induced structural change in $\alpha(Ni)_2\beta(Fe)_2$ can be transmitted from a β(Fe) subunit to either the α_1 or α_2 subunit in the high-pH region

[2] Our preliminary data on a hyperfine-shifted nonexchangeable proton resonance at 19.7 ppm are consistent with this result. We checked that the chemical shift of this resonance is temperature-dependent.

[3] If there is no structural difference between the Hb molecule in solution and crystalline states, it is rather unlikely that the other upfield resonance is derived from the E11-Val γ_1-methyl, because the separation of two upfield resonances in the α(Ni) subunits is extremely smaller than that predicted by the X-ray structure of deoxy-FeHb (Fermi, 1975). The X-ray studies also indicated that the methyl groups of F4-Leu, F7-Leu, FG3-Leu, FG5-Val, G8-Leu, and H19-Leu are located around the heme plane in both subunits (Fermi, 1975; Baldwin & Chothia, 1979). It should be described that the δ_1-methyl of FG3-Leu is located relatively close to the heme plane in deoxy-FeHb (Fermi, 1975), and this methyl group could not be ruled out from a possible candidate for upfield resonance in deoxy-like structure.

[4] We measured the absorption spectra of $\alpha(Ni)_2\beta(Fe)_2$ under various oxygen pressure and checked the presence of isosbestic points. The wavelengths of these isosbestic points were pH-dependent and different from those of FeHb, affected by the spectral changes of the α(Ni) subunits (Shibayama et al., 1986a).

but not to the other β(Fe) subunit. This speculation is also confirmed by our NMR results, where hydrogen-bonded protons do not show a significant change at low pH, upon CO binding to $\alpha(Ni)_2\beta(Fe)_2$ (compare Figure 1B with Figure 3A). At high pH, significant changes in the NMR of hydrogen-bonded protons are observed upon CO binding to $\alpha(Ni)_2\beta(Fe)_2$, indicating that the transmission of the structural changes from the β(Fe) subunits to the α(Ni) subunits breaks some intersubunit hydrogen bonds.

Electronic absorption spectra of the α(Ni) subunits, hydrogen-bonded proton resonances, and ring current shifted resonances of methyl groups in $\alpha(Ni)_2\beta(Fe\text{–}CO)_2$ show a marked pH dependence; however, we have been unable to observe a hyperfine-shifted proximal histidyl proton resonance of the α(Ni) subunits in a deoxy-like structure because of their diamagnetism. There exist certain qualitative correlations between the porphyrin electronic states and the protein structural changes (compare with Figure 3 and Figure 4A). However, the behaviors of the hydrogen-bonded protons are not as straight forward as a simple two-state model (Monod et al., 1965). Upon raising the pH, first intensity reduction and broadening of the 14 ppm resonance are observed, indicative of a slow exchange between the intersubunit hydrogen-bonded (α42 Tyr–β99 Asp) proton and solvent proton. This is followed by the intensity reduction of the 11 ppm resonance. Then, the 10.6 ppm resonance assigned to the hydrogen-bonded proton in an oxy-like structure appears (Figure 3A). The observed splitting of the resonance at around 12.7 ppm, which was assigned to the $\alpha_1\beta_1$ interface hydrogen bond between α126 Asp and α35 Tyr (Asakura et al., 1976), indicates that the strength of the hydrogen bond is possibly altered by the quaternary structure change. If one assumes equilibrium between the two quaternary structures, namely, one represented as the 14 ppm resonance and the other represented as the 10.6 ppm resonance, it may be impossible to interpret the NMR spectral changes shown in Figure 3A.

In the case of $\alpha(Fe)_2\beta(Ni)_2$, hyperfine-shifted proximal histidyl proton resonances of the β(Ni) subunits are always observed regardless of solution conditions and the ligation state of the partner α(Fe) subunits. Upon CO binding to the α(Fe) subunits, the hydrogen-bonded proton resonances at 14 and 11 ppm disappear. The remaining hydrogen-bonded proton resonances are pH-independent. On the other hand, the proximal histidyl NH resonances is slightly altered by pH in the same pH region (Figure 2B and Table I). Especially, the broader NH resonance at pH 6.5 may consist of two components at 70.5 and 75.5 ppm. Thus, the breakage of the intersubunit hydrogen bond between α42 Tyr and β99 Asp could not be synchronized with the change of the β-heme environments.

Origin of the 11 ppm Resonance. In 1970 Perutz suggested that the rupture of the intrasubunit hydrogen bond between β145 Tyr and β98 Val may be closely linked to the increased reactivity of β93 Cys-SH in an oxy-like structure (Perutz, 1970). He proposed this mechanism by assuming the dynamic movements of β145 Tyr residues upon changing the quaternary structure of Hb. The exchangeable proton resonance at about 11 ppm has been assigned to the intrasubunit hydrogen bond between β145 Tyr and β98 Val by Viggiano et al. (1978), on the basis of Perutz's mechanism. However, recent X-ray studies revealed that the β145 Tyr remains hydrogen bonded to β98 Val throughout the quaternary structural change (Baldwin, 1980; Shaanan, 1983). Thus, the movements of β145 Tyr should be much smaller than previously proposed by Perutz. If the assignment for the 11 ppm resonance is correct,[5] why cannot the 11 ppm resonance be observed in an oxy-like structure?

Shaanan (1983) also suggested that the orientation of the β145 Tyr and β93 Cys residues in oxy-Hb were different from those in deoxy-Hb. In oxy-Hb, the side chain of β93 Cys was in internalized position and in contact with the β145 Tyr residue. In deoxy-Hb, the side chain of β93 Cys was exposed and separated from the β145 Tyr residues. In both states, the O–H group of β145 Tyr formed a hydrogen bond to the C=O group of β98 Val. From these considerations, one may attempt to explain that these conformational changes near the hydrogen bond significantly shift the proton resonance and it could not be observed in an oxy-like structure. Our preliminary data on the β93 Cys-SH reactivity toward 4,4′-dithiopyridine suggest that the rate constants for CO-bound Ni–Fe hybrid Hbs are as large (fast) as those for fully CO-bound FeHb at pH 6.5 to pH 8.5 in the absence of IHP (N. Shibayama et al., unpublished results). On the other hand, $\alpha(Ni)_2\beta(Fe\text{–}CO)_2$ shows the 11 ppm resonance, whose intensity is pH-dependent, indicating that the 11 ppm resonance may not be related with the reactivity of the β93 Cys-SH. Thus we may argue against the assignment of the 11 ppm resonance to the hydrogen bond between β145 Tyr and β98 Val as proposed by Viggiano et al (1978).

In the α subunit, the penultimate α140 Tyr also forms a intrasubunit hydrogen bond to the C=O group of α93 Val throughout the allosteric transition (Shaanan, 1983). However, during the transition the phenol ring of α140 Tyr is rotated by approximately 90° to adapt a movement of β37 Trp forming a hydrogen bond to α94 Asp in a deoxy quaternary structure (Baldwin & Chothia, 1979; Fermi, 1975). Deoxy-des-Arg(α141),Tyr(α140)-Hb did not show the 11 ppm resonance at pH 6.0 in the presence of IHP, although it exhibited the distinct 14 ppm resonance (Miura & Ho, 1984). Therefore, the intrasubunit hydrogen bond between α140 Tyr and α93 Val or the intersubunit hydrogen bond between β37 Trp and α94 Asp can remain as a possible candidate for the origin of the 11 ppm resonance.

Comparison with Other Previous Results. Simolo et al. reported the characterization of Zn(II)–Fe(II) hybrid Hbs by NMR and other kinetic measurements (Simolo et al., 1985). CO-bound Zn–Fe hybrid Hbs did not show the proton resonance at 14 ppm. However, the 11 ppm resonance was observable only in $\alpha(Zn)_2\beta(Fe\text{–}CO)_2$ at neutral pH. These results are consistent with the present result that α(Fe–

[5] They assigned the 11 ppm resonance to the intrasubunit hydrogen-bonded proton between β145 Tyr and β98 Val, using Hb Osler (β145 Tyr → Asp) and McKees Rocks (β145 Tyr → terminal). Both abnormal Hbs showed the resonances at around 11 ppm in the absence of IHP, although they provided very high affinity to oxygen and noncooperativity, indicative of an oxy-like structure. Thus, the authors ruled out the possibility that these resonances at around 11 ppm were derived from the same hydrogen-bonded proton as resonated at 11 ppm in deoxy native Hb. We may here point out other results for these abnormal Hbs and carboxypeptidase A treated Hb [des-His(β146),Tyr(β145)-Hb]: (i) in the absence of IHP, the proximal histidyl $N_\delta H$ resonances of the α subunits in these deoxy abnormal Hbs showed almost the same chemical shifts as those of deoxy native Hb (Takahashi et al., 1982; Miura & Ho, 1984), suggesting that the α-heme environment cannot be classified in an oxy-like structure; (ii) the NH resonance of the β subunits showed a further downfield shift upon removal of β146 His and β145 Tyr from native Hb (Miura & Ho, 1984), indicating that the removal of these residues affects the β-heme environment much more than the α-heme environment; (iii) our preliminary results of oxygen equilibria and resonance Raman spectra for carboxypeptidase A treated Ni–Fe hybrid Hb support that this chemical modification affects the β heme rather than the α heme. Thus, we cannot easily classify the quaternary structure of Hb Osler or Hb Mckees Rocks into an oxy- or a deoxy-like structure.

$CO)_2\beta(Ni)_2$ exhibits neither the resonance at 11 ppm nor that at 14 ppm. On the other hand, $\alpha(Ni)_2\beta(Fe–CO)_2$ shows the 11 and 14 ppm resonances, whereas the 14 ppm resonance is much broader and has a comparatively smaller intensity than the 11 ppm resonance in neutral and alkaline pH regions.

Recent NMR studies by Ho and co-workers have revealed important properties of Hb intermediates in the course of oxygenation (Viggiano & Ho, 1979; Miura & Ho, 1982, 1984). They suggested that a partially ligated Hb molecule took a unique quaternary structure that cannot be classified in neither a deoxy nor an oxy quaternary structure. Our present NMR results revealed more details of structural changes in the intermediate state species. The spectral changes of hydrogen-bonded protons in $\alpha(Ni)_2\beta(Fe–CO)_2$ are not concerted, and further, the proximal histidyl $N_\delta H$ resonance of the β(Ni) subunits in $\alpha(Fe–CO)_2\beta(Ni)_2$ undergoes a slight change without showing the 14 ppm signal. The reactivity of β93 Cys-SH in CO-bound Ni–Fe hybrid Hbs was as fast as those in HbCO at pH 6.5 to pH 8.5, but NMR spectra of $\alpha(Ni)_2\beta(Fe–CO)_2$ are changed from deoxy-like to oxy-like in this pH region. These results strongly suggest that not all structural changes on the Hb molecule occur concertedly. Careful monitoring of structural change is necessary to understand the molecular mechanism of the Hb allosteric transition.

ACKNOWLEDGMENTS

We thank anonymous reviewers for very thorough reading of the manuscript and for very helpful comments.

Registry No. CO, 630-08-0.

REFERENCES

Abraham, R. J., & Swinton, P. F. (1969) *J. Chem. Soc. B*, 903–908.

Alston, K., Schechter, A. N., Arcoleo, J. P., Greer, J., Parr, G. R., & Friedman, F. K. (1984) *Hemoglobin 8(1)*, 47–60.

Asakura, T., Adachi, K., Wiley, J. S., Fung, L. W. M., Ho, C., Kilmartin, J. V., & Perutz, M. F. (1976) *J. Mol. Biol. 104*, 185–195.

Baldwin, J. M. (1980) *J. Mol. Biol. 136*, 103–128.

Baldwin, J. M., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Banerjee, R., & Cassoly, R. (1969) *J. Mol. Biol. 42*, 351–361.

Blough, N. V., & Hoffman, B. M. (1984) *Biochemistry 23*, 2875–2882.

Dalvit, C., & Ho, C. (1985) *Biochemistry 24*, 3398–3407.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237–256.

Fung, L. W. M., & Ho, C. (1975) *Biochemistry 14*, 2526–2535.

Ikeda-Saito, M., Yamamoto, H., & Yonetani, T. (1977) *J. Biol. Chem. 252*, 8639–8644.

Inubushi, T., & Becker, E. D. (1983) *J. Magn. Reson. 51*, 128–133.

Inubushi, T., Ikeda-Saito, M., & Yonetani, T. (1983) *Biochemistry 22*, 2904–2907.

La Mar, G. N., & Walker, F. A. (1979) *Porphyrins 4*, 129–135.

La Mar, G. N., Budd, D. L., & Goff, H. (1977) *Biochem. Biophys. Res. Commun. 77*, 104–110.

La Mar, G. N., Nagai, K., Jue, T., Budd, D. L., Gersonde, K., Sick, H., Kagimoto, T., Hayashi, A., & Taketa, F. (1980) *Biochem. Biophys. Res. Commun. 96*, 1172–1177.

Lindstrom, T. R., & Ho, C. (1973) *Biochemistry 12*, 134–139.

Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehmann, H., & Ho, C. (1972) *Biochemistry 11*, 1677–1681.

Mayer, A., Ogawa, S., & Shulman, R. G. (1973) *J. Mol. Biol. 81*, 187–197.

McLees, B. D., & Caughey, W. S. (1968) *Biochemistry 7*, 642–652.

Miura, S., & Ho, C. (1982) *Biochemistry 21*, 6280–6287.

Miura, S., & Ho, C. (1984) *Biochemistry 23*, 2492–2499.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.

Ogawa, S., Mayer, A., & Shulman, R. G. (1972) *Biochem. Biophys. Res. Commun. 49*, 1485–1491.

Perutz, M. F. (1970) *Nature (London) 228* 726–739.

Shaanan, B. (1983) *J. Mol. Biol. 171*, 31–59.

Shelnutt, J. A., Alston, K., Ho, J. Y., Yu, N. T., Yamamoto, T., & Rifkind, J. M. (1986) *Biochemistry 25*, 620–627.

Shibayama, N., Morimoto, H., & Miyazaki, G. (1986a) *J. Mol. Biol. 192*, 323–329.

Shibayama, N., Morimoto, H., & Kitagawa, T. (1986b) *J. Mol. Biol. 192*, 331–336.

Shulman, R. G., Ogawa, S., Wuthrich, K., Yamane, T., Peisach, J., & Blumberg, W. E. (1969) *Science (Washington, D.C.) 165*, 251–257.

Shulman, R. G., Ogawa, S., Wuthrich, K., Yamane, T., Patel, D. J., & Blumberg, W. E. (1970) *J. Mol. Biol. 53*, 143–157.

Simolo, K., Stucky, G., Chen, S., Bailey, M., Scholes, C., & McLendon, G. (1985) *J. Am. Chem. Soc. 107*, 2865–2872.

Takahashi, S., Lin, A. K. L. C., & Ho, C. (1980) *Biochemistry 19*, 5196–5202.

Takahashi, S., Lin, A. K. L. C., & Ho, C. (1982) *Biophys. J. 39*, 33–40.

Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673–3677.

Viggiano, G., Wiechelman, K. J., Chervenick, P. A., & Ho, C. (1978) *Biochemistry 17*, 795–799.