(Abbott Laboratories) for helpful discussions and communication of unpublished experimental details. We are also indebted to Dr. Richard L. Keene of The Upjohn Company, Infectious Diseases Research and to Prof. DeShong for gifts of authentic tirandamycin A, and to John Schaeck for contributions to the project. The crystal structure analyses were performed by Dr. F. J. Hollander, staff crystallographer at the UC—Berkeley X-ray Crystallographic Facility (CHEXRAY). Support for our research by a grant from the National Institutes of Health (No. GM-30759) is gratefully acknowledged.

Registry No. (±)-**1**, 85880-71-3; (±)-**9**, 103383-32-0; (±)-**10**, 103421-37-0; (±)-**12**, 103383-33-1; (±)-**13**, 103421-38-1; (±)-**13** (ethyl ester), 103368-33-8; (±)-**14**, 103368-35-0; (±)-**15**, 103368-36-1; (±)-**16**, 103368-37-2; (±)-**16**-ol (isomer 1), 103368-58-7; (±)-**16**-ol (isomer 2), 103421-42-7; (±)-**18**, 103368-59-8; (±)-**20**, 103368-60-1; (±)-**22** (isomer 1), 103368-61-2; (±)-**22** (isomer 2), 103421-43-8; (±)-**23**, 103368-62-3; (±)-**23**·Na, 103421-44-9; (±)-**23** (acid chloride), 103368-63-4; **25**, 103368-56-5; **25** (acid), 103368-64-5; (±)-**26**, 103368-65-6; (±)-**27**, 103368-66-7; (±)-**30**, 103368-38-3; **32**, 103368-39-4; **33**, 103368-40-7; (±)-**34**β, 103368-31-6; (±)-**34**β (TMS enol), 103368-32-7; **35**, 103368-42-9; (±)-**36**α, 103368-43-0; (±)-**36**β, 103421-39-2; (±)-**37**α, 103368-67-8; (±)-**38**, 103383-08-0; (±)-**39**, 103368-44-1; (±)-**40**, 103368-46-3; (±)-**41**, 103368-45-2; (±)-**43**, 103368-47-4; (±)-**44**$\alpha\alpha$, 103368-48-5; (±)-**44**$\alpha\beta$, 103421-40-5; (±)-**45**, 103368-41-8; (±)-**45** (TBDMS ether), 103368-49-6; (±)-**45** (bromosilyl derivative), 103368-50-9; (±)-**48**, 103368-51-0; (±)-**48** (disilated), 103368-52-1; (±)-**48** (desilated ketone), 103368-53-2; (±)-**49**, 103421-41-6; (±)-**49** (silyl enol), 103368-54-3; (±)-**50**, 103421-36-9; (±)-**51**, 103368-55-4; (±)-**52**, 103368-57-6; (±)-**53**, 97859-87-5; (±)-(*trans*)-ethyl 2,3-epoxybutyrate, 82769-14-0; [3*R**,4*R**,5*S**(1*S**)]-3-hydroxy-4-methyl-5-(1-iodoethyl)dihydro-2-(3*H*)-furanone, 103368-34-9; propyne, 74-99-7; hexafluroacetone, 684-16-2; methyl isopropenyl ketone, 814-78-8; ethyl vinyl ether, 109-92-2; 3-methoxy-3-methylbutyne, 13994-57-5; methyl propiolate, 922-67-8.

Supplementary Material Available: Experimental details for the synthesis and characterization of the compounds depicted in Schemes III and IV, as well as compounds **37** and **38** (5 pages). Ordering information is given on any current masthead page.

Influence of Propionate Side Chains on the Equilibrium Heme Orientation in Sperm Whale Myoglobin. Heme Resonance Assignments and Structure Determination by Nuclear Overhauser Effect Measurements

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Abstract: Sperm whale myoglobin was reconstituted with hemins methylated at the 2-, 4-, and 6- or 7-positions, and the corresponding metcyano complexes were studied by ^{1}H NMR spectroscopy. Nuclear Overhauser effects (NOEs) were observed between heme methyls attached to the same pyrrole ring and between heme methyls adjacent to a common meso position. The relative magnitudes of these effects could be relied on to identify heme methyl resonances and are proposed to be used as a simple method to reach spectral assignments. Along with selected and characteristic dipolar contacts with clearly identified peripheral protein side chains, the inter-methyl NOE allows direct determination of the heme orientation in the holoprotein. Thus, it was found that the replacement of either propionate side chain with a methyl does not affect the nature of the thermodynamically preferred isomer and does not perturb the equilibrium proportion of the two species.

The two alternate orientations of the heme in *b*-type hemoproteins involve a 180° rotation about the α,γ meso axis (A in Figure 1)[1,2] and have been shown to play key roles in the initial steps of the in vitro assembly from apoprotein and heme for myoglobin,[3] hemoglobin,[4] and cytochrome b_5.[5] In the former two proteins, preliminary evidence suggests that the in vivo assembly may proceed via similar pathways.[4,6] Moreover, not only does the initial step of the reaction between heme and apoprotein fail to distinguish between the two sides of the heme but both heme orientations remain populated to some degree at equilibrium, leading to equilibrium heme rotational disorder.[3,4] This equilibrium disorder in both mammalian myoglobin and hemoglobin A involves only 10–15% of the "reversed" heme orientation as in the lower part of A in Figure 1. However, considerably larger degrees of disorder have been found in insect hemoglobin,[7] fish myoglobin,[6] and mammalian myoglobin reconstituted with chemically modified 2,4-substituents.[8]

We have shown previously that the nature of the 2,4-substituents influences both the rate of heme reorientation and the position of the equilibrium between the two heme orientations.[8] In the present study, we extend our investigation to explore the influence of the modification of the heme 6- or 7-propionate chains on equilibrium heme orientation in sperm whale Mb. Holoproteins of sperm whale Mb at apparent equilibrium were prepared for the two modified hemes, 7-(2-carboxyethyl)-1,3,5,6,8-pentamethyl-2,4-divinylhemin and 6-(2-carboxyethyl)-1,3,5,7,8-pentamethyl-2,4-divinylhemin,[9] hereafter referred to as 6-

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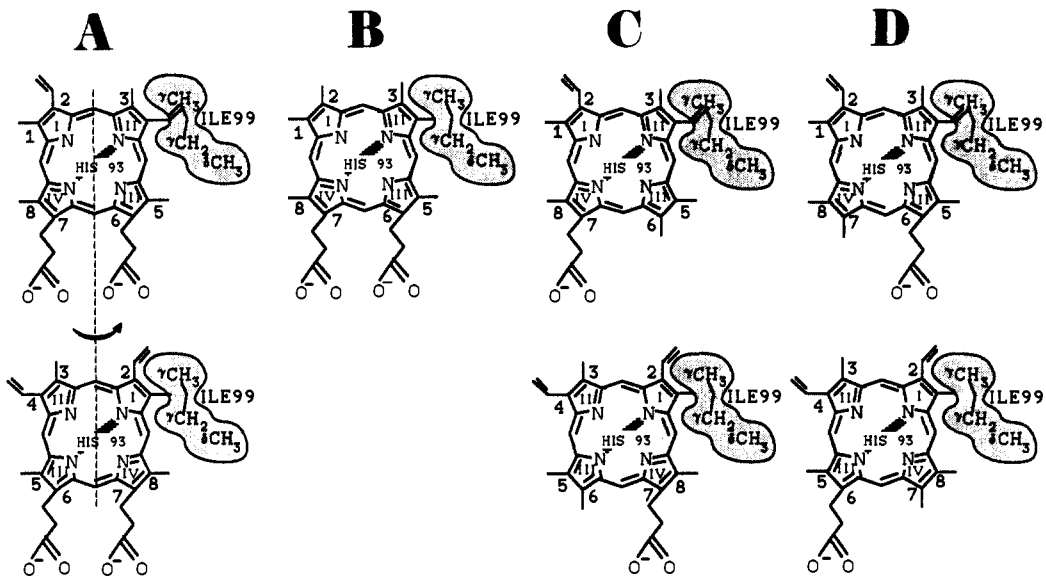

Figure 1. Structure and possible orientations of the hemins used in reconstituting the sperm whale myoglobin holoprotein. The side-chain projections of the proximal amino acids Ile-99 (FG5) and His-93 (F8) define the orientation of each hemin with respect to the protein (the proximal side is above the plane of the page). The Ile-99 is shaded, and the regions occupied by the γ- and δ-CH_3 are labeled. (A) The upper part depicts the predominant equilibrium orientation of protohemin IX as found in the native protein ("normal");[10] the lower part displays the reversed orientation, related to the normal one by a 180° flip about the α,γ axis ("reversed"). (B) The symmetrical 2,4-dimethyldeuterohemin is shown. (C) The upper part depicts the 6-methyl-6-despropionatehemin in the normal orientation; the lower part depicts the reversed orientation. (D) The upper part depicts the 7-methyl-7-despropionatehemin in the normal orientation; the lower part depicts the reversed orientation.

methyl-6-despropionatehemin and 7-methyl-7-despropionatehemin (C and D of Figure 1, respectively). We address here two questions: does a unique (or predominant) orientation exist at equilibrium for each modified heme and what is the nature of this orientation in relation to that found in the native protein.[10]

These questions and the interest in future studies of other proteins and other modified hemes require simple methods for assigning heme methyl resonances without resorting to the laborious synthesis of modified hemes with selective isotope labeling.[1,2,4-6] In an attempt to develop such general and practical methods, we have used the modified hemin 6,7-bis(2-carboxyethyl)-1,2,3,4,5,8-hexamethylhemin[11] (referred to as 2,4-dimethyldeuterohemin), which, because of its true twofold symmetry about the α,γ meso axis, can form only a single complex (Figure 1B) and therefore yields a simpler ^1H NMR spectrum.

Single-crystal structure determination has demonstrated the reversed heme orientation in erythrocruorin,[12] but heme rotational disorder has so far been detected by X-ray crystallography in only one case.[13] In solution, however, heme disorder is readily characterizable by ^1H NMR spectroscopy.[1,2,4,5] This detection method generally relies on the study of a low-spin ferric derivative and requires the unambiguous assignment of individual heme methyls. With such information, the heme orientation can be deduced by two alternative routes. The first is based on the pattern of heme methyl hyperfine shifts,[1,2] which has been proposed to depend on the orientation of the proximal histidyl imidazole plane relative to the individual pyrrole rings.[14,15] Thus, a His-F8 π-plane projection passing through pyrroles II and IV leads to large contact shifts for the 5-CH_3 and the 1-CH_3 and smaller ones for the 8-CH_3 and the 3-CH_3 (as observed in native metMbCN[2,16]). Conversely, rotating the heme by 180° about the α,γ meso axis places this His-F8 π-plane along the vector passing through pyrroles I and III (compare upper and lower rows in Figure 1), and in this orientation, the large downfield shifts are predicted (and observed in freshly reconstituted Mb[1,2] or Hb[4]) for the 8-CH_3 and the 3-CH_3. Hence, the interpretation of heme methyl shifts can lead to heme orientational information. However, such an analysis is valid provided the His π-plane projection does not pass through meso positions and is conserved relative to the protein in both forms.[14,15] This was shown to be the case for sperm whale Mb reconstituted with protohemin IX.[17]

The second and more direct method requires, in addition to the assignment of heme substituents,[2,16] the identification of signals from a protein residue near the heme periphery.[18] The demonstration of spatial proximity between assigned heme methyl and amino acid side-chain signals through the nuclear Overhauser effect[19] can yield the heme orientation regardless of the His-F8 π-plane projection. Thus, the presence of equivalent amounts of the two heme orientations, shown in A of Figure 1, could be demonstrated for freshly reconstituted Mb.[17]

The steady-state nuclear Overhauser effect,[19] designated $\eta_{i\rightarrow j}$, is given by the relation in eq 1 where η is the fractional intensity

$$\eta_{i\rightarrow j} = \sigma_{ij}/\rho_j \qquad (1)$$

change for the signal of spin j while saturating spin i, σ_{ij} is the cross-relaxation rate between spins i and j, and ρ_j is the relaxation rate for spin j obtained from exciting spin j selectively. The distance information is contained in σ_{ij}, in the form in eq 2 where r_{ij} is the length of the vector between spins i and j and τ_c is the tumbling time of the i–j vector.

$$\sigma_{ij} = -(\gamma_H{}^4\hbar^2\tau_c)/(10r_{ij}{}^6) \qquad (2)$$

Using the model protein complex reconstituted with the modified symmetric hemin, 2,4-dimethyldeuterohemin (B in Figure 1), as well as native metcyano complexes reconstituted with selectively deuterated hemins, we show that characteristic inter-heme methyl NOEs are observed between methyl groups attached to

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a single pyrrole and between methyl groups neighboring a common meso position. We demonstrate that these inter-heme methyl NOEs, in combination with NOEs between heme methyls and the readily assigned resonances of isoleucine-99 (FG5),[18] allow both location and assignment of all methyl signals as well as the unambiguous determination of the orientation of the heme in its cavity. Moreover, this method is shown to have general applicability for resonance assignment and heme orientation determination in low-spin ferric hemoproteins.

Experimental Section

Reconstitution of Proteins. Sperm whale myoglobin was purchased from Sigma Chemical Co. and used without further purification. Apomyoglobin was prepared according to the reported procedure.[8,20] Apo-Mb solution was prepared by dissolving lyophilized apo-Mb powder in chilled aqueous 0.2 M NaCl solution (pH ~7). The precipitate, if any, was removed by centrifugation, and the final protein concentration was determined from the absorption intensity (ϵ_{280} = 15 900 M^{-1} cm^{-1}).

A 10 molar equiv solution of [$\alpha,\beta,\gamma,\delta$-$^2H_4$]hemin, [1,3-($C^2H_3$)$_2$]hemin, 2,4-dimethyldeuterohemin, 6-methyl-6-despropionatehemin, and 7-methyl-7-despropionatehemin was prepared for reconstitution. These hemins are the same as those reported previously.[2,9,11] The solutions of [$\alpha,\beta,\gamma,\delta$-$^2H_4$]hemin and [1,3-($C^2H_3$)$_2$]hemin were made by dissolution in ^2H_2O, [NaO^2H] = 0.2 M. The other hemins were insufficiently soluble in alkaline aqueous medium. Therefore, a 50% aqueous pyridine/KCN solution was used to dissolve the 2,4-dimethyldeuterohemin and 6-methyl-6-despropionatehemin, while a dimethyl sulfoxide/KCN solution was necessary to dissolve the 7-methyl-7-despropionatehemin.

Each protein was reconstituted by adding to the chilled apo-Mb solution 1 molar equiv of the chilled hemin solution dropwise with continuous stirring. The reconstituted holoprotein was immediately passed through a Sephadex G-25 column and then ultrafiltered in an Amicon cell (YM5 membrane) to remove the pyridine or Me_2SO (present in <10% v/v). The protein was finally concentrated to 5 mM in the same cell, and the small volume was then exchanged with ^2H_2O, [NaCl] = 0.2 M. All the above operations were carried out at 4 °C. The sample pH was adjusted with 0.2 M NaO^2H or ^2HCl. The pH was measured with a Beckman 3550 pH meter equipped with an Ingold 620 microcombination electrode, pH values were uncorrected for the isotope effect.

The cyanometmyoglobins (pH 9.0) reconstituted with 6-methyl-6-despropionatehemin and 7-methyl-7-despropionatehemin were kept at room temperature for 3 days to equilibrate. The aquometmyoglobins reconstituted with [$\alpha,\beta,\gamma,\delta$-$^2H_4$]hemin and [1,3-($C^2H_3$)$_2$]hemin (pH 9) were kept at room temperature for 24 h to equilibrate. A fourfold excess of KCN was then added to convert them to the metcyano form. A standard 5 mM sample of native sperm whale metMbCN was also made by dissolving the native protein in 0.2 M NaCl solution in ^2H_2O and adding KCN as above. The ^1H NMR spectra of the protein complexes exhibited no changes with time over 8 months, and hence the reconstituted species are assumed to be at equilibrium with respect to the heme reorientation. 2,4-Dimethyldeuterohemin, by virtue of its twofold symmetry about the α,γ meso axis, forms only a single species.

^1H NMR Measurements. For the NMR experiments, 0.5 mL of 5 mM protein was used. ^1H NMR spectra were recorded at 35 and 45 °C on a Nicolet NT-360 FTNMR spectrometer (360 MHz) operating in the quadrature mode. Spectra were recorded at the temperature that best resolves the signals of interest. Data were collected by using double precision on 16 384 data points over a 10-kHz bandwidth. The nuclear Overhauser spectra were recorded by application of a presaturation pulse of 250 ms with the decoupler on-resonance. Corresponding reference spectra were collected under identical conditions but with the decoupler pulse off-resonance. On- and off-resonance frequencies were alternated every 128 scans. Typical spectra consisted of at least 1280 transients with a repetition rate of 1 s^{-1}. Chemical shifts for all the spectra are referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) through the residual water resonance.

Results and Discussion

The 360-MHz ^1H NMR spectra for the equilibrated metcyano complexes of sperm whale myoglobin containing native hemin, 2,4-dimethyldeuterohemin, 6-methyl-6-despropionatehemin, and 7-methyl-7-despropionatehemin are illustrated in A of Figures 2–5, respectively. The striking similarities among the spectra are quite apparent, particularly in the upfield region, where each displays a broad single proton peak, z, near −10 ppm and a pair of methyl peaks, w, x, near −3.5 ppm. The low-field regions are also similar, except for an obvious extra methyl in the region 30–12 ppm for 2,4-dimethyldeuterohemin and 6-methyl-6-despropionatehemin.

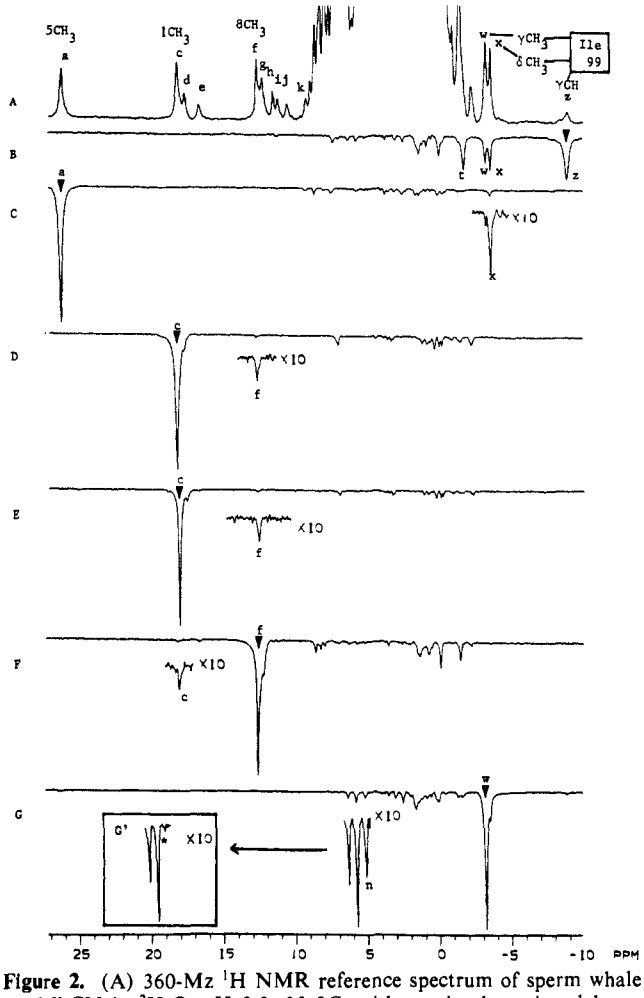

Figure 2. (A) 360-Mz ^1H NMR reference spectrum of sperm whale metMbCN in ^2H_2O, pH 9.0, 35 °C, with previously assigned heme methyls[2,16] (a = 5-CH_3, c = 1-CH_3, f = 8-CH_3) and previously assigned Ile-99 protons[18] (w = γ-CH_3, x = δ-CH_3, z = γ-CH). (B–D, F, G) Difference spectra generated by subtracting the reference spectrum described in (A) from a similar spectrum of the same sample in which one of the resonances was presaturated (saturated signals are indicated by arrows). (B) Saturate z (Ile-99 γ-CH_3). Note the NOEs to Ile-99 signals t (γ-CH′), w (γ-CH_3), and x (δ-CH_3). (C) Saturate a. Note the NOE to Ile-99 peak x (δ-CH_3). (D) Saturate c. Note the small NOE to f (8-CH_3). (E) Difference spectrum generated by the same experiment as described for D but on a sperm whale metMbCN sample that was reconstituted with [$\alpha,\beta,\gamma,\delta$-$^2H_4$]hemin. Note the NOE to f of similar magnitude to that in D. (F) Saturate f. Note the NOE to c. Off-resonance saturation of peak g can be shown not to contribute to the NOE to c. (G) Saturate w. Note NOE to peak n (3-CH_3). (G′) Inset of the 7–6 ppm region of the difference spectrum obtained in a way identical with that described for G but on a sperm whale metMbCN sample that was reconstituted with [1,3-(C^2H_3)$_2$]hemin. Note the absence of an NOE to n as indicated by the asterisk.

In all cases, the various peaks all exhibit relative intensities of 1:1 or 1:3, and the number of resolved peaks is practically the same, arguing strongly against important (≥10%) heme orientational disorder in any of the samples. In the native protein, about 8% disorder is present at equilibrium.[2-4] For 2,4-dimethyldeuterohemin–metMbCN, the twofold symmetry of the heme precludes such heme rotational disorder, and indeed, no minor intensity peaks are detected in Figure 3. Peak assignments and nuclear Overhauser effects in all of the other compounds will define an essentially unique orientation of the heme with respect to the protein matrix. The necessary assignments are most effectively pursued by considering first some aspects of the NMR spectral characteristics of the native and 2,4-dimethyldeuterohemin-reconstituted proteins.

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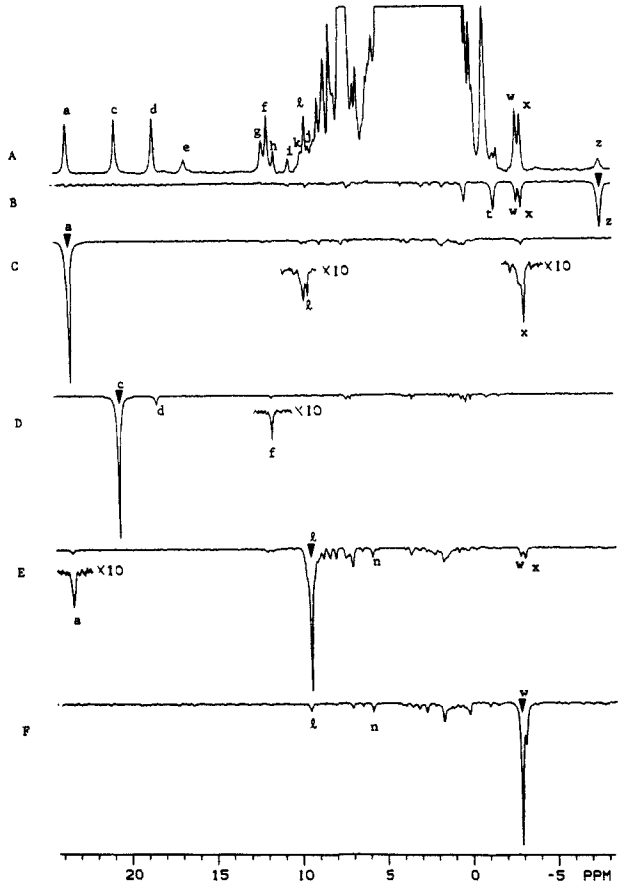

Figure 3. (A) 360-MHz ^1H NMR reference spectrum of 2,4-dimethyldeuterohemin-reconstituted sperm whale metMbCN in ^2H_2O, pH 8.5, 45 °C. (B–F) Difference spectra for the sample described in A generated as explained in Figure 2. (B) Saturate z (Ile-99 γ-CH).[18] Note NOEs to Ile-99 signals t (γ-CH′), w (γ-CH_3), and x (δ-CH_3). (c) Saturate a (5-CH_3). Note small NOE to the methyl peaks l (4-CH_3) and x (Ile-99 γ-CH_3). (D) Saturate c (1-CH_3). Note the large NOE to methyl d (2-CH_3) and the smaller one to f (8-CH_3). (E) Saturate l (4-CH_3). Note NOEs to the Ile-99 methyls w and x as well as heme methyls a (5-CH_3) and n (3-CH_3). It can be shown that the NOE to the 5-CH_3 is larger than expected due to decoupler spillage to peak k, which in turn exhibits dipolar coupling to peak a (see Figure 3C). Several of the NOEs seen in the 7–9 ppm range can also be attributed in part to decoupler spillage to peaks k and j. (F) Saturate w (Ile-99 γ-CH_3). Note NOE to l (4-CH_3) and n (3-CH_3).

Native Heme. The resolved methyl assignments (peaks a = 5-CH_3, c = 1-CH_3, f = 8-CH_3, as indicated in Figure 2) have previously been obtained by using specific isotope labeling.[2,16] The position of 3-CH_3 was not located at the time; however, it was shown to resonate somewhere in the intense diamagnetic envelope 0–9 ppm. The NOE connectivity linking peak z (γ-CH) to peaks t, w, and x, (γ-CH, γ-CH_3, and δ-CH_3, as in B of Figure 2) has led to the reported clear identification of the signals from Ile-99 (FG5).[18] Furthermore, the saturation of the 5-CH_3 (peak a in C of Figure 2) yields an NOE to peak x, confirming the heme orientation as shown in the upper portion of A in Figure 1, as discussed previously.[17]

It may be noted that, besides the relevant NOE to peak x (Ile-99 δ-CH_3), NOEs to several peaks in the diamagnetic region (0–10 ppm) are observed in the difference spectrum C of Figure 2. These effects are the manifestation of dipolar connectivities between the 5-CH_3 and other amino acid side chains located near the heme pocket. However, the assignment of the resonances relevant to heme orientation, namely, the heme methyls and Ile-99 (FG5), does not require the analysis of additional NOEs to and from the protein matrix. It should then suffice to point out that the effects observed upon saturation of a common resonance, if the heme orientation is the same, are essentially identical in all complexes considered here and consequently that they indicate unaltered

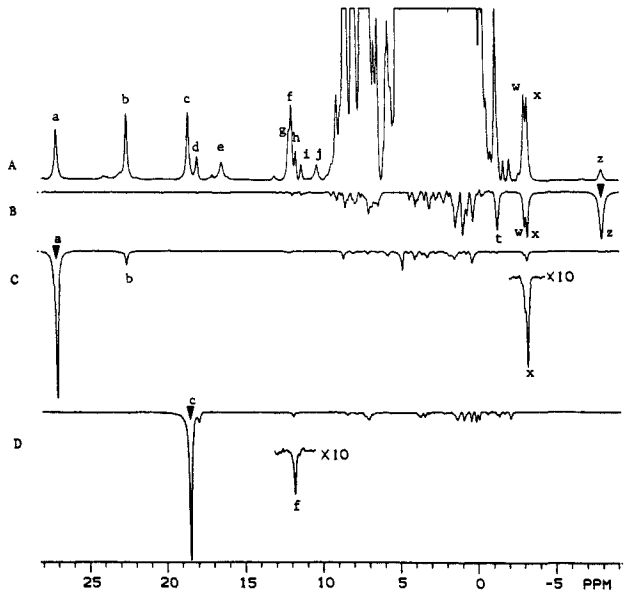

Figure 4. (A) 360-MHz ^1H NMR reference spectrum of sperm whale metMbCN reconstituted with 6-methyl-6-despropionatehemin in ^2H_2O, pH 9.0, 35 °C. (B–D) Difference spectra for the sample described in A, generated as explained for Figure 2. (B) Saturate z (Ile-99 γ-CH). Note NOEs to Ile-99 signals t (γ-CH′), w (γ-CH_3), and x (δ-CH_3).[18] (C) Saturate a (5-CH_3). Note NOEs to b (6-CH_3) and x (Ile-99 δ-CH_3). (D) Saturate c (1-CH_3). Note the small NOE to f (8-CH_3).

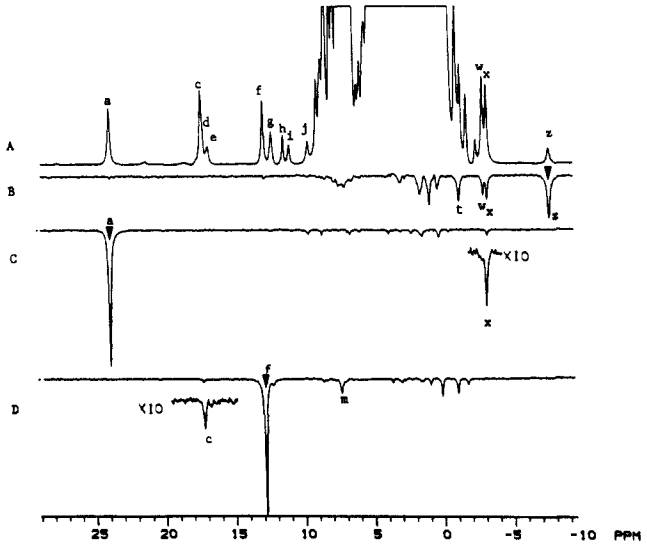

Figure 5. (A) 360-MHz ^1H NMR reference spectrum of 7-methyl-7-despropionatehemin-reconstituted sperm whale metMbCN in ^2H_2O, pH 8.7, 35 °C. (B–D) Difference spectra for the sample described in A, generated as explained for Figure 2. (B) Saturate z (Ile-99 γ-CH). Note NOEs to Ile-99 signals t (γ-CH′), w (γ-CH_3), and x (δ-CH_3). (C) Saturate a (5-CH_3). Note NOE to x (Ile-99 δ-CH_3). (D) Saturate f (8-CH_3). Note the small NOE to c (1-CH_3) and the larger NOE to m (7-CH_3).

structures. The detailed interpretation of all effects, leading to a description of that structure, must await assignment of these protein peaks based on a combination of 1-D and 2-D NMR methods. Such studies are in progress.

In Figure 2D, it is observed that the saturation of the assigned 1-CH_3 (peak c) yields a small (~−1%) NOE to the assigned 8-CH_3 (peak f). Moreover, since this NOE is undiminished when the experiment is performed on [α,β,γ,δ-$^2H_4$]hemin-reconstituted metMbCN (Figure 2E), the effect is not relayed by this proton and reflects direct inter-methyl dipolar coupling. Such NOE connectivity between heme methyls is thus indicative of positions adjacent to a single meso position. Trace F in Figure 2 demonstrates that the effect between the 1-CH_3 and the 8-CH_3 is reciprocal. As is expected from the greater distance, no such dipolar

connectivity is detected (<0.1%) between the assigned 1-CH_3 and 5-CH_3. The X-ray data place the γ-CH_3 of the amino acid side chain Ile-99 (peak w) 3.7 Å from the yet unidentified heme 3-CH_3. Saturation of peak w (Figure 2G) yields NOEs to three peaks with intensity compatible with such a distance. One of these peaks, n, exhibits a strong anti-Curie behavior (moving downfield from under the residual H^2HO at ~4.7 ppm to 5.5 ppm as the temperature is raised from 25 to 45 °C) and disappears when the identical experiment is carried out on [1,3-$(C^2H_3)_2$]hemin-reconstituted metMbCN (inset G′ in Figure 2). Since the 1-CH_3 is assigned at 18 ppm,[2,16] peak n must originate from the 3-CH_3. This locates the last elusive methyl group of native metMbCN and suggests the route for indirectly locating other unresolved heme signals in modified metMbCN complexes.

2,4-Dimethyldeuterohemin. The most obvious difference between the spectrum of the protein complex of this symmetric heme (Figure 3A) and that of the native protein (Figure 2A), besides the missing vinyl signal d,[21,22] is the presence of an additional methyl signal in the region 18–23 ppm. Regarding the two additional methyls on pyrroles I and II, the heme methyl contact shift pattern observed in the native protein predicts that the 2-CH_3 (as labeled in Figure 1B) will resonate near the 1-CH_3, which in turn should appear in the 18–22 ppm window. The 4-CH_3 can be expected near the 3-CH_3 resonance position. As found for the reference protein side chain,[18] saturating peak z (Figure 3B) identifies the Ile-99 (FG5) signals. Moreover, saturating a leads to an NOE to x (Ile-99 δ-CH_3 in Figure 3C), and hence a is defined as the 5-CH_3 according to the labels of Figure 1B. Saturating peak c (Figure 3D) yields a large NOE (~−4%) to d, establishing these methyls as neighboring groups on the same pyrrole, and the small NOE (~−1%) from c to f dictates that methyls c and f are adjacent to the same meso position. Hence, c must arise from the 1-CH_3, d from the 2-CH_3, and f from the 8-CH_3. This is consistent with the methyl hyperfine shift pattern found for the native protein.[1,2,16] Inspection of Figure 3A indicates that the additional peak at 9.4 ppm (peak l) also integrates as a heme methyl. By noting the small NOE (~−1%) from peak a (5-CH_3) to l, characteristic of methyls adjacent to the same meso position, peak l can be attributed to the 4-CH_3. This accounts for all resolved methyl signals.

Saturating peak l (4-CH_3) is expected to yield a ~−4% NOE to the 3-CH_3, which, according to the native protein data,[2,16] resonates in the diamagnetic envelope near the H_2O resonance. The result of this experiment is shown in Figure 3E, where several peaks with appropriate intensity can be observed in the difference spectrum. However, only peak n at 5.7 ppm is also observed weakly when the 2-CH_3 (d) is saturated (not shown); this effect is due to the relative position of these two methyls and, more convincingly, is detected in the difference trace (Figure 3F), when Ile-99 (FG5) γ-CH_3 (peak w) is saturated. This locates and assigns all six heme methyl resonances. We now exploit this strategy to obtain the assignments needed to determine the orientation of the hemes where the propionate side chains are replaced by methyls.

6-Methyl-6-despropionatehemin. The spectrum displays four apparent heme methyl peaks a–c and f in the low-field region (Figure 4A). In this compound, as was seen in the native protein, the saturation of peak z yields NOEs to all of the signals from Ile-99 (FG5)[18] (Figure 4B). In turn, saturation of peak a (Figure 4C) yields a strong NOE to x (Ile-99 δ-CH_3), indicating that peak a arises from the methyl group closest to Ile-99, either the 5-CH_3 or the 8-CH_3, depending on whether the heme orientation corresponds to the upper or lower part of Figure 1C. However, the fact that there is a substantial NOE (~−7%) from methyl a to methyl b demands that these two methyls be attached to the same pyrrole, and hence methyls a and b can only originate from the 5-CH_3 and the 6-CH_3, respectively. The large hyperfine shifts of the 5-CH_3 and 6-CH_3 signals are consistent with a greater spin density on pyrroles I and III. Thus, we conclude that the dominant (>90%) orientation of this modified hemin is the same as that of the hemin in native sperm whale Mb[10] (i.e., the upper orientation in Figure 1A,C). Additional signals, possibly reflecting the reversed orientation, are detected at 24, 17, and 13 ppm in the reference spectrum; they suggest the presence of about 7% of the minor component.

Table I. Chemical Shifts of Heme Methyl Groups and Ile-99 Side-Chain Resonances[a]

	sperm whale myoglobin reconstituted with			
	hemin	2,4-dimethyl-deutero-hemin	6-methyl-6-des-propionate hemin	7-methyl-7-des-propionate hemin
		Hemin[b]		
1-CH_3	18.1	21.2	18.4	17.2
2-CH_3	–	18.7	–	–
3-CH_3	5.1	5.7	5.6	5.8
4-CH_3	–	9.6	–	–
5-CH_3	26.1	24.2	26.9	23.8
6-CH_3	–	–	22.4	–
7-CH_3	–	–	–	7.3
8-CH_3	12.6	11.6	11.8	12.8
		Ile-99[b]		
γ-CH (z)	−8.8	−8.6	−8.1	−7.7
γ-CH′ (t)	−1.7	−1.8	−1.4	−1.2
δ-CH_3 (x)	−3.2	−3.2	−3.1	−3.0
γ-CH_3 (w)	−3.5	−3.5	−3.3	−3.3

[a] Chemical shifts in ppm at 35 °C, pH 9, referenced to DSS. A dash indicates that the position is not occupied by a methyl in a given heme derivative. [b] See Figure 1 for labeling of hemin and Ile-99 resonances.

That methyl peaks c and f originate from the 1-CH_3, and the 8-CH_3 is confirmed by the small NOE between them (Figure 4D), but this effect does not allow us to say which is which. However, the fact that the orientation is the same as for native hemin leads to the assignment of the more paramagnetically shifted signal c to the 1-CH_3 and f to the 8-CH_3. This can be confirmed by the observation of large and specific NOEs to the same but as yet unidentified protein signals in the upfield region (−1 to 0 ppm) of the protein complexes containing the modified as well as the native hemins.

Saturation of Ile-99 γ-CH_3 (peak w) at various temperatures (not shown) allows assignment of the 3-CH_3 based on its characteristic anti-Curie behavior. The shifts for the heme methyl and Ile-99 signals are included in Table I, together with those of the native and 2,4-dimethyldeuterohemin-reconstituted complexes.

7-Methyl-7-despropionatehemin. The number, relative intensities, and shift pattern of resolved resonances in the metcyano complex of this modified hemin (Figure 5A) are the same as for the native protein. All additional peaks have less than 0.1 proton intensity. This argues for a single dominant heme orientation. Irradiation of peak z (Figure 5B) yields the remaining Ile 99 peaks,[18] and saturation of methyl peak a again yields the NOE to peak x that locates peak a near Ile-99. In this case, there are no additional peaks with sufficient intensity in the difference trace to suggest a second methyl group on the same pyrrole; hence, peak a can be attributed only to the 5-CH_3. This is confirmed in Figure 5D, where saturation of methyl peak f yields a large NOE to an apparent methyl peak, m, at 7.3 ppm, which is not observed in any of the other samples. The intensity in the difference spectrum is consistent with the −3 to −7% NOE observed between heme methyls attached to the same pyrrole. Peak f and peak m must therefore arise from the pair of methyl groups attached to pyrrole IV, namely the 8-CH_3 and the 7-CH_3. Peak f is identified as the 8-CH_3 because of the small NOE observed to the methyl peak c (Figure 5D) and because this small effect is characteristic of methyl groups adjacent to a common meso position. Peak c must then arise from the 1-CH_3. Since the now identified 8-CH_3 does not yield an NOE to the Ile-99 peak x but peak a, the 5-CH_3, does, the dominant heme orientation is unequivocally established

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to be again the same as in the native protein (i.e., the upper orientation in Figure 1D). Saturation of the Ile-99 γ-CH_3 at variable temperature (not shown) again yields a peak with anti-Curie behavior that can be assigned to the 3-CH_3. The shifts for all heme methyls are listed in Table I.

Inter-Methyl NOE. The NOE between methyl groups attached to the same pyrrole or adjacent to a same meso position is consistent with a simple free-rotation model. In the 2,4-dimethyldeuterohemin-reconstituted metMbCN, the saturation of the 2-CH_3 causes an $\sim$-3.5% intensity change of the 1-CH_3 signal; this corresponds to an $\sim$-1.2% effect per irradiated proton. The intrinsic relaxation rate of the 1-CH_3 (peak c, Figure 3A) was measured to be 11.7 Hz under the same conditions, and thus eq 1 leads to $\sigma = -0.14$ Hz. This cross-relaxation rate implies (via eq 2 and a τ_c value of 7 ns) an experimental inter-methyl distance of $\sim$3.8 Å, while calculation of the average 1-CH_3 to 2-CH_3 distance $\langle r \rangle = \langle 1/r^3 \rangle^{1/3}$ using X-ray coordinates adjusted for the presence of the 2-CH_3 yields a value of $\sim$3.7 Å. On the other hand, the NOE from the 8-CH_3 to the 1-CH_3 is -1.0 ± 0.4%, which translates into an experimental separation of 4.7 ± 0.4 Å. This result is also comparable to the calculated average distance between the freely rotating 1-CH_3 and 8-CH_3, estimated at $\sim$5.1 Å from X-ray data.[10]

Conclusions

The homonuclear Overhauser effect has allowed us to assign all heme methyl resonances in sperm whale metMbCN and reconstituted derivatives even when the signals are buried under the intense diamagnetic envelope. This provides the value of the mean and spread of heme methyl hyperfine shifts that have been suggested to serve as a probe for the degree of imidazolate character of the proximal histidine[23] and the nature and magnitude of in-plane rhombic distortion in the protein, respectively.[16,24,25]

For the metcyano complex of proteins highly homologous to sperm whale Mb, the magnitude of inter-methyl NOEs appears characteristic and should aid significantly in allowing rapid determination of the heme orientation without resorting to isotope labels: the most shifted methyl does (8-CH_3) or does not (5-CH_3) show a small ($\sim$-1%) NOE to another heme methyl (1-CH_3). The method can be extended to any low-spin ferric hemoprotein, provided one amino acid in dipolar contact with one heme methyl can be located. Thus, the detection of NOEs between specific assigned methyl groups and the assigned protons of Ile-99 (FG5) allows the determination of the orientation of the heme in the heme cavity with respect to the α,γ meso axis.

In the two hemin analogues that have either the 6- or 7-propionate replaced by a methyl, a single heme orientation dominates ($\geq$90%) at equilibrium, and that orientation has the vinyl groups in the identical position as in the native protein.[2,10,17] Thus, we conclude that preferences in forming salt bridges between propionates and amino acid side chains (6-propionate with His-FG3 and 7-propionate with Arg-CD3) are much less important than preferences for vinyl-protein contacts in determining the equilibrium heme orientation in sperm whale myoglobin.

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Coenzyme Stereospecificity of Alcohol/Polyol Dehydrogenases: Conservation of Protein Types vs. Functional Constraints

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Abstract: A hypothesis is presented that allows prediction of the stereospecificity of alcohol and polyol dehydrogenases. The zinc-containing family with higher subunit molecular weight is *pro-R*; the family without zinc and lower subunit molecular weight is *pro-S* specific with respect to the coenzyme. New data for ethanol dehydrogenase from *Leuconostoc mesenteroides* and for glycerol dehydrogenase from *Bacillus megaterium* support this classification. Criteria for selecting systems suitable to contribute to the recent debate about functional constraints and conservation of coenzyme stereospecificity are suggested.

For the pyridine coenzyme NAD(P)-dependent dehydrogenases so far investigated it has been observed that the syn conformation of enzyme-bound nicotinamide leads to *pro-S* and the anti conformation to *pro-R* specificity of hydrogen transfer from NAD(P)H to carbonyl compounds.[1] Benner et al.[2,3] postulated that the energy difference between these conformers was used in biological evolution to put a functional constraint on enzymes catalyzing these redox reactions involving NAD(P). The function-based theory demands that reactive carbonyl compounds ($-\log K_{eq} > 11.2$) are reduced by the *pro-R* and the less reactive ones ($-\log K_{eq} < 11.2$) by the *pro-S* hydrogen of NAD(P)H. Controversial discussions have been published[4,5a,b] about the predictive potential of this new theory as compared to that of historical models, which are based on conservation of coenzyme stereospecificity by conservation of structural integrity of the proteins. According to Benner et al.,[6] the opposite stereospecificity of *Drosophila* and yeast alcohol dehydrogenase strongly supports the function-based theory, since these enzymes catalyze a reaction with low selective pressure. The equilibrium constant here lies

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