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Assignment of the Ferriheme Resonances of the High-Spin Forms of Nitrophorins 1 and 4 by ^{1}H NMR Spectroscopy: Comparison to Structural Data Obtained from X-ray Crystallography

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In this work, we report the assignment of the majority of the ferriheme resonances of high-spin nitrophorins (NPs) 1 and 4 and compare them to those of NP2, published previously. It is found that the structures of the ferriheme complexes of NP1 and NP4, in terms of the orientation of the histidine imidazole ligand, can be described with good accuracy by NMR techniques and that the angle plot proposed previously for the high-spin form of the NPs (Shokhireva, T. Kh.; Shokhirev, N. V.; Walker, F. A. *Biochemistry* **2003**, *42*, 679–693) describes the angle of the effective nodal plane of the axial histidine imidazole in solution. There is an equilibrium between the two heme orientations (**A** and **B**), which depends on the heme cavity shape, which can be altered by mutation of amino acids with side chains (phenyl vs tyrosyl) near the potential position where a heme vinyl group would be in one of the isomers. The **A**:**B** ratio can be much more accurately measured by NMR spectroscopy than by X-ray crystallography.

Introduction

The nitrophorins (NPs; nitro = NO; phorin = carrier) are a group of nitric oxide (NO)-carrying heme proteins found in the saliva of at least two species of blood-sucking insects, *Rhodnius prolixus*, the "kissing bug", which has four such proteins in the adult insect[1−5] and at least three additional NPs in the earlier stages of development,[6,7] and *Cimex lectularius*, the bedbug, which has only one NP.[8,9] These interesting heme proteins sequester NO that is produced by a NO synthase (NOS) that is similar to vertebrate constitutive NOS, which is present in the cells of the salivary glands.[10−12] NO is kept stable for long periods of time by binding it as an axial ligand to a ferriheme center.[1,3] Upon injection into the tissues of the victim, NO dissociates and diffuses through the tissues to the nearby capillaries to cause vasodilation and thereby to allow more blood to be transported to the site of the wound. At the same time, histamine, whose role is to cause swelling, itching, and initiation of the immune response, is released by mast cells and platelets of the victim. In the case of the *Rhodnius* proteins, this histamine binds to the heme iron site of each of the NPs, hence preventing the insect's detection for a period of time.[13]

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These two properties of the NPs of *R. prolixus* contribute to the transmission of the protozoan *Trypanasoma cruzi*, the vector of Chagas' disease,[14] to the victim, via the feces of the insect, which are left behind at the site of the bite[3] following the extended feeding time.

The *Rhodnius* proteins of the adult insect, which have been named NP1−NP4 in order of their abundances in the insect saliva, have been investigated by a number of techniques[1,3,15,16−30] including spectroelectrochemistry,[15,17−19] IR[15] and resonance Raman,[16] NMR,[15,18,20] electron paramagnetic resonance (EPR),[15,21] and Mössbauer spectroscopies,[22] and stopped-flow photometry.[17,23] The solid-state structures of one or more ligand complex of NP1,[15,24] NP2,[25,26] and NP4[27−32] have been determined by X-ray crystallography. The structures are unique for heme proteins in that the heme is located at the open end of a β-barrel[8,33] rather than in the more commonly observed largely α-helical globin[34] or 4-helix bundle[35−38] folds. The ferriheme molecule is bound to the protein via a histidine imidazole nitrogen, and the sixth coordination site is available to bind NO or other ligands. In the NO-off form in vitro, either water or ammonia, depending on the buffer type, is bound to the sixth site.[24,27]

Of the spectroscopic techniques for characterizing heme proteins, ^{1}H NMR spectroscopy is one of the most important because it provides information about both the geometrical and the electronic structures of the heme active site in solution. In the case of the NPs, NO bound to the Fe(III) heme center produces diamagnetic complexes at all temperatures.[1,3] In this state, all heme substituent proton resonances are buried in the protein proton resonance envelope, while the NO-free form of the NPs is high-spin ($S = {}^5/_2$).[18,20] Binding of even-electron donor ligands, such as histamine, imidazoles, pyrazoles, or cyanide, to the NO-free forms of these proteins produces low-spin ($S = {}^1/_2$) Fe(III) complexes.[15,18−21,39] The unpaired electron(s) on the metal of both $S = {}^5/_2$ and ${}^1/_2$ ferriheme proteins cause(s) shifts (called hyperfine, isotropic, or paramagnetic shifts) of the resonances from those observed in a diamagnetic protein. The two contributions to the paramagnetic shifts are the contact (through bonds) and electron−nuclear dipolar or pseudocontact (through-space) contributions; these are discussed in considerable detail elsewhere.[40−43]

We have investigated in detail the ^{1}H NMR spectra of the ferriheme center and nearby protein residues in the high- and low-spin forms of the NPs from *R. prolixus*. Early in this investigation, it was found that, of the four similarly structured proteins NP1−NP4, NP2 provides by far the simplest NMR spectra because, unlike the other three NPs, NP2 exhibits one predominant heme orientation,[20,25] and there are no chemical exchange cross-peaks observed in the NOESY spectra of this protein, unlike those of NP1 and NP4. Hence, we have reported the assignment of most of the heme resonances of NP2 in its ligand-free, high-spin ($S = {}^5/_2$) form.[18,20] Saturation transfer experiments, allowed by a favorable rate of exchange of NMeIm between individual NP2 molecules, permitted us to connect the resonances of the high-spin complex with those of the low-spin complex.[20] The same method could not be used for NP1 and NP4 because each of them shows almost equal amounts of the two isomers that result from the two possible heme orientations (Chart 1). Hence, in this work we have utilized specifically labeled hemins, where one or two of the methyls were deuterated, in order to fully assign the heme resonances of ligand-free NP1 and NP4 and compare them to those of the high-spin form of NP2. To simplify the NMR spectra

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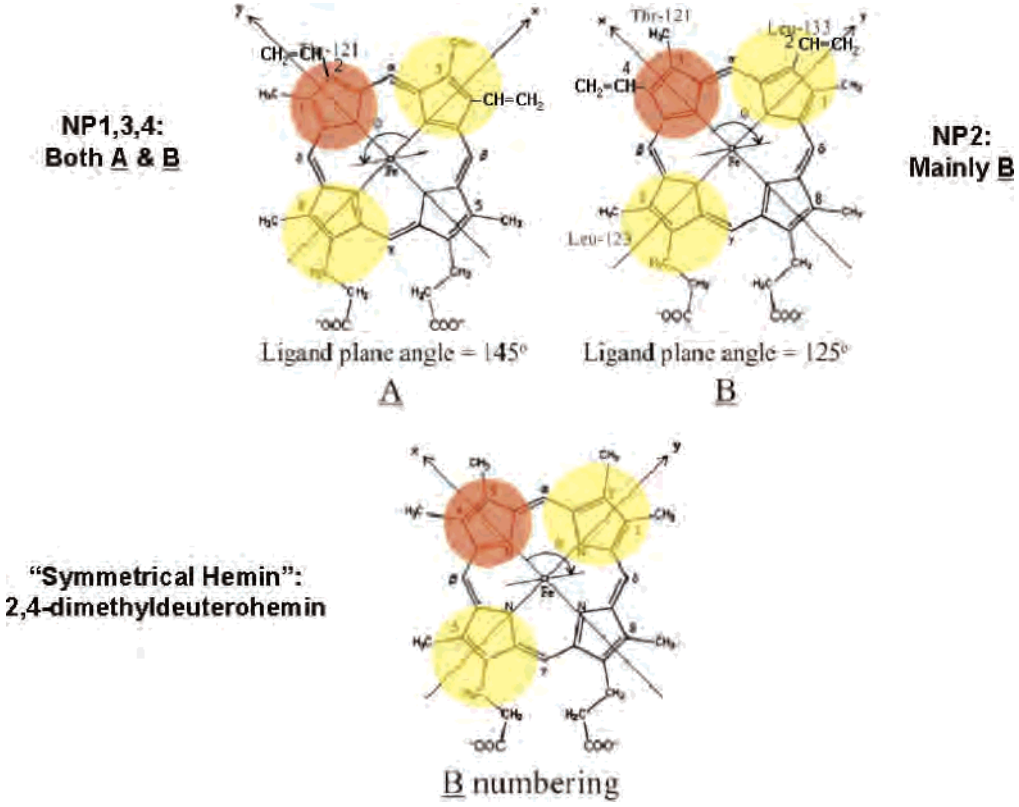
Chart 1



and show that heme disorder is the reason for the poorly resolved heme methyl resonances of NP1 and NP4, we also utilized the 2-fold-symmetric hemin, 2,4-dimethyldeuterohemin, also shown in Chart 1. Elsewhere,[39] we report the assignment of the NMR spectra of the axial ligand complexes of NP1 and NP4.

Experimental Section

Sample Preparation. The nitrophorin proteins NP1−NP4 were prepared as described previously[15,23−25] and were stored in lyophilized form at −80 °C until use. NMR samples consisted of 1−4 mM solutions of each of the proteins in D_2O-containing 30 mM phosphate buffer at pH 7.0 (uncorrected for the deuterium isotope effect). For the preparation of specifically labeled protohemin IX-containing protein samples for NMR spectroscopy, the unlabeled hemin present in NP1 or NP4 samples was first removed via acid−2-butanone extraction, as has been done previously with metmyoglobin,[44,45] methemoglobin,[45,46] and ferricytochrome b_5.[47] The apoprotein was dialyzed against phosphate buffer at pH 7.0 and then centrifuged to remove any precipitate. The apoprotein at pH 7.0 was then titrated with NaOD solutions of one of the three labeled protohemins utilized (either 5-CD_3−, 5,8-$(CD_3)_2$−, or 1,3-$(CD_3)_2$−protohemin IX) until approximately 90% of the required hemin had been added, based on the A_{280}:A_{410} ratio that was observed before unlabeled hemin removal. The the protein was readjusted periodically to pH 7.0 during this titration. The samples were concentrated and re-equilibrated with D_2O-containing 30 mM phosphate buffer at pH 7.0 (uncorrected for the deuterium isotope effect). It was found that typically only about 80−90% of the protohemin originally present in the holoprotein could be removed, and thus these reconstitution experiments always left some residual protonated-methyl protohemin present in the samples. Later in this project, it was found that the apoprotein could be refolded and purified in the absence of hemin.[18] Thus, a few remaining labeled heme complexes were prepared by titrating the apoprotein with the desired labeled hemin dissolved in NaOD as described above.

NMR Data Collection. One-dimensional (1D) ^{1}H NMR spectra were collected over the temperature range 20−30 °C on a Bruker DRX-500 spectrometer operating at 500.03-MHz proton Larmor frequency with the proton chemical shifts referenced to residual HOD.

Results and Discussion

^{1}H NMR Spectra of the High-Spin ($S = {}^5/_2$, NO-Free) Forms of the NPs. The ligand-free forms of recombinant NP1−NP4 at pH 7.0 and 25 °C have resolved ^{1}H heme and hyperfine-shifted protein resonances that extend from about +70 to −20 ppm, the high-frequency portions of which are shown in Figure 1. For NP1 and NP4, the pattern of these heme resonances is unlike that of any high-spin ferriheme protein reported previously, including those of metmyoglobin,[43,48,49] cytochromes c',[43,50,51] the resting state of horserad-

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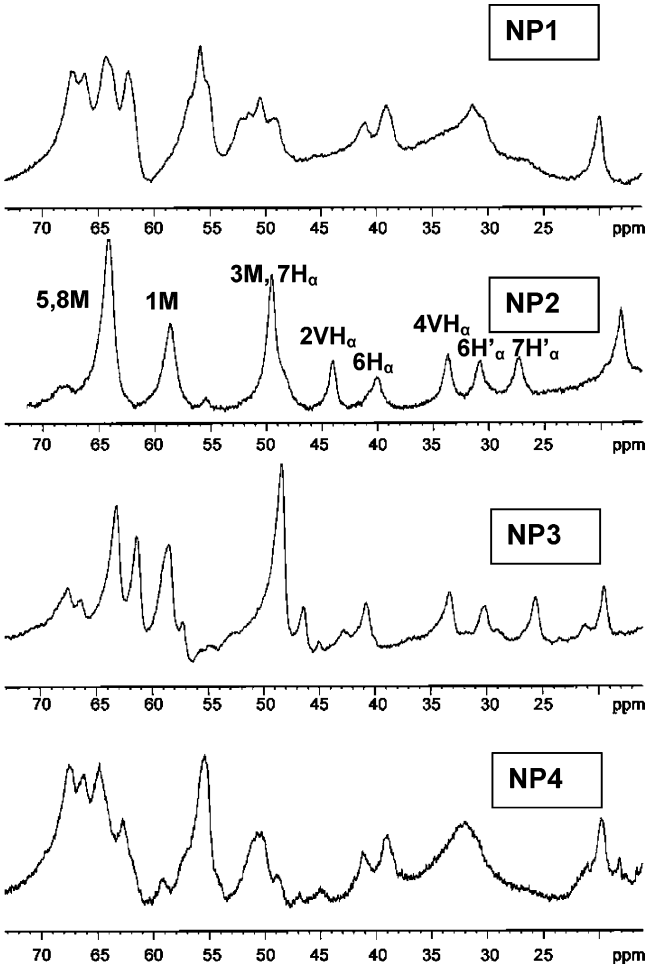

Figure 1. 1D ^{1}H NMR spectra of the high-frequency (16−73 ppm) region of the four NPs in the absence of added ligand at 25 °C, recorded at 500 MHz. The more intense resonances in the 55−68 ppm region for NP1 and NP4 and in the 49−67 ppm region for NP2 and NP3 are those of the eight heme methyls of the two heme rotational isomeric proteins (Chart 1). Resonance assignments for NP2, reported previously,[20] are given.

ish peroxidase,[43,53,54] and NP2.[20] In contrast to NP1 and NP4, as is clearly seen in Figure 1, the ^{1}H NMR spectrum of the high-spin form of NP2 is well-resolved, with unique resonances of intensities corresponding to one or three protons that are more similar in general appearance to those of previously studied high-spin ferriheme proteins,[43,48−54] especially horseradish peroxidase,[43,53,54] and even cytochrome *c*′.[43,50−52]

The strange appearance of the hyperfine-shifted ferriheme resonances of the NO-free forms of recombinant NP1 and NP4 is indicative of at least two forms of the protein being present at pH 7 in homogeneous solution, with overlapping methyl and other substituent resonances. The poor resolution of the ^{1}H NMR spectra of NP1 and NP4 is caused by the existence of nearly equal amounts of the two heme orientational isomers, the well-known "heme rotational disorder" observed in most other heme *b*-containing proteins.[43,47,53−61] This was shown by titrating the NP apoproteins with the "symmetrical hemin", trivial name 2,4-dimethyldeuterohemin IX, i.e., 1,2,3,4,5,8-hexamethyl-6,7-(dipropionic acid)porphyrinatoiron(III). Figure 2 shows the ^{1}H NMR spectra of these high-spin forms of the four proteins. In all four cases, these spectra are well-resolved and show only one set of six heme methyl resonances, albeit with four overlapping heme methyl resonances for NP2. The spectra of Figure 2 provide good evidence of almost identical high-spin ferriheme complex structures of NP1 and NP4 when the effect of heme asymmetry is excluded. The detailed study by 1D ^{1}H and 2D NMR techniques of the four NPs bound to the symmetrical hemin will be discussed elsewhere. For the moment, it is sufficient to note that the heme resonances of the high-spin Fe(III) forms of NP1 and NP4 bound to the symmetrical hemin are as well-resolved as those of NP2 when bound to protoporphyrin IX.

Using the 1D ^{1}H NMR spectrum of high-spin (in the absence of added ligand) NP2, we were able to develop a theory to explain the chemical shifts of the heme methyl resonances of high-spin ferriheme proteins,[20] which is demonstrated in Figure 3, in addition to our earlier-published theory that explains the chemical shifts of the heme methyl resonances of low-spin ferriheme proteins.[62] These angle plots are based on the contact shifts that measure the calculated spin-density distributions in the e(π) orbital of the ferriheme for which the nodal plane of that orbital is coincident with the nodal plane of the axial ligand(s)[62] and the calculated pseudocontact shifts that depend on the orientation of the magnetic axes of the heme, with counter-rotation of ligand and *x* or *y* axes.[63] Both of these contributions are related to the orientation of the nodal plane of the axial ligand but with an angular dependence of different phase and much smaller magnitude for the pseudocontact (9%) than the contact shifts for the heme methyl resonances.[62] For quantitative separation of contact and pseudocontact contributions, subtraction of the diamagnetic shifts would be necessary; these are 3.51 ± 0.03 ppm,[64] a constant to the level of accuracy possible for the chemical shifts of paramagnetic heme proteins. The plot for the high-spin hemins, shown in Figure 3, was developed in ref 20.

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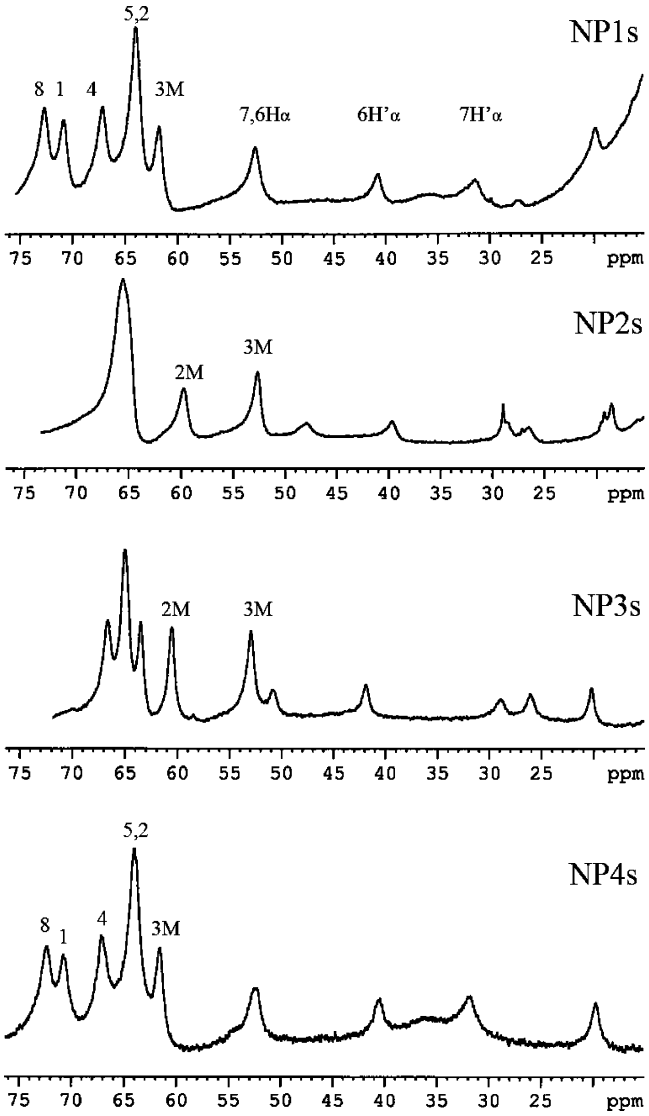

Figure 2. 1D ^{1}H NMR spectra of the high-frequency region of the symmetrical hemin complexes of the four NPs in the absence of added ligand at 25 °C, recorded at 500 MHz. The intense resonances in the 61−73 ppm region for NP1s and NP4s and in the 52−66 ppm region for NP2s and NP3s are those of the six heme methyls of the symmetrical hemin (Chart 1). The very broad resonance(s) centered at ∼36 ppm for NP1s and NP4s is (are) likely due to one or more *meso*-H of the symmetrical hemin, but they have not been assigned. Resonance assignments for NP1s, to be reported in detail elsewhere, are given.

However, note that Figure 3 does not carry numbers on the chemical shift axis because the aim of this work is not to separate contact and pseudocontact shifts but rather *to consider the order and relative spacings of the heme resonances* because these alone are sufficient to define the approximate angle of the nodal plane of the histidine imidazole ligand. The resonances of high-spin NP2 have been assigned previously[20] and are consistent with a 135 ± 1° orientation angle of the proximal histidine imidazole plane.

In the protohemin IX drawings of Chart 1, the **A** isomer is defined as having pyrrole ring II (including 4V) lying over the protein backbone C(O)CN(H) atoms of H57 (NP2) or H59 (NP1 and NP4), with that histidine ligand lying behind the plane of the heme in the pictures shown in Chart 1. With this definition, ligand plane angles are measured from the

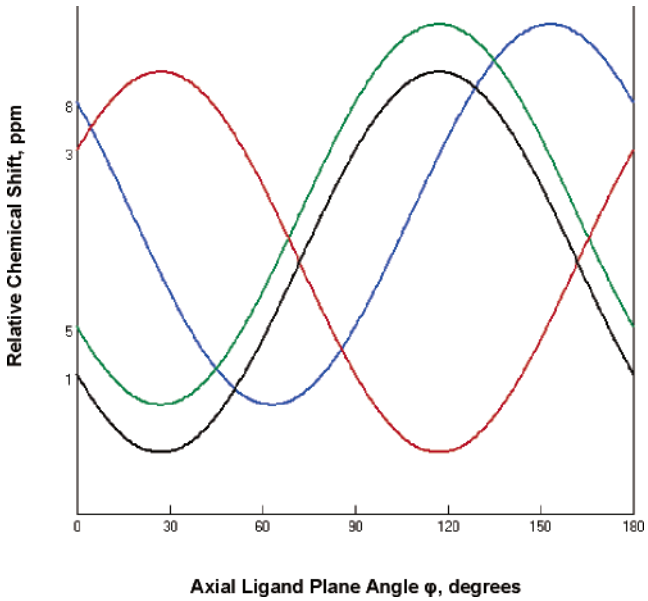

Figure 3. Plot of relative heme methyl shift vs axial ligand plane orientation, φ, for high-spin Fe(III) heme proteins. Produced by the program Shift Patterns.[20,65]

Table 1. Crystallographic and NMR Angles (φ) of the Histidine Imidazole Plane and **A**:**B** Ratio for High-Spin NP Complexes

	obtained from X-ray crystallography	obtained from NMR spectroscopy	
protein−ligand	His φ_A (φ_B)[a]	effective nodal plane[b]	**A:B**
NP1−NH_3	*142.3* (127.7) molecule I[c]	*139* (131)	∼1:1
	135.9 (134.1) molecule II[c]		
NP4−NH_3	*131.2* (138.8)		
NP4−H_2O[d]	*133.1* (136.9)[d]	*137* (133)	1.1:1
NP2−NH_3(H_2O)	135.0 (*135.0*)	∼136−137 (*135*)	1:8

[a] Measured from the N_{II}−Fe−N_{IV} axis, with positive numbers representing counterclockwise rotation. The major form (**A** or **B**) is in italics. Taken from refs 24−29 and their corresponding PDB files (2NP1, 1X8P, 1D3S, 1EUO, and 2A3F). [b] Apparent nodal plane as measured from the ^{1}H NMR shifts of the heme methyls. [c] Two molecules in the unit cell that show somewhat different geometries and even a difference in axial ligation; molecule I shows that NH_3 is not ligated, while molecule II shows that it is. [d] Crystallographic orientation inconsistent with ^{1}H methyl shifts.

N_{II}−Fe−N_{IV} axis toward and past the N_I−Fe−N_{III} axis in both cases. The 135° orientation angle is predicted by the plot of the relative chemical shifts of the heme methyls vs the angle of the imidazole plane predicted from the program Shift Patterns[20,65] (Figure 3) because at this angle the 5Me and 8Me resonances have the same chemical shift, as discussed previously.[20] The X-ray crystal structure of wild-type NP2 complexed to NH_3 shows this angle to be 135°,[25,26] while those of wild-type NP1 and NP4 show some variation in this angle, with values ranging from 130.1° to 137.8° for heme orientation isomer **A** of NP4[27−29] and from 130.8° to 139.8° for the same isomer of NP1[24] (Chart 1). The orientation angles of the His-59(57) imidazole plane for the high-spin forms of NP1, NP4, and NP2, as determined by X-ray crystallography,[24,27−29] are summarized in Table 1 and are compared to the orientation of the effective nodal plane

(65) Shokhirev, N. V.; Walker, F. A. Shift Patterns. http://www.shokhirev-.com/nikolai/programs/prgsciedu.html.

of the histidine imidazole and the **A**:**B** ratios as determined by NMR spectroscopy in this work. The latter, and their relationship to the X-ray crystallographic data, are discussed below. Because no X-ray structures of NP3 or its axial ligand complexes have yet been determined, the ^{1}H and ^{13}C NMR spectra of high-spin NP3 will be discussed elsewhere.

Ferriheme proteins isolated from various organisms, or produced by expression as holoproteins in bacteria or other hosts, usually have the equilibrium ratio of **A** and **B** orientations by the time the protein is isolated and purified, and no change in the intensity of the NMR resonances of the two occurs as a function of time. An obvious exception to this situation is outer mitochondrial cytochrome b_5, which is kinetically trapped as the 1:1 ratio of heme orientations over time periods of many months at ambient temperatures.[61,66] Although heme rotational isomerism results in only very small differences in the reduction potentials of the two cytochrome b_5 heme rotational isomers,[47] there is functional relevance to the orientation of the binding of heme to bacterial heme oxygenases that produce other than α-*meso*-carbon oxidation to CO,[58,61] and heme reorientation also serves as a probe of protein dynamics. Indeed, in recently completed work, we have shown that the equilibrium **A**:**B** ratio, **A** and **B** heme substituent chemical shifts, the equilibrium binding constants, and the on and off rates of axial ligands are different for recombinant NP2, which carries the expression−initiation Met-0 residue, and its D1A mutant, for which the Met-0 residue is cleaved off during expression.[67] Moreover, analysis of the experimental data leads to the conclusion that NO, imidazole, and histamine binding to the majority **B** isomer is more favorable than that to the minority **A** isomer.[67] Heme orientational isomerism thus appears to have important consequences for the mechanism of action of the NPs. In addition, its presence can certainly complicate the interpretation of the NMR spectral data for heme *b*-containing proteins, as is seen here for three of the four NPs (Figure 1 as compared to Figure 2).

Hence, in agreement with the complicated pattern of hyperfine-shifted heme resonances observed in the low shielding region of the ^{1}H NMR spectrum of high-spin NP1 (Figure 1), the two heme orientations are approximately equally abundant, even though this was not evident in the 2.0-Å resolution structure of NP1−NH_3.[24] This was the first NP structure solved, and it was modeled as having only one heme orientation. Only when significantly higher resolution structures of NP4[27−33] and NP2[26] and their axial ligand complexes became available was it clear that both heme orientations were observed for NP4 (and in retrospect probably for NP1 as well) but not for NP2[26] (in the latter case, the **B** isomer is favored by a ratio of 1:8[20]) and the 11% minority heme orientation is not detected by X-ray crystallography.

Because of the strong overlap of the eight methyl resonances of the **A** and **B** isomers of NP1 and NP4, which precludes the use of the saturation transfer techniques used for assignment of the heme resonances of NP2,[20] the heme methyl resonances of high-spin NP1 have been assigned using apoprotein samples reconstituted with specifically deuterated protohemins, as described in the Experimental Section and as shown in Figure 4. In the examples shown, when 5-CD_3−protohemin IX is incorporated into apo-NP1, the closely spaced resonances at 66 and 64 ppm are greatly reduced in intensity. When the doubly deuterated derivative 5,8-$(CD_3)_2$−protohemin IX is incorporated, all four peaks in the range 67−64 ppm practically disappear, while the intensity of the resonances near 62 and 56 ppm is not changed (Figure 4). Similarly, when 1,3-$(CD_3)_2$−protohemin is inserted into apo-NP1, the overlapping peaks at about 55 and 62 ppm are greatly diminished in intensity (not shown).

Using these deuterated hemins, the chemical shift orders of the high-spin ferriheme methyl resonances for the two heme orientational isomers of NP1 are found to be 8 > 5 > 1 > 3 and 5 > 8 > 1 > 3. In comparison, for NP2, the order is 5 = 8 > 1 > 3 for isomer **B**.[20] The overlap of 8Me and 5Me indicates that the angle of the axial histidine imidazole nodal plane orientation (φ) for NP2 is exactly 135° in solution (Figure 3), as was also determined from the X-ray structure in the crystalline state.[25] As shown by Figure 3, if φ is greater than 135°, the order is 8 > 5 ..., while for φ less than 135°, the order is 5 > 8 .... If $\varphi = 135° + \Delta$ for the **A** orientation (Chart 1), then assuming that the proximal histidine ligand does not change orientation from one form to the other, $\varphi = 135° - \Delta$ for the **B** orientation (Chart 1). Thus, in the case of NP1, the observed order 8 > 5 > 1 > 3 is due to the **A** orientation, while the order 5 > 8 > 1 > 3 is due to the **B** orientation. Thus, $\varphi_A > \varphi_B$, and the 5Me and 8Me resonances of isomer **A** have slightly larger chemical shifts as compared to the 8Me and 5Me resonances of isomer **B**. The orientations of the histidine imidazole planes in the two molecules in the unit cell of NP1−NH_3 have been reported as 142.3° for isomer **A** and 127.7° for isomer **B** for molecule I[24] and 135.9° for isomer **A** and 134.1° for isomer **B** for molecule II. On the basis of the chemical shifts summarized in Table 2, we suspect that the angles for the aqua complexes of NP1 **A** and **B** in solution are closer to the averages of molecules I and II for each, 139° and 131°, respectively. From the NMR shifts of the heme methyls alone, it is not possible to define which is the **A** and which is the **B** orientational isomer, in terms of the orientation of the histidine imidazole plane of H57 (NP2) or H59 (NP1 and NP4), at least for the high-spin complexes; the NMR data must be combined with X-ray crystallographic data to correctly define the **A** and **B** heme orientations.

All of the discussion of angles for the histidine imidazole plane for orientations **A** and **B** given in the previous paragraph assumes that the hemin molecule remains fixed with its N−Fe−N axes in precisely the same position in both heme orientational isomers. That this is not exactly the case is shown by the small heme methyl resonances of the **A** isomer (10−11% abundant[20]) observed for NP2 near 57, 68, and 69 ppm in Figure 1. By saturation transfer techniques using a mixture of the high-spin protein and its NMeIm

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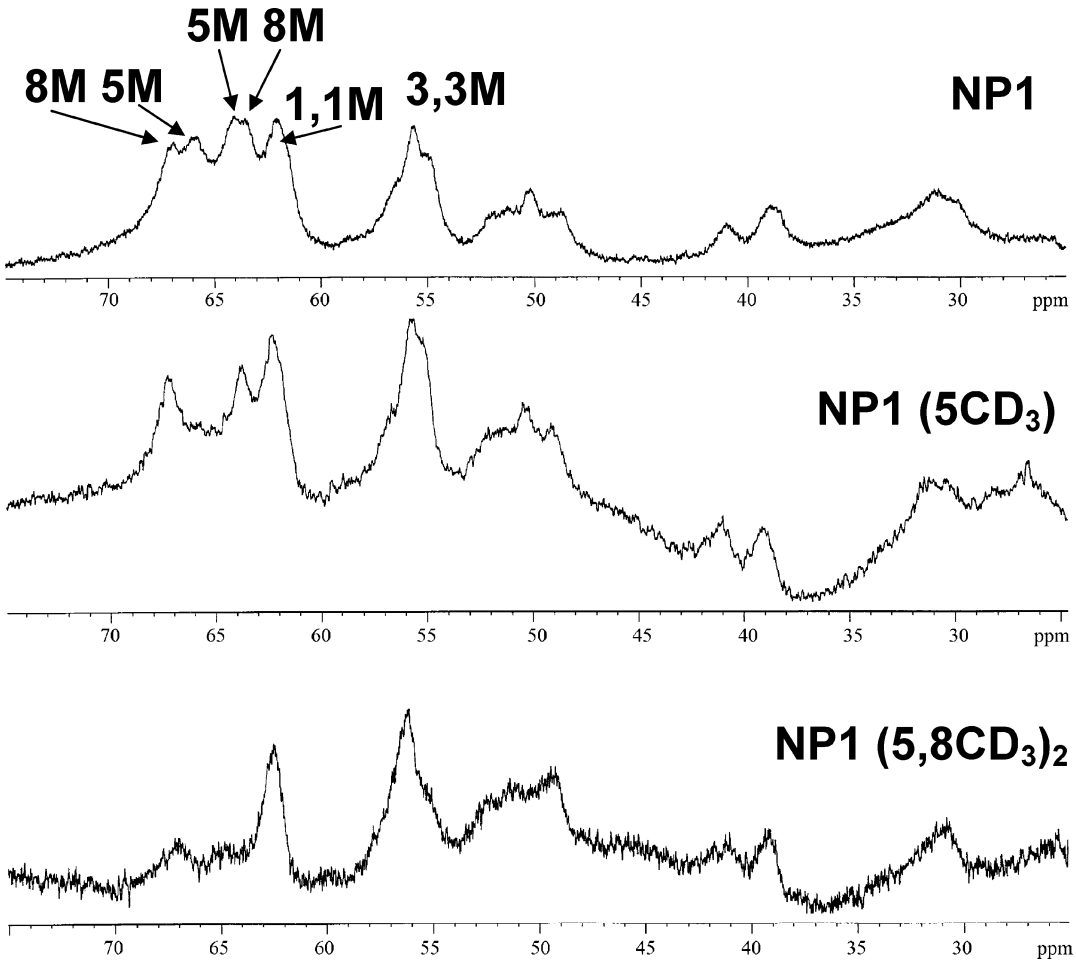

Figure 4. 1D ^{1}H NMR spectra of the high-frequency region of the native and two specifically deuterated protohemin IX complexes of NP1. Top: Native NP1 complex. Middle: 5-CD_3-labeled hemin complex. Bottom: 5,8-$(CD_3)_2$-labeled hemin complex. The first 8Me and 5Me resonances belong to the **A** isomer, while the second 5Me and 8Me resonances belong to the **B** isomer.

Table 2. Proton Chemical Shifts of the High-Spin Nitrophorins NP1 and NP4 As Compared to NP2, Recorded at 25 °C, pH 7.0

	NP1 (**A**:**B** ∼ 1:1)		NP4 (**A**:**B** ∼ 1.1:1)		NP2 (**A**:**B** ∼ 1:8)		NP2−F27Y (**A**:**B** ∼ 1:3)		NP1−Y28F (**A**:**B** ∼ 1:2)	
	A	**B**	**A**	**B**	**A**	**B**[a]	**A**	**B**	**A**	**B**
1Me	62.1	61.1	62.4?	61.5?	(55.5)[a,b]	58.6,[b] 59.5[c]	56.6	52.2	62.5	62.5
3Me	55.6	∼55.2	55.5	∼57.2	(∼53)[a,b]	49.5,[b] 50.6[c]	55.5	45.1	55.5?	54.8?
5Me	66.1	64.2	66.1	64.7	67.8[b]	64.1,[b] 63.8[c]	65.6	60.2	68.0	65.0
8Me	66.9	63.8	67.4	64.0	68.4[b]	64.1,[b] 63.8[c]	65.6	58.6	68.0	65.0
average	62.7	61.0	62.8	61.9	∼61.2	59.1,[b] 59.4[c]	60.8	54.0	63.2	62.0
spread, Δ	11.3	9.0	11.9	7.5	15.4	14.6,[b] 13.2[c]	9.0	15.1	12.5	10.2

[a] The small resonance at 55.5 ppm arises from 1Me of isomer **A**. The fourth heme methyl resonance of the **A** isomer has not been assigned but is likely at ∼53 ppm and arises from 3Me. [b] This work. T = 25 °C. [c] Taken from ref 20. T = 20 °C.

complex, we have shown that the resonance at 68.4 ppm is that of 8Me while that at 67.8 ppm is that of 5Me (not shown). These chemical shifts, as compared to those of isomer **B**, indicate that the proximal histidine imidazole plane is oriented at an angle slightly greater than 135°, while the majority **B** isomer has that imidazole plane oriented at an angle of exactly 135°. Thus, when the heme is turned over, it adjusts its seating in the heme cavity so as to avoid steric interactions between the 2- and/or 4-vinyl groups with protein side chains that are unfavorably placed for this **A** isomer. This difference in "seating" is small, estimated to be only 2−4°, and not the very large difference of ∼100° observed for the hemin cyanide complex of a bacterial heme oxygenase reported recently that gives rise to chemical exchange between the two seatings.[58] However, its effects are still clearly observable by ^{1}H NMR spectroscopy. The difference in the average heme methyl shift for the **A** and **B** isomers is an indication of the difference in seating of the two heme isomers, and it is included in Table 2. As can be seen, the **A** isomer invariably has the larger average heme methyl shift and thus the larger histidine imidazole plane angle φ.

The accuracy to which the angle of orientation of the histidine imidazole plane of high-spin ferriheme-containing proteins can be determined depends on what the angle actually is, i.e., whether one is at a crossing point of the plot shown in Figure 3 or at some other special condition or not. Hence, if 5M and 8M have exactly the same chemical shift, we know that we are at the crossing point of the angular

dependence of the 5M and 8M resonances, i.e., 135°. Because of the width of the high-spin resonances, a ±1° error seems generous for the ability to measure exact overlap, especially in view of the fairly steep slopes of the plots for the chemical shifts of those two methyl resonances at that point (Figure 3). If 1M and 8M had the same chemical shift, we would have an angle of 129 ± 1°. Thus, when two resonances of a high-spin ferriheme have the same chemical shift, the angle corresponds to the point on the plot at which the two lines cross (Figure 3). These crossings occur at 4.5°, 45°, 51°, 68.5°, 72°, 85.5°, 129°, 135°,162°, and 165.5° and are points at which the angle of the histidine imidazole plane can be determined to ±1°. At such points, the angular orientation of the histidine plane can be determined at least as accurately, and probably more so, from NMR spectra of the heme resonances of the paramagnetic protein as by X-ray crystallography. Furthermore, NMR spectra can detect small differences in the histidine imidazole plane orientation (or, more probably, small differences in heme "seating") for the **A** and **B** heme rotational isomers (Table 1) as well. Similarly, for angles where pairs of two resonances have the same difference in chemical shift (0°, 9°, 81°, 90°, and 180°) or where three of the four heme methyl resonances are equally spaced, either the three highest or the three lowest chemical shift resonances (39°, 48°, 57.5°, 65°, 70°, 75.5°, 122.4°, 132°, 141°, 158.5°, 164°, and 169°), a similar high accuracy in the determination of the orientation of the histidine imidazole plane occurs, again with a ±1° error. For cytochrome *c*, some of these angles will be shifted several degrees because of the more similar substituent effects of the 2,4-$CH(CH_3)SR$(cys) groups of hemin *c* to those of the methyls than is the case for the vinyls and methyls of protohemin IX (compare Figures 5 and 6 of ref 62). Although there are a fairly large number of special angles, for regions in between these key angles, such as for orientations of greater than 9° but less than 39°, for example, the error in determining the precise orientation of the histidine imidazole plane rises, probably to ±2−3°.

NP4 has a 1D ^{1}H NMR spectrum very similar to that of NP1 (Figures 1 and 2). Because of the strong similarity in the spectra of NP1 and NP4 and the behavior of the symmetrical hemin-reconstituted NP1 and NP4 samples whose spectra are shown in Figure 2, we expect the same order of heme methyl resonances to occur for this NP as well. The procedure that we used earlier in this study to insert the deuterium-labeled protohemins to assign the resonances of NP1 gives a very small yield of the reconstituted protein in the case of NP4 because NP4 is very sensitive to the pH; at low pH, this protein separates into two fractions, which are a monomer and a dimer (higher oligomers are said to be present in the gas phase, detected by mass spectrometry),[68] yet the protein can be crystallized in a monomeric form at pH 5.6.[69] However, by the same method as that demonstrated in Figure 4 for NP1, it was found that the heme methyl resonance order is 8Me > 5Me for the **A** isomer and also that the **A**:**B** ratio is slightly different for NP4 than for NP1 (not shown). Because of the near equality of the **A**:**B** orientation of NP4 (estimated as 60:40 from X-ray crystallography,[27] 52.4:47.6 = 1.1:1 from NMR spectroscopy), in the refinement of the high-resolution structures of this protein, the vinyl groups were modeled as having 50% occupancy at heme positions 1−4.[27−29]

However, for the crystal structure of NP4−OH_2 at pH 5.6 (PDB file 1D3S), the angles for the **A** and **B** orientational isomers are reversed from what they are for NP1−OH_2, with the **A** isomer having an angle of 130.9° and the **B** isomer having an angle of 139.2°.[29] The same is true of the NP4−NH_3 structure obtained at pH 7.5 (PDB file 1X8P). There are examples of NP4 having the **A** orientational isomer as either the smaller or larger angle, and occasionally, as for NP4−histamine, an angle very near 135°[29] (Table 1), and as is seen for all NP2 complexes whose structures have been determined thus far.[25,26] However, it should be pointed out that this reversal, for the high-resolution structure of NP4−OH_2 at pH 5.6,[29] represents a difference in the orientation of the histidine imidazole plane of 8.3° with respect to the side-chain residues of the protein, between the crystal and solution structures. Because the H-bond "anchor" of the histidine imidazole plane orientation in the structures of all of the NPs is a water molecule that is, in turn, H-bonded to the side-chain carbonyl O of Asp-70 (NP1 and NP4)[15,24,27−32] or Asn-68 (NP2)[25,26] and neither the water molecule nor Asp or Asn is optimally placed in these structures to form these H bonds, it is likely that in an aqueous solution the imidazole N−H may seek several alternative H-bonding-partner water molecules or one water molecule that is able to move somewhat freely, which yield an average orientation somewhat different from that observed in the crystal structure. An extreme example of such a difference between solid-state and solution ligand orientations is that of mouse neuroglobin, where each of the two histidine imidazole planes rotates by about 20° to decrease the dihedral angle between them from ∼60° in the solid state[70] to ∼20° in an aqueous solution.[71]

The disagreement in the His-59 orientation observed by NMR spectroscopy and X-ray crystallography may also be, in part, due to the different experimental conditions used for the two techniques, including pH and concentration, both of which are critical in the case of NP4.[68] On the basis of the ^{1}H NMR shifts of the heme substituents, we have assumed that, under the conditions that were utilized for NMR sample preparation, the **A**/**B** angles are in the same order for NP1 and NP4 because of the similarity in their chemical shifts. Thus, in Table 1, the angles for the two rotational isomers are presented as observed in the crystal structures of NP1 and NP4 (with a footnote indicating that the reverse orientation is consistent with the NMR chemical shifts of NP4−OH_2) and the average nodal plane angle is given as observed for the NMR spectra.

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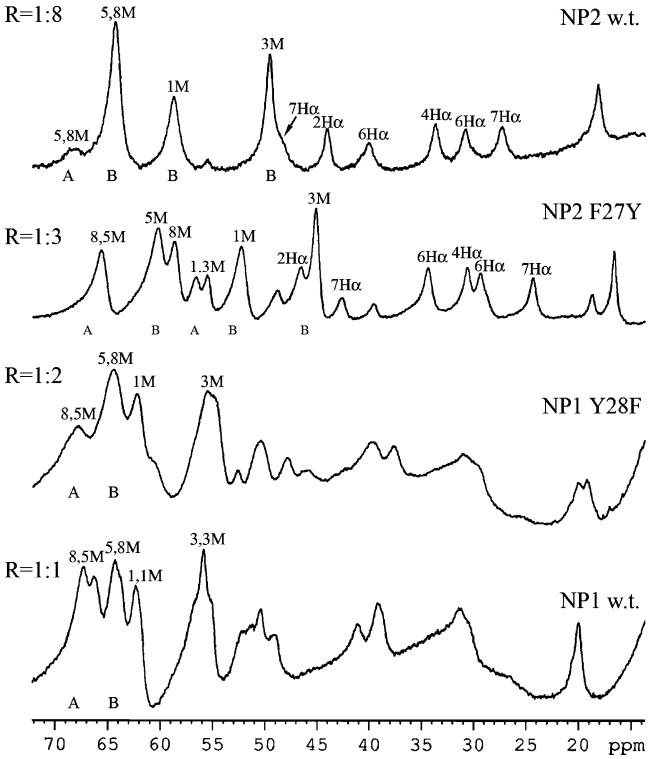

Figure 5. 1D ^{1}H NMR spectra, all at 25 °C, of high-spin NP2 wild-type (top), with the F27Y mutant of NP2 immediately below, and NP1 wild-type (bottom), with the Y28F mutant of NP1 immediately above. The ratios of **A**:**B**, as well as the patterns of hyperfine-shifted heme resonances of the NP2 mutant, are somewhat NP1-like, while those of the NP1 mutant are somewhat NP2-like.

Origin of the Differences in A:B Ratios for the NPs. As shown in Figure 1, the heme orientation ratio **A**:**B** of each of the NPs differs at least somewhat (1:1, 1:8, 1:4, and 1.1:1 respectively for NP1−NP4). We thus questioned whether this could be because of the tight packing of several phenylalanines or tyrosines around the vinyl and methyl substituents of the protohemin prosthetic group in the NP heme cavities, which change when going from NP1 and NP4 to NP2.[23−29] According to the crystal structures, Phe-27 (NP2) and Tyr-28 (NP1 and NP4) are very close to one vinyl on the proximal side of the heme. Thus, we hypothesized that the mutation of Phe-27 to Tyr would increase the **A**:**B** ratio in NP2, while the mutation of Tyr-28 to Phe would decrease the **A**:**B** ratio in NP1. Figure 5 shows the ^{1}H NMR spectra of the high-spin forms of wild-type NP2 and NP1 and their respective mutants. As can be seen, the mutants indeed have altered **A**:**B** ratios in the directions predicted. However, the effects of these two mutations are more complex than their 1D ^{1}H NMR resonance pattern and **A**:**B** ratio might suggest, in that the chemical shifts of the methyl, methylene, and α-vinyl proton resonances of the **A** and **B** isomers have changed somewhat in the mutants as well. Our interest in this work is not to discuss these differences because our detailed NMR study of these and other "belt" and distal pocket mutant proteins and their ligand complexes will be published elsewhere. However, it can be seen from the heme methyl resonance intensities that the phenylalanine/tyrosine substitution in NP1 and NP2 is responsible for a significant part of the **A**:**B** ratio difference.

Conclusions

In this work, we have shown that the structure of the NP ferriheme complexes in terms of the orientation of the heme ligand(s) can be described with good agreement with the crystal structures by NMR techniques in the high-spin form (except for NP4−H_2O) and that the plot shown in Figure 3 describes the angle of the effective nodal plane of the axial histidine imidazole in solution. The histidine imidazole plane orientation found in the solid state is, in the case of NP4−H_2O, 8.3° different from that found in an aqueous solution. This is believed to be because in the solid state the imidazole ring N−H is H-bound to a water molecule and that the water molecule is, in turn, H-bound to an aspartate (NP1 and NP4) or asparagine (NP2), but in solution it is free to take other H-bonding partners. Thus, because this H-bonding scheme seen in the solid state is common to the three NPs whose structures have been reported,[23−33] it is possible that other cases may be found where the orientation of the protein-provided histidine ligand is somewhat different in solution from found in the solid state.

There is an equilibrium between the two heme orientations (**A** and **B**) that depends not only on the heme binding ability but also on the heme cavity shape and size. An additional study of the consequences of this equilibrium between the two heme orientations will be published elsewhere.[67] The **A**:**B** ratio can be much more accurately measured by NMR spectroscopy than by X-ray crystallography.

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