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Nuclear Magnetic Resonance Studies of Hemoglobins. VII. Tertiary Structure around Ligand Binding Site in Carbonmonoxyhemoglobin†

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ABSTRACT: Proton nuclear magnetic resonance (250 MHz) (nmr) has been used to study the ring-current-shifted resonances that appear in the nmr spectra of adult and other variant human carbonmonoxyhemoglobins. Intensity measurements indicate that the three highest field resonances are methyl groups. The spectra of two β-chain mutants, Hb Sydney (E11β67 Val→Ala) and Hb Zürich (E7β63 His→Arg), having single amino acid substitutions in the heme pocket, are used to show that two of these resonances can be assigned to the βE11 valine methyls. The spectra of isolated α and β chains indicate the α- and β-chain contribution to the tetrameric hemoglobin spectrum and clearly show that the tertiary structure of the heme pocket is not significantly affected by the tetrameric association of liganded chains. These nmr studies demonstrate that the heme environments of the α and β chains in carbonmonoxyhemoglobin A are not equivalent.

The presence of ring-current-shifted resonances in the proton nuclear magnetic resonance (nmr) spectra of proteins enhances the value of nmr in the study of protein structure. Ring-current-shifted resonances occur when a proton is positioned above or below the plane of an aromatic ring so as to be influenced by the diamagnetic anisotropy of the conjugated system. This effect shifts the resonance position of the proton to higher field and the magnitude of the shift is quite dependent on the geometrical relationship between the aromatic ring and the affected proton. The greatest shifts occur at very close distances near the center of the ring and decrease as the vertical distance and the distance from the center of the ring increase as described quantitatively for benzene by Johnson and Bovey (1958) and recently for porphyrins by Shulman *et al.* (1970). The utility of these effects is well established for protein spectra in which the ring-current-shifted resonances appear outside the normal spectral region, allowing direct observation of individual residues in the protein (McDonald and Phillips, 1967; McDonald *et al.*, 1969; Sternlicht and Wilson, 1967; Ho *et al.*, 1970).

The study of the resonances in hemoproteins is uniquely propitious because the porphyrin rings in hemoproteins have large ring-current fields and the affected resonance peaks are shifted to a greater extent than can be realized by the ring-current field of aromatic amino acids (Abraham, 1961;

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McDonald *et al.*, 1969; Shulman *et al.*, 1970). In addition, the heme groups are usually the active centers of the hemoproteins and any measurable changes in shifts unambiguously reflect changes in the tertiary structure of the active center. The ability to detect structural changes in the heme pocket makes the nmr approach an important technique in the study of the structure and function of hemoproteins.

The amount of information that can be obtained from the measurements can be improved substantially by the assignment of the ring-current-shifted resonances to specific amino acid residues in the heme pocket of hemoglobin. The assignments reported here are achieved by correlating structural changes in specifically modified hemoglobins to the spectra of the ring-current-shifted resonances. In the most illuminating examples, the modifications consist of single amino acid substitutions in mutant human hemoglobins which are well characterized both by sequence studies and X-ray diffraction. The assignments to specific amino acid groups in the heme pocket impart the nmr technique with a unique capacity to assess the role that the tertiary structure of the heme pocket plays in determining the functional properties of hemoglobin in solution.

Experimental Section

Materials. Hb A[1] was prepared from fresh whole blood obtained from the local blood bank following the method of Drabkin (1946) with the cells being lysed with distilled water. Cord blood hemoglobin was prepared in the same manner from fresh cord blood obtained from Magee Womens Hospital and the Hb F fraction was isolated by the procedure of Zade-Oppen (1963) on CM-Sephadex. Hb Zürich was isolated from the heterozygous blood sample by chromatography on DEAE-Sephadex according to the procedure of Dozy *et al.* (1968). A heterozygous blood sample containing Hb Sydney was hemolyzed by the usual procedure. The α chains and the β chains were prepared using the method of Geraci *et al.* (1969). Each hemoglobin sample was dialyzed against D_2O by ultrafiltration through an Amicon UM-20E membrane. Sufficient 1.0 M potassium phosphate buffer was added subsequent to ultrafiltration so that the final solutions contained ~12% hemoglobin (w/v) in 0.1 M potassium phosphate at pD 7. The pD values were obtained by adding 0.4 pH unit to the pH value (Glasoe and Long, 1960) given by measurement with a Radiometer Model 26 pH meter equipped with a Beckman 39030 combination electrode.

Horse heart cytochrome *c* (type III) was purchased from Sigma Chemical Co. and dissolved in 0.1 M sodium phosphate buffer. The ferrous form was obtained by reduction with a 3 molar excess of sodium dithionite (Hardman and Holden Ltd.). The cytochrome *c* was dialyzed against D_2O by ultrafiltration through an Amicon XM-3 membrane. Mixtures of Hb A and cytochrome *c* were prepared by mixing known amounts of HbCO A and ferrocytochrome *c*. Concentrations were determined spectrophotometrically by means of a Cary 14 spectrophotometer using molar absortivites $\epsilon_{539\,nm} = 13.4 \times 10^3$ for HbCO A (Antonini, 1965) and $\epsilon_{550\,nm} = 29.5 \times 10^3$ for ferrocytochrome *c* (Van Gelder and Slater, 1962). Complete reduction of the sample was insured by addition of a small amount of dithionite directly into the nmr tube prior to the nmr measurement.

Method. The nmr spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer (Dadok *et al.*, 1970) by signal averaging with a Northern Scientific NS-544 digital memory oscilliscope. Each spectrum is an accumulation of 128 5-sec scans; sweeping at slower rates does not alter or improve the spectra. The spectra of the isolated chains are exceptions and represent single scans. The temperature of the probe during measurement was 32° and at this temperature the proton chemical shift of the HDO lock signal is 4.76 ppm downfield from the proton resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate. All chemical shifts included in this report are in parts per million upfield from HDO.

Results

Ring-current-shifted resonances appear in the proton nmr spectra of HbCO A in the region 4.5 to 7.0 ppm upfield from the HDO peak. The spectrum of HbCO A, shown in Figure 1, has resonances at 5.18, 5.42, 5.86, 6.48, and 6.58 ppm. A peak at 5.55 ppm appears as a shoulder to the larger peak at 5.42 ppm. Ring-current-shifted resonances also occur at 4.61 and 4.82 ppm but are not included in the figure. The intensities of the peaks at 5.86, 6.48, and 6.58 are equivalent while the larger peak at 5.42 is three to four times greater. The intensities of the peak at 4.61, 4.82, and 5.18 are more difficult to ascertain because of the influence of the base line.

The absolute intensities were determined in a mixture of HbCO A and reduced cytochrome *c*. McDonald *et al.* (1969) have shown that the nmr spectrum of reduced cytochrome *c* has a sharp ring-current-shifted resonance at 8.05 ppm which they assign to the *S*-methyl of the heme-linked methionine. The spectrum of a mixture of HbCO A and reduced cytochrome *c* in a heme ratio of 8.75 to 1 is shown in Figure 2. The ratio of the areas of the peaks at 6.48 and 6.58 ppm to the area of the peak at 8.05 ppm is 8.96 to 1. However, since the intensity of the HbCO A peaks at 6.48 and 6.58 ppm is increased in this spectrum by a one proton resonance of cytochrome *c* at 6.62 ppm, the corrected intensity ratio of the resonances of HbCO A (6.48 and 6.58 ppm) to cytochrome *c* (8.05 ppm) is 8.63 to 1, which agrees with the hemoglobin to cytochrome *c* heme ratio of 8.75 to 1. This intensity measurement indicates that the peaks at 6.48 and 6.58 ppm combined correspond to one methyl group per heme or simply that one peak represents a methyl group in the α chain and the other resonance represents a methyl group in the β chain.

The spectrum of three mutant human hemoglobins, Hb F, Hb Zürich, and Hb Sydney, are also shown in Figure 1. The spectrum of HbCO F has resonances at 5.18, 5.31, 5.55, 6.48, and 6.80 ppm with the latter two having equal intensities. Fewer differences are found in the spectrum of HbCO Zürich which has peaks at 5.14, 5.40, 5.82, 6.48, and 6.65 ppm with intensities of the latter three being equivalent. In the spectrum of the mixture of HbCO A and HbCO Sydney, the peaks at 5.80 and 6.58 ppm are reduced in intensity and a new peak occurs at 5.75 ppm.

The spectra of isolated α and β chains are shown in Figure 3. Carbonmonoxy α chains have ring current shifted resonances at 5.19, 5.31, 5.46, and 6.54 ppm with equal intensities. Carbonmonoxy PMB β chains have resonances at 5.34, 5.72, and 6.68 ppm with relative intensities 3 to 1 to 1. If the spectra of the isolated α and β chains are added together, a close approximation of the spectrum of the intact tetramer results indicating that in the carbonmonoxy forms,

[1] Abbreviations used are: Hb A, normal adult hemoglobin; Hb F, human fetal hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; PMB, *p*-chloromercuribenzoate.

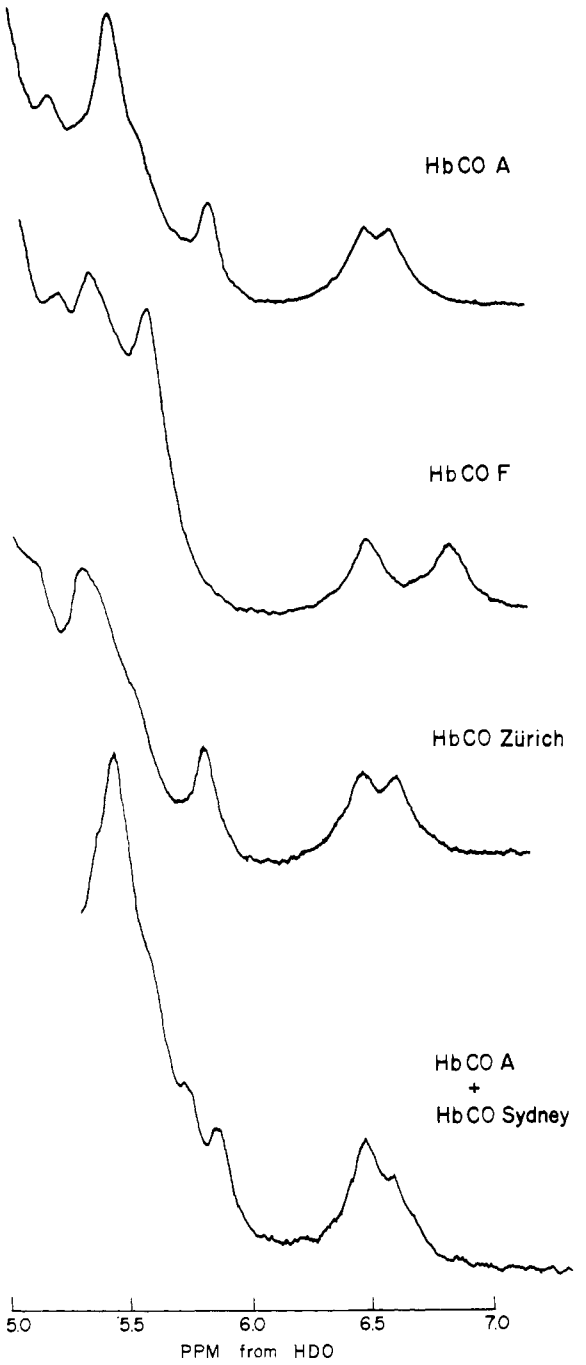

FIGURE 1: 250-MHz proton nmr spectra of carbonmonoxyhemoglobins A, F, Zürich, and Sydney at pD 7 and 32°.

the tertiary structure of the heme pocket is not greatly affected by chain association.

Discussion

In carbonmonoxyhemoglobin, a diamagnetic heme protein, the only resonance lines in the nmr spectra that lie to higher field than the aliphatic protons are those shifted by ring current fields. The shifts may be due to effects of aromatic amino acids similar to those occurring in the nmr spectrum of lysozyme (McDonald and Phillips, 1967; Sternlicht and Wilson, 1967) but are most likely due to the effects of the porphyrin rings. The porphyrin ring has a much greater ring-current

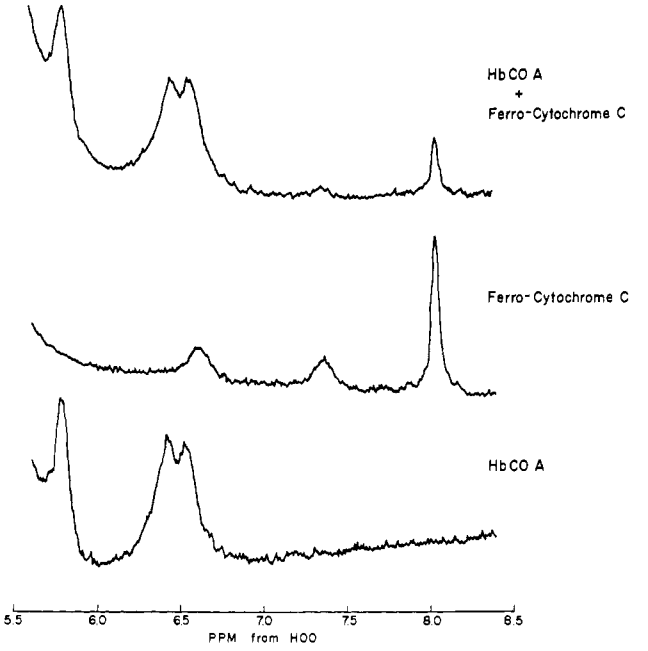

FIGURE 2: 250-MHz proton nmr spectra of ferrocytochrome *c*, HbCO A, and a mixture of ferrocytochrome *c* and HbCO A at pD 7 and 32°.

field and lines above 5.3 ppm must arise from the effects of the porphyrin system (McDonald and Phillips, 1967).

The assignments of these resonances to specific amino acid side chains in the heme pocket are based on the intensities, the spectra of mutants having amino acid substitutions in the heme pockets, the spectra of isolated chains, and calculations of expected ring-current shifts. The best evidence comes from the spectra of the mutants but the assignments are substantiated by the other data. The spectrum of HbCO Sydney contains the most information. The genetic modi-

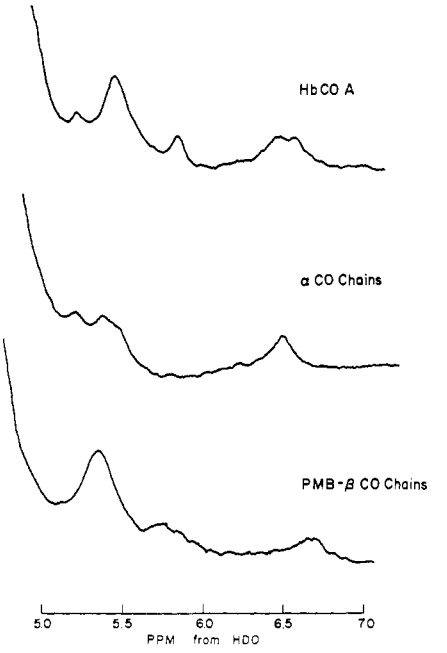

FIGURE 3: 250-MHz proton nmr spectra of HbCO A and isolated α and β chains in CO form at pD 7 and 32°.

fication in Hb Sydney is of a single amino acid; the βE11 valine is replaced by an alanine (Carrell *et al.*, 1967). Since the substitution does not affect the charge of the protein, the separation of the mutant from Hb A in a form suitable for nmr studies is not possible. For this reason, the nmr spectrum was taken on the hemolysate which contained both Hb A and Hb Sydney. The spectrum of this mixture shows the lines at 5.86 and 6.58 ppm are decreased in intensity relative to the HbCO A spectrum. This indicates that the peaks at 6.58 and 5.86 ppm are the γ_1 and γ_2 methyls of the βE11 valine and suggests that the new peak appearing at 5.75 ppm is the βE11 alanine methyl of Hb Sydney.

Another pertinent hemoglobin variant is Hb F. This hemoglobin is found in the human fetus and has normal α chains but greatly different β chains, designated γ chains (Schroeder *et al.*, 1963). The γ chains differ from normal β chains at thirty-nine amino acids but only two of these are within 4 Å of the heme (Perutz, 1969; Dayhoff, 1969). These are at position E14 and E15 where alanine and phenylalanine are replaced by serine and leucine. These residues lie adjacent to the E11 valine and their substitution should affect the E11 valine to a much greater extent than other residues in the heme pocket. In the spectrum of HbCO F, the lines assigned to the γ67(E11) valine methyls have different resonance positions than they do in the HbCO A spectrum. The line at 6.58 ppm shifts to 6.80 ppm and the line at 5.86 ppm is not observable and is probably shifted under the envelope of the peaks at 5.31 and 5.55 ppm.

Hb Zürich also has a genetically substituted amino acid in the heme pocket. In this hemoglobin, the distal histidine, β63(E7), is replaced by an arginine (Muller and Kingma, 1961). In Hb A, the distal histidine lies adjacent to the E11 valine above the heme plane (Perutz, 1969) and the replacement by the larger arginine should perturb the βE11 valine. This is evident in the nmr spectrum of HbCO Zürich in which the line normally found at 6.58 ppm is shifted to 6.65 ppm.

The assignment of the peaks to methyls is supported by the intensity measurements. The intensities, as determined by the mixture of HbCO A and ferrocytochrome *c*, indicate that the peaks at 5.86, 6.48, and 6.58 ppm each arise from one methyl group per αβ dimer. The large resonance at 5.42 ppm apparently represents a variety of residues totalling 3 or 4 methyls per αβ dimer depending on the placement of the base line. The assignment of the resonances at 5.86 and 6.58 ppm to the βE11 valine methyls agrees with the measured intensities.

These assignments are further supported by the study of the isolated chains. The ring-current-shifted nmr spectra of isolated chains are apparently the α and β components of the tetrameric HbCO A spectrum since the sum of the spectra of the isolated chains approximates the spectrum of the tetramer. This means that the resonances at 5.18, 5.30, 5.55, and 6.48 ppm are α chain residues while the resonances at 5.42, 5.86, and 6.58 ppm are β chain residues and demonstrate that the tertiary structures of the α and β heme pockets are not identical. This assignment of the resonances to α and β chains further confirms the assignment of the lines at 5.86 and 6.58 ppm to the βE11 valine methyls and also indicates the possible assignment of the 6.48-ppm resonance to an α chain methyl.

We would like to suggest that the peak at 6.48 ppm is an αE11 valine methyl. This peak is undoubtedly an α-chain residue since it is invariant in all the hemoglobins having modified β chains and is present in the spectrum of the isolated α chains. Its assignment to αE11 is suggested by calculations of the expected ring-current shift. Shulman *et al.* (1970) have calculated ring-current shifts in myoglobin using the atomic coordinates determined by Kendrew and coworkers. Similar calculations for hemoglobin are not possible since the atomic coordinates for HbCO A have not yet been determined. The atomic coordinates for acid methemoglobin are generally used to describe structurally similar oxyhemoglobin (Perutz, 1969, 1970), but the slight differences between these structures would be sufficient to give erroneous calculations of ring-current shifts. In this respect, Shulman *et al.* (1970) have indicated differences in the spectra of ring current shifted resonances in carbonmonoxy-, oxy-, and metmyoglobin and we have observed large differences between the ring-current-shifted spectra of carbonmonoxy- and oxyhemoglobin (T. R. Lindstrom and C. Ho, unpublished results). Nevertheless, we made calculations using the equation developed by Shulman *et al.* (1970) and Perutz's 2.8-Å resolution atomic coordinates for horse methemoglobin (personal communication). The results of these calculations indicate that the βE11 valine γ_1 should be shifted to highest field followed by the αE11 valine γ_1 methyl. Both this confirmation of the assignment of the 6.48 and 6.58 ppm lines to the α and βE11 valine γ_1 methyls and the calculated ring-current shifts for the other residues in the heme pocket remain problematical due to the lack of data on the structure of the heme pocket in HbCO A.

These calculations are useful because they clearly indicate that small movements of the alkyl residues are reflected by large changes in the ring-current shift and demonstrate the remarkable sensitivity of the nmr measurements to changes in tertiary structure. Diagrams constructed by Shulman *et al.* (1970) show that a 1-Å movement parallel to the heme plane can change the magnitude of the ring-current shift by more than 2 ppm and a 1-Å movement perpendicular to the heme plane gives a shift difference amounting to 1–2 ppm.

The end result of this investigation is the certain assignment of the ring-current-shifted resonances appearing at 5.86 and 6.58 ppm in the nmr spectrum of HbCO A to the γ_1 and γ_2 methyl groups of the βE11 valine. We suggest that the line at 6.48 ppm is the γ_1 methyl of the αE11 valine based on similarities in structure, ring-current-shift calculations and studies of isolated chains. The resonances comprising the larger peak at 5.42 ppm cannot be resolved and the assignment presents a more difficult problem. All of these ring-current-shifted resonances, especially those assigned to specific residues, can be expected to provide significant information about the tertiary structure of the amino acid side chains surrounding the heme groups.

These nmr results should provide a basis for relating ligand affinity to the structure of the heme pocket. Since the spectra of the isolated chains is essentially maintained in the tetramer, the differences in the chain spectra reflect a difference in the tertiary structure of the heme pockets of the α and β chains. The differences are especially significant for residues on the ligand binding site of the heme, including the E11 valines. Perutz (1970) has pointed out the differences between the E11 valines in the α and β chains of deoxyhemoglobin and has discussed the functional consequences of these different structural conformations. Our nmr results indicate different conformations of amino acid side chains, including the E11 valines, in the α- and β-heme pockets of HbCO A. These differences between the α- and β-heme pockets of liganded hemoglobin may indicate a difference in the ligand-binding properties of the hemes and need to

be recognized in the formulation of models or mechanisms of cooperative ligand binding to hemoglobin.

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Structure–Function Relationships of Neurotoxins Isolated from *Naja haje* Venom. Physicochemical Properties and Identification of the Active Site†

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ABSTRACT: Neurotoxins I and III of *Naja haje*, like other miniproteins with a high content of disulfide bridges, present an unusually high resistance to denaturing conditions at neutral pH. Optical rotatory dispersion measurements with neurotoxin I indicate 2 conformational changes at acidic pH. One of them is controlled by the unmasking of a carboxylate side chain having an apparent pK of 2.0 at 13°. Titration, nitration, and acetylation show that tyrosine-24 is masked in the native conformation of neurotoxin I. This residue is essential for the stabilization of an active structure. Its nitration abolishes toxicity. Difference spectra and fluorescence data indicate a masking of the tryptophan-28 side chain. Formylation does not abolish toxicity. Acetylation and maleylation indicate that lysine residues are essential elements of the active site. Dansylation affects selectively 2 superreactive residues, lysines-26 and -46. The kinetics of the loss of toxicity closely parallels the covalent modification of these two lysine residues.

Snake neurotoxins are made of single peptide chains of 60–74 amino acids cross-linked internally by 4 or 5 disulfide bridges. These miniproteins are well suited for comparative sequence studies, analyses of conformational properties and determinations of active sites.

Due to the increasing use of neurotoxins to study neurotransmitters–receptors interactions (Changeux *et al.*, 1970; Miledi *et al.*, 1971) it is of prime importance to understand the structure–function properties of these proteins.

Neurotoxins I and III obtained from *Naja haje* venom have been selected for this work. The sequence of neurotoxin I which consists of 61 amino acid residues and 4 disulfide bridges has been recently elucidated (Botes and Strydom,

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