NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOGLOBINS. V. THE HEME PROTON SPECTRA OF HUMAN DEOXYHEMOGLOBINS A, F, ZÜRICH, AND CHESAPEAKE*

Donald G. Davis, Nancy H. Mock
Ted R. Lindstrom, Samuel Charache†, and Chien Ho§

Department of Biophysics and Microbiology
University of Pittsburgh, Pittsburgh, Pennsylvania 15213

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Summary

The proton nuclear magnetic resonance spectra of human deoxyhemoglobins A, F, Zürich, and Chesapeake indicate that the heme groups in different subunits are magnetically nonequivalent. These results are in accord with earlier work on the cyano- and azido-derivatives of these methemoglobins. In addition, the nuclear magnetic resonance studies of Hb Chesapeake suggest that the altered functional properties of this mutant hemoglobin involve not only changes in the α_1-β_2 subunit contact region but also changes which affect the heme groups in the α-chains.

Proton nuclear magnetic resonance (NMR) studies of paramagnetic heme proteins have shown that the heme proton spectra can be used to probe the heme environment.[1-3] Magnetic interactions between the unpaired electrons of the iron atom and the heme protons produce hyperfine shifts which can be recognized by their inverse relationship to absolute temperature and are well separated from the remainder of the protein NMR spectrum.[4] This separation allows direct observation of variations in the heme environment.

Recent NMR studies have utilized these properties to probe the heme environment in a number of paramagnetic methemoglobin derivatives.[5-7]

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* This is publication No. 168 of the Department of Biophysics and Microbiology, University of Pittsburgh.

† Address: Special Hematology Laboratory, Johns Hopkins Hospital, Baltimore Maryland.

§ To whom reprint requests should be made. Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pa. 15213

These studies have demonstrated that the heme groups in the α- and β-chains are magnetically nonequivalent and that certain proton resonance lines can be assigned specifically to the α- or β- chain hemes. The assignments are based on systematic variations observed in the heme group proton resonances of different hemoglobin variants.[5-7] Further studies have shown that in some cases the magnetic nonequivalence coincides with differences in ligand affinity.[8,9]

The extension of these studies to human variants of deoxyhemoglobin is important because this paramagnetic form of hemoglobin is found in red blood cells and is capable of binding oxygen reversibly.[10,11] The use of human variants avoids the problems inherent in the study of other mammalian hemoglobins because the substitutions are of a single amino acid and the origin of the effects can be localized. The study of fetal hemoglobin is of value because the α-chains remain intact and the sequence of the γ-chains are known.

Experimental

Materials: Hemoglobins Chesapeake, Zürich, and F (fetal Hb) were isolated and purified as described earlier.[6,12] Hb A was prepared either from fresh human blood obtained from the local blood bank or from the blood samples of the heterozygotes containing Hb Zürich or Hb Chesapeake.[12] Deoxyhemoglobins A, F, Chesapeake, and Zürich were prepared from HbCO by first exchanging the CO with O_2 and then passing oxygen-free nitrogen or argon over the hemoglobin to remove oxygen. A small amount of sodium borohydride ($\sim$ 1 μmole) was added to the Hb Chesapeake solution to completely deoxygenate the hemoglobin.[13] Similar treatment of Hb A has no detectable effect on its NMR spectrum. The deoxyhemoglobin samples used were approximately 95% deoxygenated ($OD_{555}/OD_{540} > 1.2$). The hemoglobin samples were prepared at a concentration ranging from 5 to 12%(w/v) in 0.1M deuterated phosphate buffer at pD $\sim$ 7. The reagents used were the best commercially available and used without further

purification.

Method: A Bruker HFX-3 NMR spectrometer operating at a frequency of 90 MHz was used to obtain proton NMR spectra. The ambient temperature of the probe was 30°C. The residual water in each sample was used as an internal reference for calibrating the proton chemical shifts. Reported chemical shifts are the average of 5 to 15 determinations and are accurate to ± 0.1 ppm. The chemical shift of HDO is -4.72 ± 0.01 ppm (down field) from the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in aqueous solutions of pD 6.0 to 8.0. Signal to noise ratios were improved by using a Fabri-Tek Model 1074 time averaging computer.

Results and Discussion

Figure 1 shows a portion of the 90 MHz proton NMR spectra associated with some of the heme protons of the four human deoxyhemoglobins A, F, Zürich, and Chesapeake. The Hb A spectrum has three prominent lines at -17.6, -12.1, and -7.4 ppm (relative to HDO). The relative intensities indicate that the two lowest field lines are probably heme methyls and the line at -7.4 ppm a methylene. In the spectra of Hb F and Hb Zürich the lowest field lines are shifted to -16.1 and -15.4 ppm respectively while the lines at -12.1 and -7.4 ppm are unshifted. A new line appears at -11.3 ppm in the Hb Zürich spectrum. The NMR spectrum of Hb Chesapeake also differs from that of Hb A. The highest field line is missing and presumably shifted to higher field; the low field lines are at -12.5 and -17.5 ppm. Figure 2 shows expanded spectra of Hb A and Hb Chesapeake in the region where differences are observed.

The NMR results presented here are consistent with the notion that the α- and β- chain heme are nonequivalent and that variations in the heme NMR spectra can be understood, in part, by considering the effects of amino acid substitutions near the heme groups. The resultant changes in the heme proton spectra are interpreted qualitatively as alterations

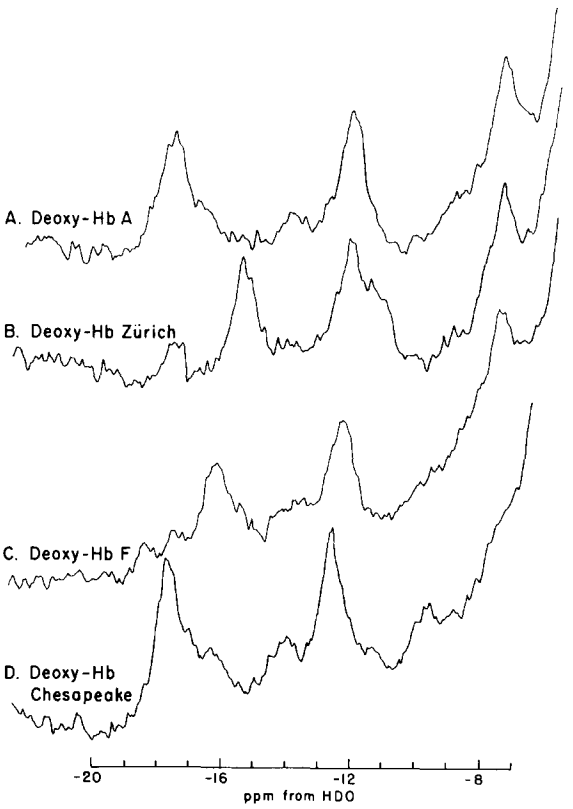

Figure 1 90 MHz proton NMR spectra of contact shifted heme resonances of deoxyhemoglobins A, Zürich, F, and Chesapeake in 0.1M deuterated phosphate at pD ∿ 7.

in the local heme environment and a rigorous discussion of the mechanism responsible for the differences must await further knowledge of heme-globin interactions. In Hb Zürich an arginine replaces the distal histidine at β63(E7)[14] and in Hb F the γ-chains replace the β-chains.[15] Because of possible altered interactions between the amino acid side chains and the heme groups, some resonance lines associated with the γ-chain could be shifted relative to those of the β-chain heme. Similar perturbations of the β-heme spectrum would be expected for Hb Zürich because the bulky, charged arginine at β63 does not fit into the heme pocket.[16] The α-chains

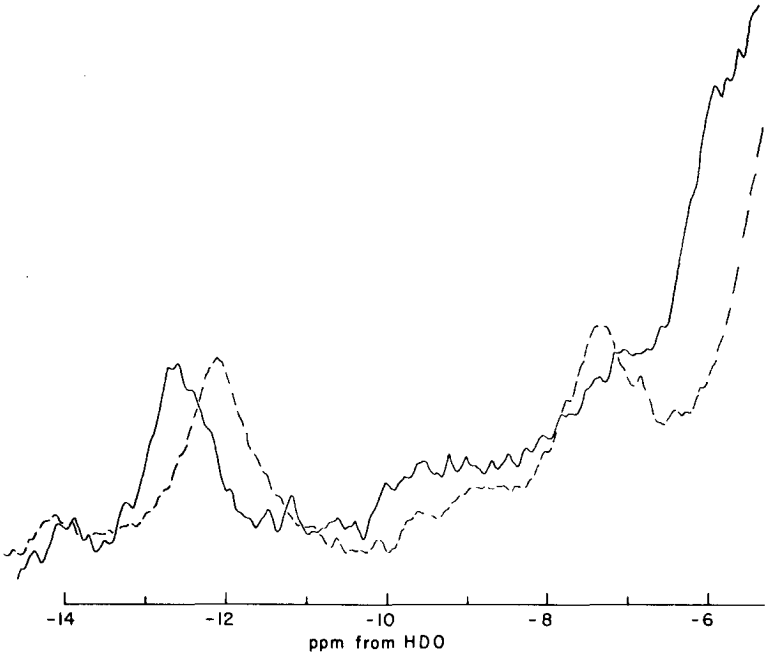

Figure 2 Superimposed 90 MHz proton NMR spectra of Hb Chesapeake (solid line) and Hb A (broken line).

are intact in both variants and the α-heme spectra should be unaffected.

The chemical shift variation of the lowest field line in the spectra of Hb Zürich and Hb F suggests that it be assigned to a β- or γ-heme methyl while the invariance of the lines at -12.1 and -7.4 ppm, especially in Hb Zürich, suggests that these lines be assigned to some of the heme protons in the α-chain. Additional support of this assignment is found in the NMR spectra of an α-chain variant, Hb Chesapeake (Figure 1D and Figure 2) in which the two lines tenatively assigned to the α-chain heme group are shifted. The line normally seen at -7.4 ppm is missing and the line at -12.1 ppm is shifted approximately 0.4 ppm down field from the corresponding line in the spectra of hemoglobins A, F, and Zürich.

Hb Chesapeake is characterized by high oxygen affinity and decreased subunit interaction (Hill coefficient, n = 1.3).[17] These properties are a consequence of the substitution of leucine for arginine at α-92(FG4).[18]

This substitution is in the α_1-β_2 contact region and is between two residues, leucine at α-91(FG3) and valine at α-93(FG5), which make contact with the heme group in the α-chain.[19] Recently Ho, et al.[12] suggested that these residues may change conformation upon oxygenation and are likely to be involved in the transfer of information between the heme groups and the α_1-β_2 subunit interface. The differences observed between the heme proton NMR spectra of Hb Chesapeake and Hb A support this conclusion. Conformational changes induced in these two residues by substitution at α-92 could produce the perturbations of the heme environment indicated by the heme proton spectrum of Hb Chesapeake.

More importantly, the heme proton spectra suggest that the altered functional properties of Hb Chesapeake are not exclusively the result of changes in the protein at the subunit interface. The spectra show that the structural modifications in the α_1-β_2 contacts are propagated to the heme environment. This interpretation differs from that obtained as a result of investigations of other non-cooperative, high affinity hemoglobins such as Hb J. Capetown* and mixed state hemoglobins. Ogawa, et al.[22] could detect no differences between the heme NMR spectra of Hb J. Capetown and Hb A. Likewise, Shulman, et al.[23] found no effects of subunit interaction on the heme proton spectra of a mixed state hemoglobin [(α-chains in deoxy-form)(β-chains in met-form)]. These two studies support the concept that alterations at the subunit interface do not affect the heme group.[22,23] However, the results of the study of Hb Chesapeake clearly indicate that a modification of the α_1-β_2 subunit interface does modify the heme environment.

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* Hb J. Capetown is a human variant in which glutamine replaces arginine at α-92 and has properties similar to those of Hb Chesapeake.[16,20,21]

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