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^{1}H NMR Study of the Influence of Hydrophobic Contacts on Protein–Prosthetic Group Recognition in Bovine and Rat Ferricytochrome b_5†

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ABSTRACT: The proton nuclear magnetic resonance spectra of the soluble fragment of native bovine and genetically engineered wild-type rat ferricytochrome b_5 reconstituted with a wide variety of hemes chemically modified at 2- and/or 4-positions have been recorded and analyzed. While all but one nonsymmetric heme yielded comparable amounts of the two heme orientations immediately after reconstitution, the relative proportion of the two orientations at equilibrium varied widely. The unpaired spin density distribution in the heme π system leads to substituent hyperfine shift patterns in these paramagnetic complexes that are completely diagnostic of the heme orientation in the protein matrix. An empirical assignment strategy is outlined and applied which allows unequivocal assignment of the absolute orientation of a derivatized heme within the protein matrix. Using a series of hemes lacking 2-fold symmetry solely due to a single substitution, the preferences for localized site occupation of vinyls, methyls, and hydrogens are developed. The large differences in relative stability of the two orientations of native protohemin in the two cytochromes b_5 is shown to result from the additivity of localized effects for the bovine protein and the near cancellation of competing effects in the rat protein. The major determinant of the heme orientation is judged to be a repulsive interaction between a vinyl and a hydrophobic cluster of amino acids including positions 23 and 25. The differences in this heme orientational preference among bovine, rat, and chicken ferricytochromes b_5 could be correlated with the relative steric bulk of the residues at positions 23 and 25. Detailed thermodynamic analysis of the orientational preferences of native protoheme reveals that, while the same orientation as found in X-ray crystal structures of bovine cytochrome b_5 predominate at 25 °C in both proteins, the preference in the bovine protein is primarily for enthalpic reasons while in the rat protein the preference is due to entropic factors.

Cytochrome b_5 is a membrane-bound electron transport protein involved in fatty acid desaturation and the cytochrome P-450 reductase system (Strittmatter et al., 1974). A soluble fragment is found as a component of the hemoglobin reductase system which is essentially identical with the tryptically cleaved microsomal protein (Hultquist et al., 1984). The soluble fragment of the bovine protein has been subjected to the most extensive studies, with details of the protein folding presented in high-resolution X-ray crystal structure of both oxidized and reduced forms (Mathews et al., 1971, 1979; Mathews, 1980). ^{1}H NMR[1] studies have provided information on the solution molecular, electronic, and magnetic properties of the protein that directly demonstrated that the protein in solution is an interconvertible heterogeneous mixture of two isomers (ratio ~ 9:1) that differ by a 180° rotation of the heme about the α,γ-meso axis, as illustrated in parts A and B of Figure 1 (Keller & Wüthrich, 1980; La Mar et al., 1981; McLachlan et al., 1986a,b). The dominant isomer in solution (Figure 1A) is the only one detected in the crystal. The two isomeric forms detectably differ in redox potential as a result of the altered peripheral interaction with the protein matrix (Walker et al., 1988). Preliminary solution ^{1}H NMR spectra of the bacterially expressed soluble fragments of rat microsomal cytochrome b_5 indicated very similar spectral parameters, including two components, but with much more similar relative stability at equilibrium (Rogers et al., 1986).

We are interested in delineating both the factors that control the heme orientational preference in the native bovine protein and the structural basis for the significantly altered preference in the rat versus bovine proteins. Preliminary ^{1}H NMR studies with a limited number of synthetic hemins with modified substituents at the 2- and 4-positions (Figures 1A,B), reconstituted into rabbit cytochrome b_5 with NMR properties in-

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[1] Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; NOE, nuclear Overhauser effect; ppm, parts per million.

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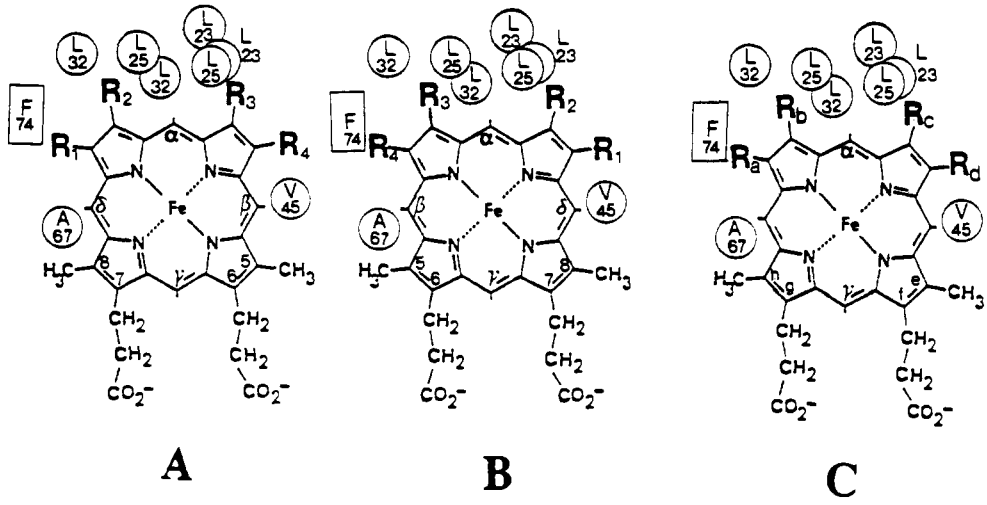

FIGURE 1: (A) Structure and labeling system of protohemin IX (**1**) with the heme orientation defined relative to the labeled amino acid residues of beef cytochrome b_5 heme cavity as found in the crystal structure. The circles shown correspond to methyl groups selected solely to uniquely identify the orientation of the heme; the square correponds to the approximate location of the aromatic ring of residue 74. (B) The heme orientation rotated 180° about the α,γ-meso axis. (C) New labeling system for hemin derivatives defined by the heme position related to specific amino acid residues irrespective of the heme orientation or substitution. The variable amino acids are as follows: for bovine, Leu 23, Leu 25, Leu 32, Phe 74; for rat, Val 23, Leu 25, Leu 32, Tyr 74; for chicken, Ile 23, Val 25, Ile 32, Phe 74.

distinguishable from those of the bovine protein, had suggested that the orientational preference for isomer A in the bovine protein is the result of steric destabilization of a vinyl at the position usually occupied by 1-methyl group in the crystal orientation (La Mar et al., 1981). However, the alternate orientation for these three hemes, deuterohemin (**2**; $R_1 = R_3 = CH_3$, $R_2 = R_4 = H$ in Figure 1A,B), pemptohemin (**3**; $R_1 = R_3 = CH_3$, $R_2 = H$, $R_4 = V$), and isopemptohemin (**4**; $R_1 = R_3 = CH_3$, $R_2 = V$, $R_4 = H$), always involves the permutative change of two substituents. In order to ascertain the localized contribution to orientational preference, it is necessary to compare the preference for altered orientation that interchanges only one pair of different substituents. To this end, we have synthesized the hemins **5**–**8**, which lack the true 2-fold rotational symmetry by solely pairwise differences of methyl versus vinyl (**5**, **6**) and methyl versus hydrogen (**7**, **8**). Determination of the relative stabilities for these four hemins **5**–**8** should provide details of the localized contributions to the heme–protein contacts that determine the preference for less symmetric hemes such as native protohemin IX (**1**).

The proposed elucidation of the factors determining heme orientation not only should be viewed as a means for understanding the detailed structure of cytochrome b_5 but can serve also as a case study of prosthetic group recognition that may mimic many of the factors that control substrate recognition by the active site of enzymes. The advantage in the present case is that both of the components are very readily subjected to systematic perturbation, the hemin by total synthesis (Smith et al., 1983) and the polypeptide chain by both selection among natural genetic variants (La Mar et al., 1981; McLachlan et al., 1986; Rogers et al., 1986) or, in the future, by site-directed mutagenesis (Bodman et al., 1986). The advantage of the study of the hemin as prosthetic groups is that the "substrate" is relatively rigid, eliminating the need for consideration of conformational change of substrate due to altered interaction with the protein matrix. Our ultimate goal is to delineate the structural consequences of the alternate protein–hemin contacts in the two orientations that must result in the differential redox properties (Walker et al., 1988).

The reconstitution of apocytochrome b_5 with hemin initially yields both orientation A and B in Figure 1, with the heme reorienting slowly to reach the equilibrium ratio of ~9 for the oxidized bovine protein. The two orientations are most readily recognized for paramagnetic ($S = {}^1/_2$), ferricytochrome b_5, where the large in-plane asymmetry in unpaired spin delocalization imparts characteristic large contact shifts to substituents on the basis of their location in the protein matrix (La Mar et al., 1981). The difference in shifts, on the other hand, originates from a complex mixture of scalar and dipolar hyperfine interactions with the unpaired spin distribution (La Mar, 1979; Satterlee, 1985) and is not readily interpreted in terms of detailed molecular structure. The reduced, diamagnetic, ferrocytochrome b_5 exhibits extraordinarily slow reorientation rates (McLachlan et al., 1986a,b) and displays relatively poorly resolved sets of signals for the alternate orientations, making it difficult to quantitate the thermodynamic properties of the heme disorder. On the other hand, chemical shift differences between alternate heme orientations and between different protein chains for the same heme orientation are readily interpreted in terms of the changed ring current shifts of amino acid residues near the heme (Keller & Wüthrich, 1972; Wüthrich, 1986). Hence we pursue a 2-fold program; herein we characterize the orientational preference and its thermodynamic basis for hemes in the labile, paramagnetic ferricytochrome b_5, and elsewhere we analyze the structural consequences of these alternate orientations in diamagnetic ferrocytochrome b_5.

The orientation for a given hemin can be determined on very limited samples of reconstituted proteins if the hyperfine-shifted signals can be assigned to their positions on the heme. In the past this was done by isotope labeling and detailed one- and two-dimensional nuclear Overhauser effect (NOE) studies (Keller & Wüthrich, 1980, 1981; La Mar et al., 1981; McLachlan et al., 1986a,b, 1988). Comprehensive isotope labeling on the present derivatized hemins is simply not practical, and limited samples preclude the lengthy NOE studies for many of our hemes. Hence we initially focus on the development of an empirical strategy for determination of the heme orientation for a given species that should have general utility for a variety of other low-spin ferric hemoproteins. This strategy relies on the characterization of chemical shifts for various functional groups at all relevant heme positions in the protein matrix and, hence, benefits from a number of symmetric hemes such as 2,4-dimethyldeutero-

hemin (**9**; $R_1 = R_2 = R_3 = R_4 = CH_3$); protohemin III (**10**; $R_1 = R_4 = CH_3$, $R_2 = R_3 =$ vinyl), and protohemin XIII (**11**; $R_1 = R_4 =$ vinyl, $R_2 = R_3 = CH_3$).

Experimental Procedures

Sample Preparation. Trypsin-solubilized beef and chicken cytochrome b_5 were isolated from fresh calf and chicken liver and purified by standard methods to an optical purity index (A_{412}/A_{280}) of ~5.8 (Reid & Mauk, 1982; McLachlan, 1989). The rat protein was made by expression of a plasmid-containing synthetic gene coding for the soluble domain of rat cytochrome b_5 in a bacterial host (Bodman et al., 1986) and was purified in a similar fashion. The purified rat protein used in this study had an optical purity index of 5.6. Bovine and rat apoproteins were prepared according to reported procedures (Teale, 1959; Reid et al., 1984). The modified hemins and a few selectively deuterated hemes used in this study were synthesized chemically as described previously (Smith & Kehres, 1983; Evans, 1977). Each apoprotein was reconstituted in situ by adding a stoichiometric amount of hemin dissolved in 0.1 M NaO^2H to a 0.4-mL solution of 0.5–1 mM apoprotein in 0.1 M deuterated phosphate buffer of pH ~7.5. Only 2,4-dimethyldeuterohemin among the modified hemins used in this study was insufficiently soluble in alkaline aqueous medium; therefore, 50% aqueous pyridine was used to dissolve the 2,4-dimethyldeuterohemin (La Mar et al., 1986), and a reconstituted holoprotein was immediately passed through Sephadex G-75 column to remove the organic component and lyophilized. The samples were finally concentrated to ~0.5–1 mM solution in ^2H_2O. 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 pH of all samples was controlled to ~7.5 by adding ^2HCl or NaO^2H as necessary. In order to achieve equilibration of the heme orientation for the native proteins at each temperature (5, 15, 25, and 35 °C), the samples were incubated for varying periods of time in a constant-temperature bath (Model KT33, Hakke).

^1H NMR Methods. ^1H NMR spectra were recorded at 25 °C on a Nicolet NT-500 spectrometer operating in the quadrature mode at 500 MHz. Data were collected (~2000 transients) by using double precision on 16 384 data points over a ±15-kHz sweep width at 0.8 s^{-1} repetition time. Chemical shifts are referenced to the residual water resonance, which, in turn, was calibrated against internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The signal-to-noise ratio was improved by apodization of the free induction decay which introduced a 10–20-Hz line broadening. The time-evolution spectra of each sample were recorded until the ^1H NMR spectra exhibited no further changes over a week at 25 °C and were assumed to be at equilibrium with respect to the heme orientation. The ratios for the two isomers were measured from the ratio of peak areas via computer simulation of the optimally resolved peaks; the uncertainties are ±10%.

The assignment of individual methyls for the various hemes not already available were confirmed by their diagnostic NOE patterns; i.e., irradiation of M_a (or m_a) gives NOEs to Phe 74 (or Tyr 74) and Leu 70 $C_{\delta 2}$-H_3. Saturation of M_b (or m_b) gives rise to the characteristic NOE patterns to Leu 32 $C_{\delta 1}$-H_3, Leu 32 $C_{\delta 2}$-H_3, Leu 25 $C_{\delta 2}$-H_3, and α-meso-H. Saturation of M_c (or m_c) gives NOEs to Leu 32 $C_{\delta 1}$-H_3, Leu 25 $C_{\delta 1}$-H_3, Leu 25 $C_{\delta 2}$-H_3, Leu 46 $C_{\delta 2}$-H_3, and α-meso-H. Saturation of M_e (or m_e) gives a specific NOE pattern to Leu 45 $C_{\gamma 1}$-CH_3, β-meso-H, and H_fs of the heme. These NOEs are consistent with the known single-crystal structure for the heme periphery of bovine cytochrome b_5 shown in Figure 1A and have been observed in its native protein (Kelle & Wüthrich, 1980; McLachlan et al., 1988).

In order to measure the temperature dependence of the ratio of the two isomers in the native proteins, spectra were collected for samples equilibrated at 5–35 °C in 10 °C intervals. The spectra for all equilibrated samples were collected at 5 °C to allow more quantitative determination of change in the equilibration constant by simply measuring the change in peak height ratios for the "quenched" reaction. It was observed that negligible reorientation took place at 5 °C during the ^1H NMR measuring time (~30 min.)

Results

The ^1H NMR spectra of the nonsymmetric hemins **1–8** all yield two sets of peaks of comparable intensity immediately after reconstitution. In each case, the ratio of intensities of the two sets changed with time, reaching equilibrium within 2–72 h. No attempt was made to quantify the rates of equilibration, with the emphasis solely on ensuring that equilibrium was reached. The two sets of peaks are readily identified by constant relative intensities within a set and are labeled M_i, V_i, and H_i, and m_i, v_i and h_i for the orientation as in A and B of Figure 1 for methyls, vinyls, and pyrrole hydrogens, respectively. The equilibrium ^1H NMR spectra of the bovine and rat proteins with native protohemin (**1**) are illustrated in Figures 2A,A′; the assignments for both isomers have been reported for the bovine protein on the basis of isotope labeling and 1D and 2D NOE studies (La Mar et al., 1981; McLachlan et al., 1986a,b, 1988). For the rat protein, essentially identical assignments are readily obtained by analogy with the bovine protein, as confirmed by NOE patterns. The ratios of major (A) to minor (B) components differ significantly between the two cytochromes, with 8.9 for the bovine but only 1.6 for the rat protein. Reconstitution with hemins **9** ($R_1 = R_2 = R_3 = R_4 = CH_3$), **10** ($R_1 = R_4 = CH_3$, $R_2 = R_3 =$ vinyl), and **11** ($R_1 = R_4 =$ vinyl, $R_2 = R_3 = CH_3$) yields only a homogeneous protein for both cytochromes b_5, as illustrated in panels B,B′, C,C′, and D,D′ of Figure 2, as is expected for these three symmetric hemins.

The equilibrium ^1H NMR traces for bovine and rat cytochrome b_5 reconstituted with hemins **2** ($R_1 = R_3 = CH_3$, $R_2 = R_4 = H$), **3** ($R_1 = R_3 = CH_3$, $R_2 = H$, $R_4 =$ vinyl), **4** ($R_1 = R_3 = CH_3$, $R_2 =$ vinyl, $R_4 = H$), and **12** [$R_1 = R_3 = CH_3$, $R_2 = R_4 = CH{=}C(CH_3)_2$] are illustrated in panels A,A′, B,B′, C,C′, and D,D′ of Figure 3, respectively. All but hemin **12** exhibit two sets of equally intense peaks intially, for which one set largely disappears with time for hemins **2** and **4**, with some ~25% of the minor form still populated at equilibrium for **3**. The equilibrium properties of these complexes have been reported previously for rabbit cytochrome b_5, whose ^1H NMR traces are essentially indistinguishable from those of the bovine protein. The dominant heme orientation of the rabbit protein with each of these hemins had been deduced to be the same as for natural protohemin IX, and hence we can conclude that orientation A dominates for both bovine and rat cytochrome b_5.

The ^1H NMR spectra of bovine and rat cytochrome b_5 reconstituted with hemins **5** and **6** are illustrated in Figure 4, both immediately after reconstitution (A,A′, C,C′) and at equilibrium (B,B′, D,D′). In each case the intially equally intense set of lines change relative intensity only slightly at equilibrium, nevertheless sufficiently to allow ready identification of the two sets of peaks M_i,V_i and m_i,v_i; in all four cases there is appreciable population of both isomers at equilibrium. The equilibrium ^1H NMR spectra for bovine and rat cytochrome b_5 reconstituted with hemins **7** and **8** are shown in

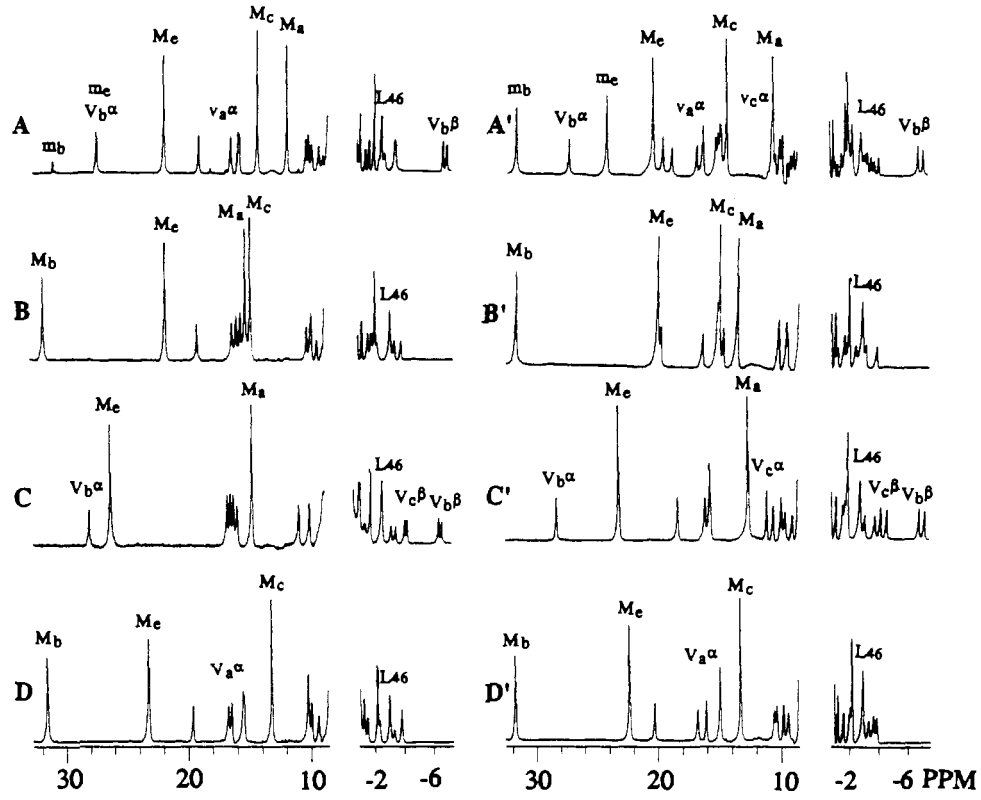

FIGURE 2: Hyperfine-shifted portions of the 500-MHz ^{1}H NMR spectra of cytochrome b_5 in ^2H_2O, 25 °C. (A) Native bovine cytochrome b_5, pH 6.90. (A′) "Native" wild-type rat cytochrome b_5, pH 7.41. (B) Bovine cytochrome b_5 reconstituted with 2,4-dimethyldeuterohemin (**9**), pH 7.59. (B′) Rat cytochrome b_5 reconstituted with 2,4-dimethyldeuterohemin (**9**), pH 7.54. (C) Bovine cytochrome b_5 reconstituted with protohemin III (**10**), pH 7.70. (C′) Rat cytochrome b_5 reconstituted with protohemin III (**10**), pH 7.75. (D) Bovine cytochrome b_5 reconstituted with protohemin XIII (**11**), pH 7.62. (D′) Rat cytochrome b_5 reconstituted with protohemin XIII (**11**), pH 7.67. Two sets of peaks represent methyl and vinyl peaks of the heme in the A and B orientations, respectively. The subscripts indicate the position of the heme side chain in the protein matrix as defined in Figure 1C.

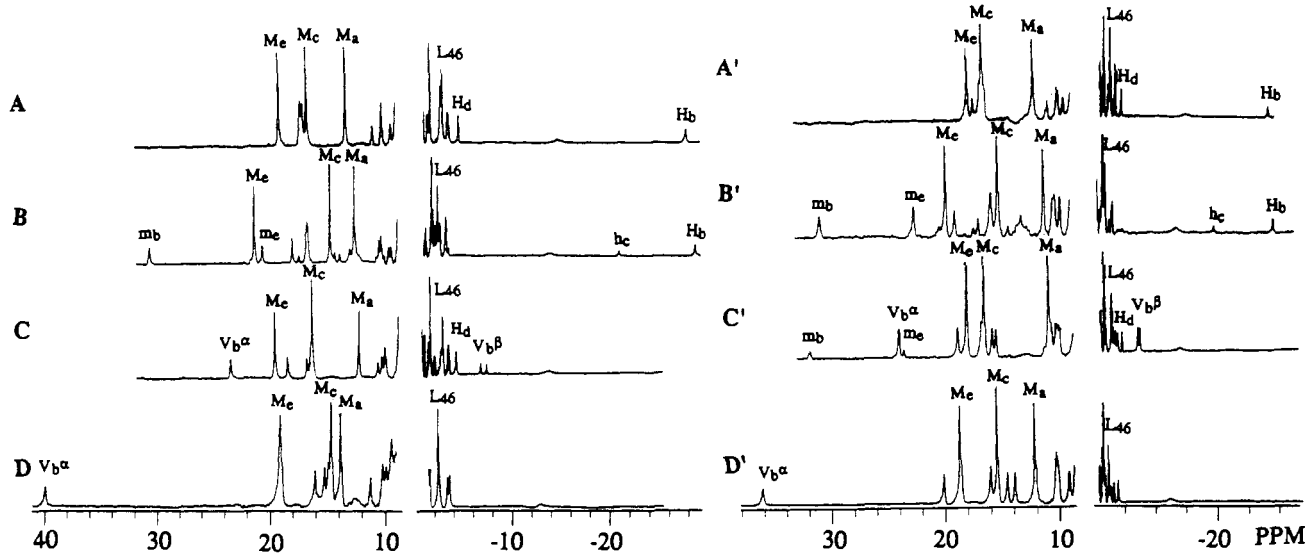

FIGURE 3: Hyperfine-shifted portions of the 500-MHz ^{1}H NMR spectra of equilibrated cytochrome b_5 reconstituted with deuterohemin (**2**), pemptohemin (**3**), isopemptohemin (**4**), and 2,4-diisobutenyldeuterohemin, (**12**) in ^2H_2O, 25 °C. (A) Bovine cytochrome b_5 reconstituted with deuterohemin, pH 8.0. (A′) Rat cytochrome b_5 reconstituted with deuterohemin, pH 7.29. (B) Bovine cytochrome b_5 reconstituted with pemptohemin, pH 7.25. (B′) Rat cytochrome b_5 reconstituted with pemptohemin, pH 7.10. (C) Bovine cytochrome b_5 reconstituted with isopemptohemin, pH 7.21. (C′) Rat cytochrome b_5 reconstituted with isopemptohemin, pH 7.10. (D) Bovine cytochrome b_5 reconstituted with 2,4-diisobutenyldeuterohemin, pH 7.41. (D′) Rat cytochrome b_5 reconstituted with 2,4-diisobutenyldeuterohemin, pH 7.41. The peak labels indicate methyl, vinyl, and pyrrole hydrogen peaks of the heme in A and B heme orientations, respectively. The subscripts indicate the position of the heme side chain in the protein matrix as defined in Figure 1C.

panels A,A′ and B,B′ of Figure 5; the methyl and pyrrole-hydrogen peaks for the two compounds are again labeled M_i, H_i and m_i, h_i.

The equilibrium constants and the free energy differences at 25 °C for each hemin in each protein were determined by the ratio of the intensities of well-resolved methyl peaks for the two isomers, for the reaction m_i, v_i, $h_i \rightleftharpoons M_i$, V_i, H_i, which will be shown (see below) to correspond to the equilibrium for the structure B $\rightleftharpoons$ A in Figure 1, i.e., the equilibrium for forming the dominant heme orientation as found in the X-ray structure (A in Figure 1). These equilibrium constants for the nonsymmetric hemins at 25 °C are listed in Table I. The

Table I: Structures of Modified Hemins and Thermodynamic Parameters for Their Orientational Preference in Cytochrome b_5

		substituents[b]				bovine		rat	
compd no.[a]	hemin	R_1	R_2	R_3	R_4	K_{eq}[c]	ΔG[d]	K_{eq}[c]	ΔG[d]
1	protohemin IX	M	V	M	V	8.9	−1.30 (−1.2)[e]	1.6	−0.29 (−0.05)
2	deuterohemin	M	H	M	H	>30	<−2.0 (−1.3)	>30	<−2.0 (−1.6)
3	pemptohemin	M	H	M	V	3.3	−0.71 (−0.56)	2.1	−0.45 (−0.21)
4	isopemptohemin	M	V	M	H	>30	<−2.0 (−1.9)	15	−1.6 (−1.4)
5	2-methyl-4-vinyldeuterohemin	M	M	M	V	2.1	−0.43	0.78	+0.14
6	2-vinyl-4-methyldeuterohemin	M	V	M	M	3.5	−0.75	1.4	−0.20
7	2-methyldeuterohemin	M	M	M	H	7.6	−1.2	7.6	−1.2
8	4-methyldeuterohemin	M	H	M	M	1.2	−0.13	1.8	−0.36
9	2,4-dimethyldeuterohemin	M	M	M	M				
10	protohemin III	M	V	V	M				
11	protohemin XIII	V	M	M	V				
12	2,4-diisobutenyldeuterohemin	M	B	M	B	>50	<−2.3	>50	<−2.3

[a] Compound numbers used in text for individual hemins. [b] Substituent as numbered in parts A and B of Figure 1; with M = methyl, V = vinyl, H = hydrogen, B = isobutenyl [CH=C(CH$_3$)$_2$]. [c] Equilibrium constant, K_{eq}, obtained from equilibrium ratio of methyl intensity, M_i/m_i, for reaction B → A. [d] Free energy for reaction B → A, obtained from $\Delta G = -RT \ln K_{eq}$ at 25 °C. [e] ΔG obtained by adding ΔGs of single substituents.

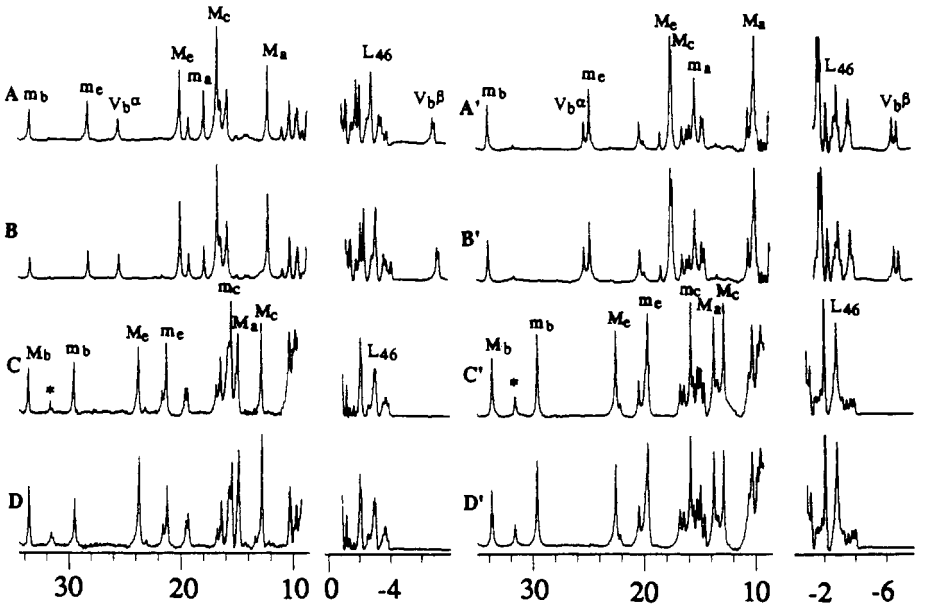

FIGURE 4: Hyperfine-shifted regions of the 500-MHz ^{1}H NMR spectra of cytochrome b_5 substituted with 2-vinyl-4-methyldeuterohemin (**6**) and 2-methyl-4-vinyldeuterohemin (**5**) in ^2H_2O, 25 °C. (A) Bovine cytochrome b_5 taken immediately after reconstitution of 2-vinyl-4-methyldeuterohemin, pH 7.38. (B) Sample A at equilibrium state. (A′) Rat cytochrome b_5 taken immediately after reconstitution of 2-vinyl-4-methyldeuterohemin, pH 7.55. (B′) Sample A′ at equilibrium state. (C) Bovine cytochrome b_5 taken immediately after reconstitution of 2-methyl-4-vinyldeuterohemin, pH 7.34. (D) Sample C at equilibrium state. (C′) Rat cytochrome b_5 taken immediately after reconstitution of 2-methyl-4-vinyldeuterohemin, pH 7.53. (D′) Sample C′ at equilibrium state. The subscripts indicate the position of the heme side chain in the protein matrix as defined in Figure 1C. (*) indicates the signal from impurity.

equilibrium constants for B → A for native bovine and rat cytochromes b_5 were determined in 10 °C intervals over the temperature range 5–35 °C; K_{eq} increased for the bovine and decreased for the rat protein as the temperature was raised. The van't Hoff plots illustrated in Figure 6 are consistent with straight lines with the following properties: $\Delta G^{298} = -1.30 \pm 0.18$ kcal/mol, $\Delta H = -1.38 \pm 0.30$ kcal/mol, $\Delta S = -0.3 \pm 0.7$ eu for the bovine, and $\Delta G^{298} = -0.29 \pm 0.04$ kcal/mol, $\Delta H = 2.36 \pm 0.39$ kcal/mol, $\Delta S = 8.9 \pm 3.9$ eu for the rat protein. It is noted that the data indicate some curvature, suggesting that ΔH may be temperature dependent. For our analysis, however, the key conclusion is the obviously different sign of the slopes for the two plots that dictates that the relative stabilities of the two orientations are inverted between the bovine and rat protein.

Discussion

Heme Orientation. The elucidation of the heme orientation could be effected on the basis of either isotope labeling or detailed NOE studies (La Mar et al., 1981, 1986; McLachlan et al., 1988; Keller & Wüthrich, 1980). However, deuterated derivatives are not available for most of these hemins. Instead, we pursue here an empirical assignment strategy that yields the identity of the orientations and use NOE studies solely to confirm those assignments as needed to determine orientation. This strategy relies on the characteristic unpaired spin distribution in a heme caused by the rhombic perturbations due primarily to the axial His bonding. This resulting heme contact shift pattern is, in a sense, independent of the heme substituents and reflects solely the position of a heme substituent relative to this perturbation in the protein matrix. This suggests that the magnitude of contact shift reflects the position of a substituent in the protein matrix and not on the particular heme. Therefore, we designate positions a–h on a general heme in the cytochrome b_5 pocket, as shown in part C of Figure 1. For the dominant crystal orientation (A in Figure 1), the positions a → h are occupied by heme position 1 → 8 (Mathews, 1980), while in the reversed orientation (B in Figure 1) a → d are occupied by 4 → 1 and e → h are occupied by 8 → 5.

In native protohemin IX (**1**) definitive assignments show that the contact shifts, and hence spin densities, are largest

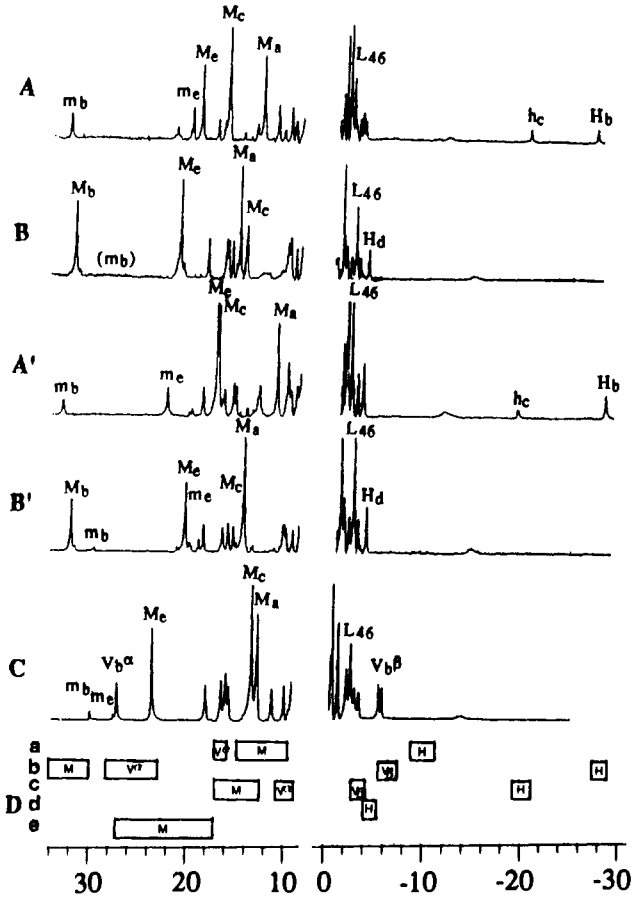

FIGURE 5: Hyperfine-shifted portions of the 500-MHz ^{1}H NMR spectra of cytochrome b_5 reconstituted with 4-methyldeuterohemin (**8**) and 2-methyldeuterohemin (**7**) in ^2H_2O, 25 °C. (A) Bovine cytochrome b_5 reconstituted with 4-methyldeuterohemin at equilibrium state, pH 7.31. (B) Bovine cytochrome b_5 reconstituted with 2-methyldeuterohemin (~50% of 3-CH_3 is perdeuterated) at equilibrium state, pH 7.56. The signal m_b is only shown in the spectrum taken immediately after reconstitution. (A') Rat cytochrome b_5 reconstituted with 4-methyldeuterohemin at equilibrium state, pH 7.36. (B') Rat cytochrome b_5 reconstituted with 2-methyldeuterohemin (~50% of 3-CH_3 is perdeuterated) at equilibrium state, pH 7.91. (C) Native chicken cytochrome b_5, pH 7.36. The peak labeling is followed as done in Figure 2 and 3. (D) The chemical shift distribution of the hyperfine-shifted region which heme side chains of cytochrome b_5 reconstituted with diversely modified hemins resonate. (a, b, c, d, and e represent the position of heme side chain relative to the labeled amino acids; M, V$^{\alpha}$, V$^{\beta}$, and H stand for methyl, vinyl α-proton, vinyl β-proton, and single proton, respectively.)

for protein positions b (2-V$^{\alpha}$, 28 ppm) and e (5-CH_3, 22 ppm), negligible (within diamagnetic envelope) for positions d (4-V$^{\alpha}$, 5 ppm) and h (8-CH_3, 3 ppm), and intermediate (just downfield of diamagnetic envelope) for positions a (1-CH_3, 11 ppm) and c (3-CH_3, 14 ppm) for the orientation as in part A of Figure 1. Consistent with this model, the reversed orientation (B in Figure 1) yields the largest shifts similarly for positions b (3-CH_3, 32 ppm) and e (8-CH_3, 26 ppm). Thus we expect low-field resolved methyl peaks (M_i, m_i) only for positions b, e (shifts 28–32 ppm) and a, c (10–15 ppm), and hence *the number of resolved methyls alone provides direct information on the* orientation *for a species* if the two orientations predict a different number of methyls occupying the positions a, b, c, and e.

The vinyl group provides additional indicators, since large low-field V$^{\alpha}$ and high-field V$^{\beta}$s occur only for position b (2-V$^{\alpha}$ 28 ppm, 2-V$^{\beta}$s −7 ppm for hemin **1** in orientation A), with intermediate shifts for position a (4-V$^{\alpha}$ 17 ppm for hemin **1** in orientation B in Figure 1). Lastly, when available, the pyrrole-H provides a diagnostic probe with very large upfield

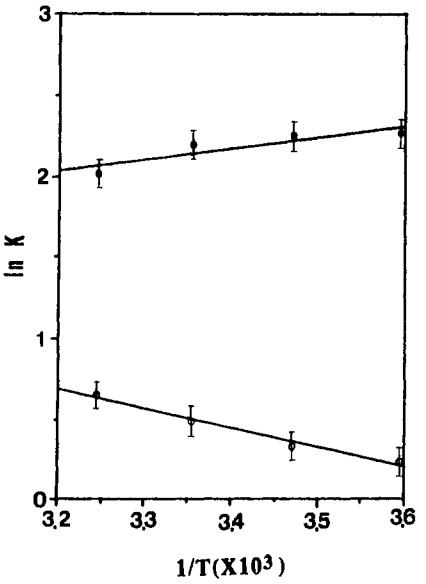

FIGURE 6: van't Hoff plot of native bovine (●) and rat (○) cytochrome b_5 for heme reorientation process (B $\overset{K}{\rightarrow}$ A) in the heme cavity.

shifts for position b (2-H at −29 ppm for orientation A in Figure 1 for hemins **2**, **3**, and **8**), less for position c (2-H at −21 ppm for orientation B for hemins **2**, **3**, and **8**), intermediate for position a (4-H ~−10 ppm) in orientation B for hemins **2**, **4**, and **7**, and minimal for position d (4-H at −5 ppm in orientation A for **2**, **4**, and **7**). These empirical rules lead to direct and unambiguous determination of orientation in every case. In order to independently confirm these conclusions, we have isotope labeled methyls in a few instances where practical and confirmed individual methyl assignment by the NOE pattern that is completely diagnostic for a given position of the heme in the protein matrix, as discussed previously (McLachlan et al., 1988).

Validation for the empirical approach is provided by a series of symmetric hemes for which rotational disorder is not possible and hence forces the select occupation of each methyl or vinyl in each of positions a–d in the protein matrix. For 2,4-dimethyldeuterohemin (**9**; panel B,B′ in Figure 2), the 2-vinyl H_{α} (V_b^{α} at 28 ppm) of the major orientation of protohemin is replaced by M_b at 31 ppm and the expected four methyls from positions a, b, c, and e are observed. In the case of protohemin III (**10**) only two methyls are resolved (M_e, M_a), since M_c is replaced by V_c^{α} when compared to protohemin IX in orientation A. Protohemin XIII (**11**; panel D,D′ in Figure 2) interchanges methyl and vinyl on pyrrole I and hence introduces M_b and V_a^{α}, but loses M_a and V_b^{α} as compared to protohemin IX in Figure 2A,A′. The resonance positions for the two orientations for the previously characterized deuterohemin (**2**), pemptohemin (**3**), and isopemptohemin (**4**) (panels A,A′, B,B′, C,C′ in Figure 3, respectively) are completely consistent with this pattern and dictate that the major orientation is as in part A of Figure 1 for each case. For the unusual hemin with 2,4-diisobutenyl groups, **12** (panel D,D′ in Figure 3), the 2-vinyl H_{α} is much further downfield than in protohemin IX, but otherwise the shift pattern is similar to that for the major isomer in panel A,A′ of Figure 2.

For 2-vinyl-4-methyldeuterohemin (**6**), the empirical rules predict three resolved methyls for both orientations A (a, b, e) and B (a, c, e) as observed. However, only orientation B can account for the low-field vinyl V_b^{α} peak ~25 ppm and upfield V$^{\beta}$s at −7 ppm. Hence both bovine and rat protein favor orientation A, indicating a clear preference for a vinyl in position b rather than c. In the case of 2-methyl-4-vinyl-

deuterohemin (**5**), orientation A predicts four methyls (a, b, c, and e) and no resolved vinyl V^{α}, while orientation B requires only three resolved methyls (b, c, e). The bovine protein favors orientation A (Figure 4C,D), but the rat protein actually has the reversed orientation B slightly more populated (Figure 4C′,D′). Hence there are only small preferences between a vinyl and a methyl for positions b and c, and they are in opposite direction in the two proteins.

The preference of methyl versus hydrogen is illustrated by the ^{1}H NMR spectra in Figure 5. For 4-methyldeuterohemin (**8**; panel A,A′ in Figure 5), orientation A predicts three resolved methyls (a, c, e) and a H_b at −29 ppm, while orientation B predicts a similar three methyls (a, b, e), but only a −20 ppm shift for h_c. The different upfield shifts, as well as the individual methyl shift pattern, clearly reveal the major isomer (60–80%) in both cases to be isomer A. The related heme, 2-methyldeuteroheme (**7**), predicts four resolved methyls (a, b, c, e) and three methyls (b, c, e) for orientations A and B, respectively. The observed methyl shift pattern (as well as the absence of a H_a peak of −10 ppm) for both bovine and rat protein (B,B′ in Figure 5) reveals overwhelmingly a single set of detectable lines that reflect heme orientation A in both cases. The individual methyls at positions a, b, c, and e for the various hemins were assigned by their characteristic NOE pattern to the protein matrix (not shown) and, in two cases, confirmed by methyl deuteration [peak M_c = 3-CH_3 of protoheme IX in rat cytochrome b_5 in Figure 2A′; and M_c = 3-CH_3 for 2-methyl-4-vinyldeuterohemin (**5**), Figure 4D,D′].

Thus it is clear that the contact shift pattern, as reflected by the essentially invariant spin density distribution of the heme that is controlled by protein-based rhombic perturbation, allows the determination of heme orientation in any protein for which a sufficient number of the peripheral substituents are resolved. To date this has been shown possible as well for cyanometmyoglobin complexes (La Mar et al., 1986, 1989). The only requirement for the general applicability of such an empirical assignment strategy is that the variable heme substituents do not perturb the mode of axial bonding of the heme to the protein. The resulting range of chemical shifts for these methyl, vinyl, and pyrrole-hydrogen substituents as a function of individual positions in the protein matrix is diagrammed in panel D of Figure 5 and should serve as a useful guide to rapid determination of orientation of other chemically modified hemins in cytochrome b_5 or for native protohemin IX in cytochrome b_5 mutants. The range of shifts for each position arises largely because, in addition to the large and dominant protein-based rhombic perturbations, the variable chemical functionalization at the periphery exerts its own small but definite asymmetry on the unpaired spin distribution, as clearly manifested by the spread in the four methyl contact shifts in model compound with axially symmetric ligands (Viscio & La Mar, 1978). The assignment strategy outlined above will work for any system and hemin where the asymmetry induced by functionalization of the heme periphery is much smaller than that induced by rhombic protein interactions. The slightly different shift patterns for bovine versus rat cytochrome with the same hemin in the same orientation (as seen most clearly for the three symmetric hemins in Figure 2) must reflect small differences in the peripheral contacts due to the substitution near the heme binding site (bovine → rat, Phe 74 → Tyr, Leu 23 → Val).

Thermodynamics of Heme Disorder. The preference for native protohemin IX (**1**) for orientation A over B, as found in both crystal and solution, was originally interpreted to reflect a steric destabilization of the 4-vinyl group at position a in the bovine protein, based on comparison among the hemins **1**–**4** (La Mar et al., 1981). This evaluation was based on the premise that primarily steric effects determine orientation. The problems in this previous analysis of these hemins was that the alternate heme orientations always interchanged two pairs of substituents. The current, more detailed data suggest a much more complicated picture. We consider initially only the bovine protein data in Table I. Comparison of K_{eq} and ΔG for competition of single vinyl versus methyl occupation of the same protein site, using hemins **5** and **6**, reveals that the a versus d or 1 versus 4 position has less preference (K = 2.1) than the b versus c (2 versus 3) position (K = 3.5). However, individually, each preference contributes to the dominant population of orientation A for the bovine protein. Interesting is the finding that the methyl versus hydrogen preference is the same as for a vinyl [H strongly prefers positions d versus a for **7** (K = 7.6)], while H slightly prefers position b versus c for **8** ($K \sim 1.2$). Hence, simple steric effects alone do not determine the heme orientation in cytochromes b_5. The individual preference of H versus CH_3 and vinyl versus CH_3 for the four above hemins, **5**–**8**, leads to reasonable estimates for the preference for orientation for those hemins involving pairs of permutations [i.e., ΔG(**1**) ~ ΔG(**5**) + ΔG(**6**); ΔG(**3**) ~ ΔG(**8**) + ΔG(**5**); ΔG(**4**) ~ ΔG(**7**) + ΔG(**6**)], indicating that the free energies for orientational preference are approximately additive. The values predicted on the basis of the additivity for ΔGs for the four hemins involving a single interchange of substituents, **5**–**8**, are given in parentheses in Table I. The linear free energy additivity works well for the systems possessing a vinyl, but much less well in the absence of vinyl [i.e., ΔG(**2**) < ΔG(**7**) + ΔG(**8**)].

For the rat protein, the vinyl versus methyl preference is smaller than for bovine for position b versus c (2 versus 3) and is *reversed from that of the bovine protein for position a* versus *d* (1 versus 4). Thus the a versus d and b versus c position preferences largely cancel to account for the small preference between the two orientations for native protohemin IX in the rat protein. Again, the free energies for the preference for individual vinyl positions add well to predict the preferences for protohemin IX. The preferences for H versus methyl for the rat protein, on the other hand, are very similar to those for the bovine protein (see Table I for hemins **2**, **7**, and **8**). The additivity of the ΔGs again works well when the hemin bears at least one vinyl, and not too well in the absence of the vinyl (compare experimental ΔG versus predicted in parentheses in Table I).

The difficulty in rationalizing these preferences, as expressed solely in K_{eq} or ΔG at 25 °C, on the basis of simple steric interactions for the substrate (hemin) interacting with the binding cavity is evidenced from the detailed analyses in the preferential binding free energy for native protohemin IX (**1**). Lowering the temperature decreases the amount of the reversed orientation (B) for bovine cytochrome b_5, while it increases it for the rat protein. This is borne out in opposite signs for the apparent enthalpy for the reaction (Figure 6). In the bovine protein, orientation A is enthalpically favored by 1.4 kcal/mol, while for the rat protein, the reversed orientation B is enthalpically favored by 2.4 kcal/mol, but orientation A dominates slightly because of apparent large entropic favor. The large amount of sample, lengthy NMR measurement time, and limited stability of most reconstituted cytochromes b_5 precluded obtaining similarly detailed thermodynamic analyses for the modified hemins. We conclude that the interpretation of changes in orientational preferences among the different hemins cannot be simply carried out on a structural

Table II: Chemical Shifts of the Resolved Heme Substituents and Amino Acid Residue Resonances for the Bovine and Rat Cytochrome b_5 Reconstituted with Chemically Modified Hemins in ^2H_2O Solution, 25 °C

		heme orientation A							heme orientation B						
hemin	protein	pH	1-*R*	2-*R*	3-*R*	4-*R*	5-CH_3	H 39[a]	L 25[b]	L 46[c]	1-*R*	2-*R*	3-*R*	4-*R*	8-CH_3
1	beef	6.90	11.5	27.4	14.4	5.4[d]	21.8	16.4	−2.1	−2.6			31.0	16.7[d]	27.5
	rat	7.41	10.7	27.4	14.4	5.1[d]	20.4	16.3	−2.2	−2.5	−0.6	10.8	31.8	16.8[d]	24.3
	chicken	7.36	12.6	25.1	13.2	4.8[d]	23.5	15.6	−1.6	−2.9			29.9		27.5
2	beef	8.00	12.9	−28.3	16.3	−4.9	18.7	16.7	−1.9	−3.2					
	rat	7.29	11.9	−28.8	16.3	−4.8	17.6	16.5	−2.0	−3.0					
3	beef	7.25	12.3	−29.2	14.4		20.0	16.4	−2.1	−2.8		−21.5	30.4		20.4
	rat	7.10	10.9	−29.2	14.9		19.4	16.6	−2.3	−2.6		−20.4	30.6		22.2
4	beef	7.21	11.9	23.2[d]	16.0	−4.5	19.3	16.5	−1.8	−3.1		30.4	−11.5		27.5
	rat	7.10	10.6	23.8[d]	16.2	−4.6	17.7	16.5	−1.8	−2.8		30.6	−9.5		23.3
5	beef	7.34	14.7	33.6	12.6		23.6	16.2	−2.1	−3.1		15.3	29.4		21.0
	rat	7.53	13.6	33.7	12.7		22.4	16.6	−2.2	−2.9		15.6	29.6		19.5
6	beef	7.38	12.0	25.3[d]	16.5		19.8	17.7	−2.0	−2.8			33.4	17.7	28.1
	rat	7.55	9.8	25.2[d]	17.2		17.3	16.3	−2.5	−3.2			33.9	15.2	24.7
7	beef	7.56	15.0	32.0	15.3	−4.6	21.1	16.4	−1.9	−3.3			29.7	−10.1	20.8
	rat	7.91	14.2	32.0	14.3	−4.5	20.2	16.3	−1.9	−3.2			29.6	−9.2	18.8
8	beef	7.31	12.9	−27.9	16.4		19.1	16.8	−2.9	−2.8		−21.1	32.7		20.1
	rat	7.36	11.0	−28.8	17.0		17.2	16.4	−2.2	−2.7		−19.9	33.0		22.2
9	beef	7.54	15.0	31.7	14.6	1.2	21.6	16.1	−2.1	−3.2					
	rat	7.54	13.4	31.7	14.8	1.4	19.9	16.2	−2.2	−3.0					
10	beef	7.70	14.7	28.1[d]	8.7[d]	−0.2	26.3	15.9	−1.9	−2.6					
	rat	7.75	12.5	28.3[d]	11.0[d]	0.2	23.1	16.0	−2.0	−2.8					
11	beef	7.62	16.3[d]	31.5	13.1	4.3[d]	23.1	16.6	−2.2	−3.0					
	rat	7.67	16.0[d]	31.8	13.2	4.5[d]	22.2	16.2	−2.2	−3.0					
12	beef	7.41	13.6	37.7[e]	16.6		18.5	15.1	−1.3	−2.4					
	rat	7.41	12.0	36.1[e]	15.2		18.4	15.8	−1.5	−2.2					

[a] His 39 $C_{\beta 1}H$. [b] Leu 25 $C_{\beta 1}H_3$. [c] Leu 46 $C_{\beta 2}H_3$. [d] The heme vinyl α-proton. [e] The heme isobutenyl α-proton.

basis (which would assume primarily enthalpic differences), and in cases where enthalpy differences dominate, it is not likely that simple steric influences determine orientation.

Protohemin IX Orientational Preferences. For the bovine complex with protohemin, ΔH favors orientation A by 1.4 kcal/mol while the rat protein favors orientation B by 2.4 kcal/mol. Thus the relative stabilities of the two orientations differ by ~3.8 kcal/mol in the two proteins. The amino acid sequence for the two proteins are highly homologous (Mathews et al., 1971) and involve only two substitutions near the heme on going from bovine → rat cytochrome b_5, Phe 74 → Tyr and Leu 23 → Val. Phe 74 is near pyrrole I (Mathews et al., 1971); it does not make van der Waals contact with 1-CH_3 in the crystal structure of bovine cytochrome b_5 but would for a vinyl at that position. Leu 23 makes van der Waals contact with 3-CH_3 in the crystal structure and would interact more strongly with a vinyl at that position. Hence it is likely that this latter interaction, which is reduced significantly in the rat protein by substitution of a smaller Val (and hence more readily accommodates a vinyl group at position c), accounts for the increased enthalpic stability of orientation B relative to A in the rat versus the bovine protein.

The difference in ΔS for the two proteins is more difficult to rationalize but may originate in differential internal immobilization of vinyls, water access to vacancies created by the differences in molecular volumes of substituents, etc. Moreover, over the limited accessible temperature, it is not ever possible to differentiate between entropic contributions to the differences in K_{eq} or simply temperature-dependent enthalpic effects. The lack of detailed thermodynamic data for the orientational preference of deuterohemin (**2**) implies it is risky to speculate on the basis for the similar preference for vinyl and hydrogen versus methyl in the protein matrix.

The likely steric destabilization for occupation of bulky side chains at position b is also supported by the essentially unique orientation for both proteins found for hemin **12** (panel D,D′ in Figure 3) with the large isobutenyl group solely at positions b and d. The alternate orientation with these groups at positions a and c in the protein matrix could not even be detected as a metastable intermediate immediately after reconstitution, as found for each case of hemins **1**–**8**.

For protohemin IX, however, our analysis clearly points to steric influence on the orientational preferences which have at least a large portion of their origin in the repulsive interaction between a vinyl and the hydrophobic cluster near position c. In order to see if this structural hypothesis can be supported by a further perturbation of the polypeptide chains, we isolated chicken cytochrome b_5 which, in comparison to bovine cytochrome b_5, has the substitutions in the heme cavity Leu 23 → Ile, Leu 25 → Val, and Leu 32 → Ile (Mathews et al., 1971), which suggest that the steric crowding near position c might be even larger than for the bovine protein, hence further destabilizing a vinyl group at position c. The ^1H NMR spectrum of chicken cytochrome b_5 is included in Figure 5C, which reveals two sets of peaks, M_i and m_i, with shift patterns very close to those for heme orientations A and B of both bovine and rat protein, respectively, but with orientation A more strongly preferred, with $K \sim 20$ and $\Delta G = \sim -1.8$ kcal/mol. This indicates that at least a major determinant of the heme orientation in cytochrome b_5 is the steric destabilization of large (vinyl) substituents at position c (Figure 1C) in the protein matrix. Parallel ^1H NMR studies on solely structural consequences of alternate heme orientations in bovine and rat reduced diamagnetic cytochrome b_5 support such steric interactions between a vinyl at position c and the hydrophobic cluster Val 23 and 25 and moreover show that it is the detailed porphyrin binding geometry, rather than the protein matrix, that relaxes to accommodate the alternate heme orientations (Pochapsky et al., 1990).

Some support for a localized differential perturbation near position c between bovine and rat cytochrome b_5 can be inferred from the heme methyl shift pattern. The three assigned amino acid peaks, His 39 $C_{\beta 1}$-H, Leu 25 $C_{\delta 1}$-H3, and Leu 46 $C_{\delta 2}$-H_3, exhibit essentially the same shift for both proteins containing all twelve hemins (Table II), reflecting the largely invariant magnetic properties of the iron center. Among the

three symmetric hemins, 2,4-dimethyldeuterohemin (**9**), protohemin III (**10**), and protohemin XIII (**11**) (Figure 2 and Table II), the one that exhibits the largest differences in heme methyl contact shift patterns between bovine and rat cytochrome b_5 is protohemin III, the only one that places a vinyl at position c. A more datailed interpretation of the shifts must await the development of a quantitative basis for the interpretation of the hyperfine shifts.

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Topography of the Membrane-Binding Domain of Cytochrome b_5 in Lipids by Fourier-Transform Infrared Spectroscopy†

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ABSTRACT: Fourier-transform infrared spectroscopy was used to examine the secondary structure of the membrane-binding domain (nonpolar peptide) of rabbit liver cytochrome b_5 in D_2O and in the presence of phospholipids and deoxycholate. In all situations, the predominant structure was α helix, but an examination of the components of the amide I band in the spectrum of the nonpolar peptide showed that the major peak was shifted from 1655 cm^{-1} in the lipids to 1650 cm^{-1} in deoxycholate. This shift to lower frequency, together with a decrease in intensity of the amide II band, is indicative of N–H to N–D exchange of the peptide backbone. A semiquantitative analysis indicated that the α helix of the peptide is over 95% exchanged in the presence of deoxycholate but is only 10% exchanged in the presence of lipid. These data suggest that the membrane-inserted portion of the peptide is α helical and is largely protected from N–H to N–D exchange by the bilayer. We suggest that this technique appears to provide a general method for determining the type of secondary structure involved in membrane interaction and the percentage of this structure which is involved in the interaction.

Cytochrome b_5, an amphipathic integral membrane protein found in the endoplasmic reticulum, has been a popular subject for model membrane studies. The protein has two distinct domains: a hydrophilic heme-containing domain and a hydrophobic membrane-binding domain (nonpolar peptide, NPP).[1] The structure of the former, in isolation, has been

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