Proton Nuclear Magnetic Resonance Investigation of Cross-Linked Asymmetrically Modified Hemoglobins: Influence of the Salt Bridges on Tertiary and Quaternary Structures of Hemoglobin†

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ABSTRACT: Asymmetrically modified hemoglobins, [α(des-Arg)β]$_A$[$\alpha\beta$]$_C$XL, [α(des-Arg-Tyr)β]$_A$[$\alpha\beta$]$_C$XL, [α(des-Arg)β(NES)]$_A$[$\alpha\beta$]$_C$XL, and [α(des-Arg)β]$_A$[$\alpha\beta$(NES)]$_C$XL, have been prepared from chemically modified human normal adult hemoglobin (Hb A) and mutant hemoglobin C (β6Glu→Lys), where the subscript A or C denotes that the $\alpha\beta$ dimer is from either Hb A or Hb C, respectively, and XL symbolizes a cross-linked hemoglobin prepared by reaction with a bifunctional cross-linking reagent, bis(3,5-dibromosalicyl) fumarate. It has been shown by X-ray crystallography that this bifunctional reagent cross-links the ϵ-amino group of the lysyl residue at position 82 of the two β chains [Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195]. Proton nuclear magnetic resonance spectra of these asymmetrically modified hemoglobins together with their parent hemoglobins, des-Arg(α141) Hb A, des-Arg(α141)-Tyr(α140) Hb A, NES-Hb A and NES-des-Arg(α141) Hb A, have been obtained over the spectral region 5–10 ppm downfield from H_2O for the exchangeable proton resonances and 50–80 ppm downfield from H_2O for the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances. The experimental results indicate that the effects on the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances at pH 6.0 of removing Arg(α141) or Arg(α141)-Tyr(α140) from one of the two α subunits are limited to within the α subunit from which the carboxyl-terminal amino acids are specifically removed. These two asymmetrically modified hemoglobins have the exchangeable proton resonance at 9.3 ppm from H_2O, which has been assigned to the hydrogen bond between α42 tyrosine and β99 aspartic acid located at the $\alpha_1\beta_2$ subunit interface. This suggests that these asymmetrically modified hemoglobins preserve the deoxy-like quaternary structure in the $\alpha_1\beta_2$ subunit interface as manifested by the presence of this intersubunit hydrogen bond. The proton nuclear magnetic resonance spectra of [α(des-Arg)β-(NES)]$_A$[$\alpha\beta$]$_C$XL and [α(des-Arg)β]$_A$[$\alpha\beta$(NES)]$_C$XL at low pH cannot be explained simply as a sum of the spectral features specific for the deoxy-like and the oxy-like quaternary structures. The present results suggest that there exist intermediate structures in which the tertiary and the quaternary structural transitions occur asymmetrically about the diad axis of the hemoglobin molecule during the course of the successive removal of the salt bridges.

The phenomenon of the cooperative oxygenation of hemoglobin (Hb)[1] (see Table I) has been investigated by a large number of scientists for several decades. In spite of considerable efforts devoted to this problem, the detailed molecular mechanism for the oxygenation of Hb is neither fully understood nor agreed upon. For a recent review on Hb, refer to Dickerson & Geis (1983). There are two general mechanisms that have been used to describe the cooperative oxygenation of Hb. The two-state allosteric model proposed by Monod et al. (1965) postulates that the Hb molecule exists in an equilibrium between two alternative quaternary structures with low and high affinities for oxygen. The cooperative oxygen binding to Hb arises from the transition between these two quaternary structures. The sequential-type model, as proposed by Koshland et al. (1966), has emphasized the importance of changes in intersubunit interactions that result from tertiary structural changes upon ligation of each subunit and thus produce ligand-induced conformational changes in the protein molecule. The main difference between these two hypothetical schemes for ligand binding is that the former postulates only two quaternary structures while the latter suggests multiple conformational states.

By comparing the atomic models of human normal adult hemoglobin (Hb A) in the deoxy form and horse oxy-like methemoglobin, Perutz (1970) proposed a stereochemical mechanism for the cooperative oxygenation of hemoglobin. In its original form, Perutz's model emphasized the link between the cooperativity and the transition between the two quaternary structures (the deoxy quaternary structure is symbolized by T and the oxy quaternary structure by R). Perutz's model allows tertiary structural changes to take place each time a subunit is oxygenated, but a single concerted quaternary structural transition (T $\rightleftarrows$ R) is responsible for the cooperativity of the oxygenation process. In light of the dovetailed nature of the $\alpha_1\beta_2$ or $\alpha_2\beta_1$ subunit interface, Perutz (1970, 1979) has maintained that there can be only two quaternary structures, namely, T and R, in the Hb molecule. According to Perutz (1970, 1976, 1979), the iron atoms in the α and β hemes that are bound to the proximal histidyl residues (F8) are out of the heme planes in deoxy-Hb. Upon oxygenation, the iron atoms move into the center of the heme planes. He further pointed out that the movement of the heme iron atom is the "trigger" mechanism to initiate tertiary and

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[1] Abbreviations: Hb, hemoglobin (see Table I for abbreviations of hemoglobins used in this work); T, low-affinity deoxy quaternary structure of Hb; R, high-affinity oxy quaternary structure of Hb; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; IHP, inositol hexaphosphate; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Tris, tris(hydroxymethyl)aminomethane; ESR, electron spin resonance; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Abbreviations and Descriptions of Hemoglobins

Hb	hemoglobin
Hb A	human normal adult hemoglobin
Hb C (β6Glu→Lys)	mutant hemoglobin in which β6 Glu is replaced by Lys
deoxy-Hb	deoxyhemoglobin
α(des-Arg)	the carboxyl terminal amino acid Arg(α141) is specifically removed from the α chain
α(des-Arg-Tyr)	the carboxyl terminal amino acids Arg(α141) and Tyr(α140) are specifically removed from the α chain
des-Arg(α141) Hb A	the carboxyl terminal amino acids Arg(α141) are removed from both α chains in tetrameric Hb A
des-Arg(α141)-Tyr(α140) Hb A	the carboxyl terminal amino acids Arg(α141) and Tyr(α140) are removed from both α chains in tetrameric Hb A
des-His(β146)-Tyr(β145) Hb A	the carboxyl terminal amino acids His(β146) and Tyr(β145) are removed from both β chains in tetrameric Hb A
Hb McKees Rocks (β145Tyr→Term)	mutant Hb McKees Rocks in which β145 Tyr and β146 His are missing
NES-Hb A	both sulfhydryl groups of Cys-(β93) in Hb A are specifically reacted with *N*-ethylmaleimide
NES-des-Arg(α141) Hb A	both sulfhydryl groups of Cys-(β93) in des-Arg(α141) Hb A are reacted with *N*-ethylmaleimide
Hb A XL	cross-linked Hb A
$(\alpha\beta)_A(\alpha\beta)_C$XL	cross-linked hemoglobin where the subscript A or C denotes that the $\alpha\beta$ dimer in parentheses comes from either Hb A or Hb C, respectively
$[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta(\text{NES})]_C$XL	cross-linked asymmetrically modified hemoglobins

eventually quaternary structural changes in the Hb molecule upon ligation. Another important feature in Perutz's stereochemical mechanism for the cooperative oxygenation of Hb is the critical roles played by the salt bridges (Perutz, 1970; Perutz & TenEyck, 1971). The salt bridges of the α chain involve the α-carboxyl of Arg(141α_1) with both the α-amino group of Val(1α_2) and the ϵ-amino group of Lys(127α_2) and the guanidinium group of Arg(141α_1) with the carboxyl group of Asp(126α_2). The β chain salt bridges involve the α-carboxyl of His(146β_2) with the ϵ-amino group of Lys(40α_1) and the imidazole group of His(146β_2) with the carboxyl of Asp(84β_2). Perutz has stated that the deoxy-Hb molecule is clamped together by these salt bridges. For a more detailed description of the salt bridges, as well as inter- and intrasubunit hydrogen bonds that stabilize the T and R structures, refer to Perutz (1970), Perutz & TenEyck (1971), and Perutz et al. (1974). The basic conceptual framework of Perutz's scheme shares many features of a two-state allosteric model, such as the one proposed by Monod et al. (1965). The purpose of this paper is to report experiments investigating the effects of the salt bridges on tertiary and quaternary structures of Hb.

Proton nuclear magnetic resonance (NMR) spectroscopy has provided a powerful tool for investigating macromolecular conformations in solution. The ^{1}H NMR spectral changes associated with the cooperative oxygenation of Hb A have been intensively investigated by this laboratory (Ho & Lindstrom, 1972; Lindstrom & Ho, 1972; Johnson & Ho, 1974; Viggiano & Ho, 1979; Viggiano et al., 1979; Ho et al., 1982). For a recent review, refer to Ho & Russu (1981). The ^{1}H NMR spectra of Hb in H_2O show several proton resonances in the spectral region 5–10 ppm from H_2O. The exchangeable proton resonance at 9.4 ppm downfield from H_2O has been assigned to the intersubunit hydrogen bond between α42(C7) Tyr and β99(G1) Asp at the $\alpha_1\beta_2$ interface of deoxy-Hb A (Fung & Ho, 1975), a characteristic feature of the deoxy quaternary structure according to Perutz's atomic model of Hb. The exchangeable proton resonance at 6.4 ppm from H_2O has been tentatively assigned to the intrasubunit hydrogen bond between β145(HC2) Tyr and β98(FG5) Val (Viggiano et al., 1978), a characteristic deoxy tertiary structure in Perutz's atomic model of Hb. These two exchangeable proton resonances have been used to investigate both tertiary and quaternary structural changes associated with the cooperative oxygenation of Hb A (Viggiano & Ho, 1979).

Deoxy-Hb A exhibits two hyperfine-shifted exchangeable proton resonances occurring about 59 ppm and 71 ppm from H_2O and resulting from the proximal histidyl $N_\delta H$ protons (LaMar et al., 1977; Takahashi et al., 1980). The proton resonance at 59 ppm has been assigned to the $N_\delta H$ of the proximal histidyl residue of the α chain and that at +71 ppm has been assigned to the corresponding position in the β chain of Hb A (Takahashi et al., 1980; LaMar et al., 1980). Nagai et al. (1982) have investigated the effect on the proximal histidyl $N_\delta H$ proton resonances of removal of the salt bridges from specific subunits. They have attempted to correlate the chemical shifts of the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances with the strain in the iron–proximal histidine bond described in Perutz's stereochemical model of Hb and to interpret the change in the chemical shifts of these resonances as a change in the covalency of this bond. Takahashi et al. (1982) have recently presented evidence to show that such simple relationships as proposed by Nagai et al. (1982) do not occur. Takahashi et al. (1982) have pointed out that the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances of deoxy-Hb are sensitive to the quaternary structure and to the details of the conformation and dynamics of the heme pockets in Hb. Information from these resonances complements nicely the information obtained from the ferrous hyperfine-shifted and exchangeable proton resonances over the spectral region 6–20 ppm downfield from H_2O.

Asymmetrically modified hemoglobins have been prepared from chemically modified Hb A and Hb C (β6Glu→Lys) by utilizing a bifunctional cross-linking reagent, bis(3,5-dibromosalicyl) fumarate (Walder et al., 1979). The modified hemoglobins prepared are $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL, $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL, $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL, and $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta(\text{NES})]_C$XL, where the subscript A or C denotes that the $\alpha\beta$ dimer is from Hb A or Hb C, respectively, XL indicates a cross-linked Hb, and NES represents the product of a reaction between *N*-ethylmaleimide and the sulfhydryl group of β93 cysteine. The bifunctional reagent bis(3,5-dibromosalicyl) fumarate was found by Walder et al. (1980) to cross-link the two β chains specifically at the β82 Lys. Hb A cross-linked by bis(3,5-dibromosalicyl) fumarate preserves its cooperative oxygen binding property with a Hill coefficient of about 2.3 (Walder et al., 1980; S. Miura and C. Ho, unpublished results). In the present work, Hb C (β6Glu→Lys) is introduced only to facilitate the isolation of specific hybrid Hb samples. For details, see Miura & Ho (1982) for our earlier work on asymmetric mixed-valency cross-linked hybrid hemoglobins. In the present ^{1}H NMR

investigation, the exchangeable proton resonances are observed in the spectral region from 5 to 10 ppm downfield from H_2O, and the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances are observed from 50 to 80 ppm downfield from H_2O. These proton resonances allow us to examine the effect of specific removal of one or two salt bridges on the tertiary and quaternary structures of Hb. We have also made a comparison between the ^1H NMR spectra of the naturally occurring mutant Hb McKees Rocks (β145Tyr→Term) and the chemically modified des-His(β146)-Tyr(β145) hemoglobin prepared from Hb A.

Experimental Procedures

Preparation of Hemoglobins and Chemically Modified Hemoglobins. 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 or heterozygous AC blood samples obtained from the Sickle Cell Society, Inc., of Pittsburgh. Organic phosphates were removed either by passage through a Sephadex G-25 column equilibrated with 0.01 M Tris buffer containg 0.1 M NaCl at pH 7.5 after dialysis against the same buffer (Berman *et al.*, 1971) or by passage through an Amberlite AG 501-X8 column. Carboxypeptidase A was purchased from Sigma, and carboxypeptidase B (DFP treated) was purchased from Worthington. Des-Arg(α141) Hb A and des-Arg(α141)-Tyr(α140) Hb A were prepared from Hb A as described by Kilmartin et al. (1975). NES-des-Arg(α141) Hb A and NES-Hb C were prepared by allowing des-Arg(α141) Hb A and Hb C to react with *N*-ethylmaleimide as described by Riggs (1961) and Benesch & Benesch (1961). Des-His(β146)-Tyr(β145) Hb A was prepared as described by Antonini et al. (1961). Hb McKees Rocks was purified as described by Winslow et al. (1976).

Preparation of Asymmetrically Modified Hemoglobins. Des-Arg(α141) Hb A, NES-des-Arg(α141) Hb A, or des-Arg(α141)-Tyr(α140) Hb A in the CO form was mixed with the same amount of HbCO C and allowed to interchange dimers for at least 1 h in an ice bath. The mixtures were then allowed to react with a stoichiometric amount of bis(3,5-dibromosalicyl) fumarate for 2 h at 37 °C in 0.01 M Bis-Tris buffer at pH 7.1 (Walder et al., 1980) under a CO atmosphere. Bis(3,5-dibromosalicyl) fumarate was synthesized as described by Walder et al. (1979). After removal of excess reagent, cross-linked Hb tetramers were separated from unreacted Hb on an Ultrogel AcA 44 (LKB) gel filtration column equilibrated with 0.1 M Tris-HCl buffer at pH 7.2 in the presence of 1 M $MgCl_2$ (Macleod & Hill, 1970). The cross-linked tetrameric Hb in which one dimer is derived from modified Hb A and the other from Hb C was isolated on a CM-52 cellulose column equilibrated with 0.035 M sodium phosphate buffer at pH 6.3 in essentially the same manner as previously reported (Miura & Ho, 1982). $[\alpha(\text{Des-Arg})\beta]_A[\alpha\beta\text{-(NES)}]_C$XL was prepared from NES-Hb C and des-Arg(α141) Hb A in a similar manner. Each purified cross-linked Hb was analyzed by $NaDodSO_4$ electrophoresis (Weber & Osborn, 1969) and by isoelectrophoresing on a polyacrylamide gel column (Righetti & Drysdale, 1971). The conversion of HbCO to HbO_2 and then to the deoxy form was accomplished by first flushing the Hb solution with O_2 in ice–water under a heat lamp and then passing N_2 through the Hb solution as described previously (Lindstrom & Ho, 1972). Deoxy-Hb samples were transferred anaerobically to N_2-filled 5-mm NMR sample tubes. After deoxygenation, a minimal amount of sodium dithionite solution (0.1 M) was added directly to the NMR sample tube with an air-tight syringe to maintain the Hb solution in the deoxy form.

Measurement of ^1H NMR Spectra. High-resolution ^1H NMR spectra of the exchangeable proton resonances located between 5 and 10 ppm downfield from H_2O were obtained on the home-built 600-MHz NMR spectrometer interfaced to a Sigma 5 computer (Bothner-By & Dadok, 1979). The signal-to-noise ratio was improved by signal averaging about 2000–4000 scans with NMR correlation spectroscopy (Dadok & Sprecher, 1974). The temperature inside the probe was 21 °C. The proton chemical shift is expressed as parts per million (ppm) relative to the water proton resonance, which is 4.99 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 21 °C.

High-resolution ^1H NMR spectra of the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton signals were obtained on a Bruker WH-300 NMR spectrometer. The signal was accumulated for about 4000 scans with 40-Hz exponential line broadening applied to increase the signal-to-noise ratio. The intense water signal was reduced by preirradiation of the water signal. The temperature inside the probe was controlled by a Bruker temperature controller. It should be mentioned that the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances of various cross-linked asymmetrically modified hemoglobins were not obtained at the same temperature as the 600-MHz ^1H NMR spectra of exchangeable proton resonances over the spectral region from 5 to 10 ppm downfield from H_2O. In order to resolve the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances due to the normal and modified chains within the tetrameric Hb, we had to vary the temperature. This is based on the finding reported by Johnson et al. (1977) that ferrous hyperfine-shifted proton resonances of deoxy-Hb A are extremely sensitive to temperature. The temperature of each sample is given in each figure.

The chemical shift scale is presently defined as positive in the low-field direction with respect to the reference H_2O signal. The accuracy of chemical shift measurements is as follows: ±0.1 ppm for the exchangeable proton resonances over the spectral region from 5 to 10 ppm downfield from H_2O and ±0.4 ppm for the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable resonances over the spectral region from 50 to 80 ppm downfield from H_2O.

All samples were prepared in 5% D_2O (v/v), 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride buffer. All Hb samples were about 10% (w/v). The pH values of the samples were measured directly on a Radiometer Model 26 pH meter equipped with an Ingold combination electrode (6030–01).

Results

Hb A and Hb A XL. The low-field ^1H NMR spectra of deoxy-Hb A and deoxy-Hb A XL in 0.1 M Bis-Tris plus 0.1 M Tris buffer and 0.2 M chloride at pH 7.4 are shown in Figure 1. Both deoxy-Hb A and deoxy-Hb A XL show two hyperfine-shifted exchangeable proton resonances at 58.9 and 71.1 ppm downfield from H_2O at 26 °C, which have been assigned to the proximal histidyl $N_\delta H$ protons of the α and β subunits in Hb A, respectively (Takahashi et al., 1980; LaMar et al., 1980). In both deoxy-Hb A and deoxy-Hb A XL, the chemical shifts of these two resonances remain constant (within 0.5 ppm) over the pH range from 6.0 to 8.6 (results not shown). On the other hand, these two resonances show large temperature-dependent chemical shifts as expected for paramagnetic-shifted proton resonances of deoxy-Hb A (Johnson et al., 1977). The chemical shifts are 56.8 ppm downfield from H_2O for the proximal histidyl residue of the α subunit and 67.5 ppm for that of the β subunit at 42 °C and

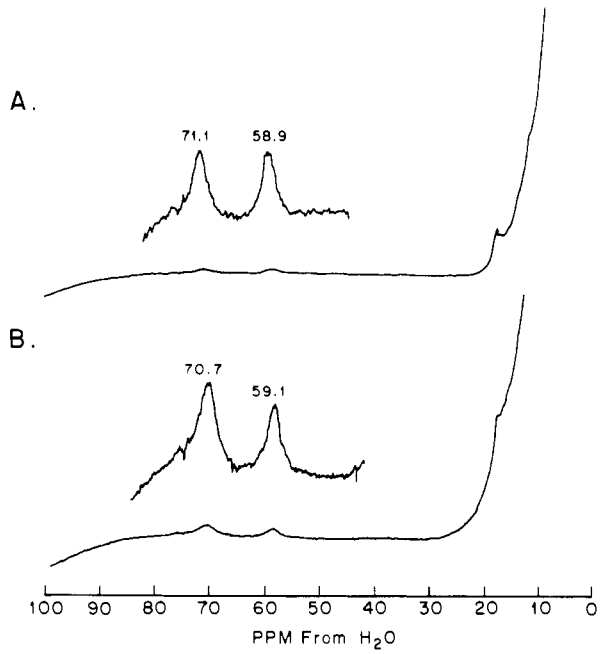

FIGURE 1: ^1H NMR spectra (300 MHz) of deoxyhemoglobins in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride at pH 7.4 in 95% H_2O and 5% D_2O at 26 °C: (A) deoxy-Hb A; (B) deoxy-Hb A XL.

57.6 ppm downfield from H_2O for the α subunit and 68.9 ppm for the β subunit at 36 °C. We have previously reported that deoxy-Hb A and deoxy-Hb A XL show similar spectra in the region between 5 and 10 ppm downfield from H_2O (Miura & Ho, 1982). In the present work, we demonstrate the spectral similarities of the proximal histidyl $N_\delta H$ proton resonances occurring between 50 and 80 ppm downfield from H_2O of deoxy-Hb A and deoxy-Hb A XL. Thus, cross-linking does not affect these spectra, and conclusions may be drawn about the effects of chemical modifications in cross-linked subunits. Nevertheless, X-ray crystallographic results suggest that there are small shifts toward the central cavity in the positions of both the E and F helices in the β subunit in deoxy-Hb A XL compared to the corresponding helices in deoxy-Hb A (Walder et al., 1980).

Des-Arg Hb A and $[\alpha(Des\text{-}Arg)\beta]_A[\alpha\beta]_CXL$. The cooperativity of des-Arg(α141) Hb A in its binding to O_2 is known to depend on the pH of the medium. Kilmartin et al. (1975) reported that at low pH, deoxy des-Arg(α141) Hb A remains in a T-like quaternary structure, while at high pH, it is converted into an R-like quaternary structure. A resemblance between the ferrous hyperfine-shifted proton resonances (arising from the heme and/or its nearby amino acid residues) of deoxy-Hb A and des-Arg(α141) Hb A at low pH has been reported (Ogawa et al., 1974; Perutz et al., 1974). A change from the T-like to the R-like quaternary structure of Hb can be detected by observing the loss of the exchangeable proton resonance at 9.4 ppm downfield from H_2O (Fung & Ho, 1975). Nagai et al. (1982) reported that the $N_\delta H$ exchangeable proton resonance from the proximal histidine in the modified α subunit of des-Arg(α141) Hb A is shifted downfield by about 13 ppm upon raising the pH from 6.9 to 9.0 and discussed this finding in light of a quaternary structural change. In the present study, the spectra of des-Arg(α141) Hb A show such pH-dependent changes in both the proximal histidyl $N_\delta H$ exchangeable proton resonances and hydrogen-bonded exchangeable proton resonances (Figure 2).

The ^1H NMR spectra of $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_CXL$ at pH 6.0 and 8.6 are also illustrated in Figure 2. At low pH, the exchangeable proton resonances in the spectral region from

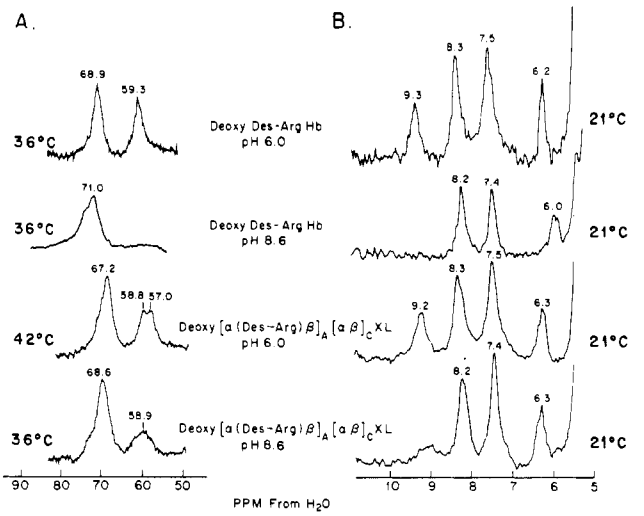

FIGURE 2: ^1H NMR spectra of deoxy des-Arg(α141) Hb A and deoxy $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_CXL$ in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride in 95% H_2O and 5% D_2O: (A) 300-MHz NMR spectra of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances (the temperature of each measurement is given in each figure); (B) 600-MHz NMR spectra of the exchangeable proton resonances at 21 °C.

5 to 10 ppm downfield from H_2O are essentially the same for deoxy des-Arg Hb A and deoxy $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_CXL$. The resonances due to the α-subunit proximal histidyl $N_\delta H$ proton of deoxy $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_CXL$ split into two peaks at 42 °C. The high-field peak shows the same chemical shift as that of the α-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance of deoxy-Hb A, presumably due to the normal α chain in the $\alpha\beta$ dimer from Hb C. The low-field peak shows the same chemical shift as that of deoxy des-Arg(α141) Hb A, presumably due to the modified α chain in the α(des-Arg) dimer from Hb A. The integrated intensity of the resonances due to the α-subunit proximal histidyl $N_\delta H$ exchangeable protons is similar to that of the β-subunit proximal histidyl $N_\delta H$ exchangeable proton signal around 70 ppm downfield from H_2O. Raising the pH to 8.6 causes about half the intensity of the α-subunit proximal histidyl $N_\delta H$ proton resonances to shift by about 10 ppm downfield as does that of deoxy des-Arg(α141) Hb A upon raising the pH. The other half of the intensity remains at a position close to that at low pH. The intensity of the exchangeable proton resonance at 9.2 ppm is reduced upon increasing pH, but the resonance at 6.3 ppm remains unchanged.

Des-Arg(α141)-Tyr(α140) Hb A and $[\alpha(Des\text{-}Arg\text{-}Tyr)\beta]_A[\alpha\beta]_CXL$. Deoxy des-Arg($\alpha$141)-Tyr($\alpha$140) Hb A is believed to be in an R-like structure in the absence of inositol hexaphosphate (IHP) and is converted to a T-like structure in the presence of IHP as judged from its ligand binding property and the reactivity of the sulfhydryl group of Cys(β93) to 4,4′-dithiopyridine (Kilmartin et al., 1975). However, the crystal structure of deoxy des-Arg(α141)-Tyr(α140) Hb A was found to be isomorphous neither with deoxy-Hb A nor with ligated Hb (Perutz & TenEyck, 1971). The ^1H NMR spectra of deoxy des-Arg(α141)-Tyr(α140) Hb A in the regions from 5 to 10 ppm and from 50 to 80 ppm downfield from H_2O in 0.1 M Tris plus 0.1 M Bis-Tris and 0.2 M chloride with and without IHP are shown in Figure 3. In the absence of IHP, the exchangeable proton resonances in the spectral region from 5 to 10 ppm downfield from H_2O show the spectral features of an R-like structure, i.e., lacking the 9.4 and 6.3 ppm resonances. Corresponding to this, the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonance from the α subunits is shifted downfield by more than 10 ppm from

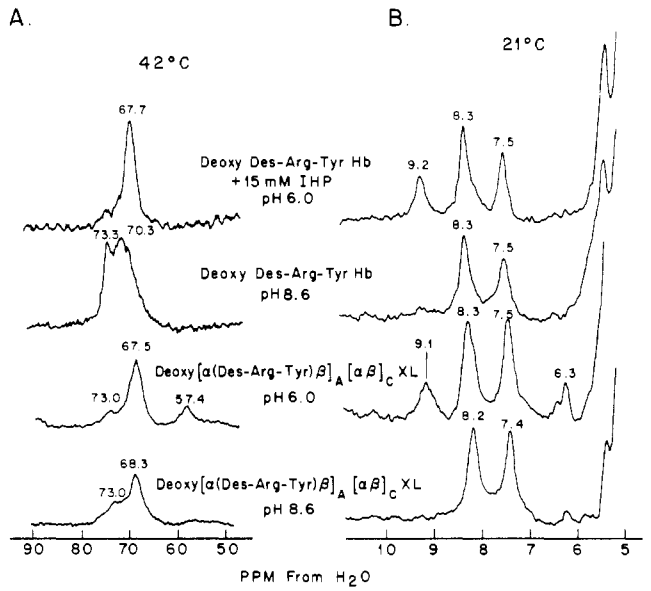

FIGURE 3: ^{1}H NMR spectra of deoxy des-Arg(α141)-Tyr(α140) Hb A and deoxy [α(des-Arg-Tyr)β]$_A$($\alpha\beta$]$_C$XL in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride in 95% H_2O and 5% D_2O: (A) 300-MHz NMR spectra of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances at 42 °C; (B) 600-MHz NMR spectra of the exchangeable proton resonances at 21 °C.

the position expected for deoxy-Hb A (Figure 3A). Addition of IHP builds up the deoxy quaternary structure marker resonance at 9.2 ppm and makes the proximal histidyl $N_\delta H$ exchangeable proton signals merge into one resonance at 68 ppm. It should be mentioned here that despite the buildup of the 9.2 ppm resonance by addition of IHP, the exchangeable proton resonance expected at 6.3 ppm from H_2O, a deoxy tertiary structural marker, is absent.

The ^{1}H NMR spectra of the asymmetrically modified Hb prepared from des-Arg(α141)-Tyr(α140) Hb A and Hb C are illustrated in Figure 3. Even at low pH, about half of the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances have already shifted downfield by about 15 ppm, but the other half of the resonances remain at a position close to that of the α-subunit proximal histidyl $N_\delta H$ proton resonance in intact Hb A. It is reasonable to assign the resonance that remains unchanged to the normal intact α subunit of $(\alpha\beta)_C$ in [α(des-Arg-Tyr)β]$_A$[$\alpha\beta$]$_C$XL. In correspondence with the shifted $N_\delta H$ proton resonance of α(des-Arg-Tyr), both resonances at 9.2 and 6.3 ppm have lost about half of their respective intensities, and they disappear when the pH is raised to 8.6. The increase in pH also causes the α-subunit histidyl $N_\delta H$ exchangeable proton resonance, normally occurring around 57 ppm downfield from H_2O at low pH, to shift downfield by about 14 ppm and the broad resonance 73 ppm downfield from H_2O to increase in intensity.

NES-Hb A and NES-des-Arg(α141) Hb A. Figure 4 shows ^{1}H NMR spectra of deoxy-NES-Hb A and deoxy NES-des-Arg(α141) Hb A in the presence and absence of IHP. As previously reported, deoxy NES-des-Arg(α141) Hb A without IHP shows a spectrum suggesting that the Hb molecule has an R-like structure, i.e., lacking a 9.4 ppm resonance (Fung & Ho, 1975) and exhibiting a downfield-shifted α(des-Arg) proximal histidyl $N_\delta H$ proton resonance (Nagai et al., 1982). Consequently, the spectrum of deoxy NES-des-Arg(α141) Hb A without IHP is quite similar to that of deoxy des-Arg(α141) Hb A at high pH (Figure 2). Addition of IHP converts this modified Hb molecule into a T-like structure with spectral features similar to those of deoxy-Hb A. These results are consistent with the oxygen binding properties of this modified

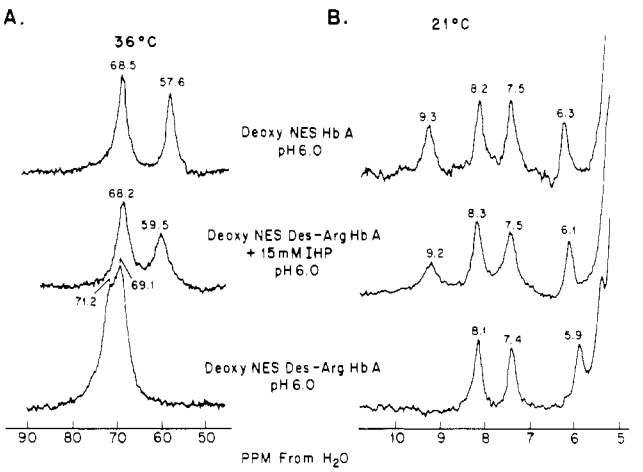

FIGURE 4: ^{1}H NMR spectra of deoxy-NES-Hb A and deoxy NES-des-Arg(α141) Hb A with and without IHP in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride in 95% H_2O and 5% D_2O: (A) 300-MHz NMR spectra of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances at 36 °C; (B) 600-MHz NMR spectra of the exchangeable proton resonances at 21 °C.

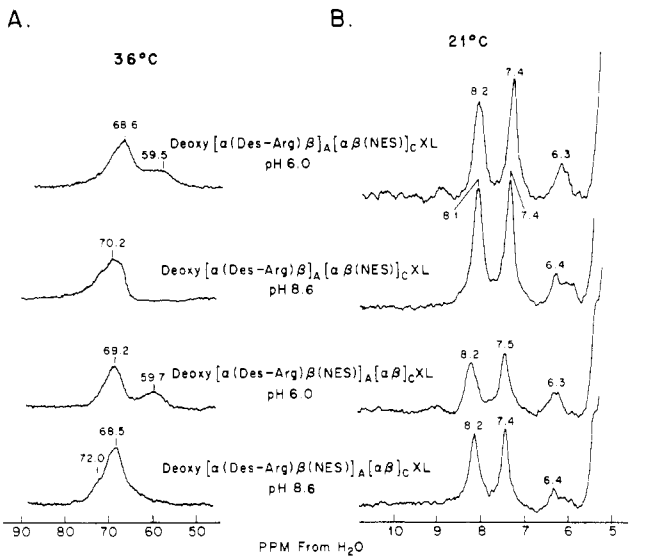

FIGURE 5: ^{1}H NMR spectra of deoxy [α(des-Arg)β]$_A$[$\alpha\beta$(NES)]$_C$XL and deoxy [α(des-Arg)β(NES)]$_A$[$\alpha\beta$]$_C$XL in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride in 95% H_2O and 5% D_2O; (A) 300-MHz NMR spectra of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances at 36 °C; (B) 600-MHz NMR spectra of the exchangeable proton resonances at 21 °C.

Hb (Kilmartin et al., 1975). However, the intensity of the proximal histidyl $N_\delta H$ exchangeable proton resonance around 60 ppm downfield from H_2O, due to the (des-Arg) subunits in NES-des-Arg(α141) Hb A, is somewhat smaller than the intensity of the resonance around 70 ppm downfield from H_2O even in the presence of IHP. Incomplete recovery of the α(des-Arg) subunit resonance in deoxy NES-des-Arg(α141) Hb A on the addition of IHP would correlate with the reduced intensity of the 9.2 ppm resonance. Attaching NES to the sulfhydryl group of Cys(β93) inhibits the formation of the salt bridge between His(β146) and Asp(β94), which is normally formed in deoxy-Hb A (Perutz et al., 1969). A ^{1}H NMR spectrum of deoxy-NES-Hb A is virtually the same as that of deoxy-Hb A. This result is consistent with the spectrum of des-His(β146) Hb A (Fung & Ho, 1975; Nagai et al., 1982).

[α(Des-Arg)β]$_A$[$\alpha\beta$(NES)]$_C$XL and [α(*Des-Arg*)β-(*NES*)]$_A$[$\alpha\beta$]$_C$*XL.* In Figure 5, the spectra of [α(des-Arg)-β]$_A$[$\alpha\beta$(NES)]$_C$XL and [α(des-Arg)β(NES)]$_A$[$\alpha\beta$]$_C$XL are

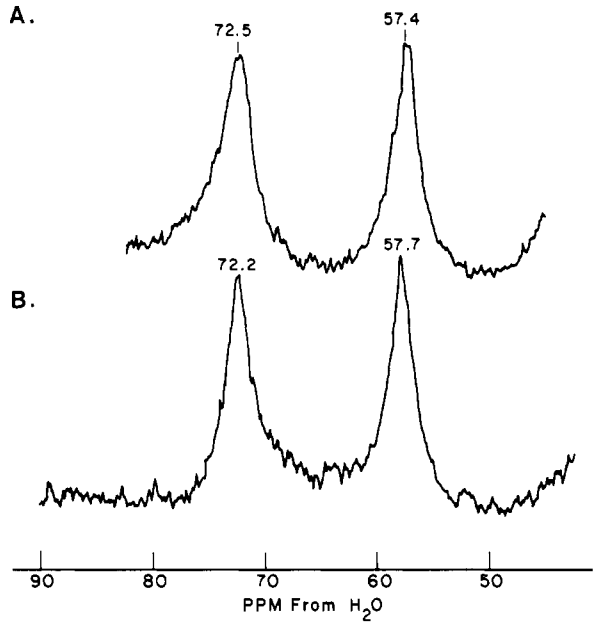

FIGURE 6: ^1H NMR spectra (300 MHz) of hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances of deoxyhemoglobins in 0.1 M Bis-Tris, 0.1 M Tris, and 0.2 M chloride with 10 mM IHP in 95% H_2O and 5% D_2O at 36 °C: (A) deoxy des-His(β146)-Tyr(β145) Hb A; (B) deoxy-Hb McKees Rocks (β145Tyr→Term).

shown. At low pH, both asymmetrically modified hemoglobins have hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances around 60 ppm downfield from H_2O, presumably due to the unmodified α subunits, but reduced in intensity due to the modified subunits. In contrast, both asymmetrically modified hemoglobins lose completely the resonance at 9.3 ppm downfield from H_2O, suggesting that the intersubunit interfaces between $\alpha_1\beta_2$ and $\alpha_2\beta_1$ are significantly altered in both hemoglobins even at low pH. It is noteworthy that the relative intensity of the proximal histidyl $N_\delta H$ exchangeable proton resonance located around 60 ppm downfield from H_2O in $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C\text{XL}$ is larger than that in $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta(\text{NES})]_C\text{XL}$. In both modified hemoglobins, raising the pH diminishes the resonance around 60 ppm and increases the intensity at 72 ppm instead. Consequently, both asymmetrically modified hemoglobins show hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances of an R-like structure.

Hb McKees Rocks and Des-His(β146)-Tyr(β145) Hb A. Figure 6 shows the spectra of deoxy des-His(β146)-Tyr(β145) Hb A and deoxy-Hb McKees Rocks (β145Tyr→Term) in the presence of IHP. Both the chemically modified and the naturally occurring equivalent mutant Hb, Hb McKees Rocks, show essentially identical proximal histidyl $N_\delta H$ exchangeable proton resonances. In the absence of IHP, both hemoglobins show a further downfield-shifted β-subunit resonance at about 73.5 ppm and no effect on the α-subunit resonances (result is not shown). Des-His(β146)-Tyr(β145) Hb A shows the same spectral change upon addition of IHP as that of Hb McKees Rocks in the spectral region from 5 to 10 ppm downfield from H_2O, indicating a structural change from an R-like to a T-like structure as previously reported (Viggiano et al., 1978). It should be mentioned that the hyperfine-shifted proximal $N_\delta H$ exchangeable proton resonances of deoxy-Hb McKees Rocks in the presence of IHP shown in Figure 6 are different from those reported by Takahashi et al. (1982). Takahashi et al. (1982) observed a splitting of the β-chain proximal histidyl $N_\delta H$ exchangeable resonance into two peaks at 71.0 and 73.6 ppm upon the addition of IHP. Such splitting is not observed in the present study. The reason for such a discrepancy is not fully understood. The sample of Hb McKees Rocks used by Takahashi et al. (1982) had been stored in a refrigerator for over a year, so that its integrity may have been uncertain. In the present study, we have used a solution of Hb McKees Rocks isolated from a fresh blood sample.

Discussion

Effect of Cross-Linking on Tertiary and Quaternary Structures of Hemoglobin. As previously reported by Miura & Ho (1982), there is essentially no difference between deoxy-Hb A and deoxy-Hb A XL in the ^1H NMR spectral region from 5 to 10 ppm downfield from H_2O arising from intra- and intersubunit hydrogen bonds in the Hb molecule. Now, the spectral similarity between the hyperfine-shifted proximal histidyl $N_\delta H$ exchangeable proton resonances at 50–80 ppm downfield from H_2O of deoxy-Hb A and deoxy-Hb A XL has been shown (Figure 1). These results show that the quaternary structure, which is manifested by hydrogen-bonded protons located in the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ subunit interfaces, and the tertiary structure, manifested by the proximal histidyl $N_\delta H$ exchangeable proton resonances, are essentially identical in cross-linked Hb A and native or unmodified deoxy-Hb A. Thus, changes in spectra seen in chemically modified cross-linked Hb's are not due to the cross-linking and may be interpreted in terms of the modified subunits.

Effect of Removing Arg(α141). The removal of the carboxyl-terminal Arg(α141) from one of the two α subunits, as seen in $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C\text{XL}$, results in a small change in the chemical shift of the proximal histidyl $N_\delta H$ exchangeable proton resonance from one of the two α subunits, when measured at low pH (Figure 2A). The shifted resonance is due to the chemically modified α subunit. However, the removal of one carboxyl-terminal Arg(α141) does not disturb the T-like structure as manifested by the resonance at 9.3 ppm, which is due to the intersubunit hydrogen bond between Tyr(α42) and Asp(β99) (Figure 2B). Thus, the small shift of the α-subunit proximal histidyl $N_\delta H$ exchangeable proton can be attributed to a small tertiary structural perturbation in the T-like quaternary structure. The removal of a carboxyl-terminal Arg(α141) from both α subunits, as seen in deoxy des-Arg Hb A, also does not disturb the T-like structure at low pH (Figure 2). The α(des-Arg) subunits in deoxy des-Arg(α141) Hb A show a spectrum similar to that of the α(des-Arg) subunit in deoxy $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C\text{XL}$ in the spectral region for the proximal histidyl $N_\delta H$ exchangeable proton of the α chain (Figure 2A). The effect at low pH of removing the carboxyl-terminal Arg(α141) from either one or both α subunits is mainly limited to the tertiary structure within the subunits from which the terminal Arg(α141) is removed.

It has been reported that the removal of the carboxyl-terminal Arg(α141) can expose the carboxyl group of Tyr(α140), which can then form a salt bridge with Val(α1) (Perutz & Ten Eyck, 1971). The newly formed salt bridge between the carboxyl-terminal carboxyl group of tyrosine on the α_1 subunit and the amino-terminal amino group of valine on the α_2 subunit must be sensitive to pH in the physiological range because the pK values of the amino-terminal valine (α1) are 7.7 for deoxy-Hb and 7.3 for oxy-Hb (Kilmartin & Rossi-Bernardi, 1971). Thus, breaking the newly formed salt bridge by increasing the pH can cause a downfield shift of the proximal histidyl $N_\delta H$ exchangeable proton resonance of the α subunit from which the carboxyl-terminal arginine was specifically removed. The tertiary structural change due to

the destruction of the salt bridge is localized within the modified subunit, and the other intact α subunit in $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL remains unaffected. The $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) subunit interface is altered upon raising the pH as a result of the tertiary structural alteration of the α(des-Arg) subunit.

Effect of Removing Arg(α141) and Tyr(α140). The removal of one Tyr(α140) from $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL causes a drastic tertiary structural change in the modified α subunit, even at low pH, without affecting the intact α subunit, as shown in Figure 3. The role of the penultimate Tyr(α140) may now be inferred as follows. The removal of the penultimate Tyr(α140) from the α(des-Arg) subunit results in the destruction of two interactions, namely, the newly formed salt bridge between the amino-terminal amino group of Val(α1) and the terminal carboxyl group of the penultimate Tyr(α140) and the hydrogen bond between the phenolic group of Tyr(α140) and the carbonyl group of Val(α93). A comparison between the spectra of $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL and $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL at low pH indicates that the two interactions in which the penultimate Tyr(α140) participates play an important role in modulating the α-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance (Figures 2A and 3A). It is reasonable to speculate that the hydrogen bond between the penultimate Tyr(α140) and Val(α93) is of primary importance to the α-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance because of the proximity of Val(α93) to the proximal histidine at α89. The spectrum of $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL at high pH shows that the newly formed salt bridge between the carboxyl group of Tyr(α140) and the amino-terminal amino group of Val(α1) also contributes to modulating the α(des-Arg) subunit proximal histidyl $N_\delta H$ exchangeable proton resonance (Figure 2A). The salt bridge between the carboxyl group of Tyr(α140) and the amino group of Val(α1) probably helps stabilize the hydrogen bond between Tyr(α140) and Val(α93) at low pH. The tertiary structural transition of the α(des-Arg-Tyr) subunit in deoxy $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL is transmitted to one of the two intersubunit interfaces between $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) so that the exchangeable proton resonance at 9.2 ppm is reduced to about half of its original intensity (Figure 3A).

The ^{1}H NMR spectrum of $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL at high pH shows no resonance in the spectral region expected for the proximal histidyl $N_\delta H$ exchangeable proton resonance of the unmodified α subunit in the $\alpha\beta$ dimer from Hb C (Figure 3A). The unmodified ($\alpha\beta$) dimer from deoxy-Hb C should have a resonance occurring $\sim$57 ppm downfield from H_2O, arising from the unmodified α subunit, and such a resonance is not observed for $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL at high pH. Thus, the present results clearly show that several features of the ^{1}H NMR spectra of asymmetrically modified hemoglobins cannot be accounted for as a spectral sum of the intact deoxy-Hb C and chemically modified Hb A.

Effect of Removing His(β146) and Tyr(β145). The spectra of deoxy des-His(β146)-Tyr(β145) Hb A support our speculation that the hydrogen bond between the phenolic group of the penultimate Tyr(α140) and the carbonyl group of Val(α93) is of primary importance to the modulation of the $N_\delta H$ exchangeable proton resonance of the proximal histidyl residue. The removal of histidine at β146 does not affect the proximal histidyl $N_\delta H$ exchangeable proton resonances from either the α or β subunit, and des-His(β146) shows essentially the same spectrum as that of deoxy-Hb A (Nagai et al., 1982). On the other hand, the removal of the penultimate Tyr(β145) from des-His(β146) Hb A causes a downfield shift of the β-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance by about 5 ppm without affecting the peak from the α subunit (results not shown).

Effect of N-Ethylmaleimide. Destruction of the salt bridge between His(β146) and Asp(β94) by reacting *N*-ethylmaleimide with one of two Cys(β93) in $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL reduces the intensity of the α-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance around 60 ppm (Figure 5A), suggesting that both salt bridges between His(β146) and Asp(β94) are intact in $[\alpha(\text{desArg})\beta]_A[\alpha\beta]_C$XL. However, the effect on the α-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance due to reacting *N*-ethylmaleimide with the β subunit in the same dimer with the α(des-Arg) subunit is less marked than the effect when the β(NES) subunit is in the other dimer (Figure 5A). It is interesting to compare these asymmetrically modified hemoglobins, $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta(\text{NES})]_C$XL and $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL, with their parent hemoglobins. Deoxy-Hb C and deoxy NES-des-Arg(α141) Hb A are the parent hemoglobins of deoxy $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL. Deoxy-Hb C exists in a T-like structure and deoxy NES-des-Arg(α141) Hb A in an R-like quaternary structure. The proximal histidyl $N_\delta H$ exchangeable proton resonances of the resulting asymmetric hybrid hemoglobin, $[\alpha(\text{des-Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL, are quite similar to the spectral sum of its parent hemoglobins at low pH (Figures 4A and 5A). On the other hand, both the parent hemoglobins for $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta(\text{NES})]_C$XL, namely, deoxy-NES-Hb C and deoxy des-Arg(α141) Hb A, are in a T-type structure at low pH, but the resulting hybrid hemoglobin does not show merely the spectral sum of its parents; rather it shows an intermediate proximal histidyl $N_\delta H$ exchangeable proton spectral feature (Figures 2, 4, and 5). The difference between these two asymmetrically modified hemoglobins may be attributed to different interactions between the two different types of dimers.

Comparison with Previous Results. Miura & Morimoto (1980) prepared asymmetrical nitroxyldeoxy hybrid hemoglobin, $(\alpha^{NO}\beta^{NO})(\alpha^{deoxy}\beta^{deoxy})$, and examined the effect on the nitroxyl radical attached to the α subunit of removing one or two carboxyl-terminal amino acid residues from one of the four subunits by monitoring the electron spin resonance (ESR) spectra of the NO radical. They found that the penultimate Tyr(α140) in the α^{NO} subunit plays an important role in modulating the triplet hyperfine signal from the α^{NO} subunit, while the removal of the penultimate Tyr(α140) in the α^{deoxy} subunit does not affect the triplet hyperfine signal from the α^{NO} subunit. These results are consistent with our present ^{1}H NMR results. However, the removal of His(β146) and Tyr(β145) from the β^{NO} subunit in $(\alpha^{NO}\beta^{NO})(\alpha\beta)$ diminished the triplet hyperfine signal from the α^{NO} subunit. The removal of His(β146)-Tyr(β145) from both β subunits in deoxy-Hb A causes a downfield shift of the β-subunit proximal histidyl $N_\delta H$ exchangeable proton resonance without affecting the signal from the proton resonance of the proximal histidyl $N_\delta H$ of the subunit.

In our previous study (Miura & Ho, 1982), the ^{1}H NMR spectra of asymmetrical cyanomet valency hybrid hemoglobins were examined. It was suggested that an asymmetric intermediate structure(s) can exist in which one of the two intersubunit interfaces ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) is altered and the other remains intact in the asymmetric valency hybrid hemoglobins with one cyanomet subunit. In the present work, it is demonstrated that there are intermediate structural states in which one of two subunits is converted to the oxy-like tertiary structure (as manifested by the proximal histidyl $N_\delta H$ exchangeable proton resonance at 60 ppm from H_2O), but the

other α subunit can remain in a deoxy-like tertiary structure. In accordance with this asymmetric tertiary structural transition from the deoxy-like to oxy-like structure, the deoxy-like quaternary structure [as manifested by the resonance at 9.3 ppm from H_2O resulting from the intersubunit hydrogen bond between α_1 and β_2 (or α_2 and β_1)] is altered somewhat.

One may attempt an explanation of the present ^1H NMR results based on the equilibrium between two quaternary structures with the observed spectral features. However, this interpretation is unlikely because there is no linear correlation between the tertiary structural marker resonance from the subunit and the quaternary structural marker resonance in the course of successive removal of the salt bridges in deoxy cross-linked Hb. In other words, the present ^1H NMR results cannot be explained as a linear combination of the two kinds of ^1H NMR spectra. Thus, the present ^1H NMR results clearly indicate that there are more than two arrangements of the subunits (quaternary structures) in the course of successive removal of specific salt bridges by means of enzymatic and chemical modifications.

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Registry No. Hb A, 9034-51-9; Hb McKees Rocks, 58985-22-1; L-Arg, 74-79-3; L-His, 71-00-1; L-Asp, 56-84-8; L-Tyr, 60-18-4; L-Val, 72-18-4; inositol hexaphosphate, 83-86-3.

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