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Assignment of Ferriheme Resonances for High- and Low-Spin Forms of Nitrophorin 3 by ^1H and ^{13}C NMR Spectroscopy and Comparison to Nitrophorin 2: Heme Pocket Structural Similarities and Differences

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Abstract

Nitrophorin 3 (NP3) is the only one of the four major NO-binding heme proteins found in the saliva of the blood-sucking insect *Rhodnius prolixus* (also called the Kissing Bug) for which it has not been possible to obtain crystals of diffraction quality for structure determination by X-ray crystallography. Thus we have used NMR spectroscopy, mainly of the hyperfine-shifted ferriheme substituent resonances, to learn about the similarities and differences in the heme pocket and the iron active site of NP3 as compared to NP2, which has previously been well-characterized by both X-ray crystallography and NMR spectroscopy. Only one residue in the heme pocket differs between the two, F27 of NP2 is Y27 for NP3; in both cases this residue is expected to interact strongly with the 2-vinyl side chain of the **B** heme rotational isomer or the 4-vinyl of the **A** heme rotational isomer. Both the high-spin ($S = 5/2$) aquo complex, NP3- H_2O , and the low-spin ($S = 1/2$) N-methylimidazole (NMeIm) complex of NP3 have been studied. It is found that the chemical shifts of the protons of both forms are similar to those of the corresponding NP2 complexes, but with minor differences that indicate a slightly different angle for the proximal histidine (H57) ligand plane. The **B** heme rotational isomer is preferred by both NP3 and NP2 in both spin states, but to a greater extent when phenylalanine is present at position 27 (**A**:**B** = 1:8 for NP2, 1:6 for NP3-Y27F, 1:4 for NP3, and 1:3 for NP2-F27Y). Careful analysis of the 5Me and 8Me shifts of the **A** and **B** isomers of the two high-spin nitrophorins leads to the conclusion that the heme environment for the two isomers differs in some way that cannot be explained at the present time. The kinetics of deprotonation of the high-spin complexes of NP2 and NP3 are very different, with NP2 giving well-resolved high-spin aquo and “low-spin” hydroxo proton NMR spectra until close to the end of the titration, while NP3 exhibits broadened ^1H NMR spectra indicative of an intermediate rate of exchange on the NMR timescale between the two forms throughout the titration. The heme methyl shifts of NP2-OH are similar in magnitude and spread to those of NP2-CN, while those of metmyoglobin-hydroxo complexes are much larger in magnitude but not spread. It is concluded that the hydroxo complex of NP2 is likely $S = 1/2$ with a mixed $(d_{\text{XY}})^2(d_{\text{XZ}}, d_{\text{YZ}})^3/(d_{\text{xy}})^1(d_{\text{xz}}, d_{\text{yz}})^4$ electron configuration, while those of met-Mb-OH are likely $S = 1/2, 3/2$ mixed spin systems.

Introduction

The nitrophorins (nitro = NO, phorin = carrier) are a group of 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 insect¹⁻⁵ and at least three additional nitrophorins in earlier stages of development,^{6,7} and *Cimex lectularius*, the bedbug, which has only one nitrophorin protein.^{8,9} These interesting heme proteins sequester nitric oxide that is produced by a nitric oxide synthase (NOS) that is similar to vertebrate constitutive NOS that is present in the salivary glands,¹⁰⁻¹² that keeps it 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, diffuses through the tissues to the nearby capillaries to cause vasodilation and thereby 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 the beginning 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 sites of the nitrophorins, hence preventing the insect's detection for a period of time.¹³ These two properties of the nitrophorins of *Rhodnius prolixus* contribute to the transmission of the protozoan *Trypanosoma cruzi*, the vector of Chagas' disease,¹⁴ to the victim, via the feces of the insect, that are left behind at the site of the bite³ following the extended feeding time.

The *Rhodnius* proteins of the adult insect, which have been named NP1–4 in order of their abundances in the insect saliva, have been investigated by a number of techniques^{1,3,15-32} including spectroelectrochemistry,^{15,17-19} infrared¹⁵ and resonance Raman,¹⁶ NMR,^{15,18,20-22} EPR^{15,23} and Mössbauer spectroscopies,²⁴ stopped-flow photometry,^{17,25} and the solid state structures of one or more ligand complexes of NP1,^{15,26} NP2,^{27,28} and NP4²⁹⁻³⁴ 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 beta-barrel,^{8,35} rather than in the more commonly-observed largely alpha-helical globin³⁶ or 4-helix bundle³⁷⁻⁴⁰ folds. The ferriheme molecule is bound to the protein via a histidine ligand, 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 buffer type, is bound to the sixth site.^{26,29}

Among the four nitrophorins, NP2 and NP3 have 76% amino acid identity, while NP1 and NP4 have higher identity, 90%. The overall identity of the four nitrophorins is only 38%. The structures of NP1, NP2 and NP4 that have been reported show very similar structures among the three proteins, and it is thus likely that the structure of NP3, which has so far resisted crystallization, will be found to be the same general beta-barrel fold, with protein residues interacting with the heme in rather similar ways. Inside the heme pocket, at a distance of up to 5 Å from the heme, the identity between NP2 and NP3 is even higher; only two amino acids are different within this radius of the heme: F27 of NP2 is a tyrosine in NP3, and N102 of NP2 is an aspartate in NP3. N102 is actually only within 5.0 Å of the iron in two (PDB files 1T68, 2ACP) of eight structures obtained thus far of NP2, close to the surface of the protein at the beginning of β strand F. All other residue differences between NP2 and NP3 are further from the Fe, on the surface of the protein, while F27 is buried at the back of the heme-binding pocket at the end of β-strand A, with C1 of the phenyl group 3.90 Å from the β-carbon of the 2-vinyl group of the heme. As a part of the work discussed in this paper, we have prepared the NP3-Y27F and the NP2-F27Y mutants to examine how this mutation affects the structure of the heme pocket.

Of the spectroscopic techniques for characterizing heme proteins, proton NMR spectroscopy is one of the most important. In the case of the nitrophorins, NO bound to the Fe(III) heme center produces diamagnetic complexes at all temperatures.^{1,3} In this state, all heme substituent resonances are buried in the protein proton resonance envelope, while the NO-free

form of the nitrophorins are high-spin ($S = 5/2$) complexes.^{18,20, 21} 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.^{15,18-20,22} The unpaired electron(s) on the metal of both $S = 5/2$ and $S = 1/2$ ferriheme proteins act as “beacons” that “illuminate” the protons in the vicinity of the metal, by causing shifts (called hyperfine, isotropic or paramagnetic shifts) of the resonances from those observed in a diamagnetic diamagnetic protein. These shifts allow much to be learned about the intimate details of the electron configuration at the iron center. The two contributions to the isotropic shifts are the contact (through bonds) and electron-nuclear dipolar or pseudocontact (through space) contributions; these are discussed in considerable detail elsewhere.⁴¹⁻⁴³

The proton NMR resonances of the ferriheme center and nearby protein residues in the two paramagnetic forms of the four nitrophorins from *R. prolixus* are being investigated in detail in our laboratory. Early in this investigation it found that of the four similar-structured proteins, NP2 provides by far the simplest NMR spectra, because unlike the other three nitrophorins, NP2 exhibits one predominant heme orientation,^{20,27} and there are no chemical exchange cross peaks observed in the NOESY spectra of this protein, unlike those of NP1 and NP4.²² Hence, we recently reported the assignment of all of the heme resonances of NP2 in its low-spin ($S = 1/2$) N-methylimidazole (NMeIm) and imidazole (ImH) complexes,^{20,22} as well as those of the ligand-free, high-spin ($S = 5/2$) form.^{18,20} Assignment of the heme resonances in both of these forms of the protein was made possible by the sharpness of the signals of the low-spin complexes, which allowed NOE cross peaks to be detected for all heme substituents (even those whose resonances were buried in the large envelope of proton resonances from the protein), and by a favorable rate of exchange of NMeIm between individual NP2 molecules, which allowed saturation transfer experiments to connect the resonances of the high-spin complex with those of the low-spin complex.²⁰ The same was not true of NP1 and NP4, because each of them shows almost equal amounts of both isomers that result from the two possible heme orientations,²¹ Scheme 1, and because the low-spin Fe(III) complexes of NP1 and NP4 have only one heme methyl resonance resolved outside of the protein envelope.²² Hence, specifically ²H-labeled hemins, where one or two of the methyls were deuterated, were utilized in order to fully assign the heme resonances of NP1 and NP4 and their axial ligand complexes.^{21,22}

As mentioned above, the crystal and molecular structures of NP1, NP2 and NP4 and some of the complexes of each of these nitrophorins have been reported. However, no structure of NP3 or any of its axial ligand complexes has been reported. Thus, NMR spectroscopy can be used in a predictive manner for NP3 and its axial ligand complexes, and because of this, NMR spectroscopic studies of NP3 are reported herein. The assignments of the heme resonances of NP2 and four of its axial ligand complexes (NMeIm, ImH, histamine and cyanide) have been reported.^{20,22} In this work the hyperfine-shifted proton resonances of the heme and nearby protein residues, which are shifted from their diamagnetic positions by the presence of unpaired electron(s) on the metal, in the two paramagnetic forms of NP3, have been investigated and the pattern of hyperfine-shifted resonances observed for this protein for a given ligand complex are compared. From this work and the previously-published work on NP2,^{20,22} NP1 and NP4,^{21,22} much is learned about the electronic and geometrical structure of NP3, even in the absence of crystallographic data.

Experimental

Sample preparation

The nitrophorin protein NP3 was prepared as described previously for the other nitrophorins,^{15,25-27} and was stored in lyophilized form at -80°C until use. NMR samples consisted of 1–3 mM solutions of the protein in D_2O containing 30 mM phosphate buffer at pH 7.0

(uncorrected for the deuterium isotope effect). To obtain the low-spin complexes, the high-spin NP3 protein was titrated with the desired ligand (cyanide, histamine, imidazole or N-methylimidazole) until the proton NMR signals in the 70–30 ppm region had just disappeared. Concomitantly, these signals were replaced by much sharper signals in the 30–10 ppm region. Especially for the cyanide complex, as reported previously for the other nitrophorins,²¹ it was found to be extremely important not to add any more ligand than necessary to cause disappearance of the high-spin resonances, and in fact, less than a stoichiometric amount of cyanide was found to be necessary for EPR measurements to avoid formation of some of the bis-cyanide complex, which has lost its protein-provided histidine ligand.

NMR data collection

NMR spectra were collected over the temperature range 10 to 37 °C with the proton chemical shifts referenced to residual water. WEFT-NOESY and HMQC spectra were obtained on a Bruker DRX-500 spectrometer operating at 500.03 MHz proton Larmor frequency. The ¹H-¹³C HMQC experiments were recorded using a 5 mm inverse-detection probe with decoupling during acquisition. A recycle time of 200 ms and refocusing time of 2.5 ms ($J = 200$ Hz) were used. The WEFT-NOESY experiments utilized 100 ms relaxation delay and 130 ms recovery-delay. The mixing time for the NOESY experiments was 12–32 ms. All 2D spectra were collected with 1024 or 2048 data points in t_2 and with 256–512 blocks in t_1 with 400–800 scans/block.

Results

Proton NMR Spectra of the High-Spin ($S = 5/2$, NO-Free) Form of NP3 as compared to NP2 and two mutants

The ligand-free forms of recombinant NP2 and NP3 at pH 7.0 and 25 °C have resolved ¹H heme and hyperfine-shifted protein resonances that extend from about 70 to –20 ppm, the high-frequency portion of which are shown in Figure 1 for NP3, and for comparison, that of NP2. Also shown are the corresponding spectra of the two residue 27 mutant proteins, NP3-Y27F and NP2-F27Y. The majority of the heme resonances of high-spin NP2 have been assigned previously,²⁰ and are consistent with the 135° orientation angle of the proximal histidine imidazole plane, in which the ligand plane is aligned along the β,δ *meso*-carbon axis in the crystal structure (PDB file 1EUO), where the **A** isomer is defined as having pyrrole ring II (including 4V) lying beneath Leu-132, with the His-57 ligand lying behind the plane of the heme. Conversely, the **B** isomer has pyrrole ring II lying beneath Ile-120, again with the His-57 ligand behind the plane of the heme, Scheme I, the well-known “heme rotational disorder” observed in the nitrophorins^{20,21} and other heme *b*-containing proteins.^{43–53} With these definitions, ligand plane angles in this work are measured from the N_{II} -Fe- N_{IV} axis toward and past the N_I -Fe- N_{III} axis for both **A** and **B**. The 135° orientation angle is seen in the crystal structure, and is independently found for the **B** isomer of NP2 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,^{54,55} shown in Supporting Information Figure S1, because at this angle the 5Me and 8Me resonances have the same chemical shift, as discussed previously.^{18,20,21} Thus, one of the first conclusions that can be made from Figure 1 is that NP3 has a somewhat different orientation of the histidine imidazole plane than is the case for NP2, i.e., the imidazole plane is oriented at an angle different from 135°. Furthermore, it can also be seen that while NP2 has only a small amount of isomer **A** present (**A**:**B** = 1:8), NP3 has a larger amount of this same isomer present, estimated, from Figure 1, as an **A**:**B** ratio of about 1:4. That it is isomer **A** that has the smaller-intensity set of two peaks at 66 and 67.5 ppm can be concluded on the basis of previous studies of not only NP2,²⁰ but also NP1 and NP4, all of which showed that the **A** heme rotational isomer has slightly larger chemical shifts than does the **B** isomer.²¹

Thus, the pattern of resonances observed in the 48–68 ppm region of Figure 1 for NP3 is totally consistent with the **A** isomer being less abundant than the **B** isomer.

To confirm that the two sets of heme resonances observed for the high-spin ferriheme proteins were the result of heme orientational disorder, in previous work the four nitrophorin apoproteins were titrated with the “symmetrical heme”, whose trivial name is 2,4-dimethyldeuterohemin IX, i.e., 1,2,3,4,5,8-hexamethyl,6,7-dipropionic acid porphyrinatoiron.²¹ The resulting ferriheme proteins of all four nitrophorins yielded ¹H NMR spectra that were well-resolved, and showed only one set of six heme methyl resonances for each protein, albeit with four overlapping heme methyl resonances for NP2.²¹ The near identical 1D NMR spectra of the symmetrical heme complexes of NP1 and NP4 in the 80–20 ppm region provided good evidence of very similar high-spin five-coordinate heme complex structures of these two proteins, even though the 1D ¹H NMR spectra of their protohemin IX complexes exhibited a poorly-resolved set of overlapping resonances due to a total of 8 heme methyls signals of similar intensity.²¹ Likewise, in the symmetrical heme-reconstituted NP3 NMR spectrum, one set of six heme methyl resonances was also observed. However, for the native protohemin IX complexes, unlike NP1 and NP4, the ratio of **A**:**B** for NP3 is not close to 1:1, and thus the ¹H NMR spectrum of high-spin NP3 is much better resolved than those of NP1 and NP4.²¹ The detailed study by 1D ¹H and 2D NMR techniques of the four nitrophorins bound to the symmetrical heme will be discussed elsewhere.⁵⁶ As for the heme methyl resonances of the protohemin IX-containing NP3, Figure 1 also exhibits sets of low- and higher-intensity resonances in the 45–20 ppm region that result from vinyl and propionate α -protons of the **A** and **B** heme orientational isomers, respectively.

To assign the heme resonances of the high-spin complex of NP3 we have used the 1D saturation transfer difference experiment, as was done previously for NP2.²⁰ The success of this experiment relies on chemical exchange between protons in identical molecular locations for the high-spin complex and the low-spin N-methylimidazole (NMeIm) complex, assuming that the protons of the low-spin complex have already been assigned. This assignment was accomplished using 2D WEFT-NOESY and HMQC data, as is described below, but for purposes of completing the discussion of the high-spin complex of NP3, we use the results of those assignments of the NMeIm complex NMR spectrum, as shown in Figure 2, where the four methyl resonances of the high-spin ferriheme complex are assigned. By this means we found that the order of heme methyl resonances of the **B** heme rotational isomer is $5 > 8 > 1 > 3$. We also found that it was possible to assign many more of the resonances of the high-spin NP3 complex, including many of those for the **A** heme rotational isomer, using the WEFT-NOESY experiment, as shown in Figure 3. Thus the propionate and vinyl protons can be assigned on the basis of their NOE cross peaks with particular methyls, as we have reported previously for NP2.²⁰ Furthermore, we can assign the side chain protons of H57, which, along with the propionate α -CH₂ and vinyl α -CH (5.59, 5.65, 5.52 Å distant, respectively) are the closest protons to the paramagnetic Fe. For NP2 and NP3 one of the β -CH₂ resonances of H57 occurs at 17–23 ppm in the proteins shown in Figure 1. As shown in the WEFT-NOESY spectrum of Figure 3, the resonance at 19.7 ppm has cross peaks to protons at +2.5 and –2 ppm. One of these is the other β -CH₂ resonance and the other is the α -CH resonance of the coordinated histidine. It is not certain at this time which is which, but these two resonances are remarkably unchanged in chemical shift from NP3 to NP2, even though the highly shifted β -CH₂ proton differs in chemical shift by 1 ppm between the two proteins (Table 1). This 1 ppm difference in chemical shift supports the fact that there is a small difference in orientation of the imidazole plane of the histidine ligand between NP3 and NP2, and the 1.7 and 1.2 ppm difference in chemical shift between this β -CH₂ resonance for the major **B** isomer as compared to the minor **A** isomer, respectively, again suggests this difference in ligand plane orientation. This conclusion is in agreement with the previous interpretations of His- β -CH₂ resonance shifts of La Mar and coworkers of various high-spin ferriheme proteins^{43,57,66} (i.e., the very short

$T_{1\rho}$ s (~ 2 ms) of one or two resonances in the 15–45 ppm region of the ^1H NMR spectrum). It is also interesting to note that the two site-directed mutants, NP3-Y27F and NP2-F27Y, have the largest difference in chemical shift for the highly-shifted $\beta\text{-CH}_2$ resonance for the **A** and **B** isomers, ~ 2 ppm in both cases, as summarized in Table 1.

Using similar strategies it was possible to assign the **A** isomer propionate resonances by assuming, as found for NP1, NP2 and NP4,^{20,21} that the two methyl resonances observed at 62.5 and 61.0 ppm are those of the 8Me and 5Me, respectively. The chemical shifts of high-spin NP3 are summarized in Table 1. The difference in chemical shifts of 5Me and 8Me for **B** (1.9 ppm) and 8Me and 5Me for **A** (1.2 ppm) corresponds to histidine imidazole plane angles of 133° and 137° , respectively, using the angle plot of high-spin Fe(III) heme proteins,²⁰ shown in Supporting Information Figure S1 obtained from the program Shift Patterns, which is available on the web.⁵⁴ In comparison, for high-spin NP2 these differences are 0 ppm and 0.6 ppm, leading to histidine imidazole plane orientations of 135° and 136° , respectively, for **B** and **A**.²⁰

In like manner, the high-spin forms of NP3-Y27F and NP2-F27Y were investigated by saturation transfer techniques to assign the paramagnetically-shifted heme resonances. The saturation transfer spectra are shown in Supporting Information Figures S2 and S3, respectively, and the WEFT-NOESY spectrum of NP2-F27Y is shown in Supporting Information Figure S4. The chemical shifts of all assigned resonances for the **A** and **B** isomers of high-spin NP3 and the NP3-Y27F and NP2-F27Y mutants are summarized in Table 1, along with those reported previously for NP2.^{20,21} In comparing NP3 and NP2, there are some differences in chemical shifts of each of the methyl resonances such that the **A** isomer of NP3 has the larger average chemical shift but smaller spread of the methyl resonances. The same is true of the **A** isomer of NP2. But notably, the 8Me of the **B** isomer of NP3 has a significantly smaller chemical shift (2.9 ppm) than does that of the **B** isomer of NP2. The spread of the methyl resonances, Δ , is a measure of the rhombicity of the heme, and it is larger for the **B** than the **A** isomer for NP2,²⁰ and also for NP3, as seen in Figure 1 and Table 1. However, for NP1 and NP4 the reverse is true.²¹ Vinyl and propionate α -protons also show some changes in chemical shifts. The three largest of these are the 2-vinyl H_α of NP3, which is shifted 2.2 ppm to lower shielding, the 6-propionate H_α' , which is shifted 2.2 ppm to higher shielding, and the 7-propionate H_α and H_α' , both of which shifted 2.9 ppm to higher shielding in NP3 as compared to their chemical shifts for NP2 at the same temperature (25°C). These shifts are not localized to a particular part of the heme ring, and thus cannot be caused by interaction with one single protein side chain, for example Y27 of NP3 as compared to F27 of NP2.

Determination of the pK_a of the Water Molecule Bound to the High-Spin Form of NP3 and NP2 at Neutral pH, and Assignment of the Heme Methyl Resonances of the Hydroxide Complex of Each

While the 5-coordinate hydroxo complexes of model hemes are invariably high-spin and readily react to form the μ -oxo dimer,⁵⁸ the 6-coordinate hydroxo complexes of heme proteins having histidine as the fifth ligand are thought to be low-spin or mixed $S = 3/2, 1/2$ spin systems.^{43,59-66} Such species have been postulated to be the product of H atom abstraction by the ferryl ($\text{Fe}=\text{O}$) centers of peroxidases and cytochromes P450,⁶⁷ which in the latter case have a cysteine thiolate instead of a histidine axial ligand. The $\text{Fe}^{\text{III}}\text{-OH}$ product should have extensive electron delocalization due to porphyrin π donation to the ferric iron.⁶⁸ Previous workers have investigated the pH-dependent deprotonation of the water molecule that is usually bound to high-spin mono-histidine-coordinated heme proteins (except for peroxidases, which are believed not to have a water molecule bound to the high-spin ferriheme resting state⁴³) by NMR spectroscopy,⁵⁹⁻⁶⁶ as we have done herein for NP2 and to the extent possible for NP3. As shown in Figure 4, for NP2 the half-point of the pH titration, is at $\text{pH}^* 10.5$. For NP3 the

same titration does not give well-resolved peaks of the hydroxo complex, but rather the high-spin aqua complex is in chemical exchange with the hydroxo complex on the NMR timescale, and the resonances of both are broad and poorly resolved. An example is shown in Supporting Information Figure S5, where only one saturation transfer experiment was possible. A rough estimate of the pK_a of NP3-OH is ~ 9.9 . It is surprising that the rate of proton exchange is so different for the two proteins, when both the aquo and hydroxo complexes have very similar chemical shifts of the heme resonances. The calculated isoelectric points⁶⁹ of NP2 and NP3 are similar (6.1 and 6.5, respectively, and thus cannot explain the difference in the rate of proton exchange.

The NP2-OH complex 1H methyl shifts were assigned by saturation transfer difference techniques, as shown in Supporting Information Figures S6a and b, and show heme methyl orders $3 > 5 > 8 > 1$ for NP2. However, the results are not as clear for NP3 because of the chemical exchange mentioned above, beyond the fact that 3Me is the methyl resonance having the largest chemical shift for NP3-OH (Figure S5), as for NP2-OH (Figure S6). The chemical shifts of the hydroxo complexes of NP2 are summarized in Supporting Information Table S1, where two single-proton resonances resolved outside the protein envelope (17.0, 14.5 ppm) are not due to the heme substituents or the histidine $\beta-CH_2$ that is assigned in Figure S6b. For NP2-OH the order of the heme methyls places the histidine imidazole plane at about 138° clockwise from the pyrrole nitrogens of rings II and IV of the **B** isomer of NP2, 3° larger than observed for the high-spin NP2 complex discussed above and as shown in the crystal structure (PDB file 1EUO). Furthermore, and more importantly, the spread of the methyl resonances (12.5 ppm) is much smaller than observed for typical low-spin complexes of the nitrophorins except for the cyanide complexes, which have similar spreads, especially for the **B** isomers of NP2-CN (10.6 ppm), NP1-CN (13.6 ppm) and NP4-CN (11.5 ppm).²² All have very similar average methyl chemical shifts (12.3 ppm for the hydroxide complexes of NP2, 12.3, 12.4, 12.1 ppm, respectively for the cyanide complexes²²). The heme methyl shifts of NP2-OH, along with those of other ferriheme proteins for which the hydroxo complexes have been reported, and for comparison some of the cyano complexes are also included are summarized in Table 2.

Proton NMR Heme Substituent Resonance Assignments for the Low-Spin NP3-N-Methylimidazole Complex

Addition of strong-field ligands such as N-methylimidazole (NMeIm) to the high-spin ($S = 5/2$) Fe(III) centers of the nitrophorins creates the low-spin Fe(III) state ($S = 1/2$), which is characterized by a much smaller range of NMR shifts (30–40 ppm) and much sharper resonances than those of the high-spin forms of the same proteins. In Figure 5 is shown the 1D 1H NMR spectrum of the NP3-NMeIm complex, the 2D 1H - ^{13}C HMQC spectrum of the same complex, and its 2D WEFT-NOESY spectrum, all recorded at 25 °C at pH 7.0. With these two 2D spectra it is possible to assign the four methyl groups and the other heme substituent resonances for heme orientation **B** and part of the resonances for heme orientation **A**. For example, the HMQC spectrum allows identification of all four methyl signals of the **B** isomer, the NMeIm methyl resonances and the pairs of proton resonances of single carbons that are due to the vinyl 4β and propionate β -protons, which, when combined with the WEFT-NOESY spectrum, lead to assignment of two methyl resonances, at -0.2 and 14.6 ppm, that have strong NOEs to the same resonance at 9.5 ppm, which must be *meso*-H δ for the **B** isomer. Likewise, one of the vinyl α -H resonances of the **B** isomer, with chemical shift 24.7 ppm, has an NOE in common with the methyl-H resonance at 12.8 ppm, at -1.8 ppm, that could be either from *meso*-H α to 2-vinyl H α and 3Me, or from *meso*-H β to 4-vinyl H α and 5Me. The same vinyl H α has a strong NOE to the methyl resonance at 14.6 ppm, which confirms the assignment of the latter resonance to 1Me; thus that at -0.2 ppm must be 8Me, and the vinyl H α resonance at 24.7 ppm must therefore be of the 2-vinyl. Thus the methyl resonance at 12.8 ppm must be

that of 3Me, all of the **B** isomer. The 2-vinyl H α also has strong NOE cross peaks to two closely-spaced resonances near -5 ppm that are well resolved in the 1D NMR spectrum, thus identifying them as the 2-vinyl H β resonances. The remaining methyl, at 16.0 ppm, must thus be 5Me, and it shows NOE cross peaks to the two propionate 6-CH₂ protons at 14.9 and 13.4 ppm, both of which are buried under more intense resonances in the 1D ¹H spectrum. With these assignments, and the knowledge that *meso*-H α and H γ , and correspondingly, *meso*-H δ and H β should have similar chemical shifts (usually within ~2–3 ppm), it is possible to find the remaining connectivities. Indeed, cross peaks common to 6- α CH₂ and 7- α CH₂ identify *meso*-H γ at -3.7 ppm. The final *meso*-H resonance, H β , with NOE to 5Me, can be seen at 6.9 ppm in the WEFT-NOESY spectrum, but it is not easy to see the common NOE cross peaks from 4-vinyl H α at 6.2 ppm at the contour level shown in Figure 5. With assignments of the major, **B**, isomer complete, it is possible to assign some of the **A** isomer resonances in the same way, but many NOE cross peaks are of low enough intensity that they cannot be observed at the contour level shown in Figure 5, and some cannot be found at all. The assignments for the **B** isomer and those that could be made for the **A** isomer are summarized in Table 3.

In addition to NOE cross peaks in the NOESY spectrum that allow assignment of the resonances of the **B** and **A** isomers, there are also chemical exchange cross peaks from the 5, 1, and 8Me resonances of the **B** isomer to chemical shifts that have no 1D counterparts, at 21.7, 18.5 and 25.7 ppm, respectively. These cross peaks, which are circled in the NOESY spectrum of Figure 5, thus indicate that there is a second orientation of the NMeIm ligand that is very low in abundance (less than 1% of the major **B** isomer), but is in dynamic exchange with the major orientation of the NMeIm ligand for the **B** isomer. Using the angle plot for low-spin ferrihemes, shown in Supporting Information Figure S7, the order of heme methyl resonances, 8 > 5 > 1 > 3 in the minor, **B'**, orientation, shows that the angle of the NMeIm plane orientation is ~53° from the x-axis of the heme, which passes through pyrrole rings 2 and 4, Scheme 1. That means that this **B'** orientation makes a near-perpendicular orientation with the H57 ligand that yields a dihedral angle of ~80°. The same phenomenon was observed for NP2 distal pocket mutant complexes with ImH as the sixth ligand.¹⁸ We suspect that part of the reason for this near-perpendicular ligand orientation is heme plane ruffling, which is well known for wild type NP2, especially for its low-spin complexes,²² but perpendicular ligand orientations are observed by NMR techniques only for NP2 distal pocket mutants in which there is significantly more space in the distal pocket.¹⁸ The mechanism of chemical exchange appears to not just be simple ligand rotation, but rather ligand jumping from one orientation to the other, which may also include ligand release and rebinding. The intensity of the **B'** methyl resonances increases as the amount of excess NMeIm is increased, suggesting that the “parallel” and “perpendicular” ligand complexes have different binding constants. It is also possible that they represent differently-ruffled species that interchange because of a vibrational mode of the heme.⁷⁰ Although NMeIm is a stronger-binding ligand to NP2-D1A than ImH⁷¹ due to its higher pK_a(LH⁺),⁷² it cannot be involved in N-H hydrogen-bond donation to backbone carbonyls of the protein that would stabilize its orientation, and this is probably why the perpendicular orientation can be observed for the NMeIm complex of NP3; however, it is not observed for the NMeIm complex of wild-type NP2.^{18,20}

Proton NMR Heme Substituent Resonance Assignments for the NMeIm complexes of NP3-Y27F and NP2-F27Y

We used the NMeIm complexes of NP3-Y27F and NP2-F27Y to assign the resonances of the high-spin complexes to see if there is any difference in the heme center structure in the mutants. Saturation transfer spectra for the NMeIm complexes of NP3-Y27F and NP2-F27Y to the respective high spin complexes were used to assign the heme methyl resonances of the high spin complex (Supporting Information Figures S2, S3). As mentioned above, the results are presented in Table 1. Assignments of the low-spin NMeIm complex resonances were made on

the basis of the WEFT-NOESY and HMQC data in the same way as for wild-type NP3. The WEFT-NOESY spectrum of NP3-Y27F-(NMeIm) is shown in Figure 6, where the assignments of the **B** isomer are marked on the 1D spectra and the assigned cross peaks are marked on the 2D spectrum. The assignments are included in Table 3. In like manner, assignments of the NP2-F27Y NMeIm complex were made by WEFT-NOESY and HMQC spectroscopy. The WEFT-NOESY spectrum is shown in Supporting Information Figure S4, and the results from this and the HMQC spectrum are summarized in Supporting Information Table S2.

Discussion

As mentioned above, the order of heme methyl resonances of the **B** heme rotational isomer of high-spin NP3 is $5 > 8 > 1 > 3$, thus indicating that the His-57 imidazole plane of NP3 has a slightly different orientation than in NP2, where that angle is 135° , i.e., the imidazole plane of H57 in NP2 lies exactly over the β,δ -*meso* carbons. As shown by Supporting Information Figure S1, if ϕ is greater than 135° the order is $8 > 5...$, while for ϕ less than 135° the order is $5 > 8...$. If $\phi = 135^\circ + x$ for the **A**-orientation (Scheme 1), then assuming that neither the proximal histidine ligand nor the heme changes orientation from one form to another, $\phi = 135^\circ - x$ for the **B**-orientation (Scheme 1). In this case the chemical shift of 8Me(**A**) should be the same as that for 5Me(**B**) and the chemical shift of 5Me(**A**) should be the same as that for 8Me(**B**). For wild type nitrophorins 1, 2 and 4,²¹ and for the NP2 distal pocket mutants studied previously,¹⁸ 8Me(**A**) and 5Me(**A**) are more shifted than 5Me(**B**) and 8Me(**B**). This may indicate that it is the heme that adjusts its position in the heme cavity for the **A** heme orientation relative to that of the **B** orientation rather than the histidine imidazole plane.

From the order and relative spacing of the heme methyl resonances in the **B** isomer of the high-spin complex, using the angle plot shown in Supporting Information Figure S1, it appears that the angle of the imidazole plane of H57 is $\phi \sim 133^\circ$, while for the **A** isomer $\phi \sim 137^\circ$; for NP2 the angles were found to be 135° for **B** and 136° for **A**. However, the chemical shifts of 8Me(**A**) and 5Me(**A**) are both larger than those of 5Me(**B**) and 8Me(**B**), Table 1, although they should simply be reversed according to the plot of Figure S1. In fact, they are larger by an amount ranging from 3.7 to 5.0 ppm for the **A** as compared to the **B** isomers of wild-type NP3 and NP2, as summarized near the bottom of Table 1. The two isomers also have a marked difference in average methyl shift, 62.5 ppm for **A**, 57.8 ppm for **B** of NP3, ~ 63.0 and 59.1 ppm, respectively, of NP2.²⁰ The same is also true of the **A** and **B** isomers of NP1 and NP4,²¹ although the average methyl shift in the angle plot for the high-spin complex is a maximum at 135° and decreases symmetrically on either side of that angle.⁵⁵ Thus there is an inherent difference in the heme pocket environment for the **A** and **B** isomers of the nitrophorins that is not explained by the angle plot. Possible differences could include 1) the effect of heme ruffling, which would place heme methyls at different out-of-plane distances from the average heme plane for the two isomers, and thus make their pseudocontact shifts different in the two heme rotational isomers, 2) the interaction of the 2- and/or 4-vinyl group(s) with protein side chains in one or the other isomers that would change the out-of-plane angle of one or both of the vinyl groups between the two isomers, thus changing the electron-withdrawing ability of the two vinyls in the two rotational isomers, or 3) other differences between the **A** and **B** isomers, such as how they interact with other residues such as I120 of NP2 and NP3 vs. T121 of NP2 and NP4. The first two effects could in fact work in concert to magnify the observed difference. The contact shifts could also in principle be changed by ruffling, although it is more difficult to visualize exactly how. Although NMR spectroscopy does not give direct information about heme ruffling, the observation of the minor “perpendicular” orientation of axial ligands in the NMeIm complex and comparison to chemical shift information for NP2 and NP2 mutants (unpublished work) leads us to expect heme ruffling in NP3, as is observed in NP2. However, looking at the way in which the ruffling would affect the **A** and **B** heme orientations for NP3, if 8Me of **A** experiences a positive pseudocontact shift due to ruffling,

then 5Me of **B** should experience the same degree of positive pseudocontact shift. This is not what is observed, but instead 8Me of **A** has a chemical shift 4.2 ppm larger than 5Me of **B**. In the same way, 5Me of **A** should experience practically no change in pseudocontact shift due to heme ruffling, and the same should be true of 8Me of **B**; however, 5Me of **A** has a chemical shift 5.0 ppm larger than 8Me of **B**, Table 1.

As for the effects of heme rotation on the out-of-plane twists of the vinyl groups, the crystal structure of NP2-NH₃ (PDB file 1EUO) shows that the 4-vinyl group of the **B** orientation (the only one seen in the crystal structure) has a torsion angle of 43.3° to the β-pyrrole carbons, while the 2-vinyl group has a torsion angle of 49.4° from its β-pyrrole carbons. The above-mentioned crystal structure suggests that the **B** orientation is much better at reducing the interaction between F42 and the 4-vinyl group, and between F27 and the 2-vinyl group than would be true for the **A** orientation. NP2 is unique in having a phenylalanine at position 27, while in NP1, NP3 and NP4 the residue at that position is tyrosine. NP4-NH₃, which was modeled in the **A** orientation (PDB file 1D2U), has the torsion angle of the 4-vinyl group as 59.8° to avoid the tyrosine ring of Y28, while the 2-vinyl group has a torsion angle of 26°. Both of these angles are very different than those shown by NP2. However, the 2- and 4-vinyl out-of-plane rotation angle should have only a secondary effect on the chemical shifts of the 5- and 8Me groups of the heme, and thus the reason for the observed differences in chemical shifts is not apparent.

In this work we prepared the F27Y mutant of NP2 and the Y27F mutant of NP3, and find that NP3-Y27F has a larger **B:A** ratio (6:1) than does wild-type NP3 (4:1), while NP2-F27Y has a smaller **B:A** ratio (3:1) than does wild-type NP2 (8:1). From the crystal structure of NP2-NH₃ (PDB 1EUO), when the 4-carbon of the phenyl ring does not interact with the 2-vinyl β-carbon of the **B** rotational isomer, it appeared that the **A** rotational isomer the 4-vinyl β-carbon *would* interact with that 4-carbon and its –OH substituent if the tyrosyl side chain is similarly positioned as observed in this 1EUO crystal structure. However, the crystal structure of NP4-NH₃ (PDB file 1D2U, 1.15 Å resolution) in the absence of added ligand shows that there is sufficient space for the 4-vinyl group near the tyrosyl side chain, and the major differences in the structure of the NP2 and NP4 proteins in this region is that NP2 is much more ruffled than NP4, and that the phenyl ring of F27 is rotated slightly toward the heme mean plane of NP2 as compared to Y28 of NP4. The arrangements of protein side chains in the heme pockets of NP2 and NP4 are shown in Supporting Information Figure S8. Both NP1 and NP4, with tyrosine at that position (Y28 in those cases) have nearly equal abundance of isomers **A** and **B**.²¹ On the basis of size, we would have expected that the slightly smaller phenyl side chain would have decreased the **B:A** ratio of NP3-Y27F while the slightly larger tyrosyl side chain would have increased the **B:A** ratio of NP2-F27Y, but the reverse is observed. In fact, NP3 itself is also out of line with the expectation that the larger tyrosine residue would disfavor the **A** isomer. We can only conclude that other residues in the heme pocket, possibly I120 of NP2 and NP3 as compared to T121 of NP1 and NP4, and/or F42 of NP2 and NP3 as compared to L44 of NP1 and NP4 play a more primary role in determining **B:A** orientation ratio, and that residue 27 has secondary effects that are opposite what we had expected.

The hydroxo complexes of NP2 and NP3 behave very differently from each other, with NP2-OH having well-resolved spectra with chemical shifts fairly similar to those of NP2-CN, while NP3-OH is in intermediate-rate chemical exchange with the high-spin aquo complex and thus cannot be characterized in detail. Although La Mar and coworkers misquoted the work of Rivera and coworkers and claimed that the latter group had assigned the ground state of the *Pseudomonas aeruginosa* heme oxygenase hydroxo complex as $S = 1/2$ with a $(d_{xz}, d_{yz})^4(d_{xy})^1$ electron configuration,⁶⁶ in fact Rivera and coworkers assigned the spin state of the *major* form of the hydroxo complex of that heme oxygenase as a mixed $S = 3/2, 1/2$ system on the basis of ¹³C chemical shifts, and they found that the d_{xy} ground state minor

species was of the order of 1% of the total of three different spin state complexes found for the hydroxo complex,⁶⁵ the third being a pure $S = 3/2$ spin state complex of somewhat larger abundance than that of the $S = 1/2$ d_{xy} ground state species, but still minor as compared to the mixed $S = 3/2, 1/2$ major species. The chemical shifts of the protons alone, presented in both papers,^{65,66} are rather different for the two proteins, and while those for *Nm*-HO-OH are fairly similar to those for NP2-OH, those for *Pa*-HO-OH are somewhat larger (methyl shifts from 24 to about 12 ppm, spread of 12–12.5 ppm, average shift of 16.6 ppm), as summarized in Table 2.

The latter shifts are, however, much smaller than those of all of the metMb-OH complexes whose chemical shifts have been reported previously, which range from nearly 40 to 20 ppm.⁵⁹⁻⁶⁴ All of these hydroxo complexes have small spreads of the four heme methyl resonances (10.5–12.1 ppm), and large average heme methyl shifts (35.3 to 33.1 ppm), with *T. akamusi* metHbV-OH having the smallest spread (10 ppm) and the smallest average shift (25.9 ppm). The order of heme methyl resonances for the metMb-OH and one metHb-OH complexes ($5 \geq 8 > 3 > 1$ for most) does not match any prediction of the angle plot that is based on a low-spin $(d_{xy})^2(d_{xz}, d_{yz})^3$ electron configurations (Supporting Information Figure S7). The large sizes of the methyl shifts and their order thus suggest strongly that the spin state is not $S = 1/2$ for metMb-OH complexes. In line with the findings of Rivera and coworkers, based on ^{13}C chemical shifts of the heme substituent resonances of *Pa*-HO-OH,⁶⁵ we suspected that the metMb-OH complexes are mixed $S = 3/2, S = 1/2$ spin state complexes with considerably more $S = 3/2$ character than *Pa*-HO-OH. Not having available the ^1H chemical shifts of pure $S = 3/2$ heme complexes (because most $S = 3/2$ ferrihemes are spin admixed $S = 3/2, 5/2$ species), trials were made using the well known $S = 5/2$ chemical shifts of the heme methyls for the same high-spin metMb complexes.^{62,73} It was found that the observed order of methyl resonances of the metMb-OH complexes, as well as the approximate values and spread of the heme methyl resonances can be obtained by averaging $1/3$ of the high-spin chemical shifts plus $2/3$ of the low-spin cyano chemical shifts of the same metMbs.^{44,74} (For example, for *Dolabella* metMb-OH a methyl chemical shift order $5 > 8 > 3 > 1$ with shifts of 40.0, 38.8, 37.2, 26.3 ppm is obtained, which yields a spread of 13.7 ppm and average methyl shift of 35.6 ppm; for sperm whale metMb-OH the order $5 > 8 > 1 > 3$ with methyl shifts of 46.0, 38.8, 29.6, 26.9 ppm is obtained, which yields a spread of 12.4 ppm for the methyl resonances and an average shift of 35.3 ppm. Thus SW metMb-OH would be better fit with a somewhat smaller high-spin contribution than $1/3$, but the data still illustrate the fact that high-spin/low-spin averaging can account for the observed shift of the hydroxo complexes.) The pure $S = 3/2$ chemical shifts should be smaller than those of the $S = 5/2$ complexes, but the order of heme methyl resonances should be the same because the mechanism of spin delocalization from the porphyrin ring to the holes in the d_{xz} and d_{yz} orbitals²⁰ should be the same for the two, with the major difference being that there will be no sigma spin delocalization to the β -pyrrole and *meso* positions because the $d_{x^2-y^2}$ orbital is empty for pure $S = 3/2$ Fe(III). Thus the amount of $S = 3/2$ character is probably significantly greater than $1/3$ for the metMb-OH complexes.

While this procedure appears to work for the metMb-OH complexes, using the high-spin ferriheme^{21,65,66} and the low-spin cyanide complex^{22,49,65,75} chemical shifts does not work for NP2-OH, NP3-OH, *Nm*-HO-OH and *Pa*-HO-OH, all of which have somewhat similar chemical shifts to those of the respective cyanide complexes (Table 2). Hydroxide is a much weaker-field ligand than cyanide, and it may not be able to support a fully low-spin electron configuration in any of these complexes. As discussed previously for the cyanide complexes, especially of heme proteins having an axial histidine ligand with its imidazole plane along the *meso*-carbon axis,²² the small values, as well as the small spread, of the methyl resonances suggests that these complexes may have at least a d_{xy} component to the ground state electron configuration. La Mar and coworkers have stated that the *Nm*-HO-OH complex has a “normal” $(d_{xy})^2(d_{xz}, d_{yz})^3$ ground state, whereas Rivera has shown by ^{13}C NMR spectroscopy

that the major form of *Pa*-HO-OH has a mixed $S = 3/2$, $S = 1/2$ ground state. From the results presented in Table 2, we suggest that it is likely that both have mixed spin ground states which include a $(d_{xz}, d_{yz})^4(d_{xy})^1$ component. For NP2-OH and *Nm*-HO-OH the mixed spin state may consist only of the two $S = 1/2$ electron configurations, with the “normal” $(d_{xy})^2(d_{xz}, d_{yz})^3$ electron configuration predominating, while *Pa*-HO-OH probably does have an $S = 3/2$ component in the mix of spin states at ambient temperatures. However, NP2-OH, NP3-OH and horse heart Mb-OH all have low-spin rhombic EPR spectra at 4.2 K (unpublished results). Further investigations of these and other hydroxo complexes, including detailed ^{13}C NMR studies, will be required in order to determine definitively what the electron configurations of these complexes are at ambient temperatures.

In model ferriheme complexes having two axial imidazole ligands, a purely ruffled heme plane structure stabilizes the perpendicular orientation of the axial ligands, although the dihedral angle between the two ligand planes is seldom exactly 90° .⁷⁶⁻⁸⁰ The observation by NMR spectroscopy (NOESY spectrum of Figure 3) of a small amount of the NP3-NMeIm complex with “perpendicular” orientation to the protein-provided ligand, H57, is interesting, for no such perpendicular orientation is observed by NMR spectroscopy for the wild-type NP2 complex. It should be mentioned, however, that in NP2 crystal structures, the axial ligand orientation is “parallel” in wild-type NP2-ImH (PDB file 1PEE), but is “perpendicular” in the crystal structure of NP2-L122V/L132V-ImH (PDB file 1PM1). In reality, neither exactly parallel nor exactly perpendicular orientations are observed for the nitrophorins; rather there is always some dihedral angle between the H57 imidazole plane and the distal ligand imidazole plane. For NP2-ImH it is 21.9° (1PEE), while for NP2-L122V/L132V-ImH it is 68.5° (1PM1). In solution using the methyl chemical shifts of the imidazole complexes and the angle plot (Supporting Information Figure S7), these angles are about $29 \pm 4^\circ$ and $88 \pm 2^\circ$, respectively. However, the perpendicular orientation is the minor one for the double mutant in solution, with the major dihedral angle for the double mutant being 23° .¹⁸ Unlike X-ray crystallography, EPR spectroscopy of the frozen solution sample of NP2-L122V/L132V-ImH at 4.2 K shows both types of ligand orientations for this complex, a major (ratio ~4:1) normal rhombic EPR signal that is characteristic of complexes with axial ligands in near-parallel planes, and a minor single feature “large g_{max} ” EPR signal that is characteristic of complexes with axial ligands in near-perpendicular planes. Observation of both signals, with ~4:1 intensity ratio indicates that the stability of the perpendicular complex increases as the temperature is lowered (unpublished results). There are more protein motions in solution at the ambient temperatures used for NMR spectroscopy, and thus the heme is apparently able to adjust to the heme cavity by changing the heme plane distortion from less to more ruffled for the “perpendicular” ligand complex. The observation of two types of axial ligand orientations for NP3-NMeIm by NMR spectroscopy became possible due to chemical exchange between them (Figure 4, NOESY spectrum), which is also an indirect observation of heme plane distortion, most likely ruffling, as seen in the crystal structures of all NP2 complexes.^{27,28}

Earlier it was shown that from the ^1H NMR shifts of the heme methyls of NP2-NMeIm, the angle plot yielded directly the orientation of the NMeIm ligand, thus indicating that it is a stronger donor than the protein-provided histidine imidazole ligand.²⁰ Thus, using the orientations of the histidine imidazole plane obtained from the high-spin forms of the proteins summarized in Table 1 and the angle plot of Supporting Information Figure S7, the dihedral angle between the planes of the two axial ligands is 30° for the **B** isomer of NP2, as compared to 34° for the major **B** isomer of NP3, 34° for the **B** isomer of NP3-Y27F, and 22° for that of NP2-F27Y (Table 3). In comparison, the **A** isomer has smaller dihedral angles, 17° for NP3 and 22° for both isomers of NP2-F27Y. The minor **B'** orientation isomer with “perpendicular” ligand orientation has a dihedral angle of 80° . All of these values are summarized in Table 2.

In conclusion, we have found that while there are differences in the chemical shifts of protons of the ferriheme ring that lead to conclusions about small differences in the orientation of the histidine ligands in the two proteins, as well as the fact that the hemes of both proteins are ruffled, the heme environment of NP2 and NP3 in both the high-spin and the low-spin NMeIm complexes is very similar. In spite of this similarity, however, the kinetics of deprotonation of the high-spin complexes of NP2 and NP3 are very different. The hydroxo complexes of NP2 and *Nm*-HO-OH⁶⁶ are probably $S = 1/2$ mixed $(d_{XY})^2(d_{XZ}, d_{YZ})^3/(d_{XZ}, d_{YZ})^4$ electron configurations, while those of *Pa*-HO-OH⁶⁵ and a number of metMb-OH⁵⁹⁻⁶⁴ are likely $S = 1/2, 3/2$ mixed spin systems, however with the metMb-OH complexes having much larger $S = 3/2$ contribution than *Pa*-HO-OH. Careful analysis of the 5Me and 8Me shifts of the **A** and **B** isomers of the two high-spin nitrophorins leads to the conclusion that the heme environment for the two isomers differs in some way that we cannot explain at the present time. Further work now underway is aimed at understanding this difference.

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Abbreviations

HMQC, Heteronuclear Multiple Quantum Coherence, a 2D NMR experiment that detects J-coupling correlations between protons and carbons to which they are bound

Hm, histamine

HO, heme oxygenase

ImH, imidazole

Mb, myoglobin

NMeIm, N-methylimidazole

NP, nitrophorin

Nm-HO, *Neisseria meningitidis* heme oxygenase

Pa-HO, *Pseudomonas aeruginosa* heme oxygenase

PDB, Protein Databank of the Research Collaboratory for Structural Bioinformatics (RCSB)

WEFT-NOESY, Water-Eliminated Fourier Transform-Nuclear Overhauser and Exchange Spectroscopy, a 2-dimensional (2D) NMR experiment that correlates protons connected by proton-proton dipolar interactions or by chemical exchange

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Supplementary Material

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Scheme I.

Figure 1. 500 MHz 1D NMR spectra of NP3, NP3-Y27F, NP2-F27Y, and NP2 HS in D₂O at pH* 7.0 and 25 °C in the absence of added axial ligand. The pair of large and small peaks in the region 17–23 ppm is due to one of the His57 β-CH₂ protons.

Figure 2.
1D saturation transfer difference NMR spectra of NP3 in the presence of a small amount of NMeIm. Recorded at 500 MHz in D₂O, pH* = 7.0 and 25 °C.

Figure 3.

WEFT-NOESY spectrum of wild-type NP3 in the absence of added axial ligand. Recorded at 500 MHz in D₂O, pH* = 7.0, 25 °C. Note the two cross peaks from the major His57 β-CH₂ proton at 19.5 ppm to two resonances near +2.5 and -2.0 ppm, which are the other β and the α-CH proton of that residue.

Figure 4.

pH titration of NP2 in the absence of added axial ligand, recorded at 500 MHz, 25 °C. pH* values (uncorrected for the deuterium isotope effect) are a) 7.10, b) 9.90, c) 10.30, d) 10.86, e) 10.99, f) plot of pH dependence vs. fraction of hydroxo complex formed (from the relative intensities of the 3Me resonance of the high-spin aquo and the hydroxo complexes). From these data the pK_a of the water molecule bound to NP2 is estimated to be 10.5 (uncorrected for the deuterium isotope effect). Note that spectrum e) shows the beginning of observable kinetic averaging of the NMR signals of the high-spin aquo and the hydroxo complexes that is quite far advanced at pH* = 9.6 for NP3 (Supporting Information Figure S5). Methyl resonance

assignments for the hydroxo complex (from Supporting Information Figure S4) are marked on d).

Figure 5.
 1D, HMQC and WEFT-NOESY spectra of wild-type NP3-NMeIm, recorded on a Bruker DRX-500 in D₂O at 25 °C, pH* = 7.0.

Figure 6.
1D and WEFT-NOESY spectra of NP3-Y27F-NMeIm, recorded at 500 MHz in D₂O at 25 °C, pH* = 7.0.

Table 1

Proton Chemical Shifts of the High-Spin Nitrophorins NP3 as Compared to NP2 and the Two Mutants, Recorded at 25 °C, pH 7.0.

	NP3 (A:B ~1:4)		NP3-Y27F (A:B ~1:6)		NP2-F27Y (A:B ~1:3)		NP2 (A:B ~1:8)	
	A	B	A	B	A	B	A	B ^a
1Me	59.0	58.4	57.3	62.4	56.5	52.1	(~60) ^d	58.6 ^d
3Me	57.2	48.3	52.5	51.3	55.4	45.1	(55.5) ^a	49.5 ^a
5Me	66.2	63.1	69.5	65.6	65.5	60.5	67.8 ^a	64.1 ^a
8Me	67.4	61.2	69.5	65.2	65.5	58.5	68.4 ^a	64.1 ^a
2H _a	...	46.3				46.9		44.1 ^b
2H _β	...	-4.1, -6.8				-6.0, -4.7		-4.8, -7.9 ^{c,b}
4H _a	...	33.2				31.1		34.0 ^b
4H _β	-8.9, -12.3	-10.7, -13.3				-11.4, -12.8		-9.8, -13.2 ^{c,b}
6H _a	55.3, 28.7	40.7, 30.1			51.6, 28.7	34.8, 29.5		41.2, 32.3 ^b
6H _β		9.9, 3.0				2.0		2.7, --, ^{b,c}
7H _a	42.8, 34.2	47.8, 25.5			51.7, 28.6	42.9, 24.6		50.6, 28.4 ^b
7H _β		7.3, 3.6				2.80, 2.75		3.1, --, ^{b,c}
H57 _β	21.2	19.5, 2.5	22.7	20.5	19.0	17.0	19.7	18.5, 2.5
Me Av., ^d	62.5, 10.2	57.8, 14.8	62.2, 17.0	61.1, 14.3	60.6, 10.1	54.1, 15.0	~63, 12.9 ^{aa}	59.1, 14.6 ^{aa}
Me order	8>5>1>3	5>8>1>3	8>5>1>3	5>8>1>3	8>5>1>3	5>8>1>3	8>5>1>3	5>8>1>3
5Me ^A — 8Me ^B ^e	5.0		4.3		7.0		3.7	
8Me ^A — 5Me ^B ^e	4.3		3.9		5.0		4.3	
H57 Im angle	137°	133°	135°	134°	135°	133°	136°	135°

^aTemperature = 25 °C; taken from Ref. 21.

^bTemperature = 20 °C; taken from Ref. 20.

^cCould not be unambiguously assigned.

^dSpread of the methyl resonances, ppm.

^eChemical shift difference between the **A** 5.8 methyl resonances and their **B** 8.5 methyl counterparts, ppm (see discussion in the text).

Table 2
Methyl Proton NMR Chemical Shifts of the Hydroxo Complexes of Histidine-bound Heme Proteins.

Protein	Temp. (°C)	His-Imidazole Plane angle (°) <i>a</i>	Methyl shift order	Methyl shifts (ppm)	Spread (ppm)	Av shift (ppm)	Ref.
NP2-OH	30	132 (138)	3 > 5 > 8 > 1	19.0, 13.0, 10.7, 6.5	12.5	12.3	TW
NP3-OH	30	--- (138)					66
Nm-HO-OH	25	134 (100–105)	3 > 8 > 5 > 1	19.5, 17.5, 8.0, (7.0) ^b	(12.5) ^b	(12.3) ^b	65
Pa-HO-OH	25	~135		24, 17, 14, (10) ^b	(14) ^b	(16.3) ^b	61
<i>Aplysia</i> met Mb-OH	30	~145–154 (?)	5 > 8 > 3 > 1	39.7, 38.2, 35.5, 27.8	12.1	35.3	60,62
<i>Dolabella</i> met Mb-OH	30	~143 (?)	5 > 8 > 3 > 1	37.8, 36.9, 32.4, 26.5	11.3	33.4	62
<i>Mustelus</i> met Mb-OH	30	~145 (?)	5 = 8 > 3 > 1	36.9, 36.9, 34.1, 26.4	10.5	33.8	62
Equine met Mb-OH	30	178 (?)	5 = 8 > 3 > 1	36.9, 36.9, 32.8, 25.8	11.1	33.1	62
SW met Mb-OH	25	178 (?)	5 = 8 > 3 > 1	38.1, 38.1, 33.6, 26.8	11.3	34.1	63
<i>T. akamusi</i> met HbV-OH	25	~164 (?)	5 > 8 > 1 > 3	30.8, 26.8, 25.3, 20.8	10.0	25.9	63
Comparison to Heme Protein-Cyanide Complexes							
NP2-CN	30	135	3 > 8 > 1 > 5	18.4, 13.4, 9.7, 7.8	10.6	12.3	22
Nm-HO-CN	25	134	3 > 8 > 5 > 1	21.4, 10.3, 9.6, 7.9	13.5	12.3	75
Pa-HO-CN	35	~135	5 > 1 > 8 > 3	27.7, 22.7, 19.0, 4.4	23.3	18.5	49
<i>Aplysia</i> met Mb-CN	145	~145–154	3 > 5 > 1 > 8	17.8, 15.7, 11.8, 9.9	7.9	13.8	44
<i>Dolabella</i> met Mb-CN	---	~145	3 > 5 > 1 > 8	17.4, 15.1, 11.8, 10.2	7.2	13.6	74
SW met Mb-CN	178	178	5 > 1 > 8 > 3	27.0, 18.6, 12.9, 4.8	22.2	15.8	44

^a Angle from the crystal structure. In parenthesis, angle of nodal plane from ¹H heme methyl shift pattern; those which have a methyl shift order not predicted by the low-spin ferrheme angle plot of Supporting Information Figure S7 are indicated by a question mark.

^b Fourth heme methyl shift not reported; estimated value in parenthesis.

Table 3

Proton Chemical Shifts of the Low-Spin NMeIm Complex of NP3 as Compared to NP2, NP3-Y27F and NP2-F27Y, all at 25 °C, pH 7.0.

Proton	NP3			NP3-Y27F		NP2-F27Y		NP2 ^a	
	A	B	B'	B	B	A	B	A	B
N-Me	16.5	16.2	---	16.3	16.3	16.4	16.3	16.4	16.1
1M	-1.4	14.6	18.5	16.1	16.1	-0.4	11.9	-0.4	13.8
3M	28.1	12.8	---	11.5	11.5	26.4	15.2	26.4	13.4
5M	---	16.0	21.7	17.4	17.4	1.88	14.1	1.88	15.4
8M	15.7	-0.2	25.7	-0.4	-0.4	14.3	1.5	14.3	1.0
2H _a	---	24.7	---	24.2	24.2	11.9	23.9	11.9	23.5 ^d
2H _β	---	-5.0, -5.3	---	-6.2, -6.5	-6.2, -6.5	-5.7, -6.0	-4.0, -4.6	-5.7, -6.0	-7.0, -7.6 ^d
4H _a	---	6.2	---	6.2	6.2	8.7	6.3	8.7	6.1
4H _β	---	1.3, 0.8	---	---	---	0.7	0.6, 1.0	0.7	0.4, 1.0 ^b
6H _a	---	14.9, 13.3	---	14.4, 14.6	14.4, 14.6	10.2, 4.0	15.1, 11.4	10.2, 4.0	13.9, 12.5
6H _β	---	-0.8, -2.5	---	-2.05, -0.5	-2.05, -0.5	-2.6, -3.2	-1.2, -3.0	-2.6, -3.2	-0.9, -2.4
7H _a	15.6, 14.7	7.9, 3.1	---	3.0, 7.4	3.0, 7.4	14.4, 10.5	8.9, 4.3	14.4, 10.5	8.0, 4.1
7H _β	-2.7, -1.3	-2.8, -3.5	---	-2.9, -3.6	-2.9, -3.6	-2.8, -3.4	-1.44, -2.96	-2.8, -3.4	-3.0, -3.7
m-α	-2.8	-1.85	---	-1.4	-1.4	-3.2	-2.2	-3.2	-3.2 ^c
m-β	---	6.9	---	6.7	6.7	---	6.9	---	6.7
m-γ	-3.2	-3.7	---	-3.0	-3.0	-3.6	-4.6	-3.6	-3.8
m-δ	7.2	9.5	---	9.9	9.9	---	9.1	---	9.5
Order	3>8>5>1	5>1>3>8	8>5>1>3	5>1>3>8	5>1>3>8	3>8>5>1	3>5>1>8	3>8>5>1	5>1>3>8
φ _{NMR} (= φ _{NMeIm}) ^e	120 ± 2°	167 ± 1°	53 ± 2°	169 ± 2°	169 ± 2°	113 ± 2°	156 ± 1°	113 ± 2°	163 ± 1°
Δφ	17°	34°	80°	34°	34°	22°	22°	22°	28°

^a Taken from reference ²⁰.

^b Cis and Trans could not be assigned with certainty.

^c Shift at 10 °C.

^d Reassigned after publication of reference ²⁰.

^e See discussion in text that indicates that the angle measured by the angle plot is that of the NMeIm ligand, which is a stronger donor than the histidine imidazole.