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Preparation and Proton Nuclear Magnetic Resonance Investigation of Cross-Linked Mixed Valency Hybrid Hemoglobins: Models for Partially Oxygenated Species[†]

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ABSTRACT: The following cross-linked mixed valency hybrid hemoglobins have been prepared from derivatives of hemoglobin C (β6Glu→Lys) and human normal adult hemoglobin: $(\alpha^{+\mathrm{CN}}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+\mathrm{CN}})_A(\alpha\beta)_C$XL, $(\alpha^{+\mathrm{CN}}{}_{\beta}{}^{+\mathrm{CN}})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+\mathrm{CN}})_A(\alpha^{+\mathrm{CN}}\beta)_C$XL, where subscripts A and C denote that the $\alpha\beta$ dimers are from human normal adult hemoglobin and mutant hemoglobin C, respectively, and XL denotes cross-linked hemoglobin. These valency hybrid hemoglobins in the carbon monoxy form are cross-linked by bis(3,5-dibromosalicyl)fumarate according to the procedure of Walder et al. [Walder, J. A., Zaugg, R. H., Walder, R. Y., Steel, J. M., & Koltz, I. M. (1979) *Biochemistry 18*, 4265–4270]. It has been shown by X-ray crystallography that this bifunctional reagent cross-links between the two lysine residues of the two β chains at position 82, thereby spanning the 2,3-diphosphoglycerate binding site, and that the cross-linked hemoglobin is cooperative in its binding of oxygen [Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216]. Proton nuclear magnetic resonance spectra of the hyperfine shifted and exchangeable proton resonances for these hybrid hemoglobins over the spectral region from 7 to 20 ppm downfield from H_2O have been obtained. The hybrid hemoglobins with one cyanomet chain per tetramer can serve as models for singly ligated species and those with two cyanomet chains can serve as models for doubly ligated intermediates during the cooperative oxygenation of hemoglobin. Upon deoxygenation of the ferrous chains in $(\alpha^{+\mathrm{CN}}\beta^{\mathrm{CO}})_A(\alpha^{\mathrm{CO}}\beta^{\mathrm{CO}})_C$XL, there are several spectral changes in the hyperfine shifted proton resonances of the α cyanomet chain, whereas in the case of $(\alpha^{\mathrm{CO}}\beta^{+\mathrm{CN}})_A(\alpha^{\mathrm{CO}}\beta^{\mathrm{CO}})_C$XL, there are no observable spectral changes in the proton resonances due to the β cyanomet chain. These two asymmetric valency hybrid hemoglobins have an exchangeable proton resonance at $\sim$+9.2 ppm from H_2O at 21 °C, due to the hydrogen-bonded proton between tyrosine at α42 and aspartic acid at β99 located in the $\alpha_1\beta_2$ subunit interface. The intensity of this exchangeable proton resonance in hybrid hemoglobins with one cyanomet chain is reduced compared to that of the original signal found in human normal adult deoxyhemoglobin, and this resonance disappears upon ligation of the ferrous chains in these two hybrid hemoglobins. On the other hand, hybrid hemoglobins with two cyanomet chains per hemoglobin tetramer have no observable exchangeable proton resonance at $\sim$+9.2 ppm downfield from H_2O. The low-spin ferric hyperfine shifted proton resonances in $(\alpha^{+\mathrm{CN}}\beta^{+\mathrm{CN}})_A(\alpha\beta)_C$XL and $(\alpha\beta^{+\mathrm{CN}})_A(\alpha^{+\mathrm{CN}}\beta)_C$XL are very similar in the CO form and can also be obtained by summing the resonances arising from $(\alpha^{+\mathrm{CN}}\beta^{\mathrm{CO}})_A(\alpha^{\mathrm{CO}})_A(\alpha^{\mathrm{CO}}\beta^{\mathrm{CO}})_C$XL and $(\alpha^{\mathrm{CO}}\beta^{+\mathrm{CN}})_A(\alpha^{\mathrm{CO}}\beta^{\mathrm{CO}})_C$XL. The spectral changes of these cross-linked mixed valency hybrid hemoglobins as a function of ligation are not concerted and cannot be explained simply by two-structure concerted models. The results imply that the intermediate structure(s) can exist during the cooperative oxygenation process of hemoglobin.

The cooperative phenomenon, commonly known as the "heme–heme" interaction in hemoglobin (Hb),[1] has been the subject of intensive research during the past two decades. There are two general models which have been used to describe the cooperative oxygenation of Hb, one known as a two-state concerted mechanism [for example, see Monod et al. (1965)] and the other known as a sequential mechanism [for example, see Koshland et al. (1966)]. Despite considerable effort devoted to the Hb molecule, the detailed molecular mechanism for the cooperative oxygenation of Hb is neither fully understood nor agreed upon by researchers. For recent discussions on this topic, refer to Shulman et al. (1975, 1982), Fung et al. (1976, 1977), Perutz (1976, 1979), Viggiano & Ho (1979), Viggiano et al. (1979), Pettigrew et al. (1980), Ho & Russu (1981), Johnson & Ackers (1982), and Ho et al. (1982).

Comparing the atomic models of human deoxyhemoglobin (deoxy-Hb) and oxyhemoglobin-like horse methemoglobin (met-Hb), Perutz (1970) proposed a stereochemical mechanism for the cooperative oxygenation of Hb. In its original form, Perutz's model emphasized the link between cooperativity and the transition between the two quanternary structures (deoxy quaternary structure is symbolized by T and oxy quaternary structure by R). Perutz's mechanism allows tertiary structural changes to take place each time when a subunit is oxygenated, but a single concerted quaternary structural transition ($T \rightleftarrows R$) is responsible for the cooperativity of the oxygenation process. Due to the dovetailed nature of the $\alpha_1\beta_2$ subunit interface, Perutz (1976) maintained that there can be only two quaternary structures (T and R). The basic conceptual framework of Perutz's scheme shares many features

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[1] Abbreviations: Hb, hemoglobin; deoxy-Hb, deoxyhemoglobin; met-Hb, methemoglobin; Hb A, human normal adult hemoglobin; T, deoxy quaternary structure of Hb; R, oxy quaternary structure of Hb; NMR, nuclear magnetic resonance; $\alpha^{+\mathrm{CN}}$ or $\beta^{+\mathrm{CN}}$, the heme iron in the α or β chain is in the ferric state and is combined with cyanide ion; α^{CO} or β^{CO}, the heme iron in the α or β chain is the ferrous state and is combined with CO; $(\alpha^{+\mathrm{CN}}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+\mathrm{CN}})_A(\alpha\beta)_C$XL, $(\alpha^{+\mathrm{CN}}\beta^{+\mathrm{CN}})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+\mathrm{CN}})_A(\alpha^{+\mathrm{CN}}\beta)_C$XL, cross-linked mixed valency hybrid hemoglobins where the subscripts A and C denote that the $\alpha\beta$ dimer comes from Hb A or Hb C and XL symbolizes cross-linked Hb; 2,3-DPG, 2,3-diphosphoglycerate; ppm, parts per million; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; IHP, inositol hexaphosphate.

of a two-state allosteric model, such as the one proposed by Monod et al. (1965).

Whether the mechanism of heme–heme interaction is more appropriately described by a two-state allosteric or sequential model is difficult to ascertain due to a lack of information about the structures and properties of the partially oxygenated species formed during the oxygenation process. Because of the cooperative nature of the oxygenation process, partially heme ligated intermediate species never account for more than a small fraction of the total Hb content. Unfortunately, it is only through a detailed description of these intermediates that the two classes of model may be operationally distinguished.

There are two general approaches to investigate the nature of partially heme ligated intermediate species formed during the ligation of Hb, namely (i) investigating the structures and properties of both synthetic and naturally occurring valency hybrid hemoglobins and (ii) investigating the structures and properties of Hb as a funciton of oxygenation. For the purpose of this paper, we shall concentrate our discussion on ^{1}H nuclear magnetic resonance (NMR) studies of these two cases. For a recent review on ^{1}H NMR studies of Hb, refer to Ho & Russu (1981).

First, both synthetic and naturally occurring valency hybrid hemoglobins have been used as models to understand the nature and roles of intermediate species formed during the cooperative oxygenation process [for example, see Brunori et al. (1970), Ogawa & Shulman (1972), Banerjee et al. (1973), Shulman et al. (1975), Fung et al. (1976, 1977), and Nagai (1977) and the reference cited therein)]. In these hybrid Hb molecules, the heme groups in either the α or β chains are selectively modified while the other heme irons remain in the ferrous state, capable of binding O_2 or CO. The synthetic valency hybrid hemoglobins $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$ have been extensively investigated by ^{1}H NMR spectroscopy [for example, see Ogawa & Shulman (1972) and Shulman et al. (1975)]. Shulman and co-workers found only two classes of hyperfine shifted ^{1}H NMR spectra for each type of cyanomet valency hybrid hemoglobins. Thus, they concluded that the two forms can be observed for the half-ligated hemoglobins and that the switch between the two forms is responsible for the cooperativity. On the other hand, Fung et al. (1976, 1977) have investigated ^{1}H NMR spectra of a naturally occurring valency hybrid hemoglobin, Hb M Milwaukee (β67E11Val→Glu). In this mutant Hb, the iron atoms in the two β chains are in the high-spin ferric state and are not functional. By monitoring the ferric hyperfine shifted proton resonances due to the β chain over the spectral region from +30 to +60 ppm downfield from HDO, it was found that the spectral changes during the oxygenation of the ferrous α chains cannot be explained as a simple spectral sum of deoxy- and oxy-Hb M Milwaukee. Thus, Ho and co-workers concluded that during the oxygenation of Hb M Milwaukee, intermediate structures other than T and R must exist and that two-structure allosteric models for Hb are not adequate to describe the oxygenation of Hb M Milwaukee. It should be emphasized that these valency hybrid hemoglobins provide only limited information about doubly ligated intermediate species and no information about the singly ligated species. Unfortunately, so far, few functional properties of the asymmetric hybrid hemoglobins have been reported (Cassoly, 1978; Miura & Morimoto, 1980) due to their rapid rearrangement through the tetramer–dimer equilibrium (Bunn & McDonough, 1974).

Another approach to gain some insights into the nature and properties of partially oxygenated species is to investigate the ^{1}H NMR spectral changes associated with the cooperative oxygenation of Hb (Ho & Lindstrom, 1972; Lindstrom & Ho, 1972; Johnson & Ho, 1974; Huang & Redfield, 1976; Viggiano & Ho, 1979; Viggiano et al., 1979; Ho et al., 1982). Viggiano & Ho (1979) monitored both the exchangeable proton resonance at ~+9.4 ppm from H_2O and the ferrous hyperfine shifted proton resonance at ~18 ppm [due to the β chain according to Davis et al. (1971) and Takahashi et al. (1980)] as a function of oxygenation of Hb. The exchangeable proton resonances at ~+9.4 ppm from H_2O have been shown as a quaternary structure marker (Ogawa et al., 1972; Mayer et al., 1973) and assigned to the intersubunit hydrogen bond between α42(C7)Tyr and β99(G1)Asp (a characteristic feature of the deoxy quaternary structure according to Perutz's atomic model of Hb) (Fung & Ho, 1975). The exchangeable proton resonance at ~+6.4 ppm has been tentatively assigned to the intrasubunit hydrogen bond between β145(HC2)Tyr and β98(FG5)Val (a characteristic feature of the deoxy tertiary structure according to Perutz's atomic model of Hb) (Viggiano et al., 1978). From these results, Ho and co-workers concluded that the ligand-induced structural changes are not concerted and that two-state allosteric models are not adequate to describe the cooperative oxygenation of Hb (Viggiano & Ho, 1979; Viggiano et al., 1979; Ho et al., 1982). It should be mentioned that these ^{1}H NMR studies do not provide a direct observation of the structures of the partially oxygenated species of Hb.

Recently, the following cross-linked mixed valency hybrid hemoglobins have been prepared from Hb A and Hb C (β6Glu→Lys) by using a bifunctional cross-linking reagent, bis(3,5-dibromosalicyl)fumarate: $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, where the subscripts A and C denote that the $\alpha\beta$ dimer comes from Hb A or Hb C and XL symbolizes cross-linked Hb. The sites of cross-linking by this bifunctional reagent have been shown by X-ray crystallography to be between the two lysine residues at β82, spanning the 2,3-diphosphoglycerate (2,3-DPG) binding site (Walder et al., 1980). Walder et al. (1980) reported that Hb A cross-linked by this reagent binds O_2 cooperatively with a Hill coefficient of approximately 2.3 (J. A. Walder, personal communication) and that this cross-linked Hb is not sensitive to 2,3-DPG. Hence, these cross-linked hybrid hemoglobins can serve as useful models for both singly and doubly oxygenated species which occur during the cooperative oxygenation of Hb. In addition, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL can provide valuable insights into the structures of partially ligated $\alpha_1\beta_1$ dimer and $\alpha_1\beta_2$ dimer within a tetrameric Hb molecule, respectively. The cross-linking process prevents the dissociation of the Hb tetramers into dimers and thus ensures that we have well-defined Hb molecules for our ^{1}H NMR studies. Hb C chains were used with Hb A chains as partners to prepare various mixed hybrid Hb molecules in order to take advantage of the extra positive charge in the β chain of Hb C (β6Glu→Lys), which facilitates the separation of the various cross-linked mixed valency hybrid hemoglobins by chromatography.

The purpose of this communication is 2-fold: (i) a report of the preparation of the cross-linked mixed valency asymmetric hybrid hemoglobins and (ii) a preliminary report of the ^{1}H NMR investigation of these hybrid hemoglobins.

Experimental Procedures

Preparation of Hemoglobins and Isolation of Subunits. Human normal adult hemoglobin (Hb A) was prepared in the usual manner from fresh whole blood obtained from the local blood bank. Hb C was isolated from homozygous CC blood obtained from the Sickle Cell Society, Inc. Minor components in the CC blood were separated on a CM-52-cellulose column. Organic phosphates were removed by passage through a Sephadex G-25 column equilibrated with 0.01 M Tris buffer containing 0.1 M NaCl at pH 7.5 (Berman et al., 1971).

Isolated α_A and β_A chains of Hb A were prepared as described by Kilmartin et al. (1973, 1975). Isolated β^C chain from Hb C was purified in the same manner as that for β_A chain. The SH groups of each isolated chain were regenerated with dithiothreitol just before the preparation of various hybrid hemoglobins.

The cyanomet chains were prepared by oxidizing the α chains at 25 °C and the β chains at 36 °C, both at pH 7.0, by the addition of a 1.05-fold excess of potassium ferricyanide in the presence of excess KCN (Tyuma et al., 1966).

The cyanomet symmetric valency hybrid hemoglobins, $(\alpha^{+CN}\beta^{CO})(\alpha^{+CN}\beta^{CO})$ and $(\alpha^{CO}\beta^{+CN})(\alpha^{CO}\beta^{+CN})$, were prepared as follows: cyanomet chains were mixed with appropriate carbon monoxy chains with a slight excess of the β chain. After the chains were allowed to stand for 1 h in an ice bath, the excess β chains were separated on a DEAE-52-cellulose column (Nagai, 1977). The purity of the samples was checked by observing the ratio of optical densities at 570 and 540 nm for the carbon monoxy samples and at 577 and 540 nm for the oxy samples.

Preparation of Mixed Valency Asymmetric Hybrid Hemoglobins. The valency hybrid hemoglobins $(\alpha^{+CN}\beta^{CO})_A(\alpha^{CO}\beta^{CO})_C$XL, $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_C$XL, and $(\alpha^{+CN}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_C$XL were prepared in the following manner: 2% (w/v) CO-Hb C was mixed with $(\alpha^{+CN}\beta^{CO})_A(\alpha^{+CN}\beta^{CO})_A$, $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{+CN})_A$, or $(\alpha^{+CN}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_A$ from 2% (w/v) Hb A and incubated for 1 h in 0.01 M Bis-Tris/Cl$^-$ at pH 7.1 in an ice bath. The mixture with excess KCN was allowed to react with a stoichiometric amount of bis(3,5-dibromosalicyl)fumarate (Walder et al., 1980) for 2 h at room temperature under a CO gas atmosphere. Bis(3,5-dibromosalicyl)fumarate was synthesized as described by Walder et al. (1979) and recrystallized twice from ethanol. After the excess reagent was removed by passage through a Sephadex G-25 column, the reaction mixture was chromatographed on an Ultrogel AcA 44 (LKB) gel filtration column equilibrated with 0.1% Tris buffer in the presence of 1 M $MgCl_2$ at pH 7.2 to separate the cross-linked tetramers from unmodified Hb. The cross-linked Hb tetramer peak which consists of three components was collected and then further separated by application to a CM-52-cellulose column equilibrated with 0.035 M sodium phosphate buffer at pH 6.5. The column was eluted stepwise to prevent dilution of the sample. The second peak from the CM-52-cellulose column contained the desired cross-linked tetrameric Hb having one dimer from Hb C and the other from Hb A and was collected and used for further measurements within 12 h. $(\alpha^{CO}\beta^{+CN})_A(\alpha^{+CN}\beta^{CO})_C$XL was prepared, starting from $(\alpha^{+CN}\beta^{CO})_C(\alpha^{+CN}\beta^{CO})_C$ and $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{+CN})_A$, in the same manner as described above. All manipulations were carried out at 4 °C unless otherwise stated; columns were operated under a CO gas atmosphere and in the presence of 2 mM KCN.

^{1}H NMR Spectra. High-resolution ^{1}H NMR spectra at 600 MHz were obtained from a superconducting spectrometer interfaced with a Sigma 5 computer (Bothner-By & Dadok, 1979). The signal-to-noise ratio was improved by signal averaging about 2000 scans using NMR correlation spectroscopy (Dadok & Sprecher, 1974). The following spectrometer settings were used: sweep rate at 8 kHz/s from 1100 to 8000 Hz downfield from H_2O, 5-Hz line broadening to improve the signal to noise ratio for the exchangeable proton resonances, and 10 Hz line broadening for the hyperfine shifted proton resonances. The proton chemical shift is expressed as parts per million (ppm) relative to the water proton resonance, which is 4.99 pmm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 21 °C. The accuracy of our chemical shift measurement is ±0.1 ppm. The ambient temperature inside the probe was 21 °C. The chemical shift scale is presently defined as positive in the low-field direction with respect to the reference signal, i.e., water proton.[2]

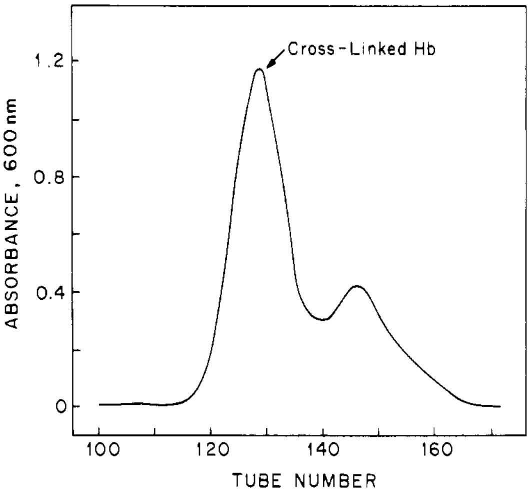

FIGURE 1: Elution profile for the separation of cross-linked hemoglobins by LKB Ultrogel AcA 44 gel filtration column (3 × 70 cm) equilibrated with 0.1% Tris buffer in 1 M $MgCl_2$ at pH 7.2.

pH Measurement and Visible Optical Spectra. pH was measured directly on a Radiometer Model 26 pH meter by using an Ingold combination electrode (No. 6030-01), without correction for the deuterium isotope effect on the glass electrode. The optical spectra of various hemoglobins and their derivatives were obtained from an Aminco DW-2 spectrophotometer.

Results

Preparation and Characterization of Mixed Valency Hybrid Hemoglobins. In high ionic strength media, such as 1 M $MgCl_2$, Hb tetramers dissociate readily into dimers (Macleod & Hill, 1970). Thus, Hb molecules which have not reacted with bis(3,5-dibromosalicyl)fumarate tend to dissociate into dimers and elute later, as illustrated in the elution profile of the reaction products on an Ultrogel AcA 44 column shown in Figure 1. Each cross-linked peak was analyzed by $NaDodSO_4$ electrophoresis (Weber & Osborn, 1969) and by isoelectrofocusing on a polyacrylamide gel column (Righetti & Drysdale, 1971). By $NaDodSO_4$ electrophoresis, two bands of equal intensity are found at the monomer and dimer positions which corresond to the α chains and cross-linked β chains, respectively. Isoelectrofocusing, which was carried out with mixtures of ampholite pH 7–9 and pH 6–8 (LKB), shows three resolved bands. One band shows the same isoelectric point as that of cross-linked Hb C, one shows that of cross-linked Hb A, and the other shows at the midpoint between

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[2] In conforming with the recommendation for the presentation in chemical journals proposed by IUPAC (1974), we have adopted the IUPAC convention; namely, the chemical shift scale is defined as positive in the low-field direction. This convention is different from that used by this laboratory prior to 1980. Hence, this change in the sign of the chemical-shift scale should be noted when referring to earlier publications reported by this laboratory.

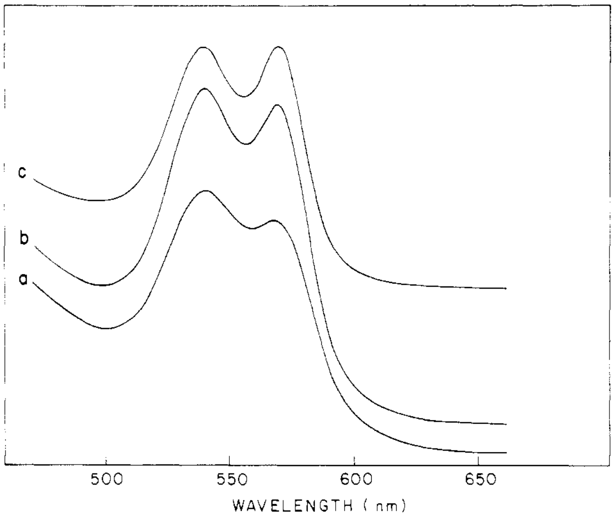

FIGURE 2: Optical spectra of cross-linked mixed valency hybrid hemoglobins in 0.035 M sodium phosphate buffer at pH 6.5: (a) $(\alpha^{+CN}\beta^{CO})_A(\alpha^{+CN}\beta^{CO})_A$XL; (b) $(\alpha^{+CN}\beta^{CO})_A(\alpha^{CO}\beta^{CO})_C$; (c) $(\alpha^{CO}\beta^{CO})_C(\alpha^{CO}\beta^{CO})_C$.

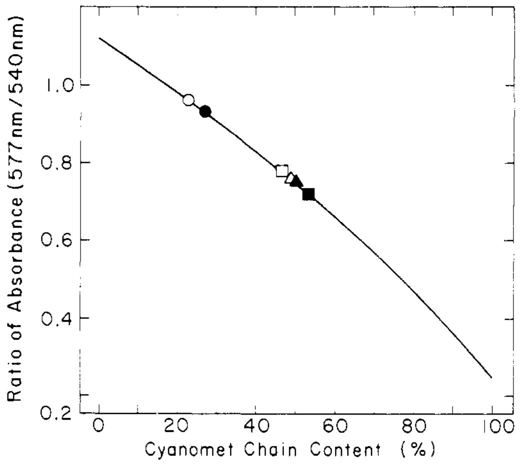

FIGURE 3: Correlation between optical spectrum (ratio of absorbance at 577 nm to that at 540 nm) and cyanomet content in cross-linked mixed valency hybrid hemoglobins in 0.1 M sodium phosphate at pH 6.8: (○) $(\alpha^{O_2}\beta^{+CN})_A(\alpha^{O_2}\beta^{O_2})_C$XL; (●) $(\alpha^{+CN}\beta^{O_2})_A(\alpha^{+CN}\beta^{O_2})_C$XL; (△) $(\alpha^{O_2}\beta^{+CN})_A(\alpha^{+CN}\beta O^2)_C$XL; (▲) $(\alpha^{+CN}\beta^{+CN})_A(\alpha^{O_2}\beta^{O_2})_C$XL; (□) $(\alpha^{O_2}\beta^{+CN})_A(\alpha^{O_2}\beta^{+CN})_A$XL; (■) $(\alpha^{+CN}\beta^{O_2})_A(\alpha^{+CN}\beta^{O_2})_A$XL. The solid line (—) is calculated by using the following millimolar extinction coefficients: $\epsilon_{540} = 1.15 \times 10^4$ for cyanomethemoglobin and $\epsilon_{577} = 1.64 \times 10^4$ for oxyhemoglobin.

them. These three bands, which are present in the cross-linked Hb tetramer peak of Figure 1, were separated on a CM-52-cellulose column. Figure 2 shows the visible optical spectra of the three components, separated on a CM-52-cellulose column, obtained for the preparation of $(\alpha^{+CN}\beta^{CO})_A$-$(\alpha^{CO}\beta^{CO})_C$XL from $(\alpha^{+CN}\beta^{CO})_A(\alpha^{+CN}\beta^{CO})_A$ and $(\alpha^{CO}\beta^{CO})_C$-$(\alpha^{CO}\beta^{CO})_C$. The ratios of the optical densities at 540 and 570 nm are 0.884, 0.947, and 1.0 for these three components. The cyanomet chain contents for the three components in Figure 2 are about 50%, 25%, and 0% in order of elution from the CM-52-cellulose column. The expected ratios of absorbance at 577 to 540 nm vs. the percent cyanomet chain content for the oxygenated hybrid hemoglobins are shown in Figure 3 and are calculated by using the following millimolar extinction coefficients: $\epsilon_{540} = 1.15 \times 10^4$ for cyanomethemoglobin and $\epsilon_{577} = 1.64 \times 10^4$ for oxyhemoglobin. The actual ratio of optical densities at 540 and 577 nm for the four kinds of oxygenated cross-linked asymmetrical valency hybrid hemoglobins and their cyanomet contents calculated from the calibration curves in Figure 3 are listed in Table I.

Table I: Relation between Measured Optical Density Ratios at 577 and 540 nm (A_{577}/A_{540}) and Percent Cyanomet Chain Content for Oxygenated Cyanomet Valency Hybrid Hemoglobins[a]

	measured OD ratio ($A_{557\text{nm}}/A_{540\text{nm}}$)	expected OD ratio ($A_{557\text{nm}}/A_{540\text{nm}}$)	calcd cyanomet chain content (%)
$(\alpha^{+CN}\beta^{O_2})(\alpha^{O_2}\beta^{O_2})$XL	0.96	0.97	23
$(\alpha^{O_2}\beta^{+CN})(\alpha^{O_2}\beta^{O_2})$XL	0.98	0.97	27
$(\alpha^{+CN}\beta^{+CN})(\alpha^{O_2}\beta^{O_2})$XL	0.87	0.87	50
$(\alpha^{+CN}\beta^{O_2})(\alpha^{O_2}\beta^{+CN})$XL	0.88	0.87	49
$(\alpha^{+CN}\beta^{O_2})_2$ and $(\alpha^{+CN}\beta^{O_2})_2$XL	0.86	0.87	53
$(\alpha^{O_2}\beta^{+CN})_2$ and $(\alpha^{O_2}\beta^{+CN})_2$XL	0.89	0.87	46

[a] The expected optical density ratios were calculated by assuming 25% cyanomet chain content for hybrid hemoglobins with one cyanomet chain and 50% for hybrid hemoglobins with two cyanomet chains.

The symmetric valency hybrid hemoglobins with and without cross-linking, namely, $(\alpha^{+CN}\beta^{CO})_A(\alpha^{+CN}\beta^{CO})_A$XL and $(\alpha^{+CN}\beta^{CO})_A(\alpha^{+CN}\beta^{CO})_A$ or $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{+CN})_A$XL and $(\alpha^{CO}\beta^{+CN})(\alpha^{CO}\beta^{+CN})$, have the same optical density ratios at 540 and 570 nm within experimental uncertainties. The deviations from the expected optical density ratio in symmtrical valency hybrid hemoglobins are consistent with the previous work and have been attributed to the optical heterogeneity between α^{+CN} and β^{+CN} chains (Ogawa & Shulman, 1972). The deviation from the expected ratio in asymmetric valency hybrid hemoglobins with one cyanomet chain are about half that in the symmetric valency hybrid hemoglobins with two cyanomet chains. Asymmetrical valency hybrid hemoglobins with two cyanomet chains have essentially the same ratio as the expected value. These results are consistent with the assumption of previous workers (Ogawa & Shulman, 1972) and, in additin, show the purity of these asymmetric valency hybrid hemoglobins.

Quaternary Structural Transitions of Cross-Linked Hb A. Figure 4 shows the exchangeable proton resonances of Hb A and cross-linked Hb A in both deoxy and CO forms over the spectral range from 5 to 10 ppm downfield from H_2O in 0.1 M Bis-Tris buffer at pH 7.4 and at 21 °C. There are no observable differences in the exchangeable proton resonances between Hb A and cross-linked Hb A. Of special interest to the present work is the disappearance of the exchangeable proton resonance at $\sim$+9.3 ppm downfield from H_2O upon binding of CO to either Hb A or cross-linked Hb A. This resonance has been assigned to the intersubunit hydrogen bond between α42Tyr and β99Asp (Fung & Ho, 1975), a characteristic featuer in the deoxy quaternary structure (Perutz, 1970). This suggests that the cross-linked Hb A undergoes the usual quaternary structural transitions upon ligation that Hb A does. This observation is in agreement with the results on O_2 binding to cross-linked Hb A reported by Walder et al. (1980). They reported that the binding of O_2 to cross-linked Hb A is cooperative (Walder et al., 1980) with a Hill coefficient of $\sim$2.3 (J. A. Walder, personal communication).

^{1}H NMR Investigation of Mixed Valency Hybrid Hemoglobins with One Cyanomet Chain. Figures 5 and 6 show the 600-Hz ^{1}H NMR spectra of cross-linked mixed valency asymmetric hybrid hemoglobins with one cyanomet chain per Hb tetramer. Figures 5A and 6A give the ^{1}H resonances of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL in D_2O over the

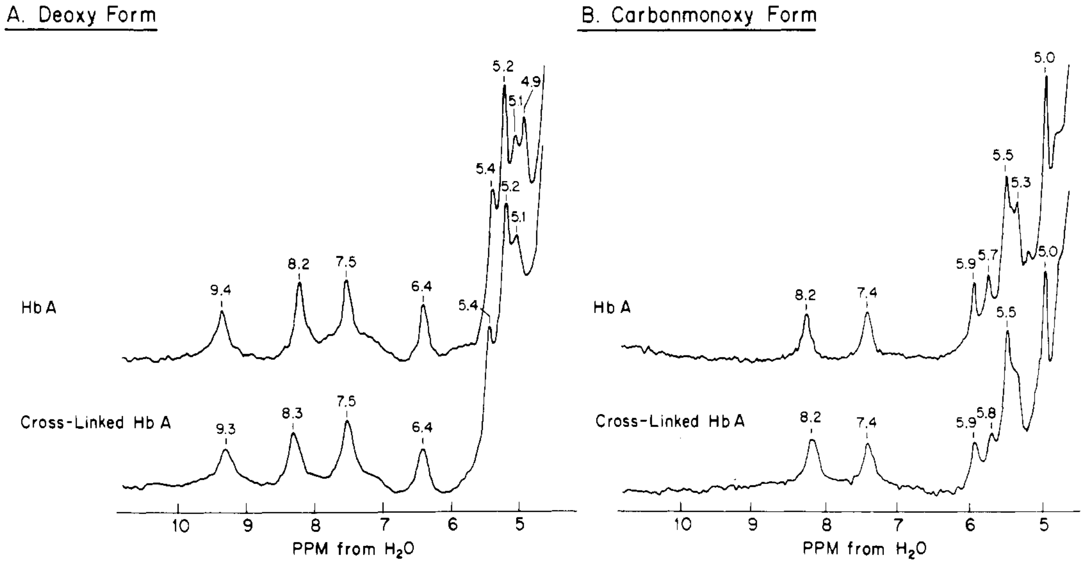

FIGURE 4: 600-MHz ^1H NMR spectra of exchangeable proton resonances in Hb A and cross-linked Hb A in the presence of 0.1 M Tris buffer at pH 7.4 in H_2O and at 21 °C: (A) deoxy form; (B) carbon monoxy form.

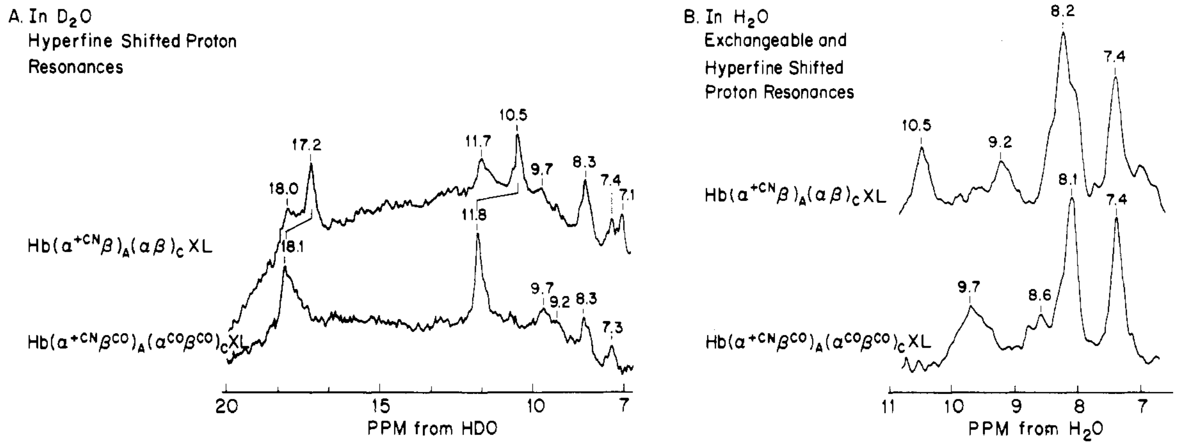

FIGURE 5: 600-MHz ^1H NMR spectra of cross-linked mixed valency asymmetric hybrid hemoglobin, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, in deoxy and CO forms, in the presence of 0.1 M sodium phosphate at pH 6.8 and at 21 °C: (A) hyperfine shifted proton resonances in D_2O; (B) exchangeable and hyperfine shifted proton resonances in H_2O.

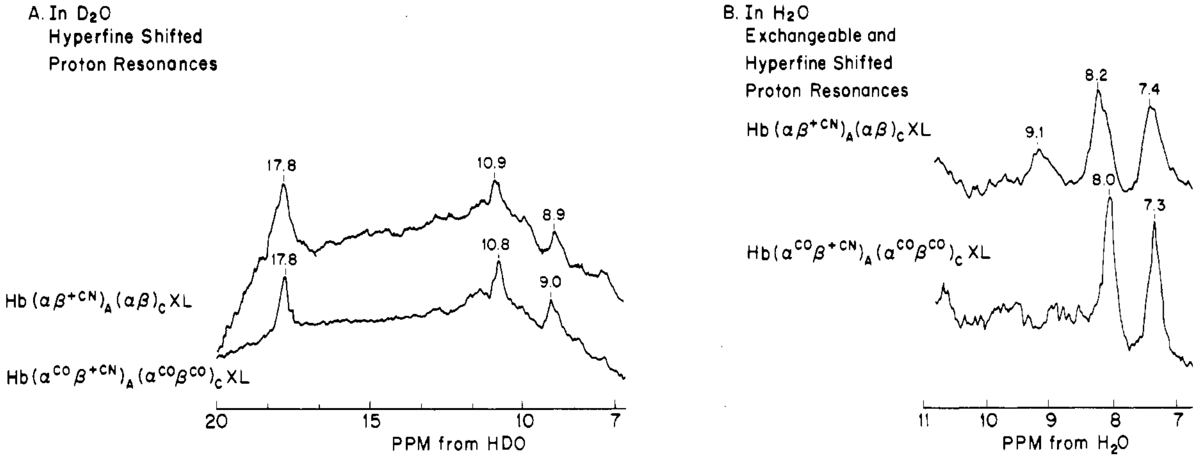

FIGURE 6: 600-MHz ^1H NMR spectra of cross-linked mixed valency hybrid asymmetric hybrid hemoglobin, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, in deoxy and CO forms, in the presence of 0.1 M sodium phosphate at pH 6.8 and at 21 °C: (A) hyperfine shifted proton resonances in D_2O; (B) exchangeable and hyperfine shifted proton resonances in H_2O.

spectral range from 7 to 20 ppm downfield from HDO. These resonances arise from the protons in the heme groups and/or the protons of the amino acid residues in the immediate surroundings of the heme group. They are shifted from their usual diamagnetic positions by hyperfine interactions between the unpaired electrons from the low-spin ferric and high-spin ferrous heme iron atoms and the nearby protons. They are very sensitive to the environment of the heme pocket [for reviews, see Ho et al. (1978) an Ho & Russu (1981)]. The signals from the subunits with high-spin ferrous heme iron are much broader than those from cyanomet (low-spin ferric) chains. The line widths of the high-spin ferrous hyperfine

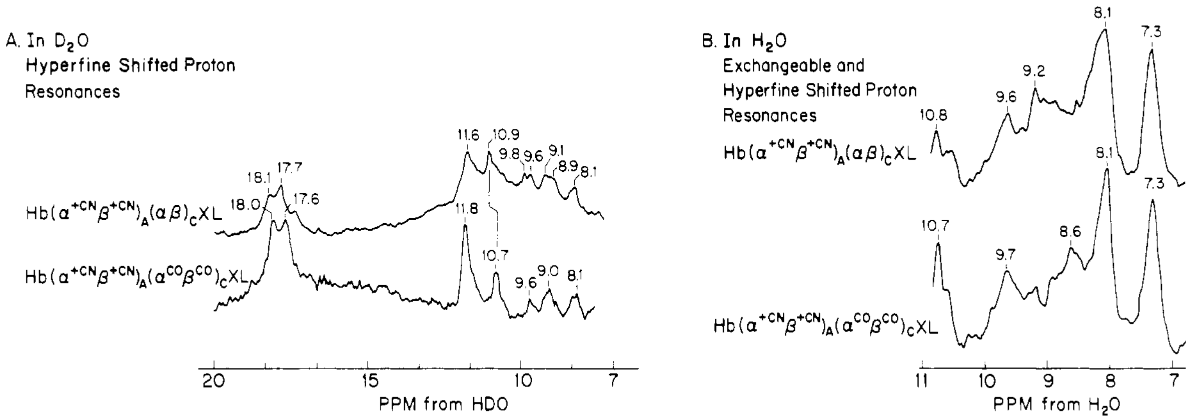

FIGURE 7: 600-MHz ^1H NMR spectra of cross-linked mixed valency symmetric hybrid hemoglobin, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_CXL$, in deoxy and CO forms, in the presence of 0.1 M sodium phosphate at pH 6.8 and 21 °C: (A) hyperfine shifted proton resonances in D_2O; (B) exchangeable and hyperfine shifted proton resonances in H_2O.

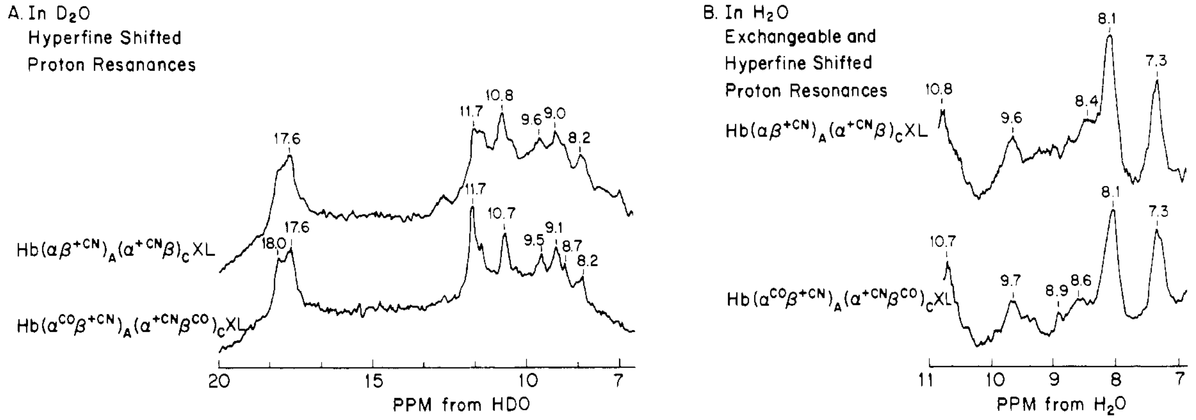

FIGURE 8: 600-MHz ^1H NMR spectra of cross-linked mixed valency asymmetric hybrid hemoglobin, $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_CXL$, in deoxy and CO forms, in the presence of 0.1 M sodium phosphate at pH 6.8 and 21 °C: (A) hyperfine shifted proton resonances in D_2O; (B) exchangeable and hyperfine shifted proton resonances in H_2O.

shifted proton resonances at 600 MHz are expected to be >1200 Hz (Johnson et al., 1977). Thus, all sharp resonances in Figures 5A and 6A come from the low-spin cyanomet chains. This conclusion is also supported by the proton resonances of $(\alpha^{+CN}\beta^{CO})_A(\alpha^{CO}\beta^{CO})_CXL$ and $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_CXL$. The resonances that occur over the spectral region from 7 to 20 ppm downfield from HDO come from the hyperfine interactions between the unpaired electrons from cyanomet chains and the nearby protons. In the CO ligated chains, the heme iron atoms are diamagnetic, and thus, no hyperfine shifted proton resonances are observable [for example, see Ho & Russu (1981)]. The binding of CO to $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ produces significant spectral changes in the low-spin ferric hyperfine shifted proton resonances of the α^{+CN} chain as shown in Figure 5A, especially in the spectral regions 10–12 and 17–18 ppm. On the other hand, the spectral changes of $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$ in going from the deoxy to the CO form are quite minor as shown in Figure 6A.

Figures 5B and 6B give the proton resonances of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ and $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$ in H_2O in both deoxy and CO forms over the spectral region from 7 to 11 ppm from H_2O. There are three different types of proton resonances shown in these two figures. The hyperfine shifted proton resonances due to cyanomet chains, such as resonances at 10.5 and 9.7 ppm in Figure 5B, come from the α^{+CN} chain. This conclusion is readily verified by comparing the corresponding spectra shown in Figure 5. The second type is due to the hyperfine shifted proton resonances of the ferrous chains. As discussed earlier, these resonances are too broad to be detectable under our NMR measurement conditions. The third type is due to the exchangeable proton resonances. In going from the deoxy to the CO form (Figures 5B and 6B), the most obvious change is the disappearance of the resonance at ~9.2 ppm. The same change occurs in Hb A or cross-linked Hb A as shown in Figure 4. It should be noted that the intensity of the resonance at ~9.2 ppm in both $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$ and $(\alpha\beta^{+CN})_A(\alpha\beta)_C$ appears to be approximately 50% that of the resonance at ~9.3 ppm in deoxy-Hb A or cross-linked Hb A (Figure 4).

^1H NMR Investigation of Cross-Linked Valency Hybrid Hemoglobins with Two Cyanomet Chains. Figures 7 and 8 show the hyperfine shifted and exchangeable proton resonances of cross-linked valency hybrid hemoglobins with two cyanomet chains, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_CXL$ and $(\alpha\beta^{+CN})(\alpha^{+CN}\beta)_CXL$, in both deoxy and CO forms over the spectral region from 7 to 20 ppm from H_2O. The meaning and origins of these proton resonances have been discussed in the last section. Overall, the 600-MHz ^1H NMR spectra of these two valency hybrid hemoglobins are quite similar to each other. There are small spectral changes in these two hybrid hemoglobins in going from the deoxy form to the CO form.

A comparison of the ^1H NMR spectra of the fully ligated, cross-linked hybrid hemoglobins with two cyanomet chains $[(\alpha^{+CN}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_CXL$ and $(\alpha^{CO}\beta^{+CN})_A(\alpha^{+CN}\beta^{CO})_CXL]$ as shown in Figures 7A and 8A with those with one cyanomet chain $[(\alpha^{+CN}\beta^{CO})_A(\alpha^{CO}\beta^{CO})_CXL$ and $(\alpha^{CO}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_CXL]$ as shown in Figures 5A and 6A clearly shows that the former spectra are the spectral sum of the latter individual spectra. Hence, we can assign the low-spin ferric hyperfine shifted proton resonances at +18.1, +11.8, +9.7,

+8.2, and +7.3 ppm downfield from HDO as due to the cyanomet α chain and those at +17.7, +10.8, +9.0, and +8.7 ppm as due to the cyanomet β chain.

The most obvious difference in the exchangeable proton resonances between the cross-linked hybrid hemoglobins with two cyanomet chains and those with one cyanomet chain is the absence of the characteristic deoxy quaternary spectral marker at ∼+9.2 ppm downfield from H_2O in the former hybrid hemoglobins as shown in Figures 7B and 8B. This suggests that the hybrid hemoglobins with two cyanomet chains per Hb tetramer do not have the usual deoxy quaternary structure as manifested by the intersubunit hydrogen bond between α42Tyr and β99Asp.

Discussion

The ratios of the optical densities at two wavelengths (540 and 577 nm) summarized in Table I are reproducible within the experrmental uncertainty and have been used to check the purity of the individual cross-linked hemoglobins. The extent of deoxygenation was checked by monitoring the ring current shifted proton resonance appearing at ∼−7.2 ppm upfield from HDO, which hsas been assigned to the methyl protons of βE11Val in HbO_2 (Lindstrom et al., 1972; Lindstrom & Ho, 1973). We have obtained the ^1H NMR spectra prior to the deoxygenation process and after the readdition of CO. Upon the addition of dithionite to deoxygenated asymmetric valency hybrid hemoglobins, the spectra are indistinguishable from those of cross-linked deoxyhemoglobins. All fully ligated spectra presented in this study have been taken after the readdition of CO. Before and after readdition of CO, the spectra are essentially identical. The ligated hybrid hemoglobins, $(\alpha^{+CN}\beta^{+CN})_A(\alpha^{CO}\beta^{CO})_C$XL and $(\alpha^{CO}\beta^{+CN})_A(\alpha^{+CN}\beta^{CO})_C$XL, cannot be differentiated by means of spectroscopic techniques (including NMR spectroscopy). However, taking the results from the asymmetrical valency hybrid hemoglobins with one cyanomet chain into account, the heme exchange rate must be slow and the effect of heme exchange should be negligible within the NMR measurement time. From these considerations, the conclusion is made that at least the major components in these asymmetric valency hybrid hemoglobin samples are as specified.

Our results on the low-spin ferric hyperfine shifted proton resonances from the cyanomet chains in the asymmetric valency hybrids with one cyanomet chain are consistent with those from the symmetric valency hybrid hemoglobins with two cyanomet chains (Ogawa & Shulman, 1972). Namely, ^1H resonances from the cyanomet α chain are observed as changing upon ligation at neighboring ferrous subunits in 0.1 M phosphate buffer at pH 6.8 and 21 °C, but those of the β chain do not (Figures 5 and 6). Only in the presence of inositol hexaphosphate (IHP) are the ^1H signals from the cyanomet β chains in symmetrical valency hybrid hemoglobins observed to undergo alterations upon ligation (Ogawa & Shulman, 1972). Unfortunately, the cross-linked Hb is not sensitive to organic phosphate because the cross-linking reagent occupies the 2,3-DPG binding site (Walder et al., 1980). Thus, the hyperfine shifted proton resonances from the cyanomet β chain are not sensitive to the ligation state of the ferrous chains in this mixed valency hybrid Hb.

As mentioned under Results, we are interested in the behavior of the exchangeable proton resonance that appears at ∼+9.2 ppm, which is due to a hydrogen bond located in the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) intersubuit interface (Perutz, 1970; Fung & Ho, 1975). X-ray crystallography data show that there are only two stable conformations in this region: one is observed in deoxy-Hb and the other in ligated-Hb (Perutz, 1976, 1979). Thus, the intersubunit hydrogen bond responsible for the peak at ∼+9.2 ppm downfield from H_2O should be formed only in the deoxy quaternary structure. However, we have found that the intensity of this resonance for valency hybrid hemoglobins with one cyanomet chain is greatly reduced compared to that in fully deoxy-Hb A or cross-linked deoxy-Hb A. The reduction of the resonance at ∼+9.2 ppm suggest that the $\alpha_1\beta_2$ subunit interface in these "partially ligated" hybrid hemoglobins is altered. One might speculate that the reduction of the signal intensity at ∼+9.2 ppm reflects the coexistence of two quaternary structures, i.e., T and R structures. This speculation is not supported by the present NMR results. For example, the hyperfine shifted proton resonances from the cyanomet α chain in $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL consist of two types of signals (Figure 5A): those that are shifted upon the addition of CO (such as signals at +10.5 and +17.2 ppm) and those that are essentially unchanged upon ligation (such as signals from +7.3 to +9.7, +11.7, and +18.0 ppm). The signals at +10.5 and +17.2 ppm have been suggested by Ogawa & Shulman (1972) as markers for the deoxy quaternary structure from their ^1H NMR investigation of symmetrical valency hybrid hemoglobins. The hyperfine shifted proton resonances from the cyanomet β chain in $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL are essentially shifted to the positions found in the oxy-like quaternary structure (Figure 6A), while in this hybrid Hb, the exchangeable proton resonance at ∼+9.2 ppm is also reduced to about half of the original intensity (Figure 6B). Therefore, these results suggest that at least one intermediate quaternary structure must be taken into account other than the oxy and deoxy quaternary structures in which almost half of the hydrogen bonds responsible for the resonance at ∼+9.2 ppm are altered. In other words, the structural changes during the ligation process are not concerted and two-structural allosteric models are not adequate to describe the cooperative oxygenation of Hb. It should be mentioned that the spectral changes observed for the high-spin ferric hyperfine shifted proton resonances of the β chains in Hb M Milwaukee upon the ligation of the ferrous α chains are consistent with our present results on cross-linked mixed valency hybrid hemoglobins with one cyanomet chain (Fung et al., 1976, 1977). As a first approximation, the following possibilities for the intermediate quaternary structure should be considered: (i) a symmetric intermediate quaternary structure about the molecular diad axis in which both of the two hydrogen bonds responsible for the resonance at ∼+9.2 ppm are equally altered and (ii) an asymmetric intermediate quaternary structure about the molecular diad axis in which one of the two hydrogen bonds remains intact and the other is broken. It is very difficult to assume that in the former case, the intensity from both hydrogen-bonded protons would be reduced to an equal extent without a change in the chemical shift. Furthermore, in the fully deoxygenated symmetric valency hybrid hemoglobins, $(\alpha^{+CN}\beta)_A(\alpha^{+CN}\beta)_A$ and $(\alpha\beta^{+CN})_A(\alpha\beta^{+CN})_A$ with IHP, the exchangeable proton resonance at ∼+9.2 ppm is missing (Figure 7; results not shown). This evidence seems to support the second possibility.

Viggiano & Ho (1979) monitored both the exchangeable and ferrous hyperfine shifted proton resonances of Hb A over the spectral region from 6 to 20 ppm downfield from H_2O as a function of O_2 saturation. They found that the spectral changes as a function of oxygenation cannot be explained simply by a two-state allosteric model and have suggested that an intermediate quaternary structure must exist during the oxygenation of Hb A. Hence, the present results on ^1H NMR studies of cross-linked mixed valency hybrid hemoglobins provide a direct, independent confirmation of our earlier conclusion. It should be emphasized that the present approach to the mechanism of cooperative oxygenation of Hb A is very different from our earlier investigation of the oxygenation of Hb A by ^{1}H NMR.

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References

Banerjee, R., Statzowski, F., & Henry, Y. (1973) *J. Mol. Biol. 73*, 455.

Berman, M., Benesch, R., & Benesch, R. E. (1971) *Arch. Biochem. Biophys. 145*, 236.

Bothner-By, A. A. & Dadok, J. (1979) in *NMR and Biochemistry* (Opella, S. J., & Lu, P., Eds.) p 169, Marcel Dekker, New York.

Brunori, M., Amiconi, G., Antonini, E., Wyman, J., & Winterhalter, K. H. (1970) *J. Mol. Biol. 49*, 461.

Bunn, H. F., & McDonough, M (1974) *Biochemistry 13*, 988.

Cassoly, R. (1978) *J. Biol. Chem. 253*, 3602.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243.

Davis, D. G., Lindstrom, T. R., Mock, N. H., Baldassare, J. J., Charache, S., Jones, R. T., & Ho, C. (1971) *J. Mol. Biol. 60*, 101.

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

Fung, L. W.-M., Minton, A. P., & Ho, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1581.

Fung, L. W.-M., Minton, A. P., Lindstrom, T. R., Pisciotta, A. V., & Ho, C. (1977) *Biochemistry 16*, 1452.

Ho, C., & Lindstrom, T. R. (1972) *Adv. Exp. Med. Biol. 28*, 65.

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

Ho, C., Fung, L. W.-M., & Weichelman, K. J. (1978) *Methods Enzymol. 54*, 192.

Ho, C., Lam, G.-H. J., Takahashi, S., & Viggiano, G. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Eaton, W. A., Collman, J. P., Gibson, Q. H., Leigh, S. J., Jr., Margoliash, E., Moffat, J. K., & Scheidt, W. R., Eds.) pp 141–149, Elsevier/North-Holland, New York.

Huang, T.-H., & Redfield, A. G. (1976) *J. Biol. Chem. 251*, 7114.

IUPAC (1974) *Inf. Bull.—IUPAC No. 38*.

Johnson, M. E., & Ho, C. (1741) *Biochemistry 13*, 3653.

Johnson, M. E., Fung, L. W.-M., & Ho, C. (1977) *J. Am. Chem. Soc. 99*, 1245.

Johnson, M. L., & Ackers, G. K. (1982) *Biochemistry 21*, 201.

Kilmartin, J. V., Fogg, J., Luzzana, M., & Rossi-Bernardi, L. (1973) *J. Biol. Chem. 248*, 7039.

Kilmartin, J. V., Hewitt, J. A., & Wooton, J. F. (1975) *J. Mol. Biol. 93*, 203.

Koshland, D. E., Nemethy, G., & Filmer, D., (1966) *Biochemistry 5*, 365.

Lindstrom, T. R., & Ho, C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1707.

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

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

Macleod, R. M., & Hill, R. J. (1970) *J. Biol. Chem. 245*, 4875.

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

Miura, S., & Morimoto, H. (1980) *J. Mol. Biol. 143*, 213.

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

Nagai, K. (1977) *J. Mol. Biol. 111*, 41.

Ogawa, S., & Shulman, R. G. (1972) *J. Mol. Biol. 70*, 315.

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

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

Perutz, M. F. (1976) *Br. Med. Bull. 32*, 195.

Perutz, M. F. (1979) *Annu. Rev. Biochem. 48*, 327.

Pettigrew, D. W., Romeo, P. H., Tsapis, A., & Ackers, G. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1849.

Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17.

Shulman, R. G., Hopfield, J. T., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325.

Shulman, R. G., Ogawa, S., & Mayer, A. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Eaton, W. A., Collman, J. P., Gibson, Q. H., Leigh, J. S., Jr., Margoliash, E., Moffat, J. K., & Scheidt, W. R., pp 205–209, Elsevier/North-Holland, New York.

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

Tyuma, I., Benesch, R. E., & Benesch, R. (1966) *Biochemistry 5*, 2957.

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

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

Viggiano, G., Ho, N. T., & Ho, C. (1979) *Biochemistry 18*, 5238.

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

Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 5074.