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HEME ORIENTATIONAL HETEROGENEITY IN DEUTEROHEMIN-RECONSTITUTED HORSE AND HUMAN HEMOGLOBIN CHARACTERIZED BY PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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The number of 2,4-H signals of met-cyano and deoxy deuteroheme-reconstituted sperm whale Mb are shown to reflect the known degree of heme rotational disorder in this modified protein. Using these unique spectral windows for the 2,4-H signals, we show that both horse and human Hb reconstituted with deuteroheme exhibit significant molecular heterogeneity which is consistent with approximately 20% heme rotational disorder within each subunit.

The interpretation of structure-function relationships of hemoglobins has relied heavily on the detailed structural information from high resolution X-ray diffraction studies (1,2) and the comparison of physico-chemical properties of hemoglobins reconstituted with modified hemes (3-7). The influence of the 2,4-substituents on the oxygen affinity has been interpreted, on the one hand, on the basis of substituent inductive effects (4,5) and, alternatively,through variable protein-heme steric interactions (6,7). Both models assume identical orientation of the heme in the heme cavity in order to make simple comparisons. In the case of deuteroheme and mesoheme (2-R, 4-R = H and ethyl, respectively, in A of Figure 1) reconstituted into horse Hb, X-ray crystallographic studies have appeared to support a heme orientation unchanged from that of the native protein (8,9).

The pattern of the heme methyl ^{1}H NMR hyperfine shifts in the met-cyano form of myoglobin has been shown to be interpretable (10) in terms of the orientation of the heme relative to the proximal histidyl imidazole π plane (11). Thus assignment of heme methyls by isotope labeling in a number of monomeric hemoproteins has established (12-14) an equilibrium between the heme orientation as found in crystals (11,15,16), and that rotated 180^o about the

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α-γ-meso axis, as shown in Figure 1. The presence of both heme orientations (heme rotational disorder), has been established not only for reconstituted Mb (10), but for the native protein as well (12,13,17). Other systems for which heme disorder is observed in solution by ^{1}H NMR (13,14), while a unique heme orientation has been proposed on the basis of X-ray studies (15,16), are Chironomus Hb and ferricytochrome b_5.

The inefficiency of reconstitution and the scarcity of isotope labeled hemes have, to date, prevented a quantitative assessment of heme disorder in tetrameric hemoglobin. In the special case of deuteroheme, however, work with both model compounds (18,19) as well as disordered sperm whale Mb (10,18,20) has revealed that the deuteroheme 2,4-H signals in each case resonate in completely unique spectral windows for both the low-spin met-cyano and high-spin deoxy hemoproteins. Thus the number and relative intensities of the 2,4-H resonances provide direct evidence on the heme orientational heterogeneity in hemoproteins. We demonstrate here by ^{1}H NMR spectroscopy that both horse and human Hb reconstituted with deuteroheme exhibit heterogeneity that is indicative of significant heme orientational disorder within each subunit.

MATERIALS AND METHODS

Hb A was prepared, reconstituted with deuteroheme, and purified as previously described by Nagai et al (21). The pH was adjusted using 2M Tris and was read directly without correction for isotope effects using a Beckman 3550 pH meter equipped with a microcombination electrode. ^{1}H NMR spectra were obtained on a Nicolet NT-360, 360 MHz, FT spectrometer. A typical spectrum used 8K points, a 14 KHz bandwidth, a 13 us 90° pulse, and an exponential multiplication of 20 Hz to enhance signal to noise. The residual solvent line was reduced by a presaturation pulse and the peaks were referenced with respect to H_2O, which in turn was calibrated against 2,2-dimethyl-2-silapentane-5-sulfonate, DSS.

RESULTS AND DISCUSSION

The spectrum of native human metHbCN is shown in A of Figure 2; the peaks a_1, b_1 and a_2, b_2 arise from the heme 5-CH_3, 1-CH_3 groups for the two subunits (22), and are indicative of the heme orientation found in the X-ray structure of both Hb and Mb (2,8,9). In B and C of Figure 2 we illustrate the hyperfine shifted region of the ^{1}H NMR spectra of human and horse deuteroheme-metHbCNs. In addition to the analogous heme methyl signals a_1, b_1 and a_2, b_2, we find a second set of minor compound peaks, most clearly resolved for c and d. These

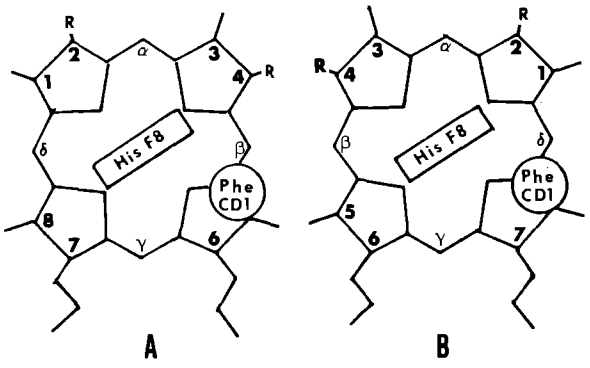

Figure 1. View from the proximal side of the orientation of the heme relative to the His F8 imidazole plane as: A, found in the X-ray structure in Mb and Hb; B, with the heme rotated by 180° about the α-γ-meso axis. The native protoheme has 2-R = 4-R = vinyl; while deuterohemin has 2-R = 4-R = H.

latter peaks exhibit shifts similar to the second component of sperm whale deuterohemin-metMbCN (peak b_1 in D of Figure 1), for which a_1, a_2, a_3 arise from 5-, 1- and 8-CH_3 for the native heme orientation (A in Fig. 1) and the second set of peaks b_1, b_2, b_3, originate from 8-, 3- and 5-CH_3 (10) of the reversed heme orientation (B in Fig. 1). The heterogeneity for deuteroheme-metMbCN is more clearly demonstrated in the upfield region where only 2,4-H resonate (18,19): a_4, a_5 arise from the major component 4-H and 2-H and b_4, b_5 arise from 2-H, 4-H of the minor component (20).

Inspection of the same 2,4-H upfield window for the two deuteroheme-metHbCNs in B and C of Figure 2 reveals that, instead of the four peaks consistent with two each 2-H and 4-H peaks for the non-equivalent subunits, seven to eight peaks are resolved. These peaks can be divided into two subsets, a_3-a_6 and b_3-b_6; in the human Hb sample, all are at least partially resolved. The areas for all members of both the a_3-a_6 and b_3-b_6 subsets are the same, with the ratio of a:b intensities ~4:1. In the horse Hb complex, the equally intense a_3-a_6 peaks are clearly resolved, but two of the four comparably intense b_5 and b_6 peaks are degenerate; the a:b intensity ratio here is also ~4:1.

Thus both the downfield (methyl) and upfield (2,4-H) portions of the ^1H NMR spectra clearly indicate protein heterogeneity with the same character-

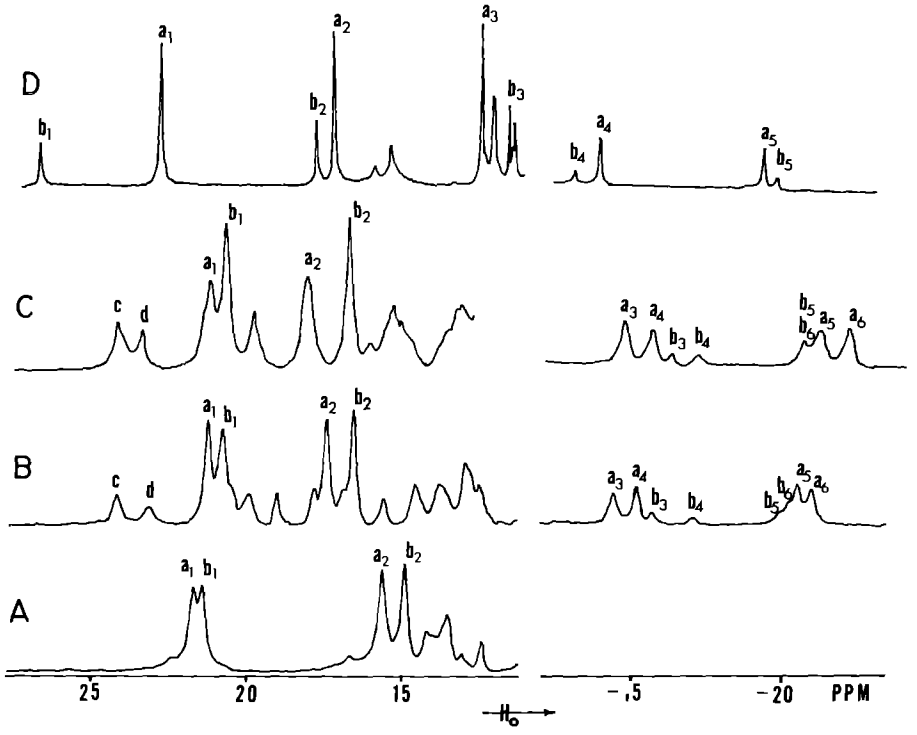

Figure 2. The hyperfine shifted regions of the 360 MHz ^1H NMR spectra of: A. human metHbCN in ^2H_2O at 35°, pH 8.0; B. human deuterohemin-metHbCN in ^2H_2O at 35°, pH 8.8; C. horse deuterohemin-metHbCN in ^2H_2O at 35°, pH 8.0; and D. sperm whale metMbCN in ^2H_2O at 40°, pH 8.6. Some of the heme methyl peaks are found in the downfield portion, while the 2,4-H signals are located solely in the illustrated upfield region.

istics shown to arise from heme rotational disorder in Mb (10,12). The downfield bias of the minor component presumed heme methyl peaks, c,d, relative to the major component peaks a_1, b_1, argue strongly for the native heme orientation for the major and the "reversed" heme orientation for the minor component, as also found in Mb (10).

The unique spectral window (18) displaying heme 2,4-H signals in high-spin Fe(II) hemoproteins, 30-70 ppm, is illustrated in A of Figure 3 for sperm whale deoxy deuteroheme-Mb. A 10% disordered deuteroheme-Mb sample yields two sets of 2,4-H peaks, a_1, a_2 and b_1, b_2, which arise from the major and minor components (A and B in Fig.1, respectively). The reduced human and horse deuteroheme-Hb spectra again exhibit too many lines (six resolved for human, seven for horse) consistent with a homogenous and unique protein. The

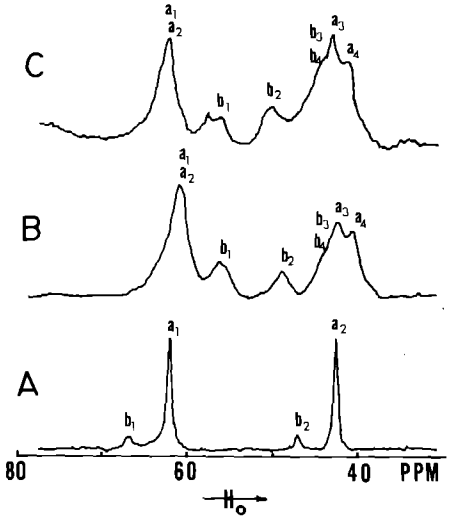

Figure 3. The 30-80 ppm region of the 200 MHz ^1H NMR spectra of: A. sperm whale deoxy deuteroheme-Mb in ^2H_2O at 25° and pH 7.6; B. human deoxy deuteroheme-Hb in ^2H_2O, at 35°C and pH 7.2; C. horse deoxy deuteroheme-Hb in ^2H_2O at 33° and pH 7.4. Only the 2-H and 4-H signals resonate in this window; the major component peaks are designated a_i, while those of the minor component are labeled b_i.

numerous lines, however, are consistent with two sets of four peaks, a_1-a_4 and b_1-b_4, as indicated in the Figure, each with comparable intensities, and a ratio of intensities a:b ~3:1. Thus the deoxy Hb spectra are also indicative of heterogeneity which is qualitatively consistent with heme rotational disorder.

Both the met-cyano and deoxy ^1H NMR spectra of deuteroheme-reconstituted human and horse Hb indicate heme disorder in solution, in contrast to the findings for the single crystals of horse deuteroheme-Hb (9). The comparable intensities of the four major component 2,4-H peaks (as well as for the four minor component peaks) indicate that the degree of disorder is very similar in the two subunits. With ~20% disorder in each subunit it is not clear whether the single crystal refinements fail to detect the disorder (9) or whether simply only the native orientation crystallizes. In the case of the Chironomus Hb, only one of the two comparably populated heme orientations (13) appears to crystallize (15). Only in the case of 2,4-dibromo-deuteroheme reconstituted into Hb have X-ray data provided unambiguous evidence for heme rotational disorder in single crystals (23).

The present results indicate that heme disorder is an important factor in interpreting the significantly reduced cooperativity reported for deuteroheme-Hb (24) and underscore the importance of establishing the heme rotational state in reconstituted hemoglobins prior to detailed interpretation of the basis for altered functional properties (4-7,24).

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