

Proton nuclear magnetic resonance characterization of heme disorder in hemoproteins

(myoglobin/hyperfine shifts/deuteroporphyrim/model compounds/deuterium labeling)

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ABSTRACT A proton NMR method is described for determining the orientation of a porphyrin within the heme pocket of a hemoprotein. The pattern of the hyperfine-shifted heme methyl resonances in low-spin ferric model compounds is demonstrated to characteristically reflect the position of a localized low-symmetry perturbation on the π system. The specific assignments via deuteration of the two interconvertible sets of methyl resonances observed for deuteroporphyrim-reconstituted sperm whale metmyoglobin cyanide lead to the conclusion that the low-symmetry perturbations on the heme due to the apo-protein contacts differ for the two protein components by a 180° rotation about the α - γ meso axis. Hence the heme in the reconstituted myoglobin is "disordered" in solution, and the altered functional properties of the reconstituted protein cannot be simply attributed to the local effect of the heme substituent. This NMR technique has applicability for determining the relative heme orientation in related hemoproteins, and may clarify the origin of doubling of heme resonances observed in several native hemoproteins.

in connection with studies of either hemoproteins reconstituted with chemically modified hemes, I, R \neq vinyl, or with certain native hemoproteins found to be heterogeneous by NMR (8, 9). Extensive structure-function relationships have been established based on *in vitro* investigations of physiological properties of Mb and Hb reconstituted with modified hemes (10). Although crystal structures have established a unique orientation for the native proteins (1-5), little evidence exists that the same or even a unique heme orientation exists in reconstituted proteins.

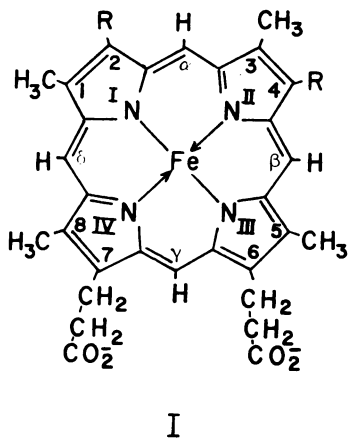
Recent proton NMR studies have revealed that the met-cyano form of several native invertebrate Hbs and Mbs (ref. 8 and unpublished observations) and one cytochrome (9) exhibit more than one set of heme methyl resonances per monomeric unit. Moreover, in at least two cases (8, 9) it was shown that the two sets of heme methyl resonances are interconvertible, indicating that the same protein exists in two distinct forms. Since the doubling of resonances was observed primarily for heme resonances, we had tentatively suggested "heme disorder" (8), as depicted in Fig. 1, as a possible origin of the molecular heterogeneity in insect Hb. Reported x-ray structural data on this protein have been interpreted in terms of a unique heme orientation in the pocket (4).

Since the occurrence of such heme disorder may have important consequences for interpretation of structure-function relationships in reconstituted as well as some native hemoproteins, it seems desirable to develop a method for ascertaining the physical origin of the two sets of NMR signals in a hemoprotein in solution. We will demonstrate here that Mbs reconstituted (10) with the modified prosthetic group, deuteroheme, I, R₂,R₄ = H, also exhibit two sets of interconvertible heme methyl resonances which can be interpreted as arising from protein forms that differ in the heme orientation (Fig. 1).

The proton NMR method for distinguishing the heme orientations in the pocket relies on the assignment of individual methyl groups via specific deuteration and comparison of the resulting shift pattern for the two protein forms (11, 12). This technique takes advantage of the large spread of the heme methyl hyperfine-shifted resonances due to the low-symmetry perturbation on the porphyrin system caused by asymmetric heme-apoprotein contacts (13). Systematic NMR studies of model compounds with variable 2,4-substituents have shown that increasing the electron-withdrawing power of the substituents significantly increases the heme methyl shift spread. Detailed analysis showed that the changes in methyl shifts reflect predominantly changes in the π contact shifts, so that the spread of the methyl shifts can be interpreted as resulting from asymmetry in the π electron distribution among the four pyrroles (14). Although substitution at both 2- and 4-positions produced large asymmetry comparable to that in metMbCN

Abbreviation: deuterio-Mb, deuteroporphyrim-reconstituted myoglobin.

Detailed three-dimensional structures of several myoglobins (Mbs) (1, 2), hemoglobins (Hbs) (1, 3-5), and cytochromes *c* (6) are available which indicate that the asymmetric porphyrin, I, R₂,R₄ = vinyl, occupies a completely unique position within



the hydrophobic heme cavity. The polypeptide chains have evolved, at least in vertebrates, so that the tertiary structural constraints appear to distinguish clearly between the two possible heme orientations depicted in Fig. 1 for sperm whale Mb (7). The pair of heme orientations differ by a 180° rotation about the α - γ meso axis, and represent an interchange on pyrroles I and II of methyl and R contacts with the apoprotein; the hydrophilic interactions of the propionic side chains remain invariant.

The possible occurrence of multiple heme orientations arises

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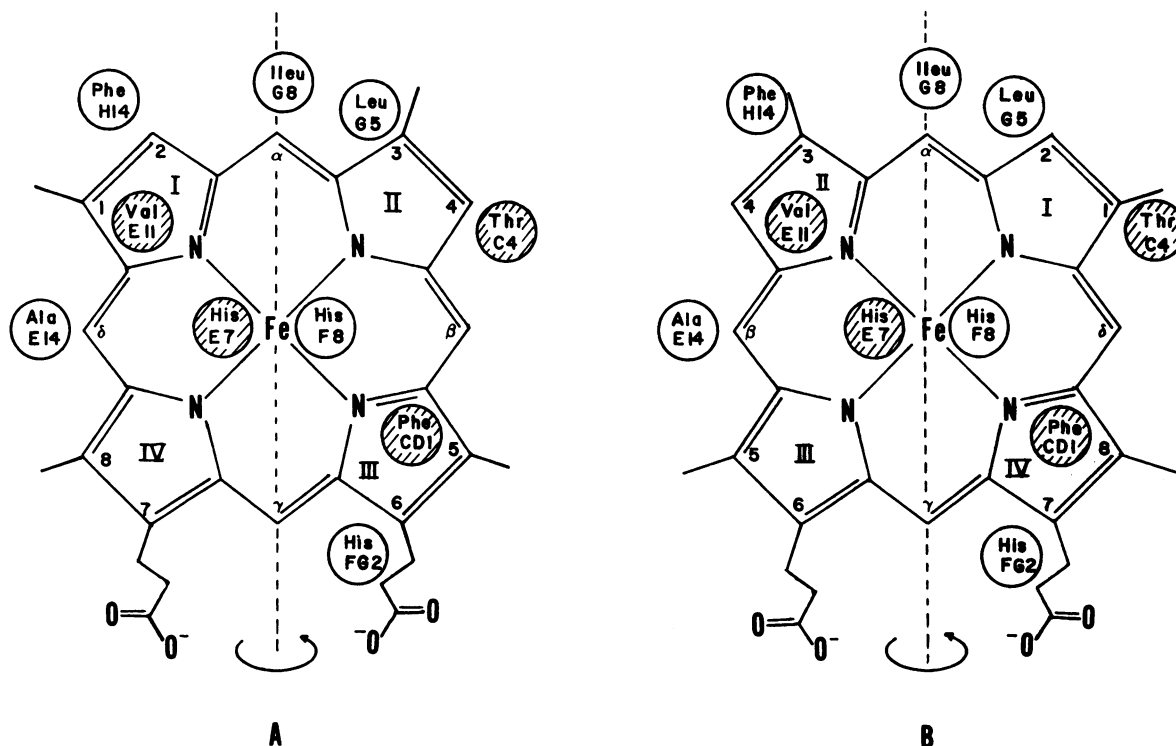


FIG. 1. Heme-apoprotein contacts for protoporphyrin in the pocket of sperm whale Mb. Open circles indicate contacts on the proximal side; shaded circles represent contacts on the distal side of the heme. Only the methyl group and propionic acid substituents are included to make the arguments applicable to deuteroporphyrin. (A) Normal orientations as found in the native protein; (B) reversed orientation, with the porphyrin rotated 180° in the heme pocket about the α - γ meso axis.

(12), the individual methyl assignments failed to match those in the protein. The use of monosubstituted porphyrins, however, results in models that both mimic the protein environment as well as permit a study of the effect on the methyl hyperfine shift pattern on moving the local perturbation about the porphyrin ring. Comparison of the assigned heme methyl shifts in models and proteins also emphasized that the asymmetry in the protein arises not from the vinyl substituents (14), but from highly characteristic asymmetric apoprotein-heme interactions within the heme pocket.

EXPERIMENTAL PROCEDURES AND RESULTS

NMR Spectra. Proton NMR spectra were recorded on a JEOL-PFT-100 spectrometer operating at 99.5 MHz in the Fourier transform mode. Transients of 100–20,000 were collected using a 20- μ sec 90° pulse, 8000 points, and a 6.25 kHz bandwidth. The probe temperature was controlled with a VT-3C temperature controller, and was maintained at 25°C for all spectra of model compounds. The temperature was maintained at 38°C for all protein spectra to facilitate resolution of the desired resonances. Chemical shifts are given in ppm relative to the internal standards tetramethylsilane in $C^2H_5O^2H$ and 2,2-dimethyl-2-silapentane-5-sulfonate in 2H_2O , with upfield shifts as positive.

Model Complexes. 2-Acetyldeuteroporphyrin and 4-acetyldeuteroporphyrin were prepared, purified, and characterized as described (15, 16). Since these porphyrins are prepared in too small amounts to make synthesis of pure methyl deuterium-labeled isomers practical, the methyl peak assignments were carried out on mixtures of acetylated deuteroporphyrins. When deuterohemin is acetylated with acetic anhydride by the method of Caughey *et al.* (17), a mixture results consisting primarily of 2,4-diacetyldeuterohemin, I, R2 = R4 = CH_3CO [Fe(III)], and minor amounts of both 2-acetyldeuterohemin, I, R2 = CH_3CO , R4 = H [Fe(III)], and 4-acetyl-

deuterohemin, I, R2 = H, R4 = CH_3CO [Fe(III)]. Using [1,3- 2H_6]deuteroporphyrin and [1,5- 2H_6]deuteroporphyrin obtained from the analogous labeled protoporphyrin (18–20), acetylation leads to the same three substituted porphyrins. De-esterification of the propionic side chains of all porphyrins, insertion of iron (21), dissolution in [2H_4]methanol, and addition of a 4-fold excess of KCN yielded the desired low-spin *bis*-cyano complexes in each case.

The proton NMR spectra of pure 4-acetyldeuteroporphinato-iron(III) dicyanide and 2-acetyldeuteroporphinato-iron(III) dicyanide are illustrated Fig. 2 A and B. Comparison of these two traces with that for a mixture of all three possible acetylated isomers (Fig. 2E) permits identification of all methyl peaks belonging to each isomer. Further comparison of the trace in Fig. 2E for the mixture of complexes with those in C and D for the mixtures of complexes made from the 1,3-deuterated and 1,5-deuterated precursor (19, 20) results in unambiguous assignment of individual methyls within each isomer. The resulting assignments for the two monoacetyl derivatives are given in Fig. 2 A and B. The assignment for the 2,4-diacetyl derivative agrees with that for the pure complex reported previously. The extent of deuterium labeling is ≈ 75 –90% in [1,5- 2H_6]deuteroporphyrin and about 50–75% in [1,3- 2H_6]deuteroporphyrin.

Myoglobins. Sperm whale apoMb was prepared from commercial Mb (Sigma) by standard methods (22). The protein was reconstituted with normal deuterohemin and the 1,3- 2H_6 - and 1,5- 2H_6 -labeled derivatives by the method of Antonini *et al.* (23). The proteins were dissolved in 0.2 M NaCl/ 2H_2O (≈ 2 –6 mM), the p 2H was adjusted to 11 with NaO 2H , and the solution was stored at 25°C for several hours. The protein is stable at pH 11 for several days without any detectable denaturation, as monitored optically and by NMR. A 5-fold excess of KCN was added to this metMbOH solution and the “pH” was adjusted to 8.5 with 0.5 M phosphate at “pH” 6.5. The pH was

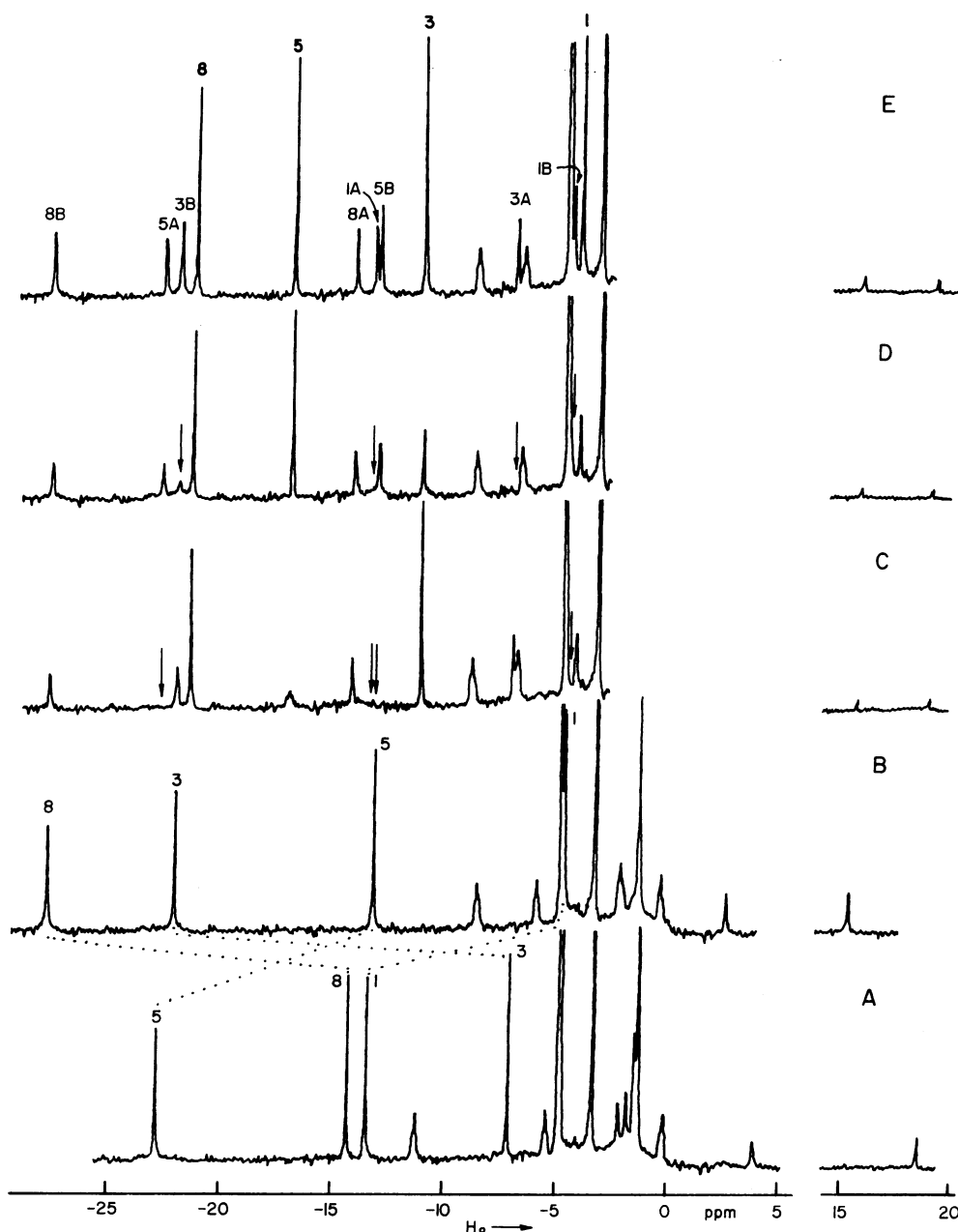


FIG. 2. Proton NMR spectra of low-spin, ferric, dicyano model compounds in $C^2H_3O_2H$ at $25^\circ C$. (A) Pure 4-acetyldeuteroporphyrin derivative; (B) pure 2-acetyldeuteroporphyrin derivative; (C) mixture of 2-acetyl-, 4-acetyl-, and 2,4-diacetyldeuteroporphyrin derivatives with deuterated 1,5-methyls; (D) mixture of same porphyrins as in C except with deuterated 1,3-methyls; (E) mixture of the same porphyrins as in C with no deuteration. Arrows indicate peaks with reduced intensity for the two monoacetyl derivatives in C and D. Peaks are assigned in E using A to designate 4-acetyl- and B to designate 2-acetyl-derivative methyl peaks.

measured with an Ingold combination electrode 6030-2 and a Beckman 3500 pH meter; readings are uncorrected for the isotope effect and are hence referred to as "pH."

When the resulting met-cyano complex of the deuteroporphyrin-reconstituted Mb, deuterio-metMbCN, is stored at "pH" lower than 8 for several hours, a proton NMR spectrum very similar to that reported previously (24) results. The trace of the downfield methyl region at $38^\circ C$ is illustrated in Fig. 3A. The prominent peaks, labeled X, result from the major component, and have been suggested to be the heme methyls. Two very small peaks, labeled Y and arising from some minor component, are also resolved. If the spectrum, however, is recorded immediately after adding KCN and setting the "pH" to 8, the proton trace in Fig. 3B results. The two minor component peaks are now much more intense than in A, and a third peak is detectable. The same trace as in B can be generated at "pH" 6 simply by first reducing deuterio-metMbH₂O with dithionite to form deuterio-deoxyMb and letting the solution sit for 1 hr, followed by rapid oxidation with ferricyanide and addition of

excess cyanide to lead directly to deuterio-metMbCN. The relative amounts of the major and minor components found initially depend upon the ratio of forms in the precursor to the met-cyano form. Within a few hours, the minor component is converted to the major component until the ratio shown in Fig. 3A results. Upon converting the minor to the major component, the sum of the methyl intensities remains constant, indicating that secondary binding sites for the heme can be discounted as the origin of the minor component.

The proton NMR spectra of the downfield region of [1,3-²H₆]deuterio-MetMbCN and [1,5-²H₆]deuterio-metMbCN recorded immediately after addition of KCN and adjusting the "pH" to 8, are given in Fig. 3 C and D, respectively. In each case, both components are present in ratios similar to that depicted in B. The peaks that have decreased intensity in both the major and minor components due to deuterium labeling are indicated by arrows. Comparison of the traces in Fig. 3 B-D leads to the methyl assignment for both components as shown in B.

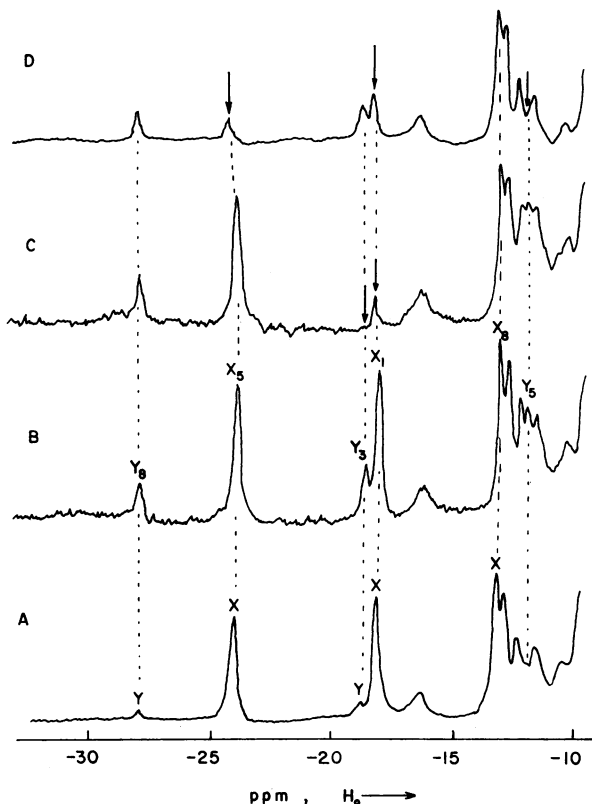


FIG. 3. Proton NMR traces of deuteroporphyrin-reconstituted sperm whale metMb cyanide, deuterio-metMbCN, at "pH" 8.5, 38°C in 0.2 M NaCl/2H₂O. (A) Protein taken to "pH" 11.1, KCN added, "pH" reduced to 7, stored for several hours, and then adjusted to "pH" 8.5; one component dominates strongly, although minor peaks, labeled Y, are detectable. (B) Protein taken to "pH" 11.1, KCN added, and "pH" readjusted to 8.5, and spectrum recorded; the minor component increased in intensity and a third peak is evident. (C) [1,3-²H₆]Deuterio-metMbCN treated as in B. (D) [1,5-²H₆]Deuterio-metMbCN treated as in B. Peaks in C and D with reduced intensity due to deuterium labeling are indicated by arrows. The presence of two isomers is also readily monitored by the 2,4-pyrrole-H peaks in the region 10–25 ppm upfield for dimethylsilapentane sulfonate (not shown). The major component resonates at +13.9 and 19.5 ppm, while the minor component resonates at +13.0 and 20.0 ppm at 38°C. The relative intensities of the two sets of 2,4-H peaks permit determination of the ratio of the two components even when methyls are deuterated.

DISCUSSION

Model compounds

The effect of moving the physical location of the low-symmetry perturbation about the porphyrin on the methyl hyperfine shift pattern of the low-spin ferric model complexes is illustrated with 2-acetyl- and 4-acetyldeuteroporphinato-iron(III)-dicyanide. These complexes differ in that the former has the electron-withdrawing substituent on pyrrole I, while the latter complex has it on pyrrole II. Hence the location of the low-symmetry perturbation differs qualitatively by a 180° rotation about the α - γ *meso* axis.

The proton NMR traces in Fig. 2 A and B reveal a spread of the heme methyl hyperfine shifts comparable to that in metMbCN (12). Moreover, in contrast to all other model compounds, 4-acetyldeuteroporphinato-iron(III) dicyanide mimics the characteristic methyl shift pattern in the protein in that 5-CH₃ is furthest downfield and 3-CH₃ is furthest upfield. Hence the 4-acetyl substituent essentially simulates the net protein perturbation on the heme π asymmetry as detected by NMR (12, 13). A more detailed interpretation of changes in

hyperfine shifts in low-spin ferric models and protein has been presented elsewhere (14).

Comparison of the traces of 2-acetyldeuteroporphinato-iron(III)-dicyanide (Fig. 2B) with that of the 4-acetyl derivative (Fig. 2A), reveals that they have comparable spreads of the methyl shifts but with basically different shift patterns. This difference is primarily that the relative position of 1-CH₃ and 3-CH₃, as well as 5-CH₃ and 8-CH₃, have interchanged, as indicated by the dotted lines in Fig. 2. The interchanged environments are not quite identical since the two complexes are not related by a true symmetry axis. However, these model compounds clearly show that the pattern of the heme methyl hyperfine shifts in low-spin ferric complexes is sensitive to the exact location of the low-symmetry perturbation.

Reconstituted Mb

The proton NMR spectrum of sperm whale metMb cyanide reconstituted with deuteroporphyrin, deuterio-metMbCN, had been reported earlier to be normal and very similar to that of native metMbCN (24). Analysis of a spectrum with very high signal-to-noise obtained several hours after addition of CN⁻ reveals the presence of two very small peaks with intensity less than a tenth of the presumed methyls (Fig. 3A). These peaks, in the absence of additional information, could be dismissed as impurities. However, if the proton trace is recorded shortly after addition of CN⁻, we find that these two minor peaks, as well as one not readily detected in A, are considerably increased in intensity (Fig. 3B). The minor component can be generated in a greater amount by first reducing deuterio-metMbH₂O with dithionite, storing the solution at 25°C for 1 hr, and then rapidly oxidizing with ferricyanide and ligating with cyanide. Upon standing, the spectrum in B slowly reverts to that in A, with the major peaks gaining in intensity at the expense of the minor peaks. The minor peaks, however, are detectable no matter how long the system equilibrates (>3 weeks).

The two sets of signals are therefore attributed to two forms of deuterio-metMbCN that are interconvertible. The time evolution of the spectra upon addition of CN⁻ is due to the fact that the two forms of the protein have different relative stabilities in the met-hydroxy, deoxy, and met-cyano forms, and the rate of interconversion between the two forms is very slow on the NMR time scale.

Since the three detectable peaks for the minor component, labeled Y, have comparable intensity, they are likely to be due to heme methyls. Inspection of the NMR traces of the two deuterium-labeled proteins in Fig. 3, [1,3-²H₆]deuterio-metMbCN (C) and [1,5-²H₆]deuterio-metMbCN (D), recorded immediately after addition of CN⁻ to the met-hydroxy form, leads to unambiguous methyl assignments in both components (Fig. 3B). The position and assignment of the three observed methyls, labeled X (the fourth is under the intense peptide backbone envelope), in the major isomer in deuterio-metMbCN are the same as in native metMbCN (12), indicating strongly that the heme orientation in the pocket in the two proteins is the same.

The assigned methyl peaks in the minor component, labeled Y, exhibit the shift pattern found in 4-acetyldeuteroporphinato-iron(III)-dicyanide and differs from that of the major component by the interchange of the environments of 5-CH₃ with 8-CH₃ and 1-CH₃ with 3-CH₃. This pairwise exchange of methyl shifts, as in the model compounds, can be directly interpreted as arising from heme orientations differing by a 180° rotation about the α - γ *meso* axis in the asymmetric heme pocket (Fig. 1). Instead of the perturbation moving by 180° about the *meso* axis, as in the models, the intact porphyrin is rotated by 180° with respect to an essentially invariant rhombic perturbation due to the apoprotein contacts.

We can therefore conclude that reconstitution of Mb with modified porphyrins, particularly deuteroporphyrin, can lead to a protein with heme disorder, so that changes in function cannot be attributed simply to the local effect of the change in substituent. The consistency in the changes in heme methyl hyperfine shift patterns in the model compounds and reconstituted metMbCNs demonstrates that the relative orientations of the heme in a class of closely related hemoproteins can be determined in solution by proton NMR.

A sizable literature has evolved on the structure-function relationships in hemoproteins based on the comparison of properties in reconstituted proteins (10). Two alternate arguments for the changes in ligand binding with variable 2,4-substituent have been discussed. On the one hand, the local inductive effect of the 2,4-substituent has been proposed to dominate (25, 26), while others have suggested that the tertiary structural changes resulting from differential apoprotein-2,4-substituent interactions give rise to variable ligand binding (27, 28). Both models assume a unique protein structure, which is contrary to the present observation. It is clear that variable heme orientation representing different heme-protein contacts must be considered in interpreting properties of reconstituted hemoproteins.

Thus, deuteroheme-reconstituted Hb has been shown (29) to have a significantly reduced Hill coefficient compared to the native protein. This result implies a specific role for the vinyls in the heme-heme interaction. However, preliminary NMR results on deoxy deuterio-Hb A reveal that, while four single proton resonances can be expected for the downfield 2,4-H, two each for the α and β chains, in fact five different resonances are observed with areas consistent with eight peaks of comparable intensity (unpublished observations). The doubling of peaks, also observed in deoxy deuterio-Mb, also likely arises from heme disorder. The inefficiency of Hb reconstitution and the limited amounts of labeled heme preclude assignment of individual methyls at this time. The evidence for a disordered solution structure is in contrast to crystallographic data (30), which have been interpreted in terms of a unique heme orientation that is identical to that of native Hb. Hence, the possibility of different solution and crystallographic structures also may have to be considered.

Although establishing heme disorder in the native insect Hbs and cytochrome b_5 will require similar studies with labeled hemes with these less readily available proteins, the appearance of methyl signals in interconverting pairs with similar chemical shifts (3, 9) suggests that rotational disorder of the heme is responsible. In cytochrome b_5 , the variable heme orientation and the concomitant change in heme-protein contacts could provide a subtle control of the path of electron transfer (31). In the insect Hbs, the disorder may be the result simply of the lack of specificity of the primitive polypeptide chain.

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