As stated above, such a geometry when confined in the cavity of the binding site should preferentially undergo the H.T.-12 process, giving the 11-cis isomer. Presently, there is no direct evidence on the existence of such a conformation in retinochrome. However, in the Diels-Alder reaction, it is common knowledge that *all-trans*-vitamin A reacts selectively in the 12-s-cis form.[28] A similar selective reaction of aporetinochrome with *all-trans*-retinal would indeed lead to the proposed structure.

Concluding Remarks

On the basis of the above discussion and that presented previously,[2] it is apparent that all chemical transformations of the chromophores of vertebrate and invertebrate visual pigments during the visual processes can be described by combinations of H.T.-*n* and B.P.-*m,n* processes. Not only has a complete rational picture linking many known but seemingly unrelated experimental results emerged but also the processes have led to proposals of molecular structures for all intermediates in the visual cycle.

We also wish to emphasize that these thoughts should be viewed as tentative proposals derived from bioorganic mechanistic reasoning assisted by the use of molecular models. It is hoped that the specifics of the conclusions will stimulate future definitive experimental or theoretical investigations in this area. Also, the current and related approaches[2-4] emphasize the role of the protein, which clearly is a factor that cannot and should not be overlooked in the design of future corroborative experiments.

(28) Robeson, C. D.; Calley, J. D.; Yeisler, J.; Stern, M. S.; Eddinger, C. C.; Chechak, N. A. J. *J. Am. Chem. Soc.* **1955**, *77*, 4111-4119.

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NMR Studies of Monoliganded Fe-Co Hybrid Hemoglobins: Their Quaternary Structure and Proximal Histidine Coordination

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Abstract: Tricobalt-substituted Fe-Co hybrid hemoglobins have been synthesized by cross-linking symmetric Fe-Co hybrid HbA and Co-substituted mutant HbC (β6 Glu→Lys) with bis(3,5-dibromosalicyl) fumarate. Carbon monoxy derivatives of these molecules can serve as important models of monoliganded hemoglobins, the intermediate species produced in the first step of oxygen binding to hemoglobin. Acceptance of the first ligand in an α subunit gave essentially no change in the proximal histidine coordination in the remaining deoxy subunits and a small alteration of deoxy quaternary structure. However, when the first ligand was bound to a β subunit, significant change in the proximal histidine coordination and complete elimination of one hydrogen bond in the deoxy quaternary structure occurred. Such substantial differences in the structures of the two monoliganded hemoglobins obtained by NMR allow us to postulate the possible course of oxygen binding. Also, the observation of asynchronous decreases in the intensities of so-called "T-state" markers and the absence of concomitant increases in the so-called "R-state" marker indicate the existence of more than two quaternary structures for Hb and contradict the two-state allosteric theory.

The applicability of the concept of allostery[1] to the ligand binding in hemoglobin (Hb) has been proven, to a first approximation, by a number of experimental data, particularly structural studies by X-ray crystallography[2] and nuclear magnetic resonance (NMR) spectroscopy.[3] However, detailed understanding of the control mechanism of ligand affinity in Hb may well be achieved only by analyzing the tertiary and quaternary structures and functional properties of the protein as a function of the degree of ligation. However, the physical and chemical characterizations of Hb species at intermediate states of ligation have been thus far elusive, owing to the difficulty in physically isolating such molecules.

CoHb, in which ferrous porphyrin is replaced by cobaltous porphyrin, has been shown not only to possess homotropic and heterotropic behaviors similar to those of natural FeHb but also to acquire physical and chemical properties derived from the Co(II) ion.[4] We had previously prepared symmetric Fe-Co hybrid Hbs such as $\alpha(Co)_2\beta(Fe)_2$ and $\alpha(Fe)_2\beta(Co)_2$. Under an anaerobic carbon monoxide (CO) atmosphere, only the ferrous subunits in these molecules are ligated, but the cobaltous subunits remain unligated.[4b] Thus, it was possible to isolate the diligated species $\alpha(Co)_2\beta(Fe\cdot CO)_2$ and $\alpha(Fe\cdot CO)_2\beta(Co)_2$ and to characterize them by various spectroscopic, thermodynamic, and kinetic techniques.[5] Recently, a cross-linking technique[6] was utilized to prepare asymmetric valency hybrid FeHbs.[7] We have applied this approach in the preparation of asymmetric Fe-Co hybrid Hbs containing three cobaltous porphyrins and one ferrous porphyrin. The reagent bis(3,5-dibromosalicyl) fumarate links the two β subunits (Lys-$\beta_1$82-Lys-$\beta_2$82) across the 2,3-diphosphoglycerate binding site.[6] Cross-linking between symmetric Fe-Co hybrid HbA and Co-substituted tetrameric HbC (β6 Glu→Lys) enables

(1) Monod, J.; Wyman, J.; Changeux, J. P. *J. Mol. Biol.* **1965**, *12*, 88.
(2) (a) Perutz, M. F. *Br. Med. Bull.* **1976**, *32*, 195. (b) Perutz, M. F. *Annu. Rev. Biochem.* **1979**, *48*, 327.
(3) (a) Ho, C.; Fung, L. W.-M.; Weichelman, K. J.; Pifat, G.; Johnson, M. E. In *Erythrocyte Structure and Function*; Brewer, G. J., Ed.; Liss: New York, 1975; pp 43-64. (b) Shulman, R. G.; Hopfield, J. J.; Ogawa, S. *Q. Rev. Biophys.* **1975**, *8*, 325.
(4) (a) Hoffman, B. M.; Petering, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *67*, 637. (b) Yonetani, T.; Yamamoto, H.; Woodrow, G. V., III *J. Biol. Chem.* **1974**, *249*, 682.
(5) (a) Ikeda-Saito, M.; Yamamoto, H.; Yonetani, T. *J. Biol. Chem.* **1977**, *252*, 8639. (b) Ikeda-Saito, M.; Yonetani, T. *J. Mol. Biol.* **1980**, *38*, 845. (c) Inubushi, T.; Yonetani, T. *Biochemistry* **1983**, *22*, 1894. (d) Inubushi, T.; Ikeda-Saito, M.; Yonetani, T. *Ibid.* **1983**, *22*, 2904. (e) Hofrichter, J.; Henry, E. R.; Sommer, J. H.; Ikeda-Saito, M.; Yonetani, T.; Eaton, W. A. *Ibid.* **1985**, *24*, 2667.
(6) Walder, J. A.; Walder, R. Y.; Arnone, A. *J. Mol. Biol.* **1980**, *141*, 195.
(7) Miura, S.; Ho, C. *Biochemistry* **1982**, *24*, 6280.

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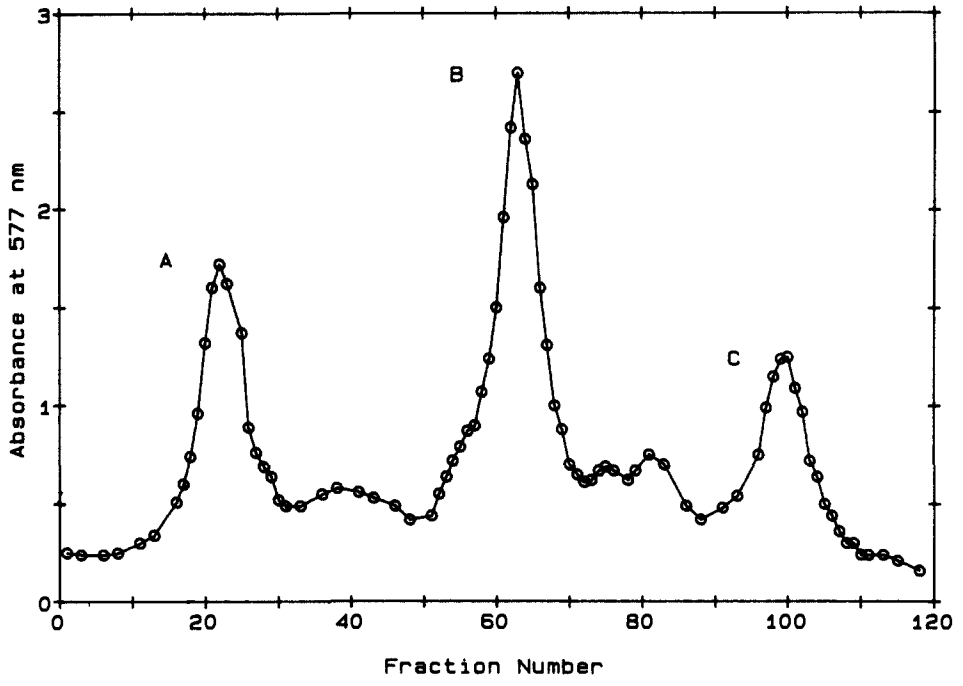

Figure 1. Elution pattern for the linear gradient of two buffers that separated the mixture of the three components from the cross-linking reaction applied to $\alpha(Co)_2\beta(Fe)_2$ and CoHbC= (A) $\alpha(Co)_2\beta(Fe)_2$AXL, pH 7.04; (B) $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_C$XL, pH 7.25; (C) CoHbCXL, pH 7.44. See the Experimental Section for details.

the isolation of the target hemoglobins, $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_C$XL and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_C$XL[8] by ion-exchange chromatography, since HbC has a higher p*I* than HbA. Under an anaerobic CO atmosphere, these molecules form monoligated states. They can serve as models of the intermediate species produced in the first step of oxygen binding to hemoglobin, and their structural and functional properties can be readily and accurately examined by physical and chemical techniques.

^{1}H NMR has been a potent tool in the characterization of the tertiary and quaternary structural changes observed upon ligand binding to the different Hb species.[9] Investigation of Co-substituted Hbs by NMR spectroscopy offers the advantage of being able to monitor two independent and strategic sites within the Hb molecule: (1) the hyperfine shift of the imidazole NH proton of the proximal histidine (His F8) coordinated to the paramagnetic porphyrin metal and (2) inter- and intrasubunit hydrogen bonds associated with the tertiary and quaternary structure of the hemoglobin molecule. The hyperfine-shifted resonances of the exchangeable proximal histidine $N_\delta H$ have been assigned in both Fe-containing and Co-substituted subunits.[5d,10] These signals revealed changes in the tertiary structure of the proximal histidine coordination to the Co(II) metal ion in the symmetric Fe–Co hybrid Hbs when they were diligated.[5d] Resonances in the exchangeable hydrogen-bonded region have been associated with the T to R quaternary transition in Hb.[11,12] Two T-state markers and one R-state marker have been assigned to specific hydrogen bonds in the inter- and intrasubunit interfaces,[13,14] and they have been used to monitor the T to R transition in a variety of Hb species.[9] In this paper, we report some unique structural features of these monoligated Hbs obtained by ^{1}H NMR spectroscopy, relate these observations to the structural transition induced by the binding of the first ligand to Hb, and discuss their implication for the allosteric model of Hb.

Experimental Section

Materials. HbA was purified from expired human blood by the method of Drabkin.[15] HbC was obtained in the same manner from homozygous CC blood. CoHbs[4b] and symmetric Fe–Co hybrid Hbs[5a] were prepared according to the previously reported methods. Tricobalt hybrid Hbs were prepared by adapting the procedure of Miura and Ho[7] with minor modifications to cross-link the appropriate symmetric Fe–Co hybrid with CoHbC. The cross-linking reaction was carried out for 4 h at 0 °C with bis(3,5-dibromosalicyl) fumarate, which was synthesized according to Walder et al.[16] Separation of the unreacted molecules from the cross-linked molecules was achieved by gel filtration column chromatography using Ultrogel AcA44(LKB).[7] Further separation of the three constituents of the cross-linked fraction was accomplished at 4 °C on a CM-cellulose column (2 × 15 cm) equilibrated with 0.01 M phosphate buffer at pH 6.7 to which a linear gradient of 0.015 M phosphate buffer at pH 6.7 and 0.020 M phosphate buffer at pH 8.0 was applied. A typical elution pattern is shown in Figure 1.

Deoxy samples were prepard by the addition of a minimal amount of sodium dithionite ($Na_2S_2O_4$) under a nitrogen atmosphere. Monoligated samples were prepared by flushing deoxy samples with CO at atmospheric pressure and then adding another aliquot of $Na_2S_2O_4$ to ensure the unligated state of the Co-substituted subunits. All samples were approximately at 0.5 mM/tetramer in 0.1 M phosphate buffer at pH 7.0.

Methods. ^{1}H NMR spectra were taken by a Bruker WH-360 spectrometer equipped with an ASPECT 2000A computer system operating at 360.04 MHz. Hyperfine-shifted signals were measured by super WEFT[17] with an 80-kHz spectral window and a 0.1-s repetition of the pulse sequence. After collection of 1024 free induction decays (fids), the data were Fourier transformed, and between 32 and 64 of these spectra were block averaged for a typical spectrum. Hydrogen-bonded resonances were obtained by a modified Redfield 2–1–4 pulse sequence in order to minimize the water signal. For each spectrum, 256 fids were accumulated, Fourier transformed, and then block averaged four to eight times. All chemical shifts were measured from the internal standard *p*-dioxane (approximately 0.05% v/v) and converted to those from sodium 3-(trimethylsilyl)propionate (TSP), assigning positive values to downfield

(8) The subscripts A and C refer to dimers from HbA and HbC, respectively, and XL signifies cross-linked.

(9) Ho, C.; Russu, I. M. *Methods Enzymol.* **1981**, *76*, 275.

(10) (a) LaMar, G. N.; Budd, D. L.; Goff, H. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 104. (b) Takahashi, S.; Lin, A. K.-A. C.; Ho, C. *Biochemistry* **1980**, *19*, 5196.

(11) Ogawa, S.; Mayer, A.; Shulman, R. G. *Biochem. Biophys. Res. Commun.* **1972**, *49*, 1485.

(12) As discussed in the later section of this paper, more than two quaternary structures may exist in the course of allosteric ligand binding to hemoglobin, so that one must be careful in using the T- and R-state terminology. In this paper, the term T-state is used to describe the quaternary structure of *fully deoxy* HbA, and R-state is used to describe the quaternary structure of *fully oxy* HbA, respectively.

(13) Fung, L. W.-M.; Ho, C. *Biochemistry* **1975**, *14*, 2526.

(14) Viggiano, G.; Wiechelman, K. J.; Cherverick, P. A.; Ho, C. *Biochemistry* **1978**, *17*, 795.

(15) Drabkin, D. L. *J. Biol. Chem.* **1946**, *164*, 703.

(16) Walder, J. A.; Zaugg, R. H.; Walder, R. H.; Steel, J. M.; Koltz, I. M. *Biochemistry* **1979**, *18*, 4265.

(17) Inubushi, T.; Becker, E. D. *J. Magn. Reson.* **1982**, *51*, 128.

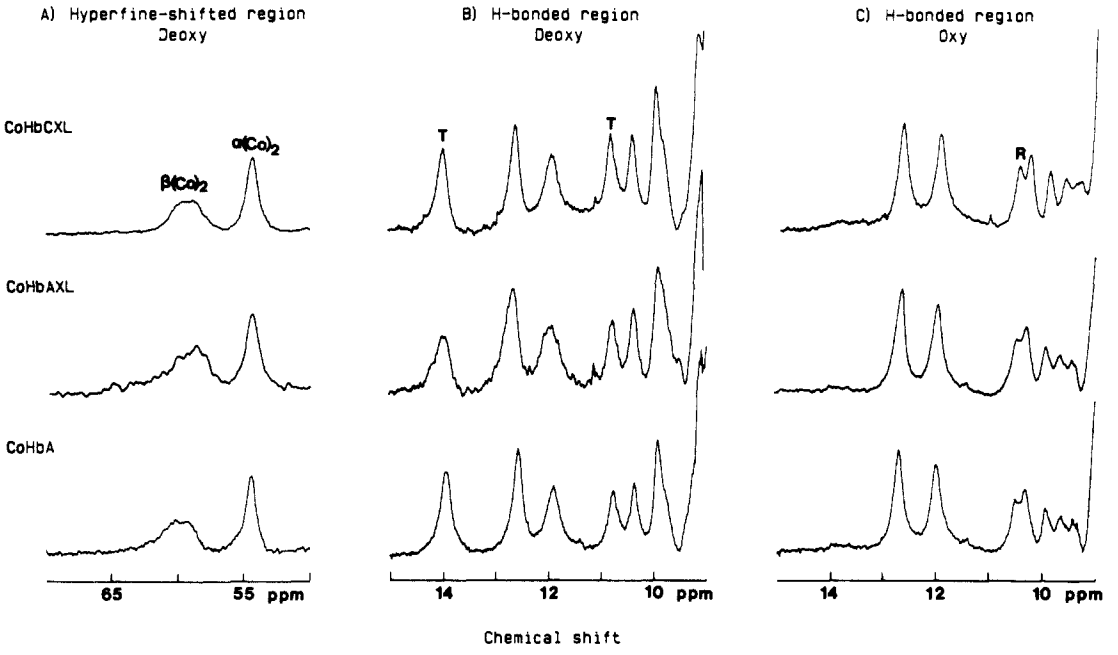

Figure 2. ^{1}H NMR spectra for CoHbA, CoHbAXL, and CoHbCXL: (A) hyperfine-shifted region for deoxy CoHbs; (B) H-bonded proton region for deoxy-CoHbs; (C) H-bonded proton region for oxy-CoHbs. All spectra were recorded at 360 MHz in 0.1 M phosphate buffer, pH 7.0 at 15 °C.

shifts. All spectra were recorded at 15 °C.

Results

1. ^{1}H NMR Spectra of CoHbAXL and CoHbCXL. The hyperfine-shifted resonances of the proximal histidine N_δ H's in deoxy-CoHbAXL and deoxy-CoHbCXL are compared with those for deoxy-CoHbA in Figure 2A. These resonances coincide with the previously assigned values for α(Co) subunits (54 ppm) and β(Co) subunits (58 ppm).[5d] No difference in the hyperfine-shifted proximal histidine region was observed between CoHbA, CoHbAXL, and CoHbCXL. As reported previously, there are no observable hyperfine-shifted signals for oxy-CoHb.[18]

The exchangeable hydrogen-bonded region exhibits peaks at 14.0 and 10.7 ppm for deoxy samples (Figure 2B) that are not present upon oxygenation of the samples. These are the T-state markers assigned to the hydrogen bonds between Tyr-$\alpha_1$42 and Asp-$\beta_2$99[13] and between Tyr-$\beta_2$145 and Val-$\beta_2$98,[14] respectively. In deoxy forms, another distinctive peak was observed at 10.3 ppm, which is commonly observed for α subunits in metal-substituted Hbs having a deoxy quaternary structure.[19] In the spectra of oxygenated samples, the R-state marker can be seen at 10.5 ppm (Figure 2C). Thus, both CoHbAXL and CoHbCXL undergo the same quaternary structural change associated with oxygenation as measured by these exchangeable ^{1}H NMR signals. The resonances at 12.0 and 12.6 ppm appeared in the spectra of both deoxy- and oxy-Co-substituted Hbs as expected from results for FeHbA.[20]

2. ^{1}H NMR Spectra of Tricobalt-Substituted Hbs. Figure 3 illustrates the ^{1}H NMR spectra of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ in deoxy, carbon monoxy, and oxy forms that correspond to unliganded, monoliganded, and fully liganded states. The two T-state markers were observed in the fully deoxy state at 14.1 and 10.8 ppm. Upon ligation of CO to the α(Fe) subunit, the signal at 14.1 ppm decreased its intensity by approximately 50% without changing its resonance position. On the other hand, the resonance at 10.8 ppm was not noticeably changed. The R-state marker for $[\alpha(Fe{\cdot}O_2)\beta(Co{\cdot}O_2)]_A[\alpha(Co{\cdot}O_2)\beta(Co{\cdot}O_2)]_CXL$ is observed at 10.5 ppm (Figure 3C). The hyperfine-shifted resonances at 56.6 and 61.0 ppm of the proximal His N_δ H's coordinated to the deoxy-Co porphyrins, corresponding to one α(Co) and two β(Co) subunits, were not changed by ligation of CO to the α(Fe) subunit. The hyperfine-shifted resonance at 61.7 ppm for the deoxy α(Fe) subunit disappeared from this region upon CO ligation because the spin state of the porphyrin Fe atom in the α subunit became diamagnetic.[21]

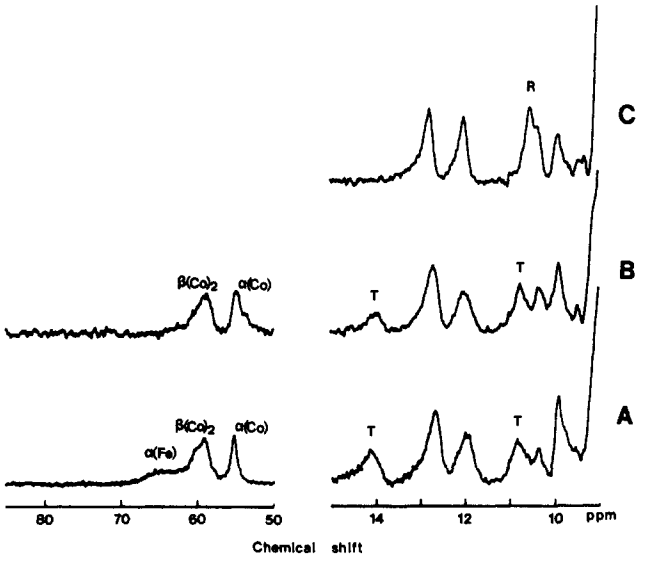

Figure 3. Hyperfine-shifted and H-bonded proton regions of 360-MHz ^{1}H NMR spectra for $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$: (A) fully deoxy form; (B) carbon monoxy form; (C) oxy form. Oxy forms of Co and Fe subunits do not exhibit observeable hyperfine-shifted resonances of proximal His N_δ H's.[18] Samples were at approximately 0.5 mM/tetramer in 0.1 M phosphate buffer, pH 7.0, and at 15 °C.

(18) Ikeda-Saito, M.; Inubushi, T.; McDonald, G. G.; Yonetani, T. *J. Biol. Chem.* **1978**, *253*, 7134.

(19) The peak at 10.3 ppm, also exchangeable with D_2O, was observed in NiHb (Shibayama, N., unpublished results), ZnHb,[12] and hybrid hemoglobins containing these metalloporphyrins in the α subunits. Also, it is not observed in the spectrum of $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_CXL$: D'Ambrosio, C., unpublished results. Although the identity of this signal has not yet been established, the peak originates from the deoxy α subunits and is associated with a deoxy quaternary structure.

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

(21) The Fe atoms in deoxy- and carbon monoxy-Hb are in the ferrous state (configuration d^6). However, these Fe(II) atoms have "high spin", S = 2, in deoxy-Hb and "low spin", S = 0, in carbon monoxy-Hb. Also, contamination by oxidized Fe subunits can be detected by the hyperfine-shifted heme peripheral signals or by the decrease in the proximal histidyl NH signal in deoxy Co subunit. Since neither of these was detected in the present experiments, the spectral changes observed by NMR are not caused by the degradation of the Hb samples.

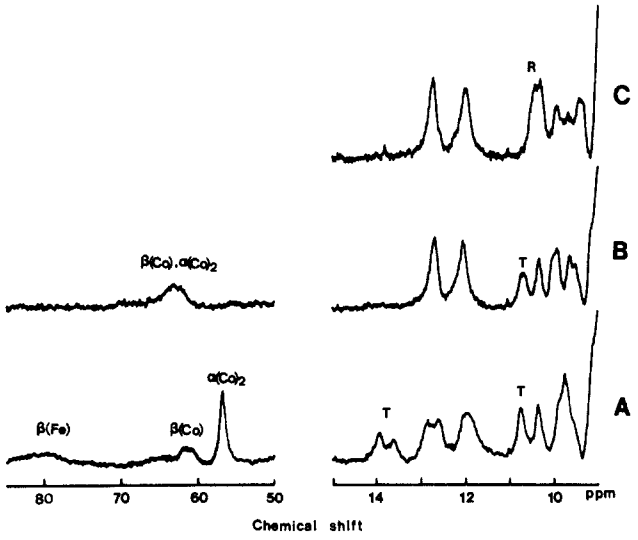

Figure 4. Hyperfine-shifted and H-bonded proton regions of 360-MHz ^{1}H NMR spectra for $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$: (A) fully deoxy form; (B) carbon monoxy form; (C) oxy form. Samples were at approximately 0.5 mM/tetramer in 0.1 M phosphate buffer, pH 7.0, and at 15 °C.

Very different changes in both the hydrogen-bonded and the hyperfine-shifted regions were observed in the complementary asymmetric hybrid Hb, $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$, as shown in Figure 4. In the fully deoxy state (Figure 4A), the lower field T-state marker, which was observed as a single peak at 14.1 ppm in the other hybrid, was split into two peaks at 13.9 and 13.6 ppm. The resonance at 12.7 ppm, which is common to both quaternary structures, was also split into two peaks at 12.9 and 12.6 ppm.[22] The second T-state marker was observed at 10.8 ppm. The ligation of CO to the β(Fe) subunit induced the complete disappearance of the twin-peaked T-state marker around 14 ppm and reduced the intensity of the other T-state marker at 10.8 ppm (Figure 4B). The R-state marker that is observed in the oxy spectrum (Figure 4C) at 10.5 ppm is not present in the spectrum of the monoliganded species. More dramatic changes were observed in the hyperfine-shifted region. Both of the resonance signals at 54.9 and 59.1 ppm of the proximal His N_δ H protons of the α(Co) and β(Co) subunits, respectively, were shifted downfield to form a single peak at 62.5 ppm upon ligation of CO to the β(Fe) subunit. The signal from the proximal His N_δ H proton of the β(Fe) subunit at 78.7 ppm disappeared from this region because of the change of the paramagnetic Fe(II) ion to the diamagnetic state upon CO ligation.

It is interesting to note that, in the region from 9 to 10 ppm, spectral changes were observed in the spectra of the oxy derivatives of both asymmetric hybrids but were observed only in the spectrum of the carbon monoxy derivative of the hybrid containing the β(Fe) subunit. The signals in this region are not assigned, and not all of them are exchangeable. The most likely source of the unexchangeable proton signals is the meso protons in diamagnetic metalloporphyrins.

Discussion

As shown in Figure 2, cross-linking does not significantly alter the NMR spectral characteristics in the hydrogen-bonded or the hyperfine-shifted proximal histidine regions of CoHb. This suggests that both the quaternary structure and the internal metal–histidine coordination are preserved in cross-linked CoHbs. Miura and Ho[7,23] reported similar results for a comparison of FeHbA and FeHbAXL, where no difference was detected in either the proximal histidine N_δ H or the hydrogen-bonded protons. Further, no difference was noticed between CoHbA and CoHbC by ^{1}H NMR spectra.[24] Therefore, we consider the cross-linked Fe–Co hybrid Hbs used in this investigation to be valid models of partially liganded Hb.

We have demonstrated that the ligation of a single CO molecule induces quite different changes in the hydrogen-bonded region of ^{1}H NMR spectra in these two asymmetric Fe–Co hybrid Hbs. The T-state marker at approximately 14 ppm decreased its intensity by about 50% in the α(Fe)-containing hybrid and disappeared completely in the complementary hybrid. This T-state marker was previously assigned to the phenolic OH of Tyr-$\alpha_1$42 hydrogen bonded to Asp-$\beta_2$99.[13] Therefore, in the former monoliganded hybrid Hb, we suggest that one of the two intersubunit hydrogen bonds in the α_1–β_2 and α_2–β_1 interfaces, possibly the one in the $[\alpha(Fe{\cdot}CO)\beta(Co)]_A$ dimer, may be broken by the structural change induced by the ligation of CO. On the other hand, the T-state marker at 10.8 ppm, which has been assigned to the intrasubunit hydrogen-bonded proton between Tyr-$\beta_2$145 and Val-$\beta_2$98,[14] was unperturbed in the α(Fe)-containing hybrid and decreased its intensity in the complementary hybrid. This observation suggests that the hydrogen bond in the α_1–β_2 interface tends to be broken before conformational change near the C-terminus of the β-subunit occurs during allosteric transition. Furthermore, it should be noted that the decreases in the T-state markers observed in these monoliganded hybrids are not compensated for by the appearance of the R-state marker. These observations directly contradict the two-state allosteric model,[1] which predicts the synchronized decrease in the intensities in the two T-state markers and the concomitant increase of the intensity of the R-state marker. Therefore, these monoliganded intermediates represent at least two quaternary structures distinct from the fully deoxy T-state and the fully ligated R-state quaternary structures. The possible existence of more than two quaternary structures in Hb was previously proposed on the basis of NMR data of monoliganded asymmetric valency hybrids[7] and diliganded symmetric Zn–Fe hybrids.[25] A comparison of the signal intensity of the T-state markers in the two monoliganded asymmetric Fe–Co hybrids indicates that the quaternary structure of $[\alpha(Fe{\cdot}CO)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ has more T-state character than its complementary counterpart, $[\alpha(Co)\beta(Fe{\cdot}CO)]_A[\alpha(Co)\beta(Co)]_CXL$. The binding of the first ligand to the β subunit induces more profound changes in the quaternary structure of Hb than the binding to the α subunit. This is also supported by the observation of spectral changes between 9 and 10 ppm for $[\alpha(Co)\beta(Fe{\cdot}CO)]_A[\alpha(Co)\beta(Co)]_CXL$, whereas no significant change was seen for $[\alpha(Fe{\cdot}CO)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$, although no further interpretation of the data in this region is possible at this time.

The difference between the two monoliganded asymmetric Fe–Co hybrids is more pronounced in the hyperfine-shifted region of the proximal His N_δ H protons. The ligation of a single CO molecule to the β(Fe) subunit in $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$ shifted the proximal His N_δ H proton peaks of the α(Co) and β(Co) subunits 7.6 and 3.4 ppm downfield, respectively, whereas no appreciable change was discerned in the complementary hybrid. We previously found a correlation between the degree of the hyperfine shift of the proximal His N_δ H proton signal and the coordination strength of the Co(II)–His bond: a larger downfield shift corresponds to a stronger Co–His coordination.[5] In model complexes, the oxygen affinity is increased with stronger coordination between the nitrogenous base and the porphyrin metal ion.[26] Thus, it is reasonable to assume that $\alpha(Fe)_2\beta(Fe{\cdot}CO)\beta(Fe)$ may have a higher affinity for the binding of the second ligand than $\alpha(Fe{\cdot}CO)\alpha(Fe)\beta(Fe)_2$. An attempt is being made to probe such a possibility in our laboratory. It has been reported that an

(22) Comparison with the preliminary results for the complete series of mono-, di-, and tricobalt-substituted Hbs indicates that this is due to the modification of tertiary structure caused by the substitution of Co porphyrin for Fe porphyrin in the β(Co) subunits.
(23) Miura, S.; Ho, C. *Biochemistry* **1984**, *23*, 2492.
(24) D'Ambrosio, C.; Inubushi, T.; Yonetani, T., unpublished results.
(25) Simolo, K.; Stucky, G.; Chen, S.; Bailey, M.; Scholes, C.; McLendon, G. *J. Am. Chem. Soc.* **1985**, *107*, 2865.
(26) (a) Collman, J. P.; Brauman, J. I.; Doxsee, K. M.; Halbert, T. R.; Hayes, S. E.; Suslick, K. S. *J. Am. Chem. Soc.* **1978**, *100*, 2761. (b) Drago, R. S.; Beugelsdijk, T.; Breese, J. A.; Cannady, J. P. *J. am. Chem. Soc.* **1978**, *100*, 5374.

asymmetric valency hybrid Hb having one cyanometheme in one of the α subunits has a larger cooperativity and a smaller affinity for the first oxygen ligand than the complementary hybrid having one cyanometheme in one of the β subunits.[27] These observations suggest that the binding of the first ligand to the α subunit in Hb may induce less pronounced changes in its quaternary structure and in its metal-His coordination than the binding of the first ligand to the β subunit, in accordance with the present NMR results. Since Adair equilibrium constants for the first and second oxygen molecules are almost unchanged in FeHb,[28] the affinities of the subunits accepting these ligands must also be similar. This implies that the first oxygen molecule binds to one of the α subunits in natural FeHb. A higher affinity for oxygen of the α subunits than that of the β subunits has been observed in FeHb by ^{1}H NMR spectroscopy.[29]

Since the Fe(II)-His bond is the only covalent linkage between the heme and the globin moieties in Hb, roles in controlling ligand affinity and in triggering the allosteric transition have been attributed to the Fe-His bond.[30] Spectroscopic parameters representing the Fe-His bond such as the resonance Raman Fe-His stretching mode at 210-225 cm^{-1}, the hyperfine-shifted ^{1}H NMR signals of the proximal His N_δ H protons, and the EPR characteristics of divalent porphyrin metal ions show significant differences between Hb molecules having a T quaternary structure and those having an R quaternary structure, so that these spectroscopic parameters have often been used as convenient quaternary state indicators. Strictly speaking, however, these spectroscopic parameters represent only the tertiary structural changes near the metal-His bond. The assumed correlation between these spectroscopic parameters and the quaternary structural state may be coincidental, and it must be analyzed with caution, as clearly demonstrated by the present ^{1}H NMR study. On the other hand, some of the ^{1}H NMR signals in the hydrogen-bonded region have been explicitly assigned to specific hydrogen bonds involved in the inter- and intrasubunit interactions that are directly related to the quaternary structure of Hb. However, the assigned T-state and R-state markers in this spectral region represent only a fraction of the total number of hydrogen bonds involved, so that some uncertainty exists as to whether or not the limited number of hydrogen bonds observable by NMR can adequately represent the quaternary structural changes of this macromolecule. Nevertheless, the observed behaviors of the hydrogen-bonded resonances, namely asynchronous decreases in the T-state markers and absence of a concomitant increase in the R-state marker upon ligation of a single CO molecule to the asymmetric Fe-Co hybrid Hbs, have convincingly demonstrated that the synchronized breakage and formation of all of the hydrogen bonds involved in the quaternary structural transition in Hb, which is predicted by the two-state allosteric mechanism, do not always take place.

(27) Miura, S.; Ikeda-Saito, M.; Yonetani, T.; Ho, C. submitted for publication.
(28) Imai, K. *Biochemistry* **1973**, *12*, 798.
(29) Viggiano, G.; Ho, C. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 3673. Oxygen titration by monitoring hyperfine-shifted resonances of the proximal histidine NH in α and β subunits of tetrameric CoHb also showed higher oxygen affinity in the α subunits than in the β subunits: Inubushi, T.; Yonetani, T., unpublished results.
(30) Perutz, M. F. *Nature (London)* **1970**, *228*, 726.

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A Stable Aryldialkoxybrominane: Synthesis, Structure, and Reactions of an Organo-Nonmetallic 10-Br-3 Species[1a]

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Abstract: The syntheses of 4-methyl-2,6-bis[1-hydroxy-1-(trifluoromethyl)-2,2,2-trifluoroethyl]bromobenzene (**7a**) and its 4-*tert*-butyl analogue **7b** are described. The oxidations of bromo diols **7** with BrF_3 give brominanes **8**, 10-Br-3 species. These first examples of organobromine(III) species are stable at their melting points (153-154 °C for **8a**, 168-170 °C for **8b**). Brominanes are strong oxidizing agents, oxidizing hydrogen bromide to bromine and aromatizing hydroaromatics such as tetralin in a controlled reaction at 130 °C. Synthesis of the iodinane analogous to brominane **8b**, 10-*tert*-butyl-3,3,7,7-tetrakis(trifluoromethyl)-4,6-benzo-1-ioda-2,8-dioxabicyclo[3.3.1]octane (**10b**), is effected by a route similar to that used for the brominane. Complete X-ray crystal structures of **8a** and **10b** are described. Both halogenanes are pseudo-trigonal-bipyramidal (Ψ-TBP) species with bond angles between the two apical halogen-oxygen bonds deviating from the ideal 180° by 12.4° (for the brominane) and 21.8° (for the iodinane). This distortion is in the direction predicted by VSEPR theory with the magnitude of the deviation determined largely by the length of the equatorial carbon-halogen bond. Reactions of the brominane and the iodinane with reducing agents and with nucleophiles are described.

Examples of organic compounds containing tricoordinate iodine, such as iodobenzene dichloride and its analogues, were described as early as 1885. Since then, many organic compounds containing iodine in oxidation states of three and five have been studied.[2] In contrast to the large numbers of iodine compounds known, no tricoordinate organobromine(III) compound had been synthesized prior to the research described in this paper. The few known

(1) (a) The N-X-L classification system is described by: Perkins, C. W.; Martin, J. C.; Arduengo, A. J.; Lau, W.; Alegria, A.; Kochi, J. K. *J. Am. Chem. Soc.* **1980**, *102*, 7753. (b) Address correspondence to Vanderbilt University, Box 1822 Station B, Nashville, TN 37235.

(2) (a) Willgerodt, C. *Die organischen Verbindungen mit mehrwertigem Jod*; Ehke: Stuttgart, Germany, 1914. (b) Banks, D. F. *Chem. Rev.* **1966**, *66*, 243.

0002-7863/86/1508-3803$01.50/0