



## SYNTHETIC AND SPECTROSCOPIC STUDIES OF HEMES AND HEME PROTEINS \*

*It is shown that the maximum amount of information can be gained from NMR studies of heme proteins if the assignments in the heme are made in a definitive fashion using regioselective deuterium labeling. An outline of synthetic methods for introduction of deuterium labels into heme methyl groups is presented, and some results of proton NMR studies using proteins reconstituted with these labeled hemes are discussed.*

\* This article is based on a plenary lecture of the same title delivered by Professor Smith at the 6th Annual Meeting of the Portuguese Chemical Society, held in Aveiro, October 18-22, 1983.

### 1 — INTRODUCTION

Heme (1), the iron(II) complex of protoporphyrin-IX, is the prosthetic group in a large number of vitally important heme proteins. Typical examples, are the hemoglobins (responsible for oxygen transport), myoglobins (which store oxygen, particularly in cardiac tissue), cytochromes (which accomplish electron transport, and are important in the respiratory chain, and in drug detoxification), catalases and peroxidases (which carry out biochemical reactions on hydrogen peroxide). It is a somewhat surprising fact that almost all of these heme proteins, which have very different biological functions, have the same prosthetic group, namely heme (1). Thus, one is forced to the conclusion that the reason heme can perform such diverse functions is because of the protein matrix in which the prosthetic group is embedded, which somehow causes the electronic structure within the prosthetic group to alter, depending upon the intended function.

Such alterations can loosely be termed "structure/function" relationships, and it is the goal of the research described briefly below to determine what is the effect upon the electronic structure of the heme when a particular apoprotein (e.g. apomyoglobin, apohorseradish peroxidase) accepts that heme into its protein matrix to form physiologically active native heme protein. All of the studies to be described are possible only because, in a simple series of steps (fig. 1), most heme proteins can be dissociated into the free heme and unwrapped apoprotein, which can afterwards be "reconstituted" [1] to give heme protein with full physiological activity. Fortunately, the original heme (fig. 1) can be removed from the heme/apoprotein mixture, and the heme protein can, if necessary, be reconstituted with a *different* heme; this different heme can be structurally at variance with the original heme with regard to its peripheral substituents, or, for the purposes of most of our studies, it can be "pseudo-identical" in that the differences are merely in the replacement of a particular substituent (e.g. methyl group) with a deuterated substituent (e.g. CD<sub>3</sub>). The probe to be used for investigation of heme electronic structure changes with apoprotein is high field proton nuclear magnetic resonance (NMR) spectroscopy. In the studies to be described, the iron atom in the heme will be at oxidation state +3, which affords materials which are paramagnetic

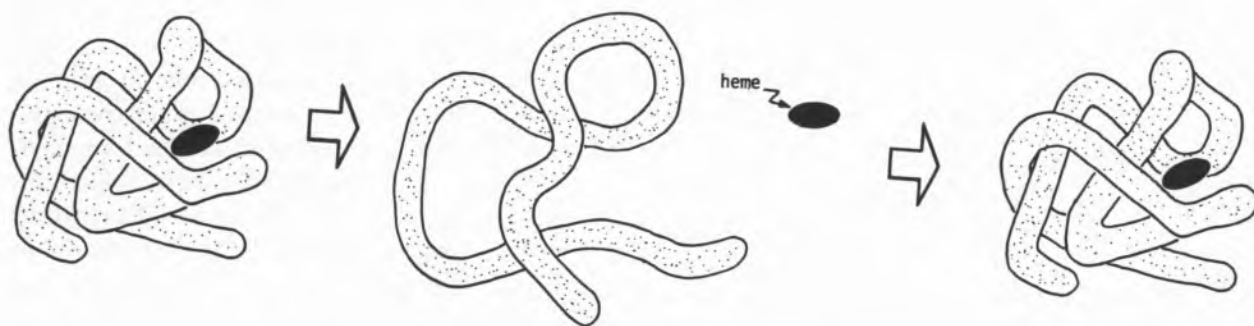


Fig. 1

*Diagrammatic representation of the dissociation of a heme protein into heme and apoprotein, followed by its reconstitution to give heme protein*

(low-spin, 1 unpaired electron; high-spin, 5 unpaired electrons). Thus, the resulting hemes and heme proteins give paramagnetic NMR spectra in which the hyperfine-shifted resonances allow one to draw direct conclusions about the spin density at the resonance being observed, and therefore about the electronic structure in the heme; it goes without saying that it is the electronic structure in the protein enfolded heme which imparts the unique function upon the system, thus allowing the heme protein to exert its own unique function. An important technical point is that the paramagnetic hyperfine shifts tend to move most of the heme resonances, and those of some protein functions close to the iron atom, away from the normal diamagnetic region (0-10 ppm) of the spectrum, which is heavily cluttered with resonances from the large protein chains. It is therefore possible to observe the critically important heme resonances, even in the presence of massive numbers of protein protons.

## 2 — HEME RESONANCE ASSIGNMENTS IN HEME PROTEINS

Though we have worked extensively on deuterium and carbon-13 labeling of all sites, for the purposes of the present discussion, only the methyls in heme (1) will be considered. It can be seen that the methyl groups at positions 1,3,5, and 8 in heme must exist as sharp three proton singlets, which should make them easy to observe; moreover, since there is one methyl group on each pyrrole subunit in (1), monitoring the chemical shifts of these lines should enable a firm picture of the electronic structure in each pyrrole ring to be obtained as the nature of the apoprotein is varied.

In fig. 2A the diamagnetic chemical shifts of the methyl peaks in protoporphyrin-IX [i.e. (1) without the iron atom] are shown in diagrammatic form; insertion of iron gives heme (1), which in its ferric oxidation state (named "protohemin") with cyanide present gives a low-spin paramagnetic species having the methyl chemical shifts shown in fig. 2B. Here, the hyperfine shifts from the diamagnetic range (fig. 2A) are obvious, and as mentioned previously, these shifts can be related directly to the spin densities in the individual pyrrole subunits, and therefore to the electronic structure in the heme. After reconstitution with apomyoglobin (fig. 1), the proton NMR spectrum of the resulting myoglobin has the methyl chemical shifts shown in fig. 2C. From the shift differences in the spectra in figs. 2B and 2C it is possible to make conclusions regarding the effect of reconstitution with apomyoglobin upon the electronic structure of the embedded heme. *However, before any conclusions can be drawn it is essential to uniquely assign each of the methyl peaks to a particular methyl group in the heme*, and this is no trivial matter. For convenience, these assignments (1,3,5,8) are included in fig. 2, but they were not known at the time we started our work. It can be seen that the average methyl chemical shift (methyl 3 in fig. 2C being assigned from deuterium NMR work) in figs. 2B and 2C is about the same, indicating that the reconstitution process has caused a simple redistribution of spin density. Other points are also obvious; for example, the ordering of the peaks from low to high field is 8,5,3,1 for the free hemin (fig. 2B), but 5,1,8,3 for myoglobin itself (fig. 2C), and assignment crossovers of this type are difficult to predict. Attempts to make such assignments indirectly using

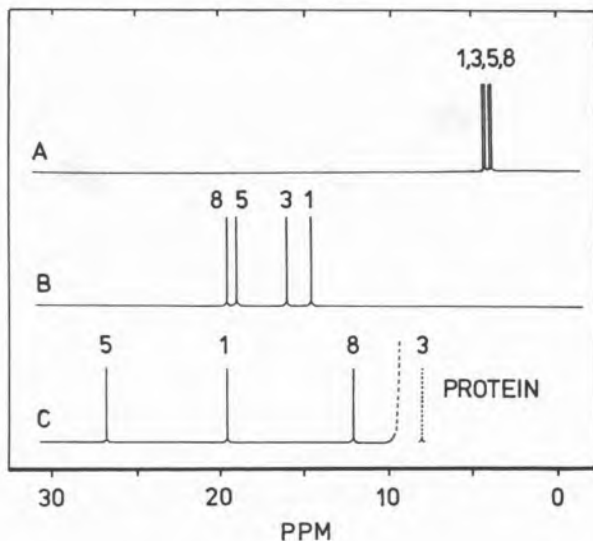


Fig. 2

Diagrammatic representation of the methyl resonances in the proton NMR spectra of: (A) protoporphyrin-IX dimethyl ester; (B) cyanoferriprothemin; (C) cyanometmyoglobin. The numbers above the peaks represent assignments obtained by deuterium labeling

a quantum mechanical model [2], using comparison of NMR and X-ray data on a cyclopropane-intercalated myoglobin [3], and using proposed hindered rotation of heme methyl groups [4], have all been resoundingly unsuccessful.

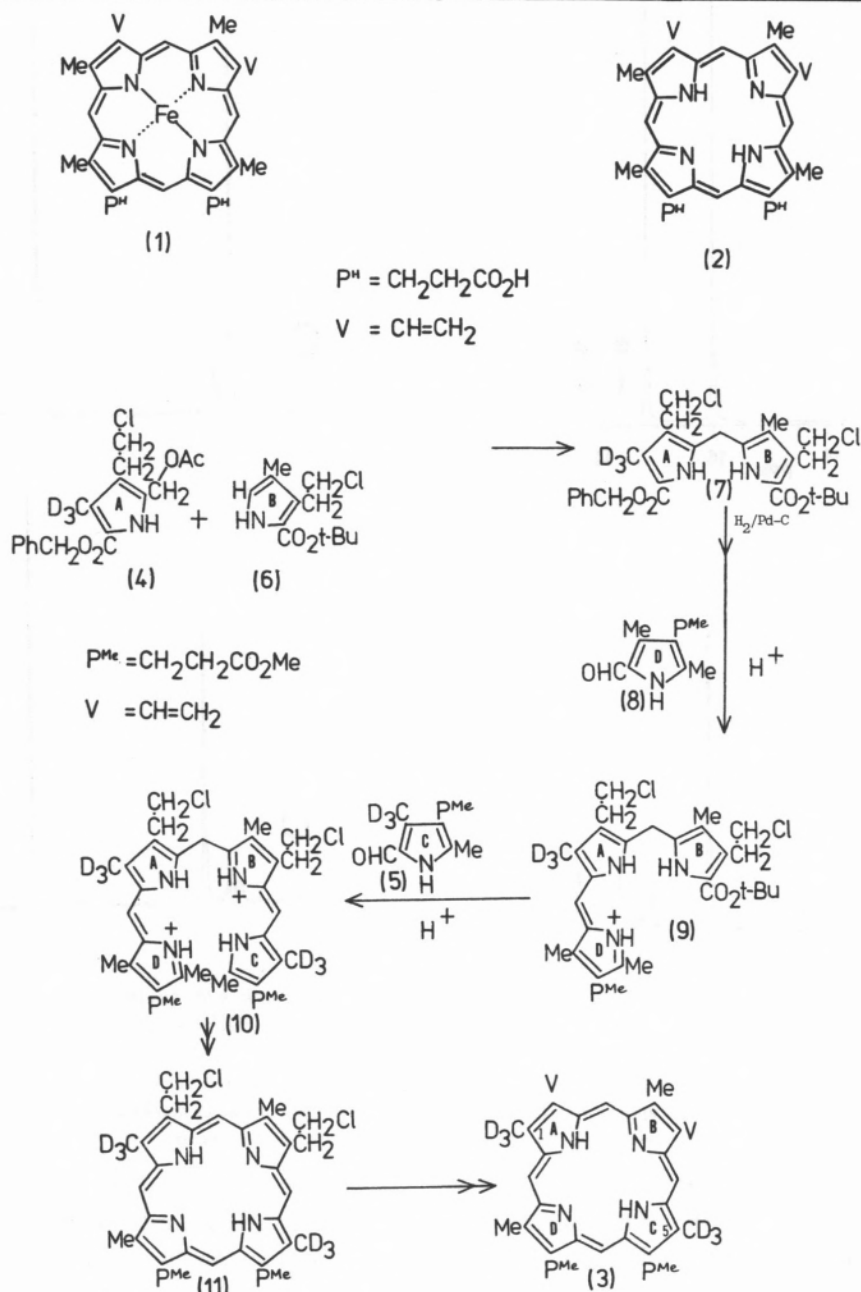
Our approach in Davis has been to make definitive assignments by way of synthetic organic chemistry. The principle was to synthesize regioselectively deuterated derivatives of protoporphyrin-IX in which methyls were replaced with  $\text{CD}_3$  groups, and which could be subsequently used in difference spectroscopy to make definitive assignments. Fig. 3 shows unlabeled cyanoferrihematin, and then the proton NMR spectra of 1,5-, 5-, and 8 methyl labeled hemins; from this, the principles used to make the assignments shown are obvious.

Scheme 1 shows the total synthesis of the 1,5-di(tri-deuteriomethyl) derivative (3) of protoporphyrin-IX (2) dimethyl ester. This route employs a novel step-wise synthesis [5] in which individual pyrrole rings are added in a 1 + 1 + 1 + 1 fashion, and at the time this approach was developed, it represented a novel departure from established approaches [6]. The pyrroles bearing deuterated methyl groups [i.e. (4) and (5)] were synthesized from acetylacetone which was deuterated in the 1 and 5 methyls by several exchanges in  $\text{D}_2\text{O}$  in presence of base [7]. Then, as shown in Scheme 1, pyrrole (4) was con-

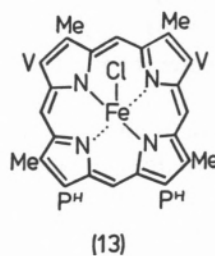
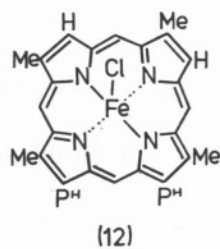
densed with (6) to give the pyrromethane (7), which after catalytic hydrogenation and acid-catalyzed condensation with the formylpyrrole (8) gave the tripyrrole (9) in excellent yield. Further condensation with the valuable deuterium labeled formylpyrrole (5) gave the a,c-biladiene salt (10) which was cyclized using copper(II) salts in hot dimethylformamide to give the porphyrin (11) after removal of chelated copper with conc. sulfuric and trifluoroacetic acids. Throughout this sequence, the vinyl groups in the required product (3) were protected in the form of 2-chloroethyl groups; these were dehydrochlorinated in base to give the required product (3) in excellent yield. Insertion of iron gave the hemin (NMR spectrum in fig. 3B), which, following ester hydrolysis, was reconstituted into myoglobin using standard methods [1]. In the course of our work, we have subsequently labeled all methyl groups, in various pair combinations and individually [7,8] and some of the results obtained from proteins reconstituted with these hemes will be described in the following section.

### 3 — SOME HEME PROTEIN NMR STUDIES

In most heme proteins, the hydrophobic (rings A,B) part of the heme becomes deeply embedded in the protein cleft, leaving the polar propionic groups to point to the outside, presumably interacting with the polar outside of the protein. There are, therefore, nominally two possible orientations for the heme in a reconstituted heme protein. These are shown in fig. 4, and they differ from each other by a  $180^\circ$  rotation about the heme alpha-gamma meso axis. Comparison of parameters from the X-ray structure of myoglobin [8] with results of our NMR assignments using labeled hemes afford a pattern which seems to hold for a large number of heme proteins. Fig. 4 shows that the proximal imidazole appears to lie along the nitrogen-nitrogen line of opposite pyrrole rings. NMR and X-ray correlations show that methyl groups on pyrrole subunits which lie along the line of the proximal imidazole (above the plane of the page in fig. 4) tend to move upfield, with the result that those methyls on rings perpendicular to this line are moved downfield. Thus, for a heme protein with the orientation shown in fig. 4A one would expect methyls 1 and 5 to be downfield and methyls 3 and 8 to be upfield. The orientation in fig. 4B would result in methyls 3 and 8 being



Scheme 1  
Total synthesis of 1,5-methyl deuterated protoporphyrin-IX  
dimethyl ester



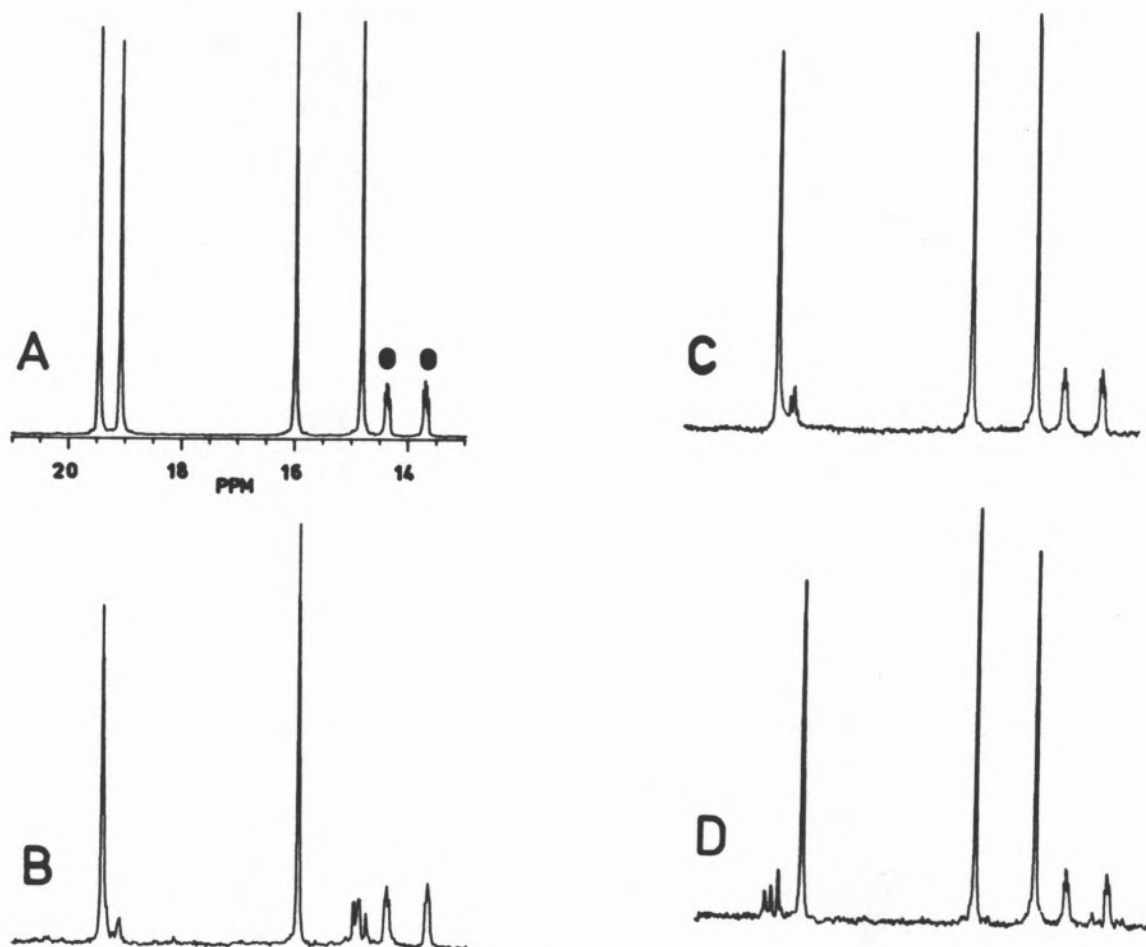


Fig. 3

360 MHz proton NMR spectra of the methyl region in the cyanoferrihemins: (A) protohemin (unlabeled); (B) 1,5-methyl labeled protohemin; (C) 5-methyl labeled protohemin; (D) 8-methyl labeled hemin. Small peaks to low-field of the labeled peaks in B, C and D are from  $\text{CH}_2\text{D}$  and  $\text{CHD}_2$  species. Peaks marked with a dot in (A) are the vinyl  $\alpha\text{-CH}$  resonances

downfield, and 1 and 5 to be upfield. Consideration of the peak order in fig. 2C shows that myoglobin has the orientation of the heme (relative to the proximal imidazole) as shown in fig. 4A.

X-ray studies appear to show that when a porphyrin is reconstituted into a protein, the heme assumes uniquely the same orientation as in the original heme protein. Our NMR studies of myoglobin reconstituted with deuteriohemin (12) (in this compound the "deutero" is a trivial name indicating replacement of the 2 and 4 vinyl groups with hydrogen, and has nothing to do with deuterium) indicate that, contrary to X-ray indications, reconstitution with deuteriohemin gives a heme protein which is orientationally heterogeneous [9]. This "heme

disorder" can be readily demonstrated in fig. 5; fig. 5A shows the down-field region in the proton NMR spectrum of sperm whale myoglobin reconstituted with deuteriohemin (12). Minor peaks, often thought to be impurities, are observed (labeled "Y"), and these can be enlarged (fig. 5B) by a minor modification of the reconstitution procedure. The extraneous peaks can be assigned using our deuterium labeled hemes (figs. 5C and 5D) to show that they correspond with minor amounts of heme which has reconstituted in the orientation which is rotated  $180^\circ$  about the  $\alpha\text{-}\gamma$  meso axis (i.e. methyls 8 and 3 are down-field; see preceding paragraph). This observation contrasts strongly with the X-ray results which indicate only one orientation in



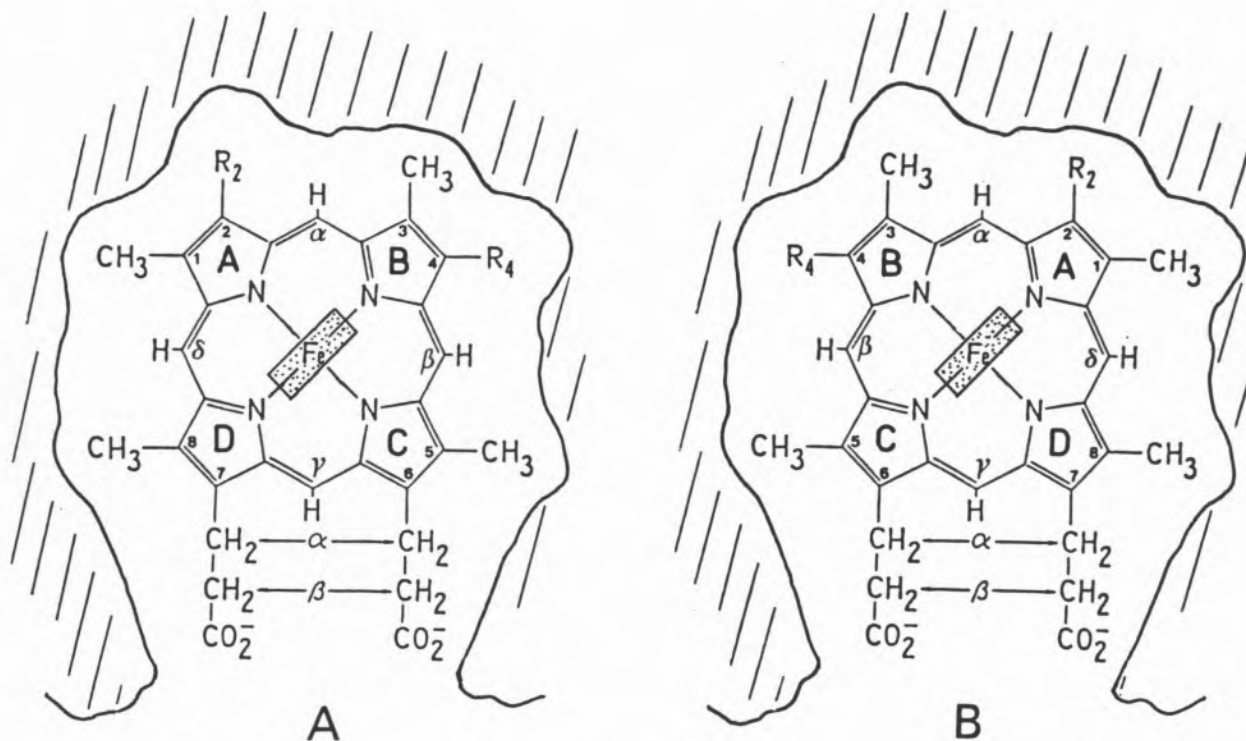


Fig. 4

Diagrammatic representation of the two possible heme orientations, relative to the proximal imidazole (rectangular box, above the plane of the paper) in common heme proteins. The orientation in (A) is as in myoglobin

this reconstituted heme protein, and points out limitations in the X-ray method, at least compared with NMR.

Since deuteriohemin-reconstituted myoglobin is not a natural heme protein, the question next arises as to whether or not the phenomenon of heme disorder can be observed in native heme proteins. Optical spectroscopy has shown [10,11], that the reconstitution step shown in fig. 1 is complete within one millisecond; that is, the unfolded apoprotein accepts the heme and folds to give native heme protein on the time scale of only one millisecond. In fig. 6 [12] the results of a heme/apomyoglobin reconstitution in an NMR tube are plotted; the species observed is metaquomyoglobin, and so, in this high-spin protein, the shifts of the methyls are between 50 and 100 ppm. In fig. 6A the spectrum of the material 8 minutes after reconstitution is given. When this is compared with native metaquomyoglobin in fig. 6D it can be seen that the protein is heterogeneous because the four methyls are doubled in the newly reconstituted myoglobin. With time, [fig. 6B (140 min); fig. 6C (800 min)] one set of methyl peaks di-

sappears to give a spectrum virtually identical with the native protein (fig. 6D). Deuterium labeling experiments definitively showed [13] that the pairs of peaks correspond to approximately equal initial amounts of the species which differ by a 180° rotation about the alpha/gamma axis. Thus, while the reconstitution process is indeed fast, one does not immediately get native protein. Clearly, formation of the critical heme-imidazole bond does not discriminate initially between the two faces of the heme, and the methyl/vinyl substituent pattern does not become important until the final stages of the protein folding to afford the holoprotein. The protein with the reversed heme orientation is a metastable form which requires, at least, partial unfolding of the protein and rupture of the heme-imidazole bond before it can become "native", and the rate of this equilibration is very slow ( $10^{-5} \text{ sec}^{-1}$ ). The spectra in fig. 6 also point out another fact; comparison of the initial spectrum (fig. 6A) with that of native protein (fig. 6D) shows that the native species does indeed have about 8% of a component (obvious peaks arrowed in the figure) in which the heme is rotated

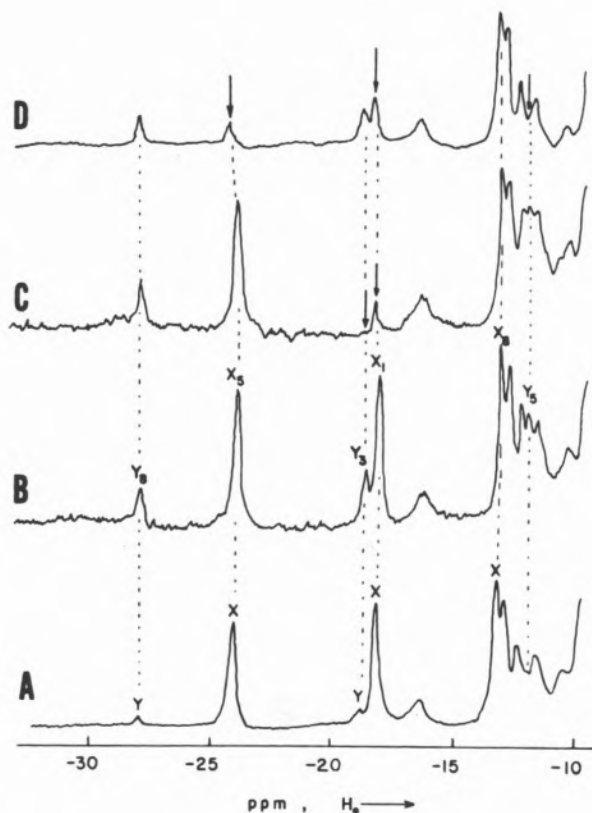


Fig. 5

Proton NMR traces of deuterohemin-reconstituted sperm whale cyanometmyoglobin at pH 8.5, 38°C, in 0.2 M NaCl/D<sub>2</sub>O. (A) Protein taken to pH 11.1, KCN added, pH reduced to 7, and the mixture stored for several hours, then taken to pH 8.5. One component dominates, although minor peaks (labeled Y) are apparent. (B) Protein taken to pH 11.1, KCN added, and pH readjusted to 8.6; the minor component has increased in intensity. (C) 1,3-methyl deuterated cyanometmyoglobin treated as in (B). (D) 1,5-methyl deuterated cyanometmyoglobin treated as in (B). Peaks in (C) and (D) with reduced intensity due to deuterium labeling are arrowed

by 180° about the alpha/gamma axis. Further proof is provided by the fact that reconstitution with the symmetrical synthetic protohemin-XIII (13) [14] affords an initial heme protein in which no extraneous doubling of peaks is apparent.

A heme protein in which heme rotational disorder is particularly striking is horseradish peroxidase. Unlike most heme proteins, reconstitution of apohorseradish peroxidase with deuterohemin (12) affords a heme protein in which considerable spectroscopic (e.g. electronic spectra of catalytic species) and physicochemical (e.g. ligand binding) differences between the natural (protohemin) and unnatural (deuterohemin) species exist. Such obvious

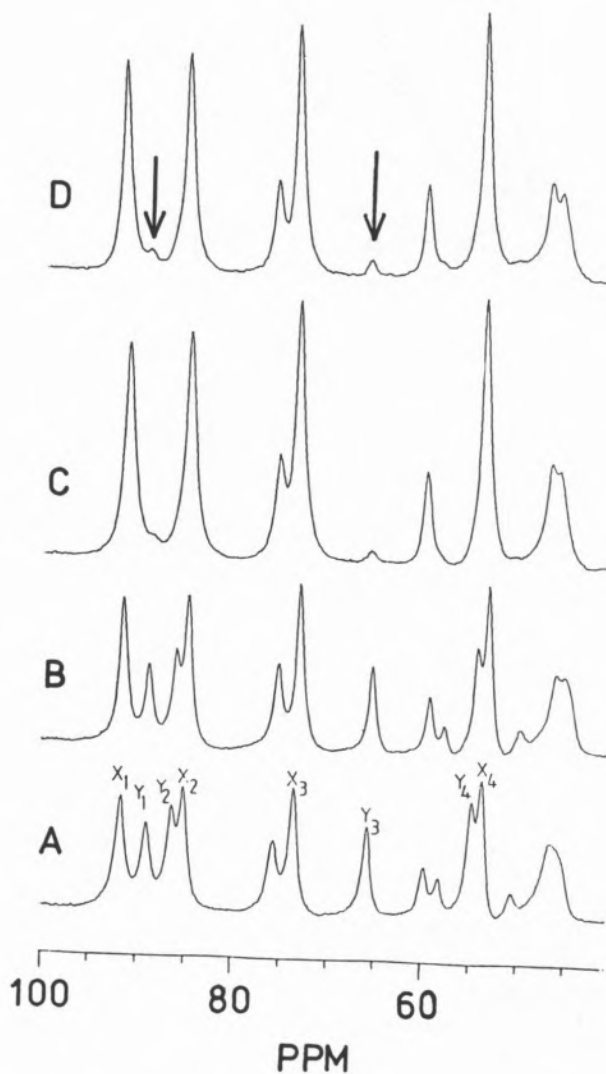


Fig. 6

360 MHz proton NMR spectra (downfield region) of metaquo-myoglobin at 25°C at various time intervals after reconstitution: (A) 8 min. (B) 140 min. (C) 800 min., pH 5.7. (D) Native protein. Peaks indicating the presence of an orientational isomer in the native protein are arrowed

anomalous behavior cannot simply be attributed to a "vinyl-for-hydrogen" substituent difference between the two proteins because virtually all other similarly reconstituted heme protein analogues have properties similar to those of the native holo-protein.

In fig. 7A, the spectrum of cyano-horseradish peroxidase (one unpaired electron) is presented, and above it the proton nmr spectra of the deuterated species which yielded the assignments in A [15]. The 8 and 3 low-field methyl assignments immediately indicate in the native protein the orientation of the

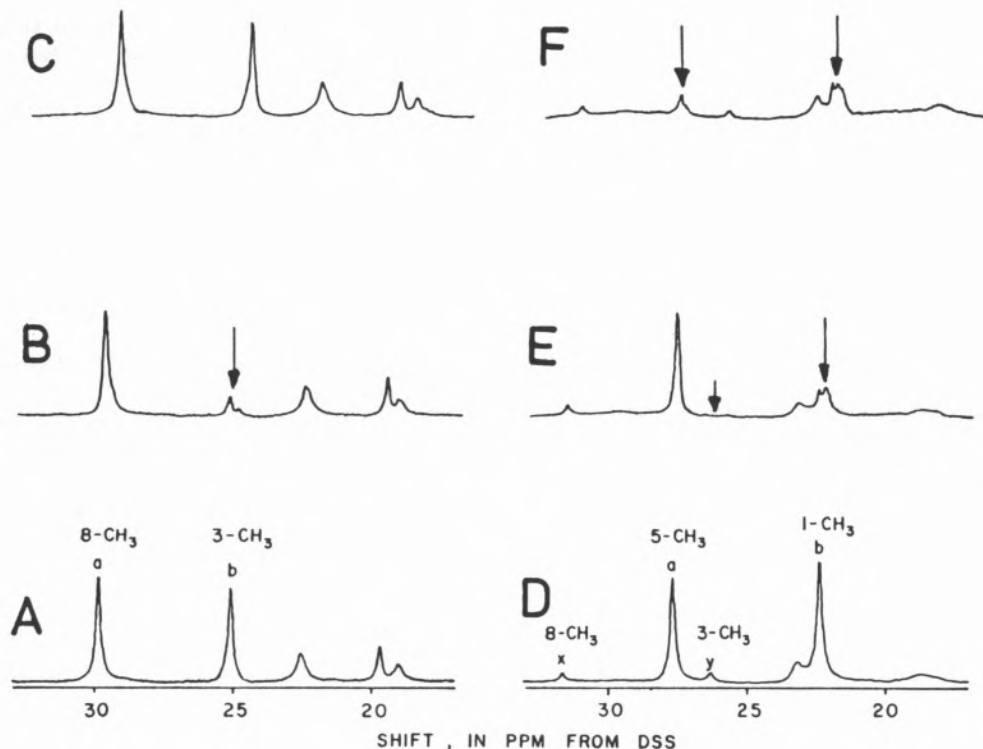


Fig. 7

360 MHz proton NMR spectra (downfield region) of: (A) Native cyano-horseradish peroxidase. (B) Cyano-horseradish peroxidase reconstituted with 1,3-methyl deuterated protohemin. (C) Cyano-horseradish peroxidase reconstituted with 1,5-methyl deuterated protohemin. (D) Deuterohemin-reconstituted cyano-horseradish peroxidase. (E) 1,3-Methyl deuterated deuterohemin-reconstituted cyano-horseradish peroxidase. (F) 1,5-Methyl deuterated deuterohemin-reconstituted cyano-horseradish peroxidase. Reduced methyl intensities due to deuteration are arrowed

heme relative to the proximal imidazole is opposite to that found in myoglobin (where methyls 1 and 5 are downfield). In fig. 7D, the proton NMR spectrum of cyano-horseradish peroxidase reconstituted with deuterohemin (12) is presented, and above it the corresponding spectra for deuterium labeled analogues. The assignments (methyls 5 and 1 downfield) clearly indicate that in this species the heme is oriented in the same configuration, relative to the proximal imidazole, as in myoglobin. Thus, the reason for the great differences between the protohemin and deuterohemin-reconstituted species is associated with the orientation of the heme, and not with simple substituent differences in rings A and B, though they are responsible for the reconstitution in the reversed orientation. Interestingly, the deuterohemin species does reconstitute with a small amount

(fig. 7D) of the "correctly oriented" heme! Observations of this type indicate several directions in which future research can proceed, and this work is presently in progress [e.g. 16] in Davis.

#### ACKNOWLEDGEMENTS

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