Proton Nuclear Magnetic Resonance Studies of Hemoglobin Providence (β82EF6 Lys→Asn or Asp): A Residue Involved in Anion Binding†

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ABSTRACT: High-resolution proton nuclear magnetic resonance studies of hemoglobins Providence-Asn (β82EF6 Lys→Asn) and Providence-Asp (β82EF6 Lys→Asp) show that different amino acid substitutions at the same position in the hemoglobin molecule have different effects on the structure of the protein molecule. Hemoglobin Providence-Asp appears to be in a low-affinity tertiary structure in both the deoxy and carbonmonoxy forms. Deoxyhemoglobin Providence-Asn has its β heme resonance shifted downfield slightly from its position in normal adult hemoglobin; however, the tertiary structures of the heme pocket of hemoglobins A and Providence-Asn are very similar when both proteins are in the carbonmonoxy form. These results are consistent with the oxygen equilibrium measurements of Bonaventura, J., et al. [(1976) *J. Biol. Chem. 251,* 7563] which show that both Hb Providence-Asn and Hb Providence-Asp have oxygen affinities lower than normal adult hemoglobin, with Hb Providence-Asp having the lowest. Our studies of the effects of sodium chloride on the hyperfine shifted proton resonances of deoxyhemoglobins A, Providence-Asn, and Providence-Asp indicate that the β82EF6 lysine is probably one, but not the only binding site for chloride ions.

The β82EF6 lysine residue is invariant in all mammalian β, γ, and δ chains of hemoglobin (Hb)[1] (Dayhoff, 1972). This lysine plays an important role in the allosteric regulation of hemoglobin since it is one of the residues involved in the binding of 2,3-diphosphoglycerate (P_2-glycerate) and inositol hexaphosphate (Ins-P_6) (Arnone, 1972; Arnone & Perutz, 1974). The β82EF6 lysine has also been implicated as a possible binding site for the inorganic phosphate anion (Arnone, 1972) and the chloride anion (Chiancone et al., 1972; Nigen & Manning, 1975). Since the β82EF6 lysine plays an important role in the allosteric regulation of normal human hemoglobin (Hb A), it should be interesting to investigate the structural implications of a mutation at this position. Hemoglobin Providence offers a unique opportunity to investigate the implications of a mutation at the β82EF6 position since it contains two hemoglobins with different amino acid substitutions at this position. Hemoglobin Providence-Asn contains an asparagine at the β82EF6 position and post-synthetic deamidation is believed to be responsible for the formation of Hb Providence-Asp (Charache et al., 1975, 1977; Moo-Penn et al., 1976). In the absence of anionic cofactors both Hb Providence-Asn and Hb Providence-Asp have oxygen affinities lower than that of Hb A, near normal cooperativity as reflected in their Hill coefficient (n) of 2.5–2.7, and a decreased Bohr effect. P_2-Glycerate and Ins-P_6 have a considerably smaller effect on the oxygen affinities of the Hb Providence mutants than on Hb A. In the case of P_2-glycerate, $\Delta \log p_{50}$ is 0.81 for Hb A, 0.42 for Hb Providence-Asn, and 0.07 for Hb Providence-Asp. For Ins-P_6, $\Delta \log p_{50}$ for Hb A is 1.29, for Hb Providence-Asn it is 0.95, and for Hb Providence-Asp it is 0.14. It is also interesting to note that the chloride ion is effective in lowering the oxygen affinity of the two hemoglobin mutants. The decrease in oxygen affinity ($\Delta \log p_{50}$) for Hb Providence-Asn is similar to that seen for P_2-glycerate (0.44) and for Hb Providence-Asp the decrease in affinity in the presence of chloride is greater than that observed for either P_2-glycerate or Ins-P_6 (0.26) (Bonaventura et al., 1976). These authors found that in both oxygen affinity and kinetic measurements Hb Providence-Asn is more similar to Hb A than is Hb Providence-Asp.

In recent years, high-resolution proton nuclear magnetic resonance (NMR) spectroscopy has proved to be a very sensitive tool for investigating structural perturbations in a number of mutants and chemically modified hemoglobins (Davis et al., 1971; Ho et al., 1973, 1975; Perutz et al., 1974; Wiechelman et al., 1974; Fung & Ho, 1975; Wiechelman et al., 1976; Fung et al., 1976, 1977). In a molecule as large as hemoglobin, one would expect the many proton resonances to overlap to give an unresolvable spectral envelope; however, the presence of the heme group results in shifting certain proton resonances away from this large envelope of resonances so they can be easily resolved. In the unliganded form of hemoglobin the heme iron is paramagnetic, and its unpaired electrons can interact with protons on the porphyrin ring and/or with amino acids positioned sufficiently close to the heme to give rise to the hyperfine (or contact) shifted proton resonances. There are three prominent hyperfine shifted proton resonances in the spectrum of deoxy Hb A which occur at −17.6, −12.4, and −7.9 ppm from HDO at room temperature (Davis et al., 1971; Ogawa & Shulman, 1972; Ho et al., 1973). Studies of several mutant hemoglobins have shown that the resonance at ca. −18 ppm can be assigned to β chain protons and the resonances at ca. −12 and −8 ppm come from protons on the α chains (Davis et al., 1971; Lindstrom et al., 1972a; Ho et al., 1973; Fung et al., 1976, 1977).

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[1] Abbreviations used: Hb, hemoglobin; Hb A, normal human adult hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; P_2-glycerate, 2,3-diphosphoglycerate; Ins-P_6, inositol hexaphosphate, p_{50}, partial pressure of oxygen at 50% saturation; NMR, nuclear magnetic resonance; ppm, parts per million; Tris, tris(hydroxymethyl)aminomethane; Bistris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; and DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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In the liganded forms of hemoglobin (HbCO and HbO_2), certain amino acid residues are close enough to the heme group to be affected by the delocalized π electrons in the porphyrin ring (McDonald & Phillips, 1967; McDonald et al., 1969; Shulman et al., 1970; Ho et al., 1970; Lindstrom et al., 1972b; Lindstrom & Ho, 1973). These ring-current shifted resonances are very sensitive to even small changes in the tertiary structure around the ligand binding sites in the CO form of hemoglobin (Lindstrom et al., 1972b; Lindstrom & Ho, 1973).

If proton NMR studies are carried out in H_2O rather than D_2O, resonances due to exchangeable NH and/or OH protons can be observed in the spectrum (Patel et al., 1970; Ogawa et al., 1972, 1974; Ho et al., 1973, 1975; Mayer et al., 1973; Breen et al., 1974; Fung & Ho, 1975). In deoxy Hb A, exchangeable proton resonances appear at −9.4, −8.3, −7.5, and −6.4 ppm from the proton resonance of H_2O at 27 °C. The resonances at −8.3 and −7.5 ppm are superimposed on the hyperfine shifted proton resonance at ca. −8 ppm. In the spectrum of HbCO A exchangeable proton resonances are observed at −8.3, −7.5, and −5.5 ppm. The resonances at −9.4 (Ogawa et al., 1972, 1974; Mayer et al., 1973; Ho et al., 1975; Fung & Ho, 1975) and at −6.4 ppm are characteristic of the deoxy quaternary structure and the resonance at −5.5 ppm is characteristic of the oxy quaternary structure (Ho et al., 1975; Fung & Ho, 1975). The resonance at −9.4 ppm is believed to originate from the hydrogen bond across the $\alpha_1\beta_2$ subunit interface in deoxy Hb A between aspartic acid-β99G1 and tyrosine-α42C7 and the −5.5 ppm resonance may originate from the hydrogen bond between aspartic acid at α94G1 and asparagine-β102G4 (Ho et al., 1975; Fung & Ho, 1975). The resonance at −8.3 ppm has been assigned to one of the hydrogen bonds made by tyrosine-β35C1, probably the one to aspartate-α126H9 in the $\alpha_1\beta_2$ subunit interface (Asakura et al., 1976). The intrasubunit hydrogen bond between the β chain penultimate tyrosine (β145HC2) and valine-β98FG5 is believed to give rise to the −6.4-ppm resonance (Viggiano et al., 1978).

In the present study we have monitored the hyperfine shifted, ring-current shifted, and exchangeable proton resonances of hemoglobins Providence-Asn and Asp to study the effects of the mutations on the structures of these two hemoglobins. We have also investigated the effects of P_2-glycerate, Ins-P_6, and chloride ions on the structures of these hemoglobins.

Experimental Section

Materials. A blood sample was obtained from the propositus, a 26-year old black female. Hemoglobins Providence-Asp (the major component) and Providence-Asn (the minor component) were isolated and purified by chromatography on DEAE-Sephadex A-50 (Pharmacia) using the isolation and purification procedure of Huisman & Dozy (1965). The DEAE-Sephadex was equilibrated at pH 7.8 with 0.05 M Tris buffer, and after the hemolysate had been applied to the column elution was started using a linear pH gradient between pH 7.8 and pH 6.8 (0.05 M Tris). Hb A was eluted first, then Hb Providence-Asn, and Hb Providence-Asp was eluted last. Cellulose acetate electrophoresis showed that Hb Providence-Asn was contaminated with Hb Providence-Asp and that Hb Providence-Asp was pure. Hb Providence-Asn was rechromatographed as before and two bands containing electrophoretically pure Hb Providence-Asp and Hb Providence-Asn were eluted. Hb A was prepared by standard procedures from fresh whole blood samples obtained from the local blood bank (Lindstrom & Ho, 1972). Hemoglobin samples were freed from organic phosphates by passage through a column of Sephadex G-25 (Pharmacia) equilibrated with 0.01 M Tris containing 0.1 M NaCl at pH 7.5 (Berman et al., 1971). Deuterium oxide (Bio-Rad) was exchanged into the sample by repeated dilution with D_2O and subsequent ultrafiltration through an Amicon UM20-E membrane.

Stock solutions of [bis(2-hydroxyethyl)imino]tris(hydroxylmethyl)methane (Bistris) (Aldrich), inositol hexaphosphate (Sigma), and 2,3-diphosphoglycerate (Calbiochem) were prepared in D_2O as described previously (Wiechelman et al., 1974). Deoxyhemoglobin was prepared from HbCO by the standard procedure used in this laboratory (Lindstrom & Ho, 1972). The pD of all solutions in D_2O was determined by adding 0.4 pH unit (Glasoe & Long, 1960) to the meter reading obtained from a Beckman Model 3500 pH meter equipped with a Beckman 39504 electrode.

Methods. ^1H NMR spectra were obtained using the MPC-HF 250-MHz superconducting spectrometer (Dadok et al., 1970) at an ambient temperature of 27 °C. The residual HDO in the samples was used as an internal reference for the proton chemical shifts. The HDO resonance is 4.83 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 27 °C. A negative value in the chemical shift indicates that the resonance is downfield from HDO and a positive chemical shift indicates that the resonance is upfield from HDO. The chemical shifts of the hyperfine shifted and the exchangeable proton resonances are accurate to ±0.1 ppm and those of the ring-current shifted resonances are accurate to ±0.05 ppm. Signal-to-noise ratios were improved by NMR correlation spectroscopy using a Sigma-5 computer interfaced to the MPC-HF 250 MHz spectrometer (Dadok & Sprecher, 1974).

Results

The hyperfine shifted proton spectra of deoxyhemoglobins A, Providence-Asn, and Providence-Asp are shown in Figure 1. In the spectrum of Hb A in Bistris, three prominent resonances occur at −17.6, −12.4, and −7.9 ppm from HDO. The β-heme resonance of Hb Providence-Asn in Bistris is shifted downfield slightly to −17.8 ppm and the β heme resonance of Hb Providence-Asp is shifted even further downfield to −18.1 ppm. The addition of P_2-glycerate or Ins-P_6 to deoxy-Hb A causes the β heme resonance to shift downfield to ca. −18.1 ppm (Ho et al., 1973). In the presence of either organic phosphate, the β-heme resonance of Hb Providence-Asn is shifted downfield to ca. −18.1 ppm, while the β-heme resonance of Hb Providence-Asp is not affected by the addition of organic phosphates. When 0.1 M NaCl is added to Hb A in 0.1 M Bistris, the β-heme resonance is shifted downfield by ca. 0.2 ppm. The position of the β-heme resonance in both hemoglobins Providence-Asn and Providence-Asp is not affected by the addition of 0.1 M NaCl. Table I summarizes the results on the effect of 0.1 M NaCl on the hyperfine shifted proton resonances of deoxy-Hb A and Hb Providence.

The ring-current shifted proton spectrum of the CO form of Hb Providence-Asn in 0.1 M Bistris is similar to that of HbCO A, while the spectrum of the HbCO Providence-Asp differs from those of HbCO A and HbCO Providence-Asn as shown in Figure 2. The resonance at +5.86 ppm in the HbCO A spectrum has been assigned to the γ_2 methyl of valine-β67E11 and the resonance at +6.58 ppm arises from the γ_1 methyl of valine-β67E11. The resonance at +6.48 ppm in the HbCO A spectrum comes from the γ_1 methyl of valine-α63E11 (Lindstrom et al., 1972b; Lindstrom & Ho, 1973). At higher pD values (pD > 7) the γ_1 methyl groups of both the α63E11 and β67E11 valines are equivalent and give rise to a single resonance at ca. +6.5 ppm (Lindstrom & Ho, 1973).

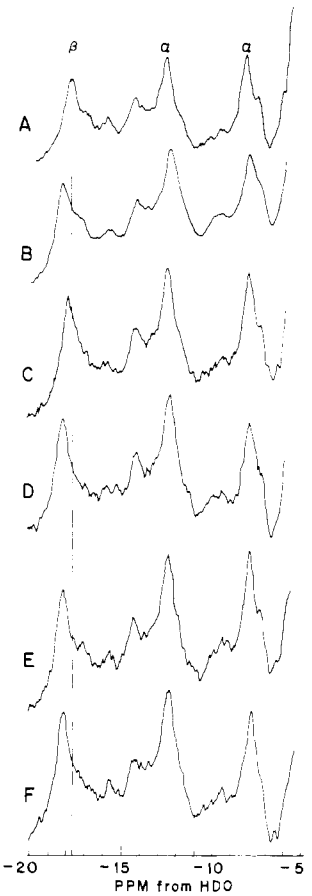

FIGURE 1: The 250-MHz ^{1}H NMR spectra of the hyperfine shifted proton resonances of deoxyhemoglobins A, Providence-Asn, and Providence-Asp in D_2O at 27 °C. (A) Hb A, 10%, in 0.1 M Bistris, pD 7.0; (B) 10% Hb A at the same conditions as A but plus 12 mM Ins-P_6 at pD 6.9; (C) 11% Hb Providence-Asn in 0.1 M Bistris, pD 6.8; (D) 10% Hb Providence-Asn at the same conditions but plus 10 mM Ins-P_6; (E) 11% Hb Providence-Asp in 0.1 M Bistris, pH 8; (F) 10% Hb Providence-Asp at the same conditions as E but plus 10 mM Ins-P_6.

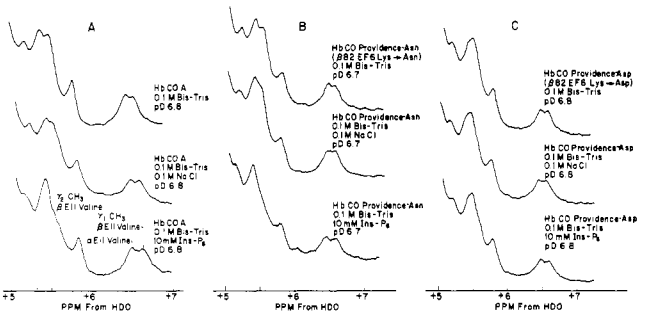

FIGURE 2: The 250-MHz ^{1}H NMR spectra of ring-current shifted proton resonances of carbonmonoxyhemoglobins A, Providence-Asn, and Providence-Asp in the presence and absence of inositol hexaphosphate in D_2O at 27 °C.

TABLE I: The 250-MHz ^{1}H NMR Spectra of Deoxyhemoglobins A, Providence-Asp, and Providence-Asn in 0.1 M Bistris in the Presence and Absence of 0.1 M NaCl.

	Chemical shift (ppm from HDO)		
	β	α	α
8% Hb A	−17.9	−12.7	−8.4
8% Hb A + 0.1 M NaCl	−18.1	−12.7	−8.3
11% Hb Providence-Asn	−17.8	−12.4	−7.9
11% Hb Providence-Asn + 0.1 M NaCl	−17.7	−12.4	−8.0
12% Hb Providence-Asp	−18.1	−12.4	*a*
12% Hb Providence-Asp + 0.1 M NaCl	−18.1	−12.4	*a*

[a] These spectra were obtained in H_2O so the α-heme resonance at ca. −8 ppm is superimposed on two exchangeable proton resonances so its position cannot be determined accurately.

The addition of P_2-glycerate or Ins-P_6 to HbCO A results in changes in the spectral region from ca. +5.2 to +5.6 ppm and a shift of the γ_1 methyl resonance of the β67E11 valine by about 0.05 ppm at a pD of 6.8 (Figure 2). The spectrum of HbCO Providence-Asp is not affected by the addition of Ins-P_6 and, even in the absence of organic phosphate, it is similar to the spectrum of HbCO A in the presence of Ins-P_6 with the exception of the γ_1 methyl resonance of the β67E11 valine resonance which is ca. 0.05 ppm downfield from its position in the spectrum of HbCO A plus Ins-P_6 (Figure 2C). The downfield region of the spectrum of HbCO Providence-Asn is altered by the addition of Ins-P_6 in a manner very similar to that seen for HbCO A; however, the γ_1 methyl resonance of the β67E11 valine is not shifted upfield as it is in the spectrum of HbCO A plus Ins-P_6 (Figure 1B). The addition of 0.1 M NaCl to HbCO A in 0.1 M Bistris causes the resonance at ca. +5.5 ppm to shift upfield by about 0.04 ppm. A similar shift in this resonance is seen in the spectrum of HbCO Providence-Asn in the presence of 0.01 M NaCl, while the spectrum of HbCO Providence-Asp is not affected by the addition of chloride (Figure 2).

The exchangeable proton resonances of both the deoxy and CO forms of Hb Providence-Asp are identical with the corresponding spectra of Hb A (Table I). This indicates that the quaternary structure of Hb Providence-Asp, as reflected in the hydrogen bonds of the exchangeable proton resonances, is similar to that of Hb A (Ho et al., 1975; Fung & Ho, 1975).

Discussion

The ^{1}H NMR studies of hemoglobins Providence-Asn and Providence-Asp show that different amino acid substitutions at the same position in the hemoglobin molecule can have different effects on the structure of the protein molecule. In deoxy Hb A the addition of either organic or inorganic phosphate causes the β-heme resonance to shift downfield by about 0.5 ppm. The structural changes which give rise to this shift in the position of the β-heme resonance have been attributed to a change in the tertiary structure of the hemoglobin molecule and are believed to be associated with the decrease in the oxygen affinity of the hemoglobin (Ho et al., 1973). It is interesting to note that the decrease in the oxygen affinities of the Hb Providence mutants appears to be related to the downfield shift of their β resonance, which is not surprising since lysine-β82EF6 is approximately 20 Å from the β-heme group. In Hb Providence-Asn the β-heme resonance has shifted downfield by ca. 0.2 ppm and its oxygen affinity at pH 7 (log p_{50} = 0.47) is somewhat lower than that of Hb A (log p_{50} = 0.30) (Bonaventura et al., 1976). The oxygen affinity of Hb Providence-Asp is still lower (log p_{50} = 0.65) (Bonaventura et al., 1976) and correspondingly the β-heme resonance is shifted downfield by about 0.5 ppm.

The addition of organic phosphates to HbCO A results in changes in the tertiary structure of the heme pocket which are reflected in the changes in the ring-current shifted proton resonances (Lindstrom & Ho, 1973). The spectrum of HbCO Providence-Asp is similar to that of HbCO A in the presence of Ins-P_6 in the region from ca. +5 to 6.6 ppm, suggesting that

this hemoglobin is in a low affinity tertiary structure even in the absence of organic phosphates. Both the deoxy and CO spectra of Hb Providence-Asp suggest that it is "locked" in a low affinity tertiary structure somewhat similar to that produced by the addition of organic phosphates to Hb A. The finding that P_2-glycerate and Ins-P_6 do not alter the spectra of either deoxy or carbonmonoxy Hb Providence-Asp is consistent with the small change in the p_{50} of this mutant upon the addition of organic phosphates (Bonaventura et al., 1976).

Our results suggest that the tertiary structures of the deoxy and CO forms of Hb Providence-Asn are more similar to those of the corresponding forms of Hb A. This is consistent with the oxygen affinity and kinetic studies of Bonaventura et al. (1976) which showed that the properties of Hb Providence-Asn are more similar to those of Hb A than Hb Providence-Asp. The slight downfield shift in the β-heme resonance of Hb Providence-Asn in Bistris may be related to the lowered oxygen affinity of this mutant. The addition of P_2-glycerate or Ins-P_6 to Hb Providence-Asn results in changes in both its deoxy and CO spectra which are presumably associated with the lower oxygen affinity induced by these allosteric affectors.

In deoxy-Hb A in the presence of 0.1 M NaCl, the β-heme resonance is shifted downfield by about 0.2 ppm from its position in the absence of chloride. Since a downfield shift of the β-heme resonance in deoxy Hb A in the presence of organic phosphates appears to be associated with a lower oxygen affinity (Ho et al., 1973), the downfield shift of this resonance in the presence of NaCl may also be related to a decrease in oxygen affinity. When 0.1 M NaCl is added to the Hb Providence mutants, there is no shift in the position of their β-heme resonances even though the p_{50} of both mutants is increased. This may suggest that the binding of a chloride ion to the β82EF6 lysine is responsible for the downfield shift of the β-heme resonance of Hb A. These results, together with the finding that increasing amounts of NaCl lower the oxygen affinities of Hb Providence-Asp and Hb Providence-Asn (Bonaventura et al., 1976), indicate that, even though the β82EF6 lysine may be one chloride binding site, there must be additional chloride binding sites on the hemoglobin molecule. This is consistent with the results of Mauk et al. (1976) who found that the effect of chloride ions on the oxygen affinity of Hb Rahere (β82EF6 Lys→Thr) was about half of the effect on Hb A.

The exchangeable proton resonances of a hemoglobin molecule can be used as indicators of the quaternary structure of the hemoglobin (Ogawa et al., 1972, 1974; Mayer et al., 1973; Ho et al., 1975; Fung & Ho, 1975). Our studies of the exchangeable proton resonances of Hb Providence-Asp and the finding that this hemoglobin has an essentially normal Hill coefficient (Bonaventura et al., 1976) show that this hemoglobin does change from the deoxy to the oxy quaternary structure. Our results suggest that the altered oxygen affinities of this mutant and Hb Providence-Asn are a result of perturbations in the tertiary structure of the heme pocket in the deoxy and/or liganded forms of these hemoglobins.

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Proton Nuclear Magnetic Resonance Studies of Hemoglobins Osler (β145HC2 Tyr→Asp) and McKees Rocks (β145HC2 Tyr→Term): An Assignment for an Important Tertiary Structural Probe in Hemoglobin†

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ABSTRACT: High-resolution proton nuclear magnetic resonance studies of deoxyhemoglobins Osler (β145HC2 Tyr→Asp) and McKees Rocks (β145HC2 Tyr→Term) indicate that these hemoglobins are predominately in the oxy quaternary structure in 0.1 M [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane buffer at pH 7. Upon the addition of inositol hexaphosphate, the proton nuclear magnetic resonance spectra of these hemoglobins become similar to those characteristic of a hemoglobin molecule in the deoxy quaternary structure. The exchangeable proton resonance which is found at −6.4 ppm from H_2O in the spectrum of normal human adult deoxyhemoglobin is absent in the spectra of these two mutant hemoglobins. Consequently we believe the hydrogen bond between the hydroxyl group of tyrosine-β145HC2 and the carboxyl oxygen of valine-β98FG5 gives rise to this resonance. This assignment allows us to use the −6.4 ppm resonance as an important tertiary structural probe in the investigation of the cooperative oxygenation of hemoglobin.

Comparison of the high-resolution proton nuclear magnetic resonance (NMR)[1] spectra of proteins in H_2O and D_2O shows that exchangeable NH and/or OH protons give rise to resonances in the low-field region of the spectra (Glickson et al., 1969; Patel et al., 1970; McDonald et al., 1971; Ogawa et al., 1972, 1974; Ho et al., 1973, 1975; Mayer et al., 1973; Breen et al., 1974; Fung & Ho, 1975). ^1H NMR studies of hemoglobin in water have revealed several exchangeable proton resonances in the spectral region from ca. −9.4 to −5.5 ppm from H_2O (Patel et al., 1970; Ogawa et al., 1972, 1974; Ho et al., 1973, 1975; Mayer et al., 1973; Breen et al., 1974; Fung & Ho, 1975). The two exchangeable proton resonances which are found at −8.3 and −7.5 ppm from the H_2O resonance at 27 °C occur in the spectra of both liganded and unliganded hemoglobins. This suggests that they are located in a region of the hemoglobin molecule which is not significantly altered by the switch in quaternary structures, most likely in or near the $\alpha_1\beta_1$ subunit interface. The resonance at −8.3 ppm is believed to come from one of the hydrogen bonds formed by tyrosine-β35C1, probably the one to aspartate-α126H9 in the $\alpha_1\beta_1$ subunit interface (Asakura et al., 1976). The origin of the resonance at −7.5 ppm is not yet ascertained.

The presence of the remaining exchangeable proton resonances appears to depend on the quaternary structure of the hemoglobin molecule and can be used as markers for their respective quaternary structures. The resonances at −9.4 and −6.4 ppm from the proton resonance of H_2O in the spectrum of deoxy-Hb A are characteristic of the deoxy quaternary structure (Ogawa et al., 1972, 1974; Mayer et al., 1973; Ho et al., 1975; Fung & Ho, 1975). The resonance at −9.4 ppm has been assigned to the proton involved in the hydrogen bond between aspartic acid-β99G1 and tyrosine-α42C7, which stabilizes the $\alpha_1\beta_2$ subunit interface in the deoxy quaternary structure (Ho et al., 1975; Fung & Ho, 1975). The resonance at −5.5 ppm in the spectrum of HbCO A and at −5.8 ppm in HbO_2 A may come from the hydrogen bond between aspartic acid at α94G1 and asparagine-β102G4, which stabilizes the $\alpha_1\beta_2$ subunit interface in the oxy quaternary structure (Ho et al., 1975; Fung & Ho, 1975).

The hydrogen bonds between the penultimate tyrosine-HC2 and valine-FG5 of either the α or β chains have been suggested as possible candidates for the −6.4 ppm resonance (Fung &

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[1] Abbreviations used: Hb, Hemoglobin; Hb A, normal adult hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; P_2-glycerate, 2,3-diphosphoglycerate; Ins-P_6, inositol hexaphosphate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Bistris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; p_{50}, partial pressure of oxygen at 50% saturation; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; rf, radiofrequency.

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