# Relationship between Heme Binding Site Structure and Heme Orientations of Two Ferrocytochrome $b_5$s. A Study in Prosthetic Group Recognition

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**Abstract:** The heme binding sites of two $b_5$ cytochromes, one isolated from beef liver and the other sequentially identical with the globular portion of rat liver cytochrome $b_5$ and expressed bacterially from a synthetic gene, have been characterized in the diamagnetic reduced state by one- and two-dimensional NMR techniques. Both proteins are heterogeneous in structure in the vicinity of the heme prosthetic group, the result of two possible heme binding orientations. Equilibrium ratios of the two orientations differ between the oxidized beef and bacterially expressed rat proteins, with a ratio of 9:1 of the two isomers in the beef and a 6:4 ratio in the rat. A kinetic (1:1) mixture of the two forms may be trapped by reduction of protein freshly prepared by reconstitution of the apoprotein with hemin. Structural differences between the beef and bacterially expressed rat protein in the vicinity of the heme pocket were determined by nuclear Overhauser effect experiments. Structural differences between the two proteins are discussed in relation to differences in heme orientational equilibria.

Because of its stability, moderate size, and ease of isolation, cytochrome $b_5$ has been the subject of numerous NMR[‖] investigations, in both the diamagnetic ferrous and paramagnetic ferric forms.[1-8] Recent publications have indicated that extensive sequential $^1H$ assignments have been made for reduced bovine cytochrome $b_5$.[9] It has been noted that cytochrome $b_5$ as isolated from beef liver is heterogeneous as a result of the heme prosthetic group being bound to the protein in one of two orientations, related to each other by a rotation of 180° about the porphyrin $\alpha$–$\gamma$ axis (Figure 1).[4] In beef liver ferricytochrome $b_5$, the two forms exist in roughly 9:1 ratio at equilibrium.[10] The more populated form at equilibrium will be referred to as isomer A and the less populated form as isomer B. Recently, we have examined the bacterially expressed soluble fragment of rat liver cytochrome $b_5$[11] and found it also to bind heme in two orientations, with an equilibrium ratio of the two isomeric forms of 6:4 in the oxidized protein.

In order to establish a structural basis for the differences in equilibrium ratios of heme orientation between the two proteins, we have undertaken an extensive $^1H$ NMR investigation of the heme binding sites of both proteins. The problem of heme orientational disorder is of intrinsic interest because it is an easily studied case of prosthetic group recognition/binding with obvious parallels to enzyme–substrate interactions. In the present case, we have been able to examine subtle structural effects on heme binding free energies because of the energetic near-equivalence of the two heme orientations ($\Delta\Delta G_{beef} = -1.30$ kcal/mol; $\Delta\Delta G_{rat} = -0.29$ kcal/mol at 25 °C).[12] By virtue of sequence-specific $^1H$ resonance assignments that have been made, a detailed structural comparison of the heme binding sites of the reduced rat and beef cytochromes is possible. This information in turn aids in understanding the roles that specific residues play in determining the extent and sense of heme orientational preference. These same resonance assignments have also proven useful for spectroscopic comparison of various site-specific mutants of the soluble rat cytochrome $b_5$.[13] All sequence-specific resonance assignments presented here are based on interproton distances derived from X-ray crystallographic data.[14,15]

The relationship between the binding orientation preferences and structure in the beef and rat proteins is not easily determined: in the oxidized forms, which equilibrate rapidly, it is possible to determine free energy differences between the two orientations.[12] Unfortunately, the paramagnetism of the oxidized form makes an equally simple interpretation of observed NOEs and relaxation parameters problematic. In the diamagnetic reduced forms, interpretation of NOE data is somewhat simplified, but the dynamics of exchange between the two orientations are slowed sufficiently that equilibrium distributions cannot be reliably established. Thus, conclusions based on spectroscopic observations in the reduced form of cytochrome $b_5$ concerning the effect of structure on the equilibrium thermodynamics of heme binding must be tempered with caution: it cannot be concluded with certainty that the equilibrium is the same in both oxidation states. Furthermore, it has been established from variable-temperature experiments that orientation A is favored at 25 °C in the oxidized rat protein

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‖ Abbreviations: DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate; COSY, two-dimensional *J*-correlated spectroscopy; fid, free induction decay; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; ppm, parts per million; WT, wild type. One letter amino acid abbreviations: A, alanine; F, phenylalanine; H, histidine; I, isoleucine; L, leucine; V, valine; W, tryptophan; Y, tyrosine.

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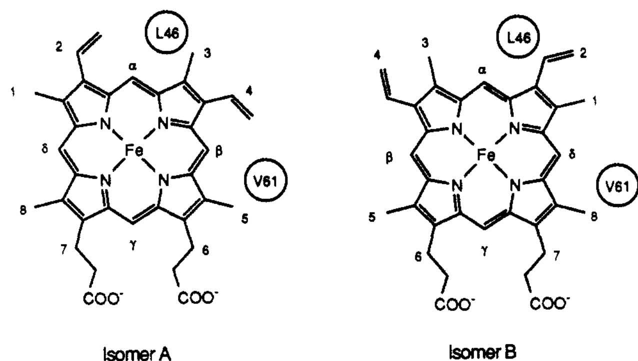


**Figure 1.** Isomerism in the heme binding site of cytochrome $b_5$ due to rotation of bound heme around the $\alpha-\gamma$ axis. Isomer A is the form most populated at equilibrium in the ferric state of both the beef and bacterially expressed rat proteins.

primarily for entropic reasons, while in the beef protein, the same isomer is favored enthalpically, with a decrease in the percentage of isomer A as temperature increases.[12] A simple explanation for these observations is unlikely. However, these observations do not preclude a structural explanation for heme binding orientational preferences, and in fact, present results point toward such an explanation.

The spectroscopic consequence of the existence of two orientations of the heme in the binding site of cytochrome $b_5$ is the doubling of virtually every resonance corresponding to a heme binding site proton. This complicates the assignment problem significantly, especially in the case of the beef protein, in which isomer B makes up only 10% of the total protein, making isomer B resonances difficult to assign. The slowness of interconversion of isomeric forms in the reduced protein makes it possible to "freeze" the kinetic (1:1) mixture of isomers by reduction of cytochrome $b_5$ freshly prepared from the apoprotein by reconstitution with protohemin.[10] Assignments of most of the porphyrin proton resonances for isomer B of the reduced beef protein have been made in the freshly reconstituted protein, as well as many resonances corresponding to amino acid side chains in the heme binding site. Because the preparation of apoprotein and subsequent reconstitution invariably leads to some denaturation of protein, two-dimensional NMR experiments on the reconstituted beef protein are problematic, and repurification must be avoided in order to prevent reequilibration of the two isomeric forms. Therefore, assignments in the reconstituted beef protein have been made using one-dimensional NOE experiments.

The bacterially expressed rat liver cytochrome $b_5$ is on the other hand suitable for two-dimensional NMR studies, since the heme is bound to the rat protein in both orientations to comparable extents at equilibrium, and assignments for both isomeric forms may be made in the protein as isolated. These assignments have therefore been made using phase-sensitive NOESY and COSY data.

## Experimental Procedures

**Protein Isolation and Purification.** The trypsin-resistant water-soluble fragment of microsomal cytochrome $b_5$ containing the heme binding site was isolated from fresh beef liver, solubilized, and purified as described previously.[16] The corresponding soluble fragment of rat liver cytochrome $b_5$ was isolated as the holoprotein from bacterial cell-free extracts of *Escherichia coli* (strain TB-1) harboring a plasmid construct containing a synthetic gene coding for the 98 amino acid polypeptide corresponding to the globular soluble portion of rat liver cytochrome $b_5$.[11] Purification of this protein was performed as for the beef protein, however, without the necessity of detergent solubilization or trypsin digest. Apoprotein was prepared and reconstituted with protohemin as described previously.[10]

Samples of ferrocytochrome $b_5$ in deuterated phosphate buffer (0.1 M, pH 7.0) were prepared for NMR spectroscopy by reduction with 10-$\mu$L aliquots of 1 M ($^2H_2O$) sodium dithionite. Sample concentrations were typically 1–2 mM for one-dimensional and 4–6 mM for two-dimensional NMR experiments. Sample pH was established with a Beckmann 3550 pH meter equipped with an Ingold combination microelectrode. pH values are not corrected for isotope effect.

**$^1$H NMR Methods.** One-dimensional $^1$H spectra were obtained with a Nicolet NT 500 spectrometer (UCD) operating in quadrature detection mode with a proton frequency of 500 MHz, or on a GN 500 spectrometer (UIUC) operating at 500 MHz. A typical acquisition consisted of 3000 transients with a spectral width of ±5000 Hz and 8K complex data points. A 60° pulse width and 2-s postacquisition delay were used. Steady-state one-dimensional NOE spectra were obtained by selectively saturating the resonance of interest for 500 ms with the decoupler on-resonance. Reference spectra were collected in an identical fashion with the decoupler off-resonance. Reference and NOE spectra were obtained in an interleaved fashion. NOE difference spectra were generated by subtracting the reference spectrum from the on-resonance spectrum. Sample temperature was maintained at 25 °C by a 0.1 °C pyrometer. Chemical shifts are referenced to the residual $^1H^2HO$ peak. Signal to noise was improved by multiplication of the fid with a 5-Hz exponential line-broadening function or resolution enhanced by multiplication of the fid by a phase-shifted sine bell.

Two-dimensional phase-sensitive NOESY and COSY spectra were obtained by using standard pulse sequences on a GN-500 500 MHz spectrometer (UIUC) operating in quadrature mode. The method of States was used to obtain phase sensitivity in the $\omega_1$ dimension.[17] Typically, 2 × 96 transients, each with 1024 complex points over a sweep width of ±4000 Hz, were obtained at each of 300 $t_1$ values for NOESY and 2 × 64 transients at each of 512 $t_1$ values were obtained for COSY data sets. NOESY spectra were acquired with $\tau_m$ = 200 ms. Based on interproton distances derived from the crystal structure,[14] no evidence for indirect NOEs due to spin diffusion was observed at this mixing time. Probe temperature was maintained at 25 ± 0.1 °C. Data processing was performed on a Nicolet 1280 microcomputer. GE GEM software was used for all data manipulations. Prior to transformation in the $t_2$ dimension, an unshifted sine bell was applied to COSY data, while a Gaussian function was applied to NOESY data. After transformation in $t_2$ and transposition, a 45° phase-shifted sine bell was applied to COSY and NOESY data sets in the $t_1$ dimension prior to Fourier transformation. Chemical shifts are referenced to the internal $^1H^2HO$ peak.

## Results and Discussion

**Assignment of Resonances to Isomeric Forms.** There is a substantial body of evidence for ascribing the heterogeneity of the heme binding site of cytochrome $b_5$ to rotation about the heme $\alpha-\gamma$ axis. Besides the NOE evidence described here and in previous publications,[1-8] we have noted that samples of beef apocytochrome $b_5$ reconstituted with [8-$C^2H_3$]protoporphyrin IX show the absence of two resonances normally seen in the protiated material, one from each isomer. Those resonances in protiated 8-$CH_3$ samples exhibit cross relaxation with polypeptide resonances corresponding to protons expected to be close to the 8-methyl if isomeric configurations are as described in Figure 1.

The resolved upfield and downfield portions of the 500-MHz $^1$H NMR spectrum of native beef ferrocytochrome $b_5$ is shown in Figure 2A. Figure 2B shows the corresponding spectrum of the beef protein freshly prepared by reconstitution of apoprotein with Fe–protoporphyrin IX. In both spectra, several resonances are shifted upfield from their random coil positions due to ring current shielding by the heme and aromatic amino acid side chains. The downfield region (8.5–10 ppm) of the native beef ferrocytochrome $b_5$ spectrum contains a number of exchangeable proton resonances not seen in the spectrum of the reconstituted protein. These protons exchange only slowly with solvent in the holoprotein, but exchange rapidly in the apoprotein. A number of nonexchangeable resonances also appear in this spectral region that have been previously assigned as heme meso protons. The heme methyl and vinyl resonances of the beef protein isomer A have also been previously assigned.[3] Table I summarizes the assignments for isomer A made previously and in the present effort. The lettering system for resonance identification in Table I is the same used in previous publications and is maintained for simple cross-reference with those works.[7]

Comparison of the native and freshly reconstituted beef ferrocytochrome $b_5$ spectra in Figure 2A and B reveals some differences. As one might expect, the ratios of integrations of the

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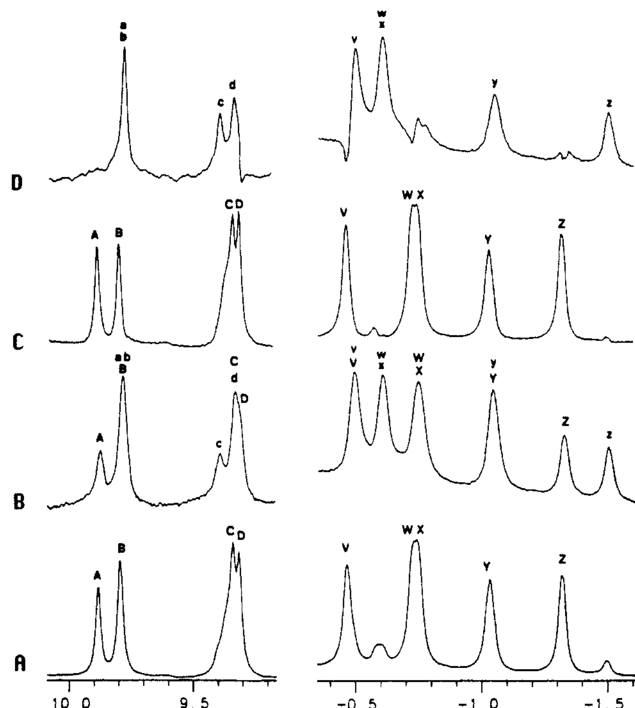


**Figure 2.** The resolved downfield and upfield resonances of the 500-MHz $^1$H NMR spectra of beef ferrocytochrome $b_5$ (0.1 M deuterated phosphate buffer, pH 7, 25 °C). (A) Native beef ferrocytochrome $b_5$, (B) disordered beef ferrocytochrome $b_5$ freshly reconstituted from the apoprotein, (C) computer-generated difference spectrum of isomer A of beef ferrocytochrome $b_5$, (D) computer-generated difference spectrum of isomer B of beef ferrocytochrome $b_5$. Labels refer to assignments in Tables I and II. Chemical shift axis is in ppm from DSS (referenced to the residual HDO resonance).

**Table I.** Assignments for Isomer A Resonances, Cytochrome $b_5$ (ppm)[a]

| assignment | beef | WT rat |
|---|---|---|
| α-meso (D) | 9.31 | 9.30 |
| β-meso (B) | 9.78 | 9.80 |
| γ-meso (C) | 9.33 | 9.36 |
| δ-meso (A) | 9.88 | 9.90 |
| 2-HC$_\alpha$ (F) | 7.43 | 7.42 |
| 2-HC$_\beta$(trans) (I) | 5.57 | 5.40 |
| 2-HC$_\beta$(cis) (H) | 5.51 | 5.07 |
| 2-HC$_\alpha$ (E) | 8.30 | 8.30 |
| 4-HC$_\beta$(trans) (G) | 5.90 | 6.04 |
| 4-HC$_\beta$(cis) | | 5.96 |
| 1-CH$_3$ (M) | 3.35 | 3.33 |
| 3-CH$_3$ (L) | 3.37 | 3.43 |
| 5-CH$_3$ (K) | 3.48 | 3.55 |
| 8-CH$_3$ (J) | 3.67 | 3.67 |
| L25 γ-CH (O) | 1.10 (1.63) | 1.31 |
| L25 δ$_2$-CH$_3$ (Q) | 0.75 (1.02) | 0.65 |
| L25 δ$_1$-CH$_3$ (V) | −0.39 (0.20) | −0.36 |
| L46 γ-CH (R) | 0.18 (−0.09) | 0.25 |
| L46 δ$_2$-CH$_3$ (W) | −0.63 (−0.52) | −0.65 |
| L46 δ$_1$-CH$_3$ (X) | −0.65 (−0.62) | −0.72 |
| V61 α-CH (N) | 3.18 (3.79) | 3.31 |
| V61 β-CH (S) | 0.15 (1.80) | 0.37 |
| V61 γ$_1$-CH$_3$ (P) | 0.80 (0.98) | 0.83 |
| V61 γ$_2$-CH$_3$ (Z) | −1.22 (−1.10) | −1.08 |
| I76 δ$_2$-CH$_3$ (Y) | −0.93 (−1.13) | −0.97 |

[a] Letters in parentheses refer to labels in Figure 1 and in refs 7 and 10. Numbers in parentheses are chemical shifts obtained from ring current calculations using X-ray crystal coordinates.[18]

peaks A–D, previously assigned to the isomer A heme meso protons,[3] to those of peaks a–d, assigned to the isomer B meso protons, are much larger in the native protein than in the freshly reconstituted material, reflecting the difference between the kinetic and equilibrium ratios of the two isomeric forms. Careful spectral subtraction of the kinetic and equilibrium spectra yields the spectra of the individual isomeric components of the beef protein (Figure 2C and D). Although the difference between the kinetic and equilibrium ratios in the bacterially expressed rat cytochrome $b_5$ is not as great (1:1 vs 3:2), a similar experiment permits assignment of resonances to the appropriate isomeric forms of the rat protein.

**Table II.** Assignments for Isomer B Resonances, Cytochrome $b_5$ (Chemical Shifts in ppm from DSS)[a]

| assignment | beef | WT rat |
|---|---|---|
| α-meso (d) | 9.33 | 9.47 |
| β-meso (b) | 9.79 | 9.73 |
| γ-meso (c) | 9.39 | 9.37 |
| δ-meso (a) | 9.79 | 9.73 |
| 2-HC$_\alpha$ (f) | 8.01 | 7.89 |
| 2-HC$_\beta$(trans) (h) | 6.23 | 6.23 |
| 2-HC$_\beta$(cis) | | 5.69 |
| 4-HC$_\alpha$ (e) | 8.19 | 8.37 |
| 4-HC$_\beta$(trans) (g) | | 4.56 |
| 4-HC$_\beta$(cis) | | 5.23 |
| 1-CH$_3$ (j) | 3.52 | 3.54 |
| 3-CH$_3$ (m) | 2.86 | 2.88 |
| 5-CH$_3$ (i) | 3.63 | 3.67 |
| 8-CH$_3$ (k) | 3.49 | 3.56 |
| L25 γ-CH | | 1.27 |
| L25 δ$_1$-CH$_3$ (w) | −0.49 | −0.51 |
| L25 δ$_2$-CH$_3$ | | 0.64 |
| L46 γ-CH | | 0.23 |
| L46 δ$_2$-CH$_3$ (v) | −0.39 | −0.79 |
| L46 δ$_1$-CH$_3$ (x) | −0.49 | −0.59 |
| V61 α-CH (l) | 3.12 | 3.25 |
| V61 β-CH (o) | −0.07 | 0.21 |
| V61 γ$_1$-CH$_3$ (n) | 0.75 | 0.85 |
| V61 γ$_2$-CH$_3$ (z) | −1.39 | −1.22 |
| I76 γ$_2$-CH | | 0.18 |
| I76 γ$_2$-CH′ | | 0.42 |
| I76 δ$_1$-CH$_3$ (y) | −0.93 | −0.93 |

[a] Letters in parentheses refer to labels in Figure 1 and in refs 7 and 10.

**Sequence-Specific Assignments. Beef Ferrocytochrome $b_5$.** Resolved amino acid side-chain $^1$H and heme substituent $^1$H resonances (except for heme propionate CH$_2$ protons) have been assigned previously for isomer A of beef ferrocytochrome $b_5$ (Table I).[3] Assignments were made by a combination of NOE, saturation transfer between the oxidized and reduced forms, ring current calculations, and spin-decoupling experiments. All of the previous assignments were made to porphyrin and methyl groups in the heme binding site, with one exception: Resonance Y was assigned to the δ-CH$_3$ of I76, its upfield position due to shielding by the indole ring of W22.

In order to assign the corresponding resonances in isomer B of the beef ferrocytochrome $b_5$, a series of steady-state 1-D NOE experiments were performed on the metastable freshly reconstituted protein. Some spectral overlap is observed; resonances W and X (isomer A) are degenerate, as are isomer B resonances w with x and v with V. Three nonexchangeable downfield resonances arising from heme meso protons (a, b, and B) are heavily overlapped. Nevertheless, all the heme proton resonances except those of 6- and 7-propionate protons could be identified in isomer B by comparison of patterns of NOEs observed upon saturation of resolved resonances in the reconstituted protein with those in the equilibrium mixture. Multiplet analysis, difference NOE patterns, and comparison with two-dimensional NMR data for the rat protein also permitted assignment of a number of amino acid side chains in the heme pocket of isomer B. Assignments for isomer B of beef ferrocytochrome $b_5$ are summarized in Table II. Detailed analyses of these difference NOE experiments are available as supplementary material.

**Bacterially Expressed Rat Ferrocytochrome $b_5$.** As noted above, the presence of both isomers of the rat ferrocytochrome $b_5$ in nearly equal quantities made it convenient to assign resonances in that protein by 2-D techniques (Figures 3 and 4). A number of

**Table III.** Resonance Assignments for Unresolved Spin Systems in WT Rat Ferrocytochrome $b_5$[a]

| | | |
|---|---|---|
| Y30 | A | $\delta$-H 7.21 (7.30), $\epsilon$-H 6.92 (6.93) |
| | B | $\delta$-H 7.14, $\epsilon$-H 6.87 |
| F35 | A | $\delta$-H 6.52 (6.85), $\epsilon$-H 6.22 (6.36), $\zeta$-H 6.97 (6.67) |
| | B | $\delta$-H 6.56, $\epsilon$-H 6.29, $\zeta$-H 7.13 |
| A54 | A | $\beta$-$CH_3$ 1.75 (1.79), $\alpha$-CH 5.1 (4.69) |
| | B | $\beta$-$CH_3$ 1.88, $\alpha$-CH 5.1 |
| F58[20] | A | $\epsilon$-H 5.2 (6.10), $\zeta$-H 5.53 (6.45) |
| | B | $\epsilon$-H 5.155, $\zeta$-H 5.545 |
| H63[20] | A | $\delta_2$-H 0.35 |
| | B | $\delta_2$-H 0.42 |
| Y74 | A | $\delta$-H 6.46 (6.62), $\epsilon$-H 7.21 (7.19) |
| | B | $\delta$-H 6.56, $\epsilon$-H 7.27 |
| W22[b] | I | $\zeta_2$-H 6.785, $\eta_2$-H 6.52, $\zeta_3$-H, 5.94, $\epsilon_3$-H 6.79 |
| | II | $\zeta_2$-H 6.765, $\eta_2$-H 6.50, $\zeta_3$-H 5.92, $\epsilon_3$-H 6.65 |

[a] Chemical shifts in ppm; values in parentheses are calculated chemical shift values from ring current shift calculations. [b] Although two sets of resonances are seen for W22, the two sets could not be unambiguously assigned to a particular isomeric form.

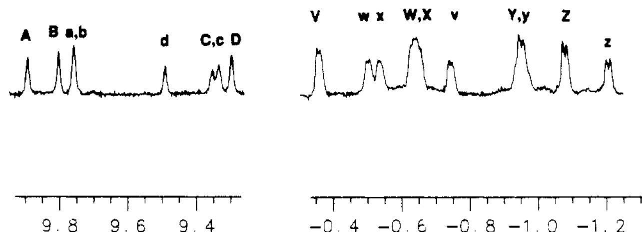


**Figure 3.** Upfield and downfield regions of 500-MHz $^1$H NMR spectrum (25 °C in 0.05 M deuterated phosphate buffer, pH 7) of bacterially expressed rat ferrocytochrome $b_5$. fid was multiplied by a 20° phase-shifted sine bell prior to transformation in order to accentuate multiplicities. Lables refer to resonance assignments in Tables I and II. Chemical shift axis is in ppm from DSS (referenced to the residual HDO resonance).

sequence-specific resonance assignments have been made based on spin-system connectivities and by comparison of observed NOEs with those predicted from the crystal structure.[14,15]

The assignments of heme $^1$H resonances in both isomeric forms of the rat protein could in many instances be made by direct comparison with beef assignments or logically, based on the porphyrin structure and observed NOEs. Once the heme substituent proton resonances were assigned, NOEs observed to these resonances, combined with COSY data, allowed assignment of a number of spin systems in the vicinity of the heme. Tables I and II summarize these assignments. Assignment of portions of the L46, L25, and V61 spin systems described previously for the beef protein[3] have been corroborated and extended by the two-dimensional experiments in the rat protein. A number of other unresolved amino acid spin systems have also been assigned in the rat protein by using two-dimensional data.

Two alanine spin systems are located within NOE distance of porphyrin substituents, A54 and A67. A54 is readily located by NOE connectivity with the 3-$CH_3$ and 4-$HC_\alpha$ vinyl (isomer A), 2-$HC_\beta$ vinyl (isomer B), and L25 $\delta_2$-$CH_3$ protons, as well as by close correspondence between calculated and experimental ring current shifts.[3,18] COSY data provide connectivity to the $\alpha$-proton of A54 for both isomers. Assignment of the A67 spin system is described in the supplementary material.

The assignments of many of the aromatic resonances of oxidized beef cytochrome $b_5$ have been published.[6] We have made corresponding assignments of aromatic resonances in the reduced rat cytochrome $b_5$, based on NOE and COSY connectivity. The absence of paramagnetic shifts in the reduced protein also permits the assignment of all of the aromatic resonances of F35 and some ring proton resonances of F58, neither of which could be assigned by two-dimensional methods in the oxidized protein because of proximity to the paramagnetic center.[6]

The spin system of W22 is readily identified by COSY connectivity and NOE to I76 $\delta$-$CH_3$. NOEs from L46 $\delta_1$-$CH_3$, $\delta$-meso, and 1-$CH_3$ (isomer A), and $\beta$-meso and 4-$HC_\alpha$ (isomer B) prompt assignment of a phenylalanine spin system to F35. NOEs from the 1-$CH_3$ and 4-$HC_\alpha$ and 4-$HC_\beta$ vinyl protons (isomer A) to a tyrosine spin system identify the aromatic resonances of Y74. NOEs from Y74 and L25 $\delta_2$-$CH_3$ in both isomers in turn identify another tyrosine side chain, Y30. Interestingly, we noted that the doubling effects of heme isomerism were apparent in high-resolution two-dimensional spectra of the rat protein in the spin system of the W22 indole, although this spin system is 18 Å from the heme iron in the crystal structure.

The ring proton resonances of F58 proved difficult to locate. Based on the crystal structure, NOEs from a number of assigned protons, including the L25 $\delta_1$- and $\delta_2$-$CH_3$s and heme 2-vinyl protons, should make identification of the F58 spin system straightforward. However, only weak NOEs are observed to the L25 $\delta_1$- and $\delta_2$-$CH_3$s from aromatic resonances other than Y30. Weak COSY cross peaks connect two of the resonances responsible for these NOEs, making them likely to be due to F58 ring protons. No resonance attributable to a third proton or set of protons for the F58 ring was located in either isomer. Examination of high-resolution one-dimensional $^1$H NMR spectra indicates that the resonances assigned to F58 are significantly broadened with respect to aromatic resonances assigned to residues more distant from the heme (these more extensive aromatic resonance assignments are listed in the supplementary material). Such broadening is also observed to a lesser degree for the F35 side-chain resonances. This may indicate that motion in the heme pocket is fairly restricted, and that ring flips of the F58 and F35 phenyl groups occur at an intermediate rate on the chemical shift time scale.[20]

**Structural Comparison of the Heme Binding Sites of Beef and Rat Cytochrome $b_5$s.** The binding of heme to apocytochrome $b_5$ is an easily studied example of prosthetic group recognition. Forces driving heme binding are considerable, including formation of two dative covalent Fe–N bonds, a hydrogen bond between a heme propionate and a serine residue (S64),[14] dispersion interactions between heme pocket side chains and porphyrin substituents, and favorable entropic changes upon desolvation of the porphyrin from aqueous solution. Superimposed on these large driving forces are the more subtle factors controlling heme orientation. These factors might be discerned by structural comparison of two proteins such as beef and rat cytochrome $b_5$s, in which the equilibrium ratios of the two binding orientations differ.

There is a high degree of sequence homology between the beef and rat cytochrome $b_5$s in the vicinity of the heme binding site, with only two variant residues, residue 23 (valine in the rat and leucine in beef) and residue 74 (tyrosine in the rat and phenylalanine in the beef protein). The F → Y replacement is relatively isosteric, although this does not preclude residue 74 from involvement in heme orientational equilibria. The $CH_3$ groups of L23 in the beef protein, however, are in van der Waals contact with the heme 3-$CH_3$ in isomer A (and the 2-vinyl group in isomer B) and form part of a structurally important hydrophobic "well"

(18) Ring current shift calculations were based on the crystal structure coordinates of the oxidized soluble fragment of beef cytochrome $b_5$ reported by F. S. Mathews and E. W. Czerwinski (revised 5/31/84, Brookhaven Crystallographic Database) and performed by using the SHIFTS program, kindly provided by Dr. Dave Case of the Research Institute of Scripps Clinic, La Jolla, CA. The Johnson–Bovey eight-loop model factor was used in calculations.

(19) Lee, K.-B.; La Mar, G. N.; Pochapsky, T. C.; Sligar, S. G., unpublished results.

(20) Recently, a nearly complete set of resonance assignments for the A form of reduced beef cytochrome $b_5$ has been published: Guiles, R. D.; Altman, J.; Kuntz, I. D.; Waskell, L. *Biochemistry* **1990**, *29*, 1276–1289. We note that our assignments agree with those of Guiles et al. with the exception of the assignment of F58. We were unable to verify the assignment described by Guiles in our own spectra. Furthermore, significant upfield ring current shifts are expected to the F58 ring if the crystal structure is accurate, and these are not observed in the assignment of Guiles et al. We also note that our assignment of the H63 $\delta_2$ imidazole proton was made solely on the basis of NOE data and was not verified by COSY data. However, the agreement between our assignment and that of Guiles et al. for this resonance prompts our inclusion of it in Table III.

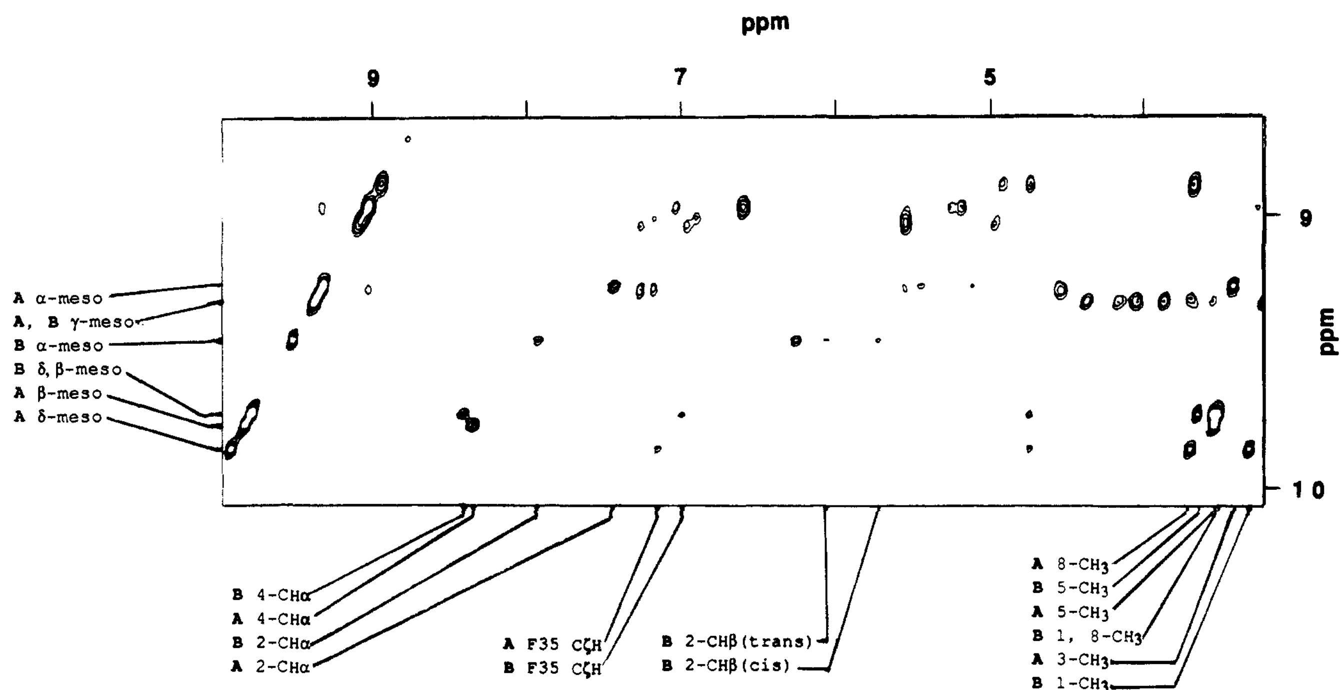


**Figure 4.** Portion of 500-MHz phase-sensitive NOESY ($\tau_m$ = 200 ms, 25 °C in 0.05 M deuterated phosphate buffer, pH 7) of bacterially expressed rat ferrocytochrome $b_5$. Shown are cross peaks to the heme meso protons in both isomers. Chemical shift axes are in ppm from DSS (referenced to the residual HDO resonance).

in the heme binding site made up of the side-chain methyl groups of residues 23, L25, L46, and A54. Extensive $^1$H NMR studies of the kinetically labile oxidized forms of the beef and rat proteins indicate a number of steric interactions between the closely packed methyls of this pocket and the heme 2-vinyl group in isomer B, which might disfavor this heme binding orientation.[12] Mathews noted that in the more populated isomer A the sterically larger 2-vinyl group fits into a cavity near the surface of the protein.[15]

Further insight into the differences of equilibrium binding orientation ratios between the beef and rat proteins can be gained by comparing chemical shift and NOE data between the two reduced species in both isomeric forms. Table I reveals little chemical shift difference between isomer A resonances of the rat and beef proteins. NOE patterns in beef and rat isomer A are also quite similar, and solely on the basis of these considerations, it is not possible to discern the resonances corresponding to the methyls of the 23 residue (which have been identified in the oxidized protein).[19] On the other hand, some resonances in isomer B show significant chemical shift differences between the beef and rat proteins. Chemical shifts for the L46 methyl protons, nearly degenerate in isomer A of both proteins, become nondegenerate in isomer B, with a splitting between $\delta_1$-$CH_3$ and $\delta_2$-$CH_3$ of 0.1 ppm in the beef and 0.2 ppm in the WT rat protein. The L46 $\delta$-$CH_3$s in isomer B of the rat protein are significantly more ring-current shifted than the corresponding resonances in the beef isomer B. Note that the L46 $\delta$-$CH_3$s are elements of the "well" noted above and, as such, might be expected to exhibit cross relaxation with the heme subsituent occupying that well (3-$CH_3$ in isomer A, 2-vinyl in isomer B). Furthermore, if differences in degree of steric interaction between the 2-vinyl group and the elements of the hydrophobic "well" are important in determining the differences in equilibrium ratios of the two isomeric forms between beef and rat, these differences should be reflected in the observed NOEs to the 2-vinyl spin system in isomer B of the two proteins. In the rat isomer B, large NOEs are observed between the 2-$HC_\beta$ (trans) proton and the $\alpha$-meso, with substantial NOEs observed to the 2-$HC_\alpha$ proton from the $\alpha$-meso. From L46 $\delta_2$-$CH_3$, a significant NOE is observed to the 2-$HC_\beta$(trans) resonance, with only very small effects at the 2-$HC_\alpha$ and 2-$HC_\beta$(cis). From L25 $\delta_1$-$CH_3$, there are moderate effects observed to both of the 2-$HC_\beta$ protons, but only small effects at the 2-$HC_\alpha$. The observed effects describe a 2-vinyl orientation as shown in Figure 5A for the rat isomer B.

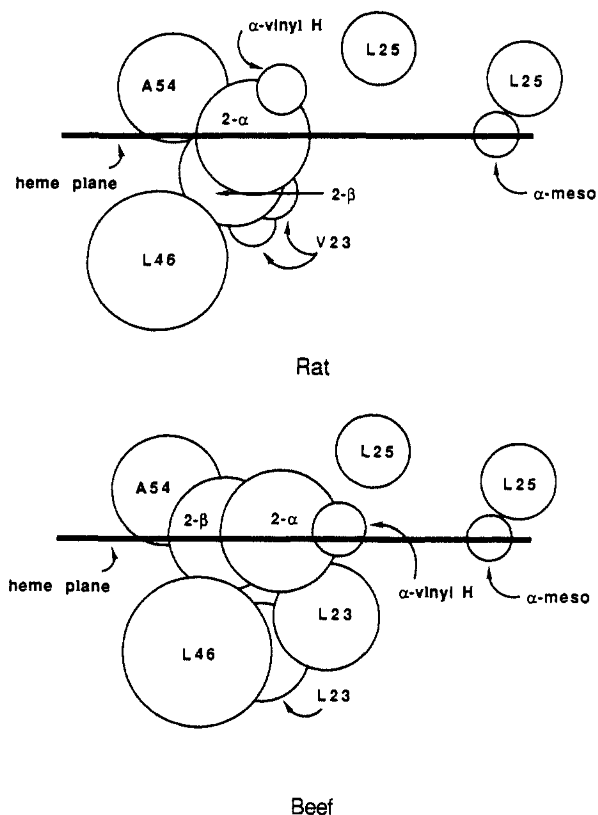


**Figure 5.** Heme 2-vinyl orientations in isomer B of beef and rat proteins. Orientations of the vinyl groups are based on intensities of NOEs observed between L46 $\delta_2$-$CH_3$, L25 $\delta_1$-$CH_3$, 2$\alpha$- and 2-$\beta$-vinyl, and $\alpha$-meso protons as described in the text. The more open pocket environment provided by the rat protein (A), in which residue 23 is a valine, permits the 2-vinyl group to be more readily accommodated than the beef protein (B), in which steric interaction with the L23 $CH_3$s forces a less favorable orientation of the 2-vinyl group in-plane with the porphyrin ring.

In isomer B of the beef protein, saturation of the resonance assigned to L46 $\delta_2$-$CH_3$ does not give rise to NOEs at the 2-$HC_\beta$(trans). Saturation of the $\alpha$-meso resonance gives a large effect at the 2-$HC_\alpha$ vinyl proton and a small effect at the 2-

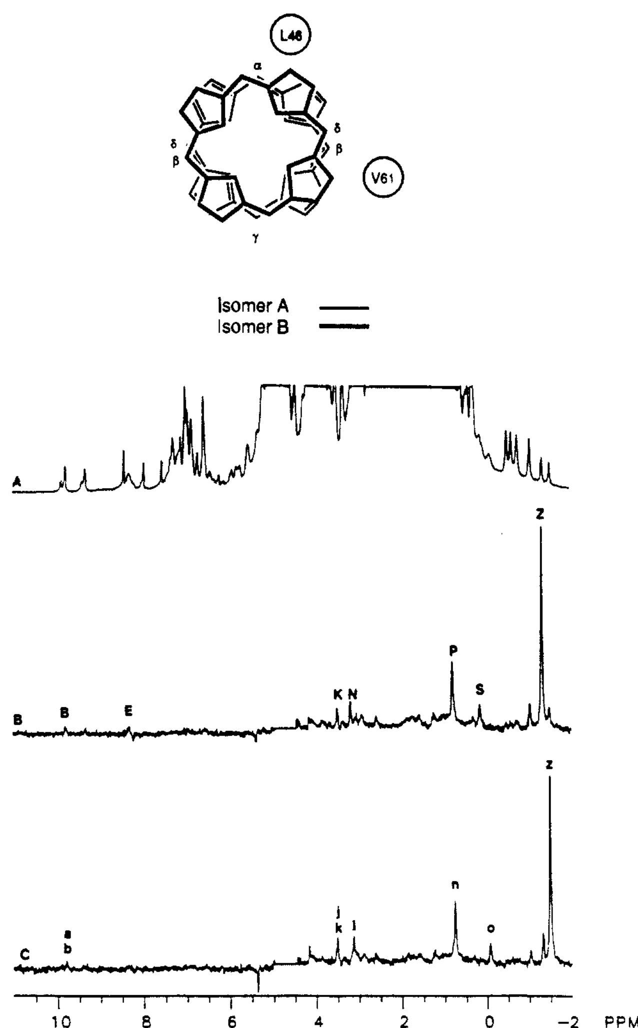


**Figure 6.** Proposed radial displacement of the heme about the normal axis in isomer B relative to A. The displacement is proposed based on comparison of NOEs observed between heme methyl and meso protons and the V61 $\gamma_1$-$CH_3$ in the two isomers of beef ferrocytochrome $b_5$ (see text). Also shown are $^1H$ NOE difference spectra of a kinetic mixture of isomers of reconstituted beef ferrocytochrome $b_5$ at pH 7 and 25 °C obtained by saturation of the V61 $\gamma_1$-$CH_3$ resonances in both isomers. (A) Reference spectrum. (B) Steady-state NOE spectrum following saturation of the previously assigned V61 $\gamma_1$-$CH_3$ (Z). NOEs are observed to 5-$CH_3$ (K), V61 $\alpha$-CH (N), V61 $\gamma_1$-$CH_3$ (P), and V61 $\beta$-CH (S) and smaller NOES to the heme $\beta$-meso-H (B) and 4-$\alpha$ vinyl (E). (C) Steady-state NOE difference spectrum following saturation of the minor peak z gives rise to NOEs at peak n, V61 $\gamma_2$-$CH_3$ (isomer B). Other NOEs observed are at V61 a-CH (l), V61 $\gamma_1$-$CH_3$ (n), and V61 $\beta$-CH (o) additional NOEs are observed to the heme protons, the composite meso proton peak b, $\delta$-meso-H (a,b), and the heme 1- and 8-$CH_3$s (j,k). The NOE observed to peak i,j is ascribed primarily to the 8-$CH_3$, since the decreased NOE to the $\delta$-meso H is inconsistent with an increased NOE to the 1-$CH_3$ (all relative to NOEs observed in isomer A).

$HC_\beta$(trans). Based on this information, the differences in the vicinity of the 2-vinyl group of isomer B of the beef and rat protein are related to the planarity of the heme 2-vinyl group. By use of molecular graphics, and maintaining all crystal structure coordinates except those for the side chain of residue 23, the substitution of a valine for a leucine at this site in the beef protein creates a more open site into which the vinyl of the porphyrin fits, as shown in Figure 5B. Porphyrin vinyl orientations shown are those predicted to be most populated by observed NOEs.

Other insights into structural differences between the isomeric forms are obtained by comparison of NOEs observed to the V61 $\gamma_2$-$CH_3$ in both isomers. Based on intraresidue NOEs from V61 $\gamma_2$-$CH_3$ to the $\beta$-CH and $\alpha$-CH in both isomers of the beef protein, there is little or no conformational change for the V61 side chain upon going from isomer A to isomer B in either protein. Differences in the chemical shift of V61 $\gamma_2$-$CH_3$ between isomer A to isomer B might be due to a displacement of the pophyrin ring itself, resulting in an alteration of ring current shielding experienced by V61 $\gamma_2$-$CH_3$. The differences in chemical shift must be interpreted with care, since ring current shifts are directional, and displacement in one direction made lead to a very different shift than the same displacement in another direction. However, there is an increase in the NOE observed from the V61 $\gamma_2$-$CH_3$ to the heme 8-$CH_3$ in beef isomer B with respect to that observed to the 5-$CH_3$ resonance in isomer A. Simultaneously, a decrease is seen for the NOE observed to the heme meso proton ($\delta$-meso in isomer B, $\beta$-meso in isomer A) from the V61 $\gamma_2$-$CH_3$ in isomer B relative to isomer A (Figure 6). In the absence of different dynamics of the two isomers leading to different relaxation times for corresponding nuclei, these changes are interpreted to indicate a slight counterclockwise rotational displacement of the heme with respect to its normal axis upon going from isomer A to isomer B as viewed from the distal face (Figure 6). It may be that in order to accommodate the more planar 2-vinyl group in isomer A of the beef protein, the heme itself is forced to shift. This is in line with observations in the ferric protein that the side chains of the heme binding site are quite closely packed, and it is the porphyrin, rather than the amino acid side chains in the pocket, that must move to accommodate steric interactions.[19] A full analysis of this heme rotational displacement will require the use of a complete NOE buildup data set.

The present studies clearly indicate that amino acid side-chain positions in the heme binding sites differ only slightly between the two orientations of the heme in either the rat or beef cytochrome $b_5$s. The difference in free energy between the two isomeric forms is evidently too small in either protein to perturb significantly the equilibrium packing of the side chains in the heme binding site. Rather, it is the prosthetic group itself that must accommodate to the topology of the binding site by changes in vinyl conformation and perhaps by angular displacement about the heme normal. This result is perhaps not surprising in light of the evident close packing of the heme pocket amino acid side chains. The heme pocket is not static [$k_f = 3.0 \times 10^{-5}$ $s^{-1}$ (25 °C) for heme reorientation in beef ferrocytochrome $b_5$[10]], but slow isomeric interconversion, coupled with the broad resonances observed for F35 and F58, indicates that the heme pocket is fairly close packed in reduced cytochrome $b_5$. Side-chain motions are likely to be highly cooperative, and equilibrium positions well-defined. Considerable reorganization would be required for even relatively small displacements. Clearly, the free energy difference between the two isomeric forms is insufficient to cause such reorganization in either the beef or rat protein.

A converse observation is that it appears that only one kinetically approachable minimum energy conformation (or more exactly, ensemble of microstates) of the heme pocket suffices to bind both orientations of the porphyrin, and that this minimum is not dependent on specific interaction with heme substituents for its definition (although the presence of the macrocycle might be required). A vital question to be answered is whether or not the topology of the heme binding site is largely defined prior to heme binding, or if the equilibrium packing of side chains in the binding site is reached as part of the binding process. If the latter is true, then heme binding is more properly considered as a final stage in protein folding. Current efforts in these and other laboratories are directed toward answering these questions.[21]

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**Supplementary Material Available:** Summary of NOEs for beef and rat ferrocytochromes $b_5$ (cyt $b_5$), table of chemical shift assignments for rat cyt $b_5$, $^1H$ NMR spectra of beef cyt $b_5$, and NOESY and COSY spectra of rat cyt $b_5$ (19 pages). Ordering information is given on any current masthead page.

(21) LeComte, J. T. J.; Moore, C. D. *Biochemistry* **1990**, *29*, 1984–1989.