Proton Nuclear Magnetic Resonance Studies of Hemoglobin Malmö: Implications of Mutations at Homologous Positions of the α and β Chains†

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ABSTRACT: The abnormal human hemoglobin Malmö (β97FG4 His→Gln) has been studied and its properties are compared with those of normal adult hemoglobin A. The data presented here show that the ring-current shifted proton resonances of both HbCO and HbO_2 Malmö are very different from the corresponding forms of Hb A. The hyperfine shifted proton resonances of deoxy-Hb Malmö do not differ drastically from those of deoxy-Hb A. This result, together with the finding that the exchangeable proton resonances of the deoxy form of the two hemoglobins are similar, suggests that unliganded Hb Malmö can assume a deoxy-like quaternary structure both in the absence and presence of organic phosphates. We have also compared the properties of Hb Malmö with those of Hb Chesapeake (α92FG4 Arg→Leu). This allows us to study the properties of two abnormal human hemoglobins with mutations at homologous positions of the α and β chains in the three-dimensional structure of the hemoglobin molecule. Our present results suggest that the mutation at βFG4 has its greatest effect on the tertiary structure of the heme pocket of the liganded forms of the hemoglobin while the mutation at αFG4 alters the deoxy structure of the hemoglobin molecule but does not alter the tertiary structure of the heme pockets of the liganded form of the hemoglobin molecule. Both hemoglobins undergo a transition from the deoxy (T) to the oxy (R) quaternary structure upon ligation. The abnormally high oxygen affinities and low cooperativities of these two hemoglobins must therefore be due to either the structural differences which we have observed and/or to an altered transition between the T and R structures.

Over the past few years high-resolution proton nuclear magnetic resonance (NMR)[1] spectroscopy has become an increasingly powerful tool for studying protein molecules in solution. The hemoglobin molecule is particularly well suited for study by NMR since the heme group is able to shift several resonances away from the envelope of protein resonances in both the liganded and unliganded forms of hemoglobin. Even though NMR studies of normal human adult hemoglobin (Hb A) have yielded much useful information, hemoglobins with altered properties caused by a single amino acid substitution offer an even greater chance to study structure–function relationships in the hemoglobin molecule. Single amino acid substitutions have the greatest effect when the amino acid in question is either in contact with the heme group, or located in the $\alpha_1\beta_2$ subunit interface. All known hemoglobins with mutations in the $\alpha_1\beta_2$ interface have altered oxygen affinities and decreased heme–heme interactions (Perutz and Lehmann, 1968). X-Ray crystallographic studies show that the $\alpha_1\beta_2$ interface in the oxy-like methemoglobin crystal differs significantly from the corresponding subunit interface in deoxy-Hb. The two subunits appear to shift by 13° relative to each other in the transition from the oxy to the deoxy quaternary structure. This quaternary structural transition results in a slight loosening of the $\alpha_1\beta_1$ interface, but these two subunits do not shift relative to each other (Perutz and Ten Eyck, 1971). The $\alpha_1\beta_2$ interface is connected to the heme groups so any changes in the region of the interface could be expected to affect the heme environment (Perutz, 1969; Davis et al., 1971; Ho et al., 1973).

In Hb Malmö, the histidine residue at β97FG4 in the $\alpha_1\beta_2$ subunit interface has been replaced by a glutamine (Lorkin et al., 1970). The effect of this mutation is to raise the oxygen affinity of the hemoglobin molecule and reduce its cooperativity, which is reflected in the Hill coefficient (n) of ~1.4 (Boyer et al., 1972; Zak et al., 1976) as compared with $n \sim 3$ for normal hemoglobin.

There are several characteristic regions of the hemoglobin molecule which can be studied by ^{1}H NMR. The ring-current shifted resonances are due to protons of amino acid residues that are close enough to the porphyrin ring to be affected by the local magnetic fields produced by delocalized π electrons in the heme groups (McDonald and Phillips, 1967; McDonald et al., 1969; Shulman et al., 1970; Ho et al., 1970; Lindstrom et al., 1972b; Lindstrom and Ho, 1973). The positions of these resonances are very sensitive to small alterations of the amino acid residues relative to the iron atom at the center of the heme group (Lindstrom et al., 1972b; Lindstrom and Ho, 1973). Consequently these resonances serve as a sensitive monitor of the tertiary structure of the heme pockets of liganded hemoglobins.

In deoxyhemoglobin, unpaired electrons of the paramagnetic iron atom interact with protons on the porphyrin ring and/or protons of amino acids positioned sufficiently close to the iron atom to give rise to the hyperfine (or contact) shifted resonances (Davis et al., 1971; Ogawa and Shulman,

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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb, hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; Tris, tris(hydroxymethyl)aminomethane; IHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; Bis-Tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol; NADP, nicotinamide adenine dinucleotide phosphate; CMC, carboxymethyl-cellulose; T, deoxyhemoglobin quaternary structure; R, oxyhemoglobin quaternary structure; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

1972). In hemoglobin A, the prominent hyperfine shifted proton resonances are found at −17.6, −12.2, and −7.9 ppm from the residual HDO resonance at 25 °C (Davis et al., 1971). Studies of various mutant hemoglobins have shown that the resonance at ca. −18 ppm is due to β-chain heme protons, while the resonances at ca. −12 and ca. −8 ppm arise from heme protons on the α chain (Davis et al., 1971; Lindstrom et al., 1972a). When ligands bind, the hemes become diamagnetic and their resonances return to their normal positions buried in the aromatic and/or aliphatic resonances of the hemoglobin molecule.

When ^1H NMR studies are carried out with H_2O rather than D_2O as the solvent, additional resonances due to exchangeable NH and/or OH protons can be observed in the hemoglobin molecule (Patel et al., 1970; Ogawa et al., 1972; Ho et al., 1973; Mayer et al., 1973; Breen et al., 1974; Ogawa et al., 1974; Ho et al., 1975; Fung and Ho, 1975). In deoxy-Hb A, exchangeable proton resonances appear at −9.4, −8.3, −7.5, and −6.4 ppm from H_2O at 27 °C. In the HbCO A spectrum, exchangeable proton resonances are observed at −8.3, −7.5, and −5.5 ppm (Fung and Ho, 1975). The resonances at −9.4 (Ogawa et al., 1972; Mayer et al., 1973; Ogawa et al., 1974; Fung and Ho, 1975) and at −6.4 ppm are characteristic of the deoxy (T) quaternary structure, while the resonance at −5.5 ppm is characteristic of the oxy (R) quaternary structure (Fung and Ho, 1975). The resonance at −9.4 ppm is believed to originate from the hydrogen bond across the $\alpha_1\beta_2$ 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 and Ho, 1975).

We have studied the various regions of the Hb Malmö molecule using high resolution proton NMR spectroscopy at 250 MHz. The study of this particular mutant Hb is especially relevant since comparing its properties with those of Hb Chesapeake (α92FG4 Arg→Leu) allows us to compare the structural and functional implications of a mutation at a homologous position in the α and β chains of the hemoglobin molecule. Our present results indicate that an amino acid substitution in position FG4 of the β chain has its greatest effect on the structure of the liganded form of the Hb molecule while a mutation at residue FG4 of the α chain alters the structure of the unliganded form of the Hb molecule. For a detailed report on Hb Chesapeake, refer to Wiechelman et al. (1974).

Experimental Procedure

Materials. Red blood cells containing Hb Malmö were obtained by centrifuging whole blood samples which had previously been treated with carbon monoxide. The cells were then washed four times with isotonic saline and lysed with distilled water. The stroma and lipids were removed by extraction with xylene. Carboxymethyl-cellulose (CM52, Whatman) was equilibrated with 0.01 M sodium phosphate buffer, pH 6.5, and poured into a chromatographic column (8 × 45 cm) to a length of 30 cm. Two grams of the hemoglobin solution was applied to the column, and about 4 l. of sodium phosphate buffer, 0.01 M at pH 6.8, was run through the column. When the effluent was completely clear and only one band was present at the top of the column, the buffer was switched to 0.01 M sodium phosphate, pH 6.95. After approximately 4 l. of this buffer had run through the column, the Hb Malmö band had moved about 6 cm down the column away from the other Hb components. The upper portion of the CMC resin, which contained hemoglobins A and A_2, was removed with small volumes of the pH 6.95 buffer. The Hb Malmö was then eluted from the column in a concentrated zone with a saturated solution of KCl in 0.1 M Na_2HPO_4. Hb Malmö obtained by this method was completely free of any contamination with other hemoglobin pigments (Figure 1). Hemoglobins Malmö and A are present in approximately equal proportions in the blood specimen. Hb A was isolated by standard methods from fresh whole blood samples obtained from the local blood bank (Lindstrom and Ho, 1972). When Hb Malmö contained appreciable amounts of methemoglobin, it was anaerobically reduced using sodium dithionite (Mannox Brand, Holdman and Hardman, Miles Platting, Manchester, England). Excess dithionite was removed by passing the hemoglobin solution through a column of Sephadex G-25 (Pharmacia) equilibrated with CO. Comparison with Hb Malmö samples which had not been treated with dithionite showed no differences in the properties of the hemoglobin molecule. Hemoglobin was freed from organic phosphates by passing through a column of Sephadex G-25 equilibrated with 0.01 M Tris buffer 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 UM-20E membrane. In samples where H_2O was the solvent, the hemoglobin solution was dialyzed exhaustively against deionized H_2O to reduce the concentration of salts in the sample. Stock solutions of Bis-Tris (Aldrich), inositol hexaphosphate (IHP) (Sigma), and 2,3-diphosphoglycerate (DPG) (Calbiochem) were made in D_2O as described previously (Wiechelman et al., 1974). The hemoglobin samples were made deoxy by the standard procedure

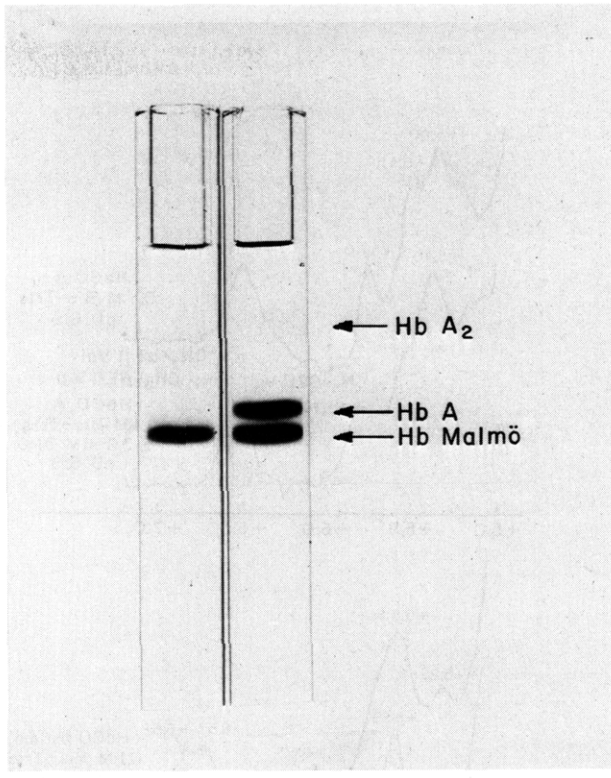

FIGURE 1: Isoelectric focusing of Hb Malmö before and after purification on 7% polyacrylamide gels containing ampholines (LKB) in the pH range 6–8. Electrophoresis was run at 400 V (1 mA/tube) for 5 h at 2 °C. For details on gel polymerization, refer to Dale and Latner (1968).

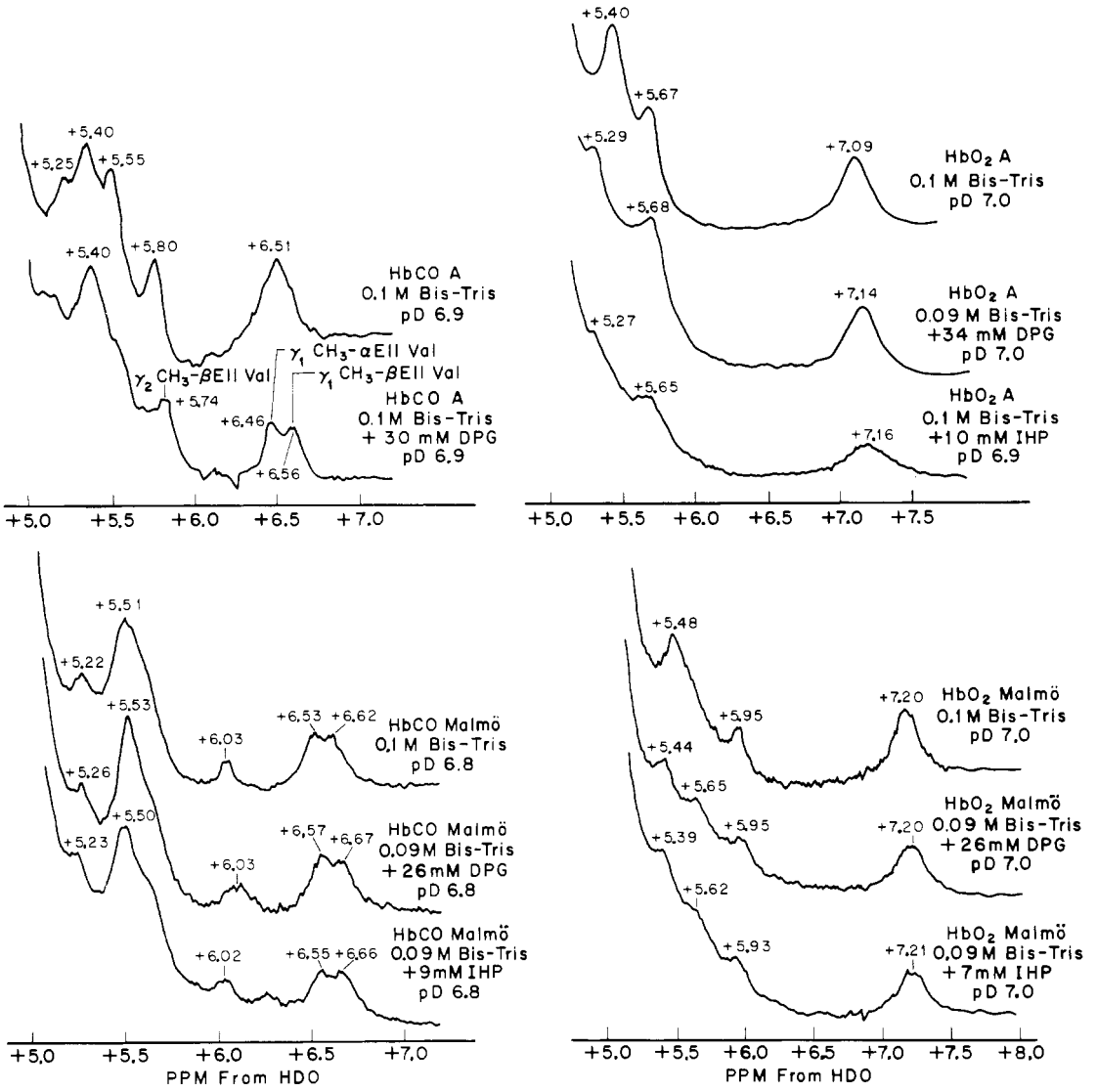

FIGURE 2: The 250-MHz ring-current shifted proton resonances of oxy- and carbonmonoxyhemoglobins A and Malmö in 0.1 M Bis-Tris at pD 6.9 and 27 °C in the absence and presence of organic phosphates. The assignments of the γ_1 and γ_2 methyl groups of the E11 valine resonances of HbCO A in the presence of 30 mM DPG are from Lindstrom et al. (1972b).

used in this laboratory (Lindstrom and Ho, 1972). The pD of all solutions in D_2O was determined by adding 0.4 pH unit (Glasoe and Long, 1960) to the meter reading of a Radiometer Model 4 pH meter equipped with a Beckman 39030 combination electrode.

To prepare samples for the CO saturation studies, appropriate amounts of deoxyhemoglobin were mixed with HbCO in 5-mm NMR sample tubes. The oxygen saturation studies were carried out in the presence of the methemoglobin reductase system of Hayashi et al. (1973) with NADPH substituted for NADP. All of the materials used in the reductase system were purchased from Sigma. Appropriate amounts of air were injected into 5-mm tubes containing deoxy-Hb Malmö and the samples were equilibrated for at least 30 min before NMR spectra were taken. The degree of ligand saturation was measured by monitoring the decrease in the deoxy peak at 757 nm using a Zeiss PMQ II spectrophotometer. The absorbance was measured directly through the 5-mm sample tubes which were held in the light path by a specially designed holder. The concentrations of Hb used for the NMR studies varied from 8 to 14 g %.

Methods. NMR spectra were obtained with the MPC-HF 250-MHz superconducting spectrometer (Dadok et al., 1970) at an ambient temperature of 27 °C. Proton chemical shifts are referenced with respect to the residual H_2O in the sample. The HDO signal is −4.83 ppm from the proton resonance of sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) at 27 °C. The negative sign of the chemical shifts indicates that the resonance is downfield from the HDO resonance and a positive sign indicates that the resonance is upfield from HDO. The chemical shifts of the hyperfine shifted resonances and exchangeable proton resonances are accurate to ±0.2 ppm and the chemical shifts of the ring-current shifted resonances are accurate to ±0.05 ppm. Signal-to-noise ratios were improved by the NMR correlation spectroscopy technique with a Sigma 5 computer interfaced to the MPC-HF 250-MHz spectrometer (Dadok and Sprecher, 1974).

Results

Figure 2 shows the 250-MHz ring-current shifted proton resonances of the oxy and CO forms of Hb Malmö and Hb A. These resonances differ significantly between the two hemoglobins, indicating that the tertiary structure of the

heme pockets in the liganded forms of Hb Malmö is altered by the mutation. The resonance at ca. +5.8 ppm from HDO in the HbCO A spectrum has been assigned to the γ_2 methyl of valine β67(E11) while the resonance at ca. +6.5 ppm in Bis-Tris buffer arises from the γ_1 methyl group of both the α63(E11) and β67(E11) valines (Lindstrom et al., 1972b; Lindstrom and Ho, 1973). In the presence of organic phosphate, such as DPG or IHP, the resonance at ca. +6.5 ppm splits with the βE11 valine resonance being shifted upfield. This reflects a movement of the βE11 valine closer to the iron atom at the center of the heme group (Lindstrom and Ho, 1973). In the spectrum of HbCO Malmo in 0.1 M Bis-Tris, the upfield resonance is split into two peaks at +6.53 and +6.62 ppm and the γ_2 E11 valine resonance is shifted upfield to +6.03 ppm. There are also significant differences in the remainder of the ring-current shifted resonances. In contrast to HbCO A, the addition of DPG or IHP to HbCO Malmö does not appear to affect the tertiary structure of the heme pocket. In HbO_2 A, the ring-current shifted resonances are very different from the resonances of HbCO A (Lindstrom and Ho, 1973) and the addition of DPG or IHP does not drastically alter the HbO_2 spectrum (Figure 2). Resonances are found at +5.40, +5.67, and +7.09 ppm in HbO_2 A and at +5.48, +5.95, and +7.20 in HbO_2 Malmö (Figure 2). The addition of DPG or IHP to HbO_2 Malmö causes significant changes in the ring-current shifted resonances, with an additional peak appearing at about ca. +5.6 ppm.

The hyperfine shifted proton resonances of deoxy-Hb Malmö are found at −17.8, −11.9, and −7.9 ppm from HDO in the absence of organic phosphate (Figure 3) and, in the presence of DPG or IHP, the β-heme resonance is shifted to −18.4 ppm while the α-heme resonances are relatively unaffected.

By comparing the intensities of the α-heme resonance at ca. −12 ppm and the β-heme resonance at ca. −18 ppm as a function of increasing ligand saturation, it is possible to monitor the relative affinities of the α and β hemes for ligand (Lindstrom and Ho, 1972; Johnson and Ho, 1974). When the hyperfine shifted proton resonances of Hb Malmö are monitored upon increasing saturation with CO, both in the absence and presence of organic phosphate, the α- and β-heme resonances lose intensity at nearly the same rate. From these results we can conclude that the affinity of the α and β hemes for CO is nearly the same. Partial oxygen saturation studies of Hb Malmö show that, both in 0.1 M Bis-Tris buffer and in the presence of DPG, the areas under the α- and β-heme resonances disappear at about the same rate, indicating that oxygen binds randomly to the α and β hemes under these conditions. In the presence of IHP, the area under the α-heme resonance disappears faster than the area under the β heme. This preferential decrease in the α heme area is probably caused by the α heme having a higher affinity for oxygen in the presence of IHP. In these studies the methemoglobin reductase system of Hayashi et al. (1973) was used to prevent the formation of significant amounts of methemoglobin during the course of the experiment. It has been shown that this system does not affect the results of oxygen saturation studies of hemoglobins A and Chesapeake in the presence of DPG or IHP (Wiechelman et al., 1974).

The exchangeable proton resonances of hemoglobins Malmo and A are shown in Figure 4. In the deoxy forms of both hemoglobins, the hyperfine shifted resonances still appear in the spectra at about −17.7, −12.0, and −7.9 ppm

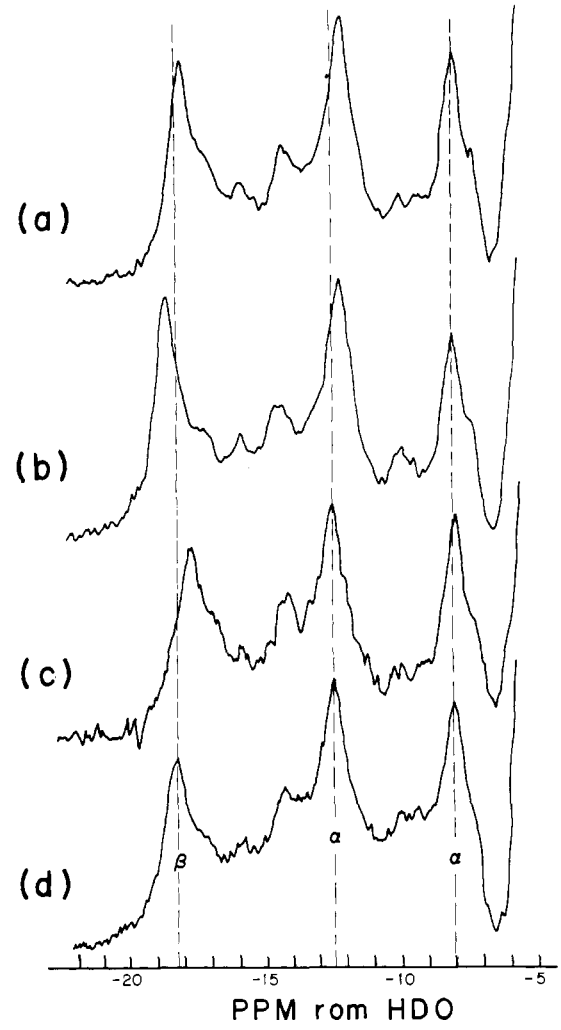

FIGURE 3: The 250-MHz hyperfine shifted proton resonances of deoxyhemoglobins A and Malmö with and without IHP at pD 6.9 and 27 °C. (a) 10% Deoxy-Hb Malmö in 0.1 M Bis-Tris; (b) 9% Deoxy-Hb Malmö in 0.1 M Bis-Tris plus 9 mM IHP; (c) 15% Deoxy-Hb A in 0.09 M Bis-Tris; and (d) 15% Deoxy-Hb A in 0.09 M Bis-Tris plus 9 mM IHP.

from H_2O. In addition, exchangeable proton resonances appear at −9.3, −8.2, −7.6, and −6.5 ppm. The two resonances at −8.2 and −7.6 ppm are superimposed on the hyperfine shifted resonance at −7.9 ppm. In HbCO A the hyperfine resonances have been shifted to their normal position in the envelope of aromatic and aliphatic resonances and only the exchangeable proton resonances at −8.3, −7.4, and −5.5 ppm remain in the spectrum (Ho et al., 1975; Fung and Ho, 1975). The resonances at −8.2, −7.4, and −5.4 ppm are also present in HbCO Malmö, and the shoulder at ca. −5.9 ppm in the HbCO A spectrum appears to be more pronounced in HbCO Malmö. The exchangeable proton resonances of HbO_2 Malmö and A are very similar with the resonances occurring at −8.2, −7.3, and −5.9 ppm (results not shown).

Discussion

Examination of the ring-current shifted proton resonances of Hb Malmö shows that the tertiary structures of the heme pocket of the liganded forms of this mutant hemoglobin differ significantly from those of the corresponding forms of Hb A. In HbCO Malmö in Bis-Tris buffer, the βE11 valine resonances at +6.03 and +6.62 ppm have been

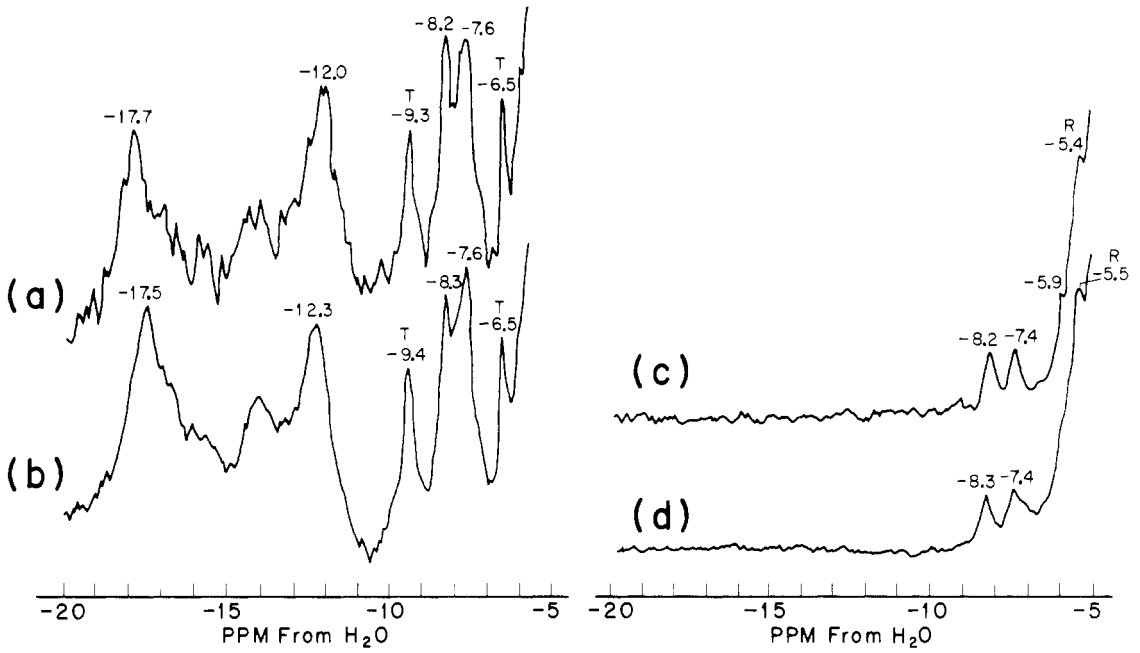

FIGURE 4: The 250-MHz spectra of the exchangeable proton resonances of the deoxy and CO forms of hemoglobins A and Malmö in 0.1 M Bis-Tris in H_2O at pH 6.6 and 27 °C. (a) 8% Deoxy-Hb Malmö; (b) 18% Deoxy-Hb A; (c) 8% HbCO Malmö; and (d) 12% HbCO A.

shifted upfield from their normal positions in HbCO A. This indicates that both of the γ-methyl groups of the βE11 valine are positioned closer to the porphyrin ring in Hb Malmö than in Hb A (Lindstrom and Ho, 1973). The site of the mutation in Hb Malmö at the FG corner of the β chain is on the proximal side of the heme group, while the E11 valine lies on the distal side of the heme pocket. Thus, the structural perturbations created by the amino acid substitution at the $\alpha_1\beta_2$ subunit interface in liganded Hb Malmö are transmitted through the β chain to the opposite side of the heme pocket. A similar shift of the γ-methyl groups of the E11 valine is seen in the ring-current shifted spectra of HbCO Kempsey and HbCO Yakima where the mutation is at β99G1 (Ho et al., 1973).

The addition of DPG or IHP to HbCO A causes significant alterations in the ring-current shifted proton resonances, while the effects of the organic phosphates on the resonances of HbO_2 A are less severe. The situation in Hb Malmö is somewhat different, however, since the addition of DPG or IHP to the carbonmonoxy form of this hemoglobin does not alter the ring-current shifted resonances significantly. The addition of DPG or IHP to HbO_2 Malmö causes a new resonance to appear in the NMR spectrum at +5.65 ppm from HDO. Thus, it appears that the binding of organic phosphates to the liganded forms of Hb Malmö affects the tertiary structure of this hemoglobin differently than it affects the tertiary structure of liganded Hb A.

The exchangeable proton resonances at −9.4 and −6.4 ppm are characteristic of the T (or deoxy) quaternary structure (Ho et al., 1975; Fung and Ho, 1975). The presence of these two resonances in the spectrum of deoxy-Hb Malmö indicates that this hemoglobin can assume a T-like quaternary structure in the absence of organic phosphates. The positions of the hyperfine shifted proton resonances of deoxy-Hb Malmö also indicate that the hemoglobin molecule is in a T-like quaternary structure. Perutz et al. (1974) have found that there is a set of hyperfine shifted resonances which is characteristic of the T quaternary structure and a different set of resonances characteristic of the R (or oxy) quaternary structure. For hemoglobins in a T-like conformation, resonances are found at ca. −18, ca. −12, and ca. −8 ppm. For hemoglobins which are in a R-like conformation in the unliganded form, the hyperfine shifted resonances are found at ca. −15, −11.4, and ca. −8 ppm (Perutz et al., 1974).

In nearly all of the $\alpha_1\beta_2$ mutant hemoglobins studied to date, a mutation in one chain alters the heme environment of both the abnormal and the normal chains (Davis et al., 1971; Lindstrom et al., 1973; Ho et al., 1973). The small shifts of the β-heme resonance and the α-heme resonance at −12 ppm in the Hb Malmö spectrum relative to Hb A (Figure 3) are probably due to small differences in the tertiary structures of the heme pockets of the two hemoglobins in the deoxy form. The addition of IHP to most $\alpha_1\beta_2$ mutant hemoglobins causes the hyperfine shifted resonances to become more similar to those of Hb A (Lindstrom et al., 1973; Ho et al., 1973; Perutz et al., 1974; Ho et al., 1975). In Hb Malmö even after the addition of IHP, the β-heme resonance is still ca. 0.3 ppm downfield from the corresponding resonance of Hb A.

Partial saturation studies of Hb Malmö show that CO binds to the α and β hemes with little, if any, preference. This is similar to the case of Hb A where there are no large differences in the affinities of the α and β hemes for CO (Johnson and Ho, 1974). Oxygen binds to the α and β hemes of Hb Malmö randomly in the absence of organic phosphates and in the presence of DPG and binds preferentially to the α hemes in the presence of IHP. There is more difference in the affinities of the α and β hemes in the case of oxygen binding to Hb A where oxygen binds preferentially to the α hemes in the presence of both DPG and IHP (Johnson and Ho, 1974).

It is of particular interest to study Hb Malmö since its properties can then be compared with those of Hb Chesapeake (α92FG4 Arg→Leu). The amino acid substitutions in hemoglobins Malmö and Chesapeake are homologous in the three-dimensional structure of the hemoglobin molecule. Both involve residue FG4, the fourth amino acid in the

connecting region between helices F and G. X-Ray crystallographic studies of deoxy- and oxy-like methemoglobin have shown that the $\alpha_1\beta_2$ subunit interface is dovetailed so that the CD region of one chain fits into the FG region of the opposite chain. The change in quaternary structure causes the two subunits to rotate relative to each other with the dovetailing of the CD region of the β chain with the FG part of the α chain remaining nearly the same, whereas the dovetailing of the CD part of the α chain and the FG region of the β chain is altered (Perutz, 1970).

Oxygen equilibrium studies of Hb Chesapeake have shown that this hemoglobin has an oxygen affinity about six to seven times higher than that of Hb A and a Hill coefficient (n) of 1.2–1.3 (Nagel et al., 1967; Imai, 1974). Addition of DPG or IHP to Hb Chesapeake results in a decrease in the oxygen affinity and an increased cooperativity with n = 2.15 in the presence of IHP (Imai, 1974). The Hill coefficient for Hb Malmö in the absence of phosphate is 1.44, and its oxygen affinity is intermediate between the affinities of hemoglobins A and Chesapeake (Boyer et al., 1972; Zak et al., 1976).

In Hb Chesapeake in 0.1 M Bis-Tris buffer, the hyperfine shifted resonances are found at −17.4 and −12.8 ppm from HDO, with the −7.9 ppm α-chain resonance missing (Davis et al., 1971; Wiechelman et al., 1974). This indicates that the deoxy structure of this hemoglobin differs from that of deoxy-Hb A. In contrast, the ring-current shifted proton resonances of the liganded forms of Hb Chesapeake are very similar to the corresponding forms of Hb A, so the tertiary structure of the heme pockets of liganded Hb Chesapeake must be very similar to those of Hb A (Wiechelman et al., 1974). In Hb Malmö, where the mutation of the FG4 amino acid residue is in the β chain, the structural perturbations resulting from the mutation are manifested primarily in the liganded form of the hemoglobin molecule.

The α and β chains within the Hb Chesapeake tetramer are functionally more equivalent in their reactions with ligands than the α and β chains of Hb A. In the presence of IHP, oxygen binds preferentially to the α hemes of Hb Chesapeake, but in Bis-Tris buffer or in the presence of DPG oxygen binds to the hemes randomly. In both the absence and presence of organic phosphate, CO binds randomly to the α and β hemes of Hb Chesapeake (Wiechelman et al., 1974).

Our NMR studies of hemoglobins Malmo and Chesapeake show that an amino acid substitution at position FG4 of the β chain has a different effect on the hemoglobin molecule than a mutation at position FG4 of the α chain. In Hb Malmö, the structural features of the deoxy-Hb molecule that we have monitored by NMR suggest that this form of the hemoglobin molecule is not altered drastically by the mutation. This is not the case for Hb Chesapeake, since in this hemoglobin the hyperfine shifted resonances of both the α and β chains differ significantly from those of Hb A. The greatest effect of the mutation in Hb Malmö is seen in the liganded form of the hemoglobin molecule where the ring-current shifted resonances are very different from those of both Hb A and Hb Chesapeake. The effect of the amino acid substitution at position FG4 of both the α and β chains appears to have a somewhat similar effect on the relative affinities of the α and β hemes for ligands.

There is some evidence that the size and charge of the amino acids involved in the mutation in an abnormal hemoglobin help to determine the effect of the mutation on the functional properties of the hemoglobin. Studies of several $\alpha_1\beta_2$ mutant hemoglobins with different amino acid substitutions at the same position in the hemoglobin molecule show that the effect of the mutation on the functional properties of the hemoglobin molecule depends on the nature of the amino acid substitution. Mutations in hemoglobins Chesapeake (α92FG4 Arg→Leu), Kempsey (β99G1 Asp→Asn), and Kansas (β102G4 Asn→Thr) have more drastic effects on their functional properties than the mutations in hemoglobin J Capetown (α92FG4 Arg→Gln), Yakima (β99G1 Asp→His), and Richmond (β102G4 Asn→Lys) (Nagel et al., 1967; Novy et al., 1967; Bonaventura and Riggs, 1968; Reed et al., 1968; Efremov et al., 1969; Ogawa et al., 1970; Davis et al., 1971; Nagel et al., 1971; Pulsinelli, 1973; Lindstrom et al., 1973; Ho et al., 1973; Shulman et al., 1973; Ho et al., 1975). Studies of the deoxy forms of some of these hemoglobins by NMR to monitor the structural perturbations show that there may be some correlation between the extent of the structural perturbation and the effect on the functional properties of the hemoglobin molecule. In Hb Yakima, whose functional properties are altered less than those of Hb Kempsey (Novy et al., 1967; Reed et al., 1968), the hyperfine shifted resonances are more similar to those of Hb A (Ho et al., 1973; Ho et al., 1975). In Hb J Capetown, the observation that the deoxy form of the hemoglobin, monitored by the hyperfine shifted proton resonances, is not altered as drastically as the structure of Hb Chesapeake (Ogawa et al., 1970; Davis et al., 1971) is consistent with the finding that Hb J Capetown has an oxygen affinity only slightly higher than that of Hb A and a value of n = 2.3 (compared with $n \sim 3$ for Hb A and n = 1.3 for Hb Chesapeake; Nagel et al., 1971).

Precise oxygen equilibrium measurements have shown that K_4, the Adair constant for the binding of the fourth oxygen molecule to hemoglobin, is very similar for Hb A and Hb Chesapeake both in the absence and presence of organic phosphate (Imai, 1974). This result is consistent with the finding that the tertiary structures of the heme pocket of the two hemoglobins monitored by the ring-current shifted proton resonances are very similar (Wiechelman et al., 1974). Our finding that the ring-current shifted proton resonances of the liganded forms of Hb Malmö differ significantly from the spectra of the corresponding forms of Hb A suggests that the tertiary structures of the heme pockets of Hb Malmö differ from those of Hb A. Since there appears to be a correlation between the tertiary structure of the heme pocket and ligand affinity (Lindstrom and Ho, 1973), our results may suggest that K_4 for Hb Malmo differs from that of Hb A.

Our NMR studies show that residues FG4 of the α and β chains, which are homologous in the three-dimensional structure of the hemoglobin molecule, are not structurally equivalent. An amino acid substitution in the β chain at position FG4 (Hb Malmö) has its greatest effect on the tertiary structure of the heme pocket of the liganded form of the hemoglobin molecule. A mutation of the α chain at position FG4 (Hb Chesapeake) alters the deoxyhemoglobin structure but has no effect on the tertiary structure of the heme pocket of the liganded form of the hemoglobin molecule.

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