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NMR Studies of Nitrophorin Distal Pocket Side Chain Effects on the Heme Orientation and Seating of NP2 as Compared to NP1

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Abstract

The nitrophorins (NP) of the adult blood-sucking insect *Rhodnius prolixus* fall into two pairs based on sequence identity (NP1,4 (90%) and NP2,3 (79%)), which differ significantly in the size of side chains of residues which contact the heme. These residues include those in the distal pocket of NP2 (I120) and NP1 (T121) and the “belt” that surrounds the heme of NP2 (S40, F42), and NP1 (A42, L44). To determine the importance of these residues and others conserved or very similar for the two pairs, including L122(123), L132(133), appropriate mutants of NP2 and NP1 have been prepared and studied by ¹H NMR spectroscopy. Wild-type NP2 has heme orientation ratio (A:B) of 1:8 at equilibrium, while wild-type NP1 has A:B ~1:1 at equilibrium. Another difference between NP2 and NP1 is in the heme seating with regard to His57(59). It is found that among the distal pocket residues investigated, the residue most responsible for heme orientation and seating is I120(T121). F42(L44) and L106(F107) may also be important, but must be investigated in greater detail.

1. Introduction

The nitrophorins (nitro = NO, phorin = carrier) are a group of nitric oxide (NO) carrying heme proteins found in the saliva of blood-sucking insects [1]. One such insect is *Rhodnius prolixus* (the “kissing bug”), which has a number of salivary nitrophorins that store NO *via* a ferriheme-nitrosyl complex and keep it stable for long periods of time [2, 3]. Upon injection into the tissues of the bug's victim, dilution and pH elevation cause dissociation of NO. The NO diffuses through the tissues to the nearby capillaries to cause vasodilation (and inhibition of platelet aggregation) to thereby allow more blood to be transported to the site of the wound [4 – 6]. In addition, the heme sites of the *Rhodnius* nitrophorins are able to bind histamine, which is released by mast cells and platelets of the victim in response to the wound. This histamine would otherwise cause swelling, itching, and the beginning of the immune response; hence its binding to the nitrophorins prevents the insect's detection for a period of time, so that it may obtain its fill of blood [7]. These properties of the nitrophorins of the adult insect *Rhodnius prolixus* assist the bug in obtaining a sufficient blood meal. Of the four *Rhodnius* nitrophorins of the adult insect, which have been named NP1–4 (in order of their decreasing abundances in the adult insect saliva), NP2 is unique, for in addition to

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its NO-releasing and histamine-binding roles, it also has anticoagulant activity, through heme-independent inhibition of the factor Xase complex [8 – 10].

The solid state structures of *Rhodnius* nitrophorins bound to various ligands have been reported for NP1 [11, 12], NP2 [13, 14] and NP4 [15 – 20]. These structures show the heme to be located inside but at the open end of a β -barrel, with the propionate groups protruding into the aqueous medium. This structure is unique for heme proteins, which are more commonly α -helical globin or 4-helix bundle folds [21, 22]. The ferriheme prosthetic group is bound to the protein *via* a histidine ligand, His57 for NP2 and NP3, His59 for NP1 and NP4. The sixth coordination site is available to bind NO or other ligands. Ligand binding has been investigated by a number of techniques [12, 23 – 34], including infrared [12], resonance Raman [23], NMR [12, 24 – 31] and EPR [35] spectroscopy, stopped-flow kinetics [32] and spectroelectrochemistry [25, 32 – 34]. The nitrophorins transport NO *via* a ferriheme-nitrosyl complex, in which NO is bound to heme with stabilities that facilitate release upon dilution ($K_d \sim$ micromolar) [32]. Typically, other heme proteins have much more positive midpoint potentials than the nitrophorins (NP $E_m = -259$ to -318 mV at pH 5.5, -274 to -325 mV at pH 7.5 in the absence of NO; $+154$ to $+48$ mV at pH 5.5, $+127$ to -20 mV at pH 7.5 when bound to NO [33, 34]) and the nonnitrophorin ferriheme-nitrosyl complexes are unstable with respect to reduction to Fe(II)-NO in the presence of excess NO [36], which bind NO irreversibly ($K_d \sim$ picomolar [32, 33]). E_m values for heme proteins other than the nitrophorins, in the absence of NO, are in many cases in the 0 mV range vs. SHE (horse heart myoglobin) [12], about 300 mV more positive than the NPs, and the midpoint potentials of these proteins in the presence of NO cannot be measured because of autoreduction, but clearly, they are much more positive than those of the nitrophorins.

The four nitrophorins have a wide range of NO binding and release rates, in two pairs of similar-sequence proteins (Supporting Information Fig. S1): NP1 and NP4 have NO on-rates $k_f \sim 1.5$ and $2.2 \mu\text{M}^{-1}\text{s}^{-1}$ at 25°C , while NP2 and NP3 have much faster on-rates which had to be measured at much lower temperature, $k_f \sim 7.3$ and $7.0 \mu\text{M}^{-1}\text{s}^{-1}$ at 12°C , in both cases nearly independent of pH over the range 5.0–8.0 for the major fast phase of each of these reactions [32]. In contrast, the off rates of the two sets of proteins behave quite differently over the same pH range, $k_d \sim 0.2$ and 0.14 s^{-1} at pH 5.0, $k_d \sim 2.2$ and 2.6 s^{-1} at pH 8.0, for the fast phases of these reactions, respectively, a change of a factor of 11 and 20 for NP1 and NP4 over this pH range, while NP2 has a much smaller change in off rate, $k_d \sim 0.05 \text{ s}^{-1}$ at pH 5.0, 0.12 s^{-1} for the fast phase at pH 7.5, 25°C , a change of less than a factor of 3 for NP2 over this pH range [32]. The equilibrium dissociation constants for NO have been measured separately at pH 5.5 and 7.5 as $K_d = 120$ and 830 nM (NP1), 50 and 200 nM (NP4), 1.0 and 5.0 nM (NP2), at 27°C [33, 34] (not determined for NP3). The reason for the presence of the four NPs in the adult insect saliva, and for the ranges of their rate and equilibrium constants, are believed to be a means of spreading out the release of NO over a longer time period than would be the case if there were only one nitrophorin protein [2].

In contrast to the NO rate and equilibrium constants, the histamine rate and equilibrium constants at pH 7.5 are all quite similar for NP1, NP4 and NP2, $k_f = 1.5 \times 10^6$, 2.3×10^6 and $3.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_d \sim 0.43$, 0.29 and 0.56 s^{-1} , and $K_d = 9.1$, 6.7 , 10 nM , respectively (R. E. Berry, unpublished results). The reasons for the differences in rate and equilibrium constants for the binding of NO to the four NPs is rooted in the differences in protein sequences of the two pairs of nitrophorins, the interaction of the heme with the protein substituents which are very close by, and the behavior of the A–B and G–H loops that partially close the ligand escape route for NP1 and NP4 at low pH, but are open at high pH [20]. In contrast, for NP2 (and probably NP3), the A–B and G–H loops do not appear to change conformation significantly as a function of pH [37], which thus removes most of the pH dependence of the rate and equilibrium constants for these two NPs. Apart from the loop dynamics of the two

pairs of NPs, the binding of NO to all four of these proteins is strongly affected by differences in the interactions of nearby protein residues with the heme. The present paper uses NMR spectroscopy to investigate the differences in these interactions for one of each of the two pairs of nitrophorins in the absence of NO.

The nitrophorins all have two conserved leucine residues in the distal pocket where NO, histamine and other ligands can bind, which have their side chains positioned above and in contact with the heme near the iron binding site, as shown in Fig. 1, where they possibly contribute to the extensive distortion of the heme from planarity [17]. This distortion is particularly extreme in NP2, but is also present, although to a lesser extent, in NP1 and NP4. Unfortunately it has not been possible to obtain crystal structures of NP3 thus far, but because of the similarity in the protein sequences of NP2 and NP3 (Supporting Information Fig. S1) we surmise that NP3 may have a similarly nonplanar heme. This nonplanarity is believed to affect the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ midpoint potential, E_m , and thus the reactivity of the ferriheme center toward NO [25]. The Leu122(123) side chain is situated near the opening to the ligand binding pocket, while the Leu132(133) side chain is buried deep inside the pocket.

In addition to the two conserved leucine residues in the nitrophorin distal pocket there is a third residue which also is positioned above and in contact with the heme near the distal ligand binding site, also deep inside the distal pocket. This residue is an isoleucine (I120) for NP2 and NP3 (Fig. 1), but a threonine (T121) for NP1 and NP4. Because of the difference in size and hydrophilicity of the side chains of Ile and Thr, we wondered what the role of this residue is in determining the thermodynamic and kinetic properties of the nitrophorins, in particular the heme seating and the ratio of heme orientations, **A** and **B** (Fig. 1). Thus, among the mutants we have prepared are the I120T and I120V mutants of wt NP2 and the T121S, T121L and T121I mutants of wt NP1. And to check the effect of hydrophilicity we have prepared mutants of the L122V,L132V double mutant as Val to Thr (i.e., from wild-type NP2, these are the L122T,L132V and L122V,L132T double mutants of NP2).

In addition to the three distal pocket side chains of all nitrophorins, a number of residues are positioned like a “belt” around the inside of the beta-barrel in such a way that they may interact with the heme to affect the heme **A:B** orientational ratio and degree of ruffling, or in extreme cases, the actual seating of the heme. Among these, there are several sequence differences between NP1, NP4 on one hand and NP2, NP3 on the other which are somewhat different in size, for example, A42 as compared to S40, L44 as compared to F42, and F107 as compared to L106, respectively. Thus we have investigated those differences in residues between the two groups of nitrophorins.

The effect of mutations of the residues very close to the heme on the **A:B** isomer ratio of NP2 and NP1 is a major focus of this paper. The reason for the usually unequal ratio of **A:B** in the nitrophorins as well as all other heme *b* (protohemin)-containing proteins, is the interaction of the unsymmetrically-placed 2- and 4-vinyl groups with bulky protein side chains, and hence the **A:B** ratio is an equilibrium constant which measures the seemingly rather subtle effect of one extra carbon at the 2- and 4- positions of protohemin in the interaction of this prosthetic group with the protein in which it is bound. As shown in Fig. 1, the fact that protohemin does not have a 2-fold rotation axis that passes through the heme plane means that every protohemin (heme *b*)-containing heme protein has two possible isomers present in the solution; only in the cases where either **A** or **B** is so highly favored that the other isomer cannot be detected does the NMR spectrum of a *b* heme protein have only one set of heme resonances. Wüthrich, Keller and coworkers first noted in 1972 that there was some heterogeneity in the heme resonances in the NMR spectra of horse myoglobin and insect hemoglobins [38], and La Mar and coworkers later showed that for

Chironomus thummi thummi monomeric hemoglobins this heterogeneity was due to the two heme orientations [39]. Most mammalian myo- and hemoglobins strongly favor the **A** heme orientation, with sperm whale Mb [40] and human hemoglobin β chains [41] having only several percent **B** heme orientation at equilibrium, while all cytochromes b_5 have various ratios of **A**:**B** (20:1, chicken microsomal [42]; 9:1 human erythrocyte [43]; 1:1.6, human outer mitochondrial membrane [46]; 9:1, bovine microsomal and erythrocyte [44, 45]; 1:1.6, rat microsomal [42]; 1:1.2, rat outer mitochondrial membrane [46]; ~1:1, house fly microsomal [47]). NP1 and NP4 both have **A**:**B** ratios of about 1:1, while NP2 and NP3 have **A**:**B** ratios of 1:8 and 1:4 at equilibrium for the high-spin forms, respectively [26], and NP2(D1A) has an **A**:**B** ratio of 1:14 [29], which amounts to a ΔG difference of 6.6 kJ/mol for the stability of the two heme orientations at 300 K. Although the X-ray crystal structures of most cytochromes P450 are modeled with only one orientation for the heme, it is likely that some percentage, probably variable among this class of proteins of the other heme orientation is present; wt NP2 structures are modeled as having only the **B** heme orientation [13, 14], although NMR spectroscopy clearly shows an **A**:**B** ratio of 1:8 [24], but current X-ray crystallographic structures of the mitochondrial cytochrome bc_1 complex are beginning to resolve the minor orientation of the b hemes (EA Berry, personal communication). Only cytochromes c have no known cases of the two heme rotational isomers being present, because there are elaborate protein mechanisms for the stereospecific attachment of two cysteines of the proteins to the α -carbons of the 2- and 4-vinyl groups of protoheme to form heme c [48 – 50].

One of the aims of this work is to determine which protein side chains are responsible for the differing **A**:**B** heme orientational ratios of the four major nitrophorins, and whether these differing equilibrium constants lead to differences in the properties of these proteins, such as the rate of NO binding and release. Several investigations have shown differences in properties between the **A** and **B** isomers of heme proteins. These differences are very dependent on the particular heme protein, so that although O_2 affinity, which was originally thought to depend on heme orientation [51], has been shown to be independent of heme orientation for sperm whale myoglobin [52, 53], it does depend on heme orientation for *Chironomus thummi thummi* HbIII and HbIV [54]. In addition, the position of the $S = 1/2$, $S = 5/2$ spin equilibrium for both *Chironomus thummi thummi* met HbN₃ [55] and ferricytochrome b_{562} [56] is influenced by heme orientation, and there is a difference of 26 mV in midpoint potential for the **A** and **B** heme isomers of the erythrocyte form of bovine cytochrome b_5 [44]. In addition, there is functional relevance to the binding of heme to bacterial heme oxygenases that produce other than α -meso-carbon oxidation to CO [57 – 59], and heme reorientation also serves as a measure of protein dynamics, for example, for the case of recombinant wild-type NP2 as compared to NP2(D1A), where the half-lives of heme reorientation are 2 h and 43 h, respectively [29]. We have not as yet investigated the differences in rate of binding/release of NO from the **A** and **B** orientational isomers of the nitrophorins, but this is a project that we will undertake in the near future.

In this work we report the 1H NMR spectra of the wild-type NP2 and NP1 and their distal pocket and several belt mutants, in the high-spin, NO-off state, as well as the imidazole (ImH) complexes (which, unlike histamine, a physiological ligand of the NPs, can bind ImH at several angles relative to the H57(59) protein-provided axial ligand imidazole plane) of some of the distal pocket and belt mutants.

2. Materials and Methods

2.1. Protein Sample Preparation

Cloning and expression plasmids used were those reported previously [13, 25, 29]. Additional mutant genes (for the L122A, L132A, I120T, I120V, S40A and (L122A,L132A)),

(L122T,L132V) and (L122V,L132T) double mutants of NP2, and the T121I, T121L, T121S, L44F and A42S mutants of NP1) were prepared according to standard genetic engineering methods, and expressed as reported previously [13, 25]. The *E. coli*-expressed mutant NP2 proteins were purified as described previously [13, 25], and were stored in lyophilized form at -80°C until use. The mutant proteins were preliminarily characterized by UV-visible and mass spectrometry (MALDI-TOF, Matrix-Assisted Laser-Desorption/Ionization-Time Of Flight).

2.2. NMR data collection

NMR samples were prepared in D_2O containing 50 mM phosphate buffer at $\text{pH}^* = 7.0$.[§] NMR spectra were collected over the temperature range 15 to 35°C with the proton chemical shifts referenced to residual water. NOESY (Nuclear Overhauser and Exchange Spectroscopy) and HMQC (Heteronuclear Multiple Quantum Correlation) spectra were obtained on a Bruker DRX-500 or a DRX-600 spectrometer operating at 500.03 or 600.13 MHz proton Larmor frequency, respectively. The $^1\text{H}\{^{13}\text{C}\}$ HMQC experiments were recorded at a proton Larmor frequency of 500 MHz using a 5 mm inverse-detection probe with decoupling during acquisition. A recycle time of 200 ms and refocusing time of 2.5 ms ($J = 200$) were used. The WEFT-NOESY experiments, recorded at 500 MHz, utilized 100 ms relaxation delay and 130 ms recovery-delay. The mixing time for the NOESY experiments was 23–30 ms. All 2D spectra were collected with 1024 or 2048 data points in t_2 and with 256–512 blocks in t_1 with 400–512 scans/block.

2.3. EPR spectroscopy

EPR spectra were obtained on frozen protein solutions containing wt NP2 and its L $\rightarrow$ V distal pocket mutants with an excess of each of the desired ligands, at 4.2 K on a Bruker ESP-300E EPR spectrometer operating at X-band using 0.2 mW microwave power, 100 kHz modulation frequency and 2–4 G modulation amplitude. A Systron-Donner frequency counter was used to measure the microwave frequency for precise calculation of g-values.

2.4. NP1(L44F) and NP2(L106F) model building

The models of the NP1(L44F) and NP2(L106F) mutations were built using the software SWISS-PdbViewer [60] (version 4.0.1, including a version of the GROMOS 43B1 force field [61]). The nitrophorin X-ray structures (PDB code 1D3S for NP4 and 1EUO for NP2) were used as the modeling template, in which rotamers were manually selected that did not result in clashes. The structures were refined in SWISS-PDBViewer for local energy minimization (200 cycles of steepest descents followed by 200 cycles of conjugated gradients), first of the mutated residue and then including all the residue side chains within $5\text{\AA}$. The position of the heme was not refined.

3. Results

3.1. NMR Spectra of M0-containing NP2 Distal Pocket L $\rightarrow$ V, L $\rightarrow$ V,T, L $\rightarrow$ A and I120 Mutants

All mutants were prepared using the wild-type NP2 gene, which retains the M0 that results from the start codon of the recombinant gene. The NMR spectra of NP2(L122V), NP2(L132V) and the double mutant NP2(L122V,L132V) and their ImH and 2MeImH complexes have been reported previously [25]. In Fig(s). 2a and 2b are shown the 75–15 ppm chemical shift part of the ^1H NMR spectra at pH 7 and 25°C of high-spin wild-type NP2 [25], the NP2(L122V) and NP2(L132V) single mutants [25], the NP2(L122V,L132V)

[§]pH* denotes the fact that the solvent is D_2O , and that the pH values measured with a pH meter are uncorrected for the deuterium isotope effect.

double mutant [25], and the two possible L→V,T double mutants, NP2(L122A), NP2(L132A), NP2(I120T), NP2(I120V) and NP2(S40A), all recorded at pH* 7.0 and 25 °C, except for the spectrum of NP2(L132A), which was recorded at 30 °C. It can be seen that the unique resonances that are observed in the 75–15 ppm region are similar in general appearance to those of the ligand-free wild type NP2, NP2(L122V), NP2(L132V) and the NP2(L122V,L132V) double mutant [25]. The major difference between the valine and alanine distal pocket mutants of NP2 is the significant broadening of the resonances of the latter. For wt NP2 the pK_a of the water molecule bound to the heme is 10.5 [28], and that of NP3 is similar [28]. Thus it is unlikely that differences in the NMR spectra result from deprotonation of that water molecule at pH* 7.0. Rather, the broadening appears to be a sign of dynamics, but we have been unable to characterize its nature; the NP2(L122A,L132A) double mutant was too unstable to investigate, and is thus not included in Fig. 2b.

The excellent resolution of the 1D proton NMR spectra and lack of dynamics of the L132V single and (L122V,L132V) double mutants and the two L→V,T double mutants make it possible to observe at least three of the *meso*-H resonances in the 20–32 ppm region of the proton NMR spectra of the high-spin complexes (these resonances have not been assigned to individual *meso* positions). These chemical shifts are indicative of the high-spin heme being bound to a sixth ligand, a water molecule [62]. Other mutants, notably NP2(L122V) and – (I20T), show hints of these *meso*-H resonances, but wt NP2, its S40A mutant, and the L→A mutants do not. The wt NP2 sample and several others were recorded at 600 MHz, where the *meso*-H should have been more clearly resolved if the exchange rates were directly related to those of the L132V and (L122V,L132V) double mutants by the difference in NMR frequency, but they are not resolved. In the cases of wt NP2 and NP2(S40A) it is not clear why this should be the case, although it is possible that the kinetics of water exchange are faster, so that the coordination number of the iron is changing on the NMR time scale (–60 to +30 ppm expected for 5- to 6- coordination [62], 90 ppm. At 500 MHz 90 ppm = 45,000 Hz = $4.5 \times 10^4 \text{ s}^{-1}$ for coalescence of the two no-exchange signals, or 22 microseconds for the lifetime at the coalescence point. To be in the slow-exchange regime, with the broadened *meso*-H signals being at their no-exchange positions, this would require a lifetime of approximately 10–100 times as long [63], or roughly 220 microseconds to 2.2 milliseconds for water molecules on the high-spin Fe(III) nitrophorin complex, to be able to observe the resonances in the 20–30 ppm region of the proton NMR spectra of the high-spin complexes ($k_{ex} \sim 4.5 \times 10^3 - 4.5 \times 10^2 \text{ s}^{-1}$). For shorter lifetimes (faster rates) the resonances would broaden beyond detection, long before they approach coalescence at a lifetime of 22 microseconds.

The 1D saturation transfer difference experiments between the high-spin protein and its low-spin 2MeImH complex, in a solution containing 0.5 equivalent of 2MeImH, are shown in Supporting Information Fig. S2. The assignment of the low-spin 2MeImH complex spectrum, necessary to fully understand the saturation transfer experiments of Fig. S2, is shown in Supporting Information Fig. S3. The order of heme methyl resonances of the low-spin 2MeImH complex is 5>8>1>3. The chemical shifts of the 2MeImH complex are tabulated in Supporting Information Table S1.

It can be seen in Supporting Information Fig. S2 that the order of methyl resonances for the **B** isomer of high-spin NP2(L132A) is also 5>8>1>3. This order, and the relative spacing between methyl resonances, can be used to estimate the angle of the His57 imidazole plane with respect to the heme substituents: In work that began with a study of the relationship between axial ligand plane orientation and hemin methyl shifts for low-spin ferriheme proteins [64], and was later extended to high-spin ferriheme proteins [24], it was shown that the axial ligand nodal plane orientation determines the nodal plane orientation of the $3e(\pi)$ orbital containing the unpaired electron that is NOT involved in spin delocalization to the

His57 imidazole ligand, and thus determines the order of heme methyl shifts for a wide range of ferriheme proteins because of the effect of that nodal plane orientation on a combination of the contact and pseudocontact shifts. A program was created (“Shift Patterns”) which predicts the order and relative spacing of heme methyl resonances for protohemin (and “symmetrical hemin”, 2,4-dimethyldeuteroheemin) complexes of both low-spin and high-spin heme proteins [65]. According to the “angle plot” of axial ligand plane orientation for high-spin ferriheme proteins (Supporting Information Fig. S4) the angle of the His57 imidazole plane is smaller than in the wild-type high-spin NP2 ($\phi < 135^\circ$, probably about 133° , since the 5-methyl and 8-methyl shift lines cross at 135° [65], the angle found for wt NP2 [24], and it is therefore easy to estimate the His-imidazole plane orientation for this complex). This is the only NP2 mutant found thus far for which the **B** heme orientation has 5M with larger chemical shift than 8M. In contrast, the order of methyl resonances of NP2(L132V) is $8>5>1>3$ [25], which indicates that the angle of His 57 imidazole plane is larger than in wild type NP2; the same order is observed for the L122V,L132V double mutant, although in this case, the spacing is slightly larger than for the L132V mutant. (The spacing between 8M and 5M is thus a measure of the seating angle of the heme vs. the histidine imidazole plane of His57(59) [24, 64, 65], and the $8M>5M$ order indicates that the His-imidazole angle is greater than 135° , as listed in Table 1.)

The smaller angle of the nodal plane of NP2(L132A) (133°) as compared to NP2(L132V) (137°) [25] suggests that either the His57 imidazole plane has rotated clockwise by about $\sim 4^\circ$ or the heme seating has effectively rotated counterclockwise by $\sim 4^\circ$ for one of these mutants relative to the other. To determine which has occurred, we note first that the orientation of the His57 imidazole plane is constant, with a water molecule bridging between the imidazole N-H and the side chain carbonyl of Asn68, in all crystal structures reported for NP2 [13, 14]. In fact, the His57 N---Asn69 O distance which represents a hydrogen-bond between His57 imidazole NH and Asn69 side chain O is the same (4.52 ± 0.02 Å) for the six structures of the orthorhombic crystal form, but somewhat larger and more variable (4.61 ± 0.10 Å) for those of the tetragonal crystal form, where there are significant protein-protein interactions near the entrance to the heme binding pocket [14]. Since these two side chains are on different β strands (β_C for H57, β_D for N68), a very constant orientation of the histidine imidazole plane in all NP2 complexes is expected, which thus suggests that the seating of the heme changes slightly as a function of the size of the distal pocket side chains. However, there are additional changes in the heme methyl patterns of the L→V and L→A mutants, in that the spacings between 5,8M and 1M and 3M are quite different for the two series of mutants. This gives rise to different average heme methyl chemical shifts for the two, as will be discussed further below.

A different method than that used for NP2(L132A), discussed above and shown in Supporting Information Fig(s). S2 and S3, was used for the assignment of the ^1H resonances of the high-spin complexes of NP2(L122V,L132T) and NP2(L122T,L132V) mutants containing 0.5 equivalent of added imidazole (ImH), as shown in Fig. 3 and in Supporting Information Fig. S5, respectively. The binding constants of the ImH ligand to these two mutant proteins are smaller for the two L→V,T mutants ($K_{\text{eq}} = 10^{7.4} \text{ M}^{-1}$ for ImH binding to wt NP2 at pH 7.5, $K_{\text{eq}} \sim 10^{7.0} \text{ M}^{-1}$ for ImH binding to the NP2(L122T,L132V) double mutant and $10^{6.4} \text{ M}^{-1}$ for ImH binding to the NP2(L122V,L132T) double mutant (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted), that the ImH complexes can be used at temperatures near 35°C to assign the NOESY/EXSY (Exchange Spectroscopy) spectra of samples having less than a stoichiometric amount of ligand present (NMR sample protein concentration = 2 mM, ImH concentration ~ 0.5 – 1.0 mM,) because of chemical exchange between the same protons in the high-spin and the low-spin forms. (The cross peaks are the concrete evidence of the existence of this chemical exchange.) Assuming the spectrum of

the low-spin complex has already been assigned, that of the high-spin complex can readily be assigned, as shown in each of these two figures.

Since the NP2(L122A) single mutant proteins did not form a stable enough complex with 2MeImH to allow assignment of the low-spin complex, the saturation transfer experiment to assign the high-spin resonances was inconclusive, as were NOESY/EXSY experiments. Assuming the order of heme methyl resonances to be the same as found for NP2(L132A), the heme methyl shifts of the high-spin NP2(L122A) mutant are shown in Table 1 for the **B** isomer, with comparison to wild-type NP2 and the L→V and (L→V,T) double mutants. Only one set of heme resonances, of isomer **B**, was observed in the NMR spectrum of NP2(L122A,L132A) double mutant, but there are additional broad resonances that are shifted to larger chemical shifts of up to ~78 ppm at 25 °C, and thus the NMR spectrum of this mutant is not shown in Fig. 2a or 2b, nor are the shifts listed in Table 1. These resonances grow in intensity for a given NMR sample over a period of several months, and their chemical shifts are so different that it is difficult to interpret them in terms of the second isomer. Insertion of the “symmetrical hemin,” 2,4-dimethyldeuterohemin, instead of protohemin IX (heme *b*) causes some of the large chemical shift resonances to disappear, but others remain in approximately the same positions (not shown). Thus we conclude that the double L→A mutation probably destabilizes the protein to the point that some sort of partial denaturation occurs over time that gives rise to the additional resonances that are beyond our ability to understand and interpret.

The average chemical shift of the four methyl resonances of the L→V mutants is larger as compared with NP2 wild-type, but for the L→A mutants it is smaller (Table 1). In like manner, the spread of heme methyl resonances of the L→V mutants is larger as compared to NP2 wild-type, but the spread of the L→A mutant methyl resonances is smaller. The average heme methyl chemical shift (dotted line in Supporting Information Fig. S4) is a result of two factors: (1) the ruffling of the heme plane, which is unusually large for the nitrophorins as compared to other ferriheme proteins, and (2) the fact that the four methyls of protohemin are not arranged symmetrically, and thus the average methyl chemical shift varies with the orientation of the Hisimidazole plane (hence, a different axial ligand plane orientation will produce a different average methyl chemical shift). The difference in the appearance of the pattern of heme methyl resonances of the L→V and L→A mutants indicates that the heme complex symmetry is changed in different directions: L→V mutation appears to make the ferriheme complex somewhat more asymmetric, but L→A mutation appears to make it much more symmetric, both with respect to the difference in the spread of the chemical shifts. The ratio of the two heme orientational isomers (**A**:**B**) of the NP2(L132A) mutant is very similar to that of the wild-type and valine mutants (~1:8), but the NP2(L122A) mutant shows a somewhat larger **A**:**B** ratio (~1:6). In contrast, NP2(I120T) and NP2(I120V) have very different **A**:**B** ratios of 1:1.5 and 1:2, respectively, as compared to 1:8 for the wild-type, as discussed further below.

The “belt” mutant, NP2(S40A), has a very similar NMR spectrum to that of wild-type NP2 (Fig. 2b), a similar **A**:**B** ~1:8, and an NMR spectrum in which the lines are much sharper than those of wt NP1, thus showing that the A42S mutation allows a less mobile seating of the heme in both orientations. Studies of the ImH complex of NP2(S40A), discussed below, were carried out with a fresh sample, in which the **A**:**B** orientational ratio had not reached equilibrium.

3.2. His57 β-CH chemical shifts and their interpretation

The highly-shifted His β-CH proton, which is found near 20 ppm for the **A** and **B** heme orientations of the high-spin forms of NP2 and its mutants, are marked in Fig(s). 2a and 2b. Such a large chemical shift must have a very large contact shift component, since the

calculated pseudocontact shifts of the His57 β -CH proton nuclei are of the order of 8 ppm (M. E. Zaballa, L. A. Abriata, R. E. Berry, F. Yang, H. Zhang, F. A. Walker, A. J. Vila, manuscript in preparation). In several of these mutants, obviously different chemical shifts were observed for this proton for the two heme orientations, **A** and **B**. For mutants in which it is certain that **A** and **B** resonances have been unambiguously assigned, it is found that the **A** heme orientation shows the larger His57 β -CH chemical shift, while the **B** heme orientation shows the smaller. These differences in chemical shift suggest that the His imidazole plane has somewhat different angles for the two heme orientations, so that the amount of spin density at the highly-shifted His β -CH for **A** vs. **B** is different. This suggests that the His side chain is changing its rotamer angle somewhat for **A** relative to **B** for some mutants, but not for others. The crystal structures of NP2-L [13, 14] and NP4-L [15 – 20] complexes where L = H₂O, NH₃, ImH, histamine, CN[−] and others show a range of angles for the His β -CH which is most closely aligned with the orbital of the unpaired electron (i.e., perpendicular to the imidazole plane of His57). These range from 31.9 to 37.4° dihedral angles from the normal to the imidazole ring for the orthorhombic structures, and 32.0–39.1° dihedral angles for the tetragonal structures of NP2 [13, 14]. For NP4 these dihedral angles range from 28.1 to 37.7° [15 – 20]. If the dihedral angles can vary that much in the solid state, it is likely that there can be at least this amount of variation in the angles in solution. These ranges could give a difference in the amount of spin density at that proton, and thus change its chemical shift (i.e., mainly its contact shift), while the other β -CH is nearly in the plane of the imidazole ring (angles of 3.6–10.8° for NP2 [13, 14], 1.1–9.9° for NP4 [15 – 20]), so that proton has essentially zero spin density and is found at −2.6 ppm for wt NP2. Wild-type NP2 does not show two His β -CH signals for the **A** and **B** isomers, nor do its L122V or S40A mutants or its L122V,L132V double mutant (although in that latter case the amount of **A** is so small that it may simply not be possible to detect the second β -CH resonance), while NP2(I120T) shows almost equal amounts of two β -CH resonances, NP2(L132V), -(L122A), -(L132A) and the -(L122V,L132T) and -(L122T,L132V) double mutants also show two β -CH resonances in the same proportion as observed for the 5,8 methyl resonances in each case, and NP2(I120V) exhibits a somewhat misshapen His β -CH resonance which suggests the presence of two overlapping resonances. As mentioned above, for all cases where two resonances are observed, the **A** β -CH resonance has the larger chemical shift.

The most interesting of all of these His β -CH resonances with different chemical shifts for **A** and **B** is the case of NP2(I120T), where the large native Ile side chain has been replaced by the much smaller Thr side chain found for NP1 and NP4. Ile120 sits right above 3M of the **B** heme orientation of NP2, with the CD2 methyl carbon within 3.50 Å of 3M. For the **A** orientation, the CD2 methyl carbon would be within 3.50 Å of the 2-vinyl α -C, and that is obviously an unfavorable position for that protein side chain, for I120 has a major effect on the **A**:**B** ratio, as shown by the fact that NP2(I120T) has nearly equal amounts of **A** and **B** (Fig.2b), estimated as a ratio of 1:1.5.

3.3. NMR Spectra of the Distal Pocket High-Spin NP2(I120T) and NP2(I120V) Mutants, as compared to the NP1 Mutants T121S, T121L and T121I

Inside the distal heme pocket of NP2 and NP3, Ile120 sits above pyrrole ring II in the major heme rotational isomer **B** (Fig. 1), whereas NP1 and NP4 have Thr121 in that position, a much smaller side chain. In these cases Thr121 is almost equally above pyrrole ring I and II, because the **A** and **B** heme rotational isomers are almost equally stable for the latter two nitrophorins [25, 27]. Unlike the NP2(L122V,A) or -(L132V,A) mutations, the I120V mutation changes the ratio of the two heme orientations (**A**:**B**) significantly, from 1:8 to about 1:1.5 for the I120T and about 1:2 for the I120V mutant, respectively, after equilibration of the samples for eight days. The ratio is the same for the high-spin protein

(Fig. 2b) and its low-spin ImH complex, discussed further below. The chemical shifts of the **B** heme orientational isomer of the I120T and I120V mutants of NP2 are included in Table 1, and the chemical shifts of both heme orientational isomers are listed in Supporting Information Table S2.

An interesting observation that is evident within the wild-type NP2 mutants, and which follows through for the other mutants of this work, is that the introduction of a hydroxyl group near the heme leads to a decrease in the spread of the heme methyl resonances, while the removal of a hydroxyl leads to an increase in that spread. Thus, the **B** heme orientation of wt NP2 has a spread of 14.6 ppm, and the **B** orientation of many other mutants are similar, for example, the L122V,L132V mutant has a similar spread (14.4 ppm). In comparison, the I120V mutant has a spread of 13.5 ppm, but NP2(I120T) has a spread of 9.3 ppm. To be more systematic, we prepared the L→T,V mutants of NP2(L122V, L132V) for comparison to that L→V double mutant, because valine and threonine differ only in the replacement of one methyl group of Val by a hydroxyl group, as close to an isostructural mutation as is possible. It was found that the **B** isomer of NP2(L122T,L132V) has a spread of 10.9 ppm and the **B** isomer of NP2(L122V,L132T) has a spread of 12.6 ppm, as compared to the **B** isomer of NP2(L122V,L132V), with a heme methyl spread of 14.4 ppm. Although in many cases the **A** heme orientation is of low enough abundance that it is difficult to find the 3M resonance, in those cases where it can be found, the spread of the heme resonances of the **A** heme orientation is invariably larger than that of the **B** heme orientation, but is smaller for mutants in which a hydroxyl group has been introduced. Going in the opposite direction, NP2(S40A) has an increased spread of 16.0 ppm as compared to 14.6 ppm for wt NP2 (also as close to an isostructural mutation as possible). The same type of behavior was observed for NP1 mutants (Fig. 4); NP1(A42S) shows a smaller spread of the heme methyl resonances (8.3 ppm for **A**, 9.5 ppm for **B**), and NP1(T121L) shows a much larger spread (14.2 ppm for **A** and 12.7 ppm for **B**), as compared to that of NP1 (11.3 ppm for **A**, 9.0 ppm for **B**, Table S3). With these findings in mind, it is interesting to reconsider the NP2(L122A) and NP2(L132A) single mutants, which also have smaller spreads for their methyl resonances, probably because of the likely presence of polar water molecules in the distal pocket, which thereby increases the hydrophilicity.

In Fig. 4 are shown the ¹H NMR spectra of the high-spin forms of wild-type NP1 (which, like wt NP2 [29], contains the M0 from the start codon of the recombinant gene which is not cleaved by the *E. coli* methionine aminopeptidase) and its T121S, T121L and T121I mutants, as well as belt mutant A42S. In this paragraph we will discuss the wild-type and T121 mutants. In NP1(T121S) the methyl shift pattern looks similar to that of wt NP1. In both heme orientational isomers the axial ligand orientation ϕ is 135° or very close to it, while as reported previously [26], wt NP1 has a slight difference in heme seating for the **A** and **B** heme orientations, of the order of 2–4°, and it has since that time also become apparent that there is some dynamics within each of these heme seatings that provides additional line broadening for the NMR spectrum of wt NP1 [30]. Both of these features are apparent as the ¹H NMR spectra of NP1(T121L) and NP1(T121I) are compared to that of wt NP1 and its T121S mutant, since both of these mutants have considerably sharper resonances than does the wild-type protein. Thus, there is something about the shape of the heme pocket and heme plane symmetry of wt NP1 which makes the heme fit more loosely in the pocket, and allows more motion for both **A** and **B**, but this is relieved, perhaps in different ways, by either of these T121 to large side chain mutants. In addition, we note that the equilibrium **A**:**B** ratio of the T121I and T121L mutants does not change a lot, but remains around 1.3:1, fairly close to that of wt NP1. The NP1(T121I) mutation also seems to affect the **A** isomer symmetry (ruffling distortion) [27] more than **B**, with 5M~8M (~135°), and the spacing between 5M and 1M is very small or zero. The difference in appearance of the HS NP1(T121I) NMR spectrum is because in both isomers the 5M and 8M have the

same chemical shifts, 66.4 ppm, which is in between the same resonances in wt NP1. The abnormal intensity of the His59- β CH resonance at ~20 ppm is probably due to overlap with one or two propionate resonances. The chemical shifts of the heme methyls for NP1 and its mutants are summarized in Supporting Information Table S3.

Wild-type NP1 and its mutants provide an introduction to some new features, which are not observed for wild-type NP2 and its mutants; these new features are a region of very broad resonances of the high-spin heme substituents that extends from about 45 ppm to about 25 ppm. Apart from this broad region, there are generally two well-resolved peaks of relative area 1 (or two pairs of peaks whose sum in each case accounts for a relative area of 1), with little or no sign of the four additional peaks of relative area 1. These resonances are from the two α -CH vinyl resonances and the four α -CH₂ propionate resonances for one predominant heme orientation, and in a case of 1:1 ratio of **A**:**B**, double that many resonances. We have found that the two (or four in the case of **A**:**B** = 1:1) sharp resonances are invariably the 2- and 4- α -CH vinyl protons. For wt NP2, with **A**:**B** = 1:8, only the major **B** signals are well resolved; however, for NP1, with **A**:**B** = 1:1, we see, apart from two well-resolved resonances from the 2-vinyl and 4-vinyl α -H, only very broad overlapping resonances. This suggests some sort of dynamics, as has already been postulated to occur for high-spin NP1 and NP4 in terms of changes in heme seating [30].

For cases in which the heme methyl and α -vinyl resonances are relatively sharp but the majority of the α -CH propionate signals are very broad, a different kind of dynamics, one which affects only the side chains themselves, is suggested. If there are two relatively sharp signals or pairs of signals, but the other four are very broad or completely unresolved, then the possibility that one or both of the propionates is (are) undergoing some kind of motional averaging is suggested, probably a libration about the heme pyrrole(β), CH₂(α) bond. Such a motion would carry the β -CH₂ and carboxyl groups, as well as the α -CH₂ protons of each (both if they are both free to move in this way) propionate(s) through an arc, such that the projection of the C-H vector for each α -CH₂ proton with respect to the d_π orbital of the heme iron which interacts with the π orbital of the histidine imidazole ligand could readily change from parallel to perpendicular to that π orbital of the porphyrin ring (i.e., tracing out at least half of a Karplus curve). This would thereby change the amount of spin delocalization to the two CH₂ protons and thus their chemical shifts over at least the range observed for cases where the propionate C-H resonances are well-resolved and have been assigned, for example, for wt NP2. In that case of NP2, one 7H α resonance was found at 48 ppm while the other was found at 27.5 ppm, and 6H α resonances were found at 40 and 31 ppm [26]. If the projection of the α -C-H vectors on the d_π orbital which is involved in π spin delocalization to the porphyrin ring (rather than the one which is involved in spin delocalization to the His57-imidazole ring) were changing rapidly on the NMR timescale, then one would expect to see one averaged resonance for the two 7H α protons at about 38 ppm and one for the two 6H α protons at about 36 ppm. However, we do not know where the two 7H α and two 6H α resonances for the **A** and **B** heme orientational isomers would actually occur for the NP1 mutants, if they were resolved. What is observed are broad signals whose maximum is at about 32–30 ppm for wt NP1 and its mutants (Fig. 4). This difference in maximum of the broad signals from what the NP2 propionate α -CH resonance average chemical shift would be suggests that the no-exchange chemical shifts of the individual protons are somewhat different for wt NP1 and its mutants than they are for wt NP2 and its mutants. (We also note that the chemical shifts of the six 1-H signals between 50 and 25 ppm for wt NP2 and its mutants are not at the same chemical shifts for all mutants, as shown in Fig(s). 2a and 2b.)

3.4. NP1(L44F) Chemical Exchange

Residue 42 for NP2 and the corresponding residue 44 for NP1 are quite interesting, in that there are residues at the two sites 42/44 and 106/107, which are the reciprocal of each other in NP2 vs. NP1. As shown in Fig. 5, top, NP2 has F42 and L106, while NP1 has L44 and F107; in each case the larger Phe is paired with the smaller Leu, such that the amount of space utilized by the combination may be fairly constant with respect to their effect on the orientation of the heme. We have made one of the two non-complementary mutants, NP1(L44F), whose high-spin 1D and NOESY/EXSY spectrum are shown in Fig. 6, discussed below. While the orientation of the phenyl group of F42 of NP2 provides ample space for a 4-vinyl group of the **B** orientation, a vinyl group of the **A** heme orientation would seem to be too tight to fit. The ^1H NMR spectrum of the high-spin form of the non-complementary mutant NP1(L44F), shown in Fig. 4, has been reported to exhibit significant chemical exchange [28], although the resonances were not assigned at that time. From our general knowledge of NP1 we know that there are two orientational isomers, **A** and **B**. We also know that the heme methyl resonances of **A** are more shifted than those of **B**, and that the order of heme methyl resonances for wild-type NP1 **A** is $8>5>1>3$ and for **B** $5>8>1>3$ [26]. Here we present a detailed assignment of the resonances with the help of the “Shift patterns” program [65].

First we note that the spectra of Fig. 4 were recorded at 25 °C, while the NOESY/EXSY spectrum of Fig. 6 was recorded at 35 °C; both samples have the same **A**:**B** ratio, which is ~1:1. The difference in temperature could, and probably does, affect the order of several closely-spaced resonances. Seven EXSY cross-peaks are observed in the NOESY/EXSY map shown in Fig. 6, and thus all eight methyls, four from each isomer, are in chemical exchange, with one coincidence, likely that for 5,8M of **B**, based on the shape of the spectrum in Fig. 4. The resonances of all methyls are intense enough that it is in some cases difficult to tell which is the “major” and which is the “minor” species in this chemical exchange process. The vinyl and propionate α -CH resonances should also show EXSY cross peaks, but they are already broadened by other chemical exchange processes, and hence do not show additional cross peaks. We notice that 3M gives two cross peaks, one at smaller, the other at larger chemical shift. According to the “Shift Patterns” plot for high-spin ferrihememes (Supporting Information Fig. S4) [65] it is clear that one isomer has a 3M partner at a smaller angle, and the other at a larger angle. As we go to larger angle (from 135°), the 3M chemical shift increases and at the same time, the 8M chemical shift also increases, while the 5M and 1M chemical shifts decrease. If the angle becomes smaller than 135°, the chemical shifts of 3M and 8M decrease, while those of 5M and 1M increase (see Supporting Information Fig. S4). Notice that 5M and 1M always go in the same direction, and also, that 5M and 8M, in this region of the plot, go in opposite directions. Keeping these trends in mind, along with the fact that for wt NP1 the order of heme methyl resonances was $8>5$ for **A** and $5>8$ for **B** [26], a reasonable assignment is that peaks for 3M(**A**) and 3M(**B**) overlap at about 48.1 ppm and have partners at ~43.7 ppm (**B**) and 55 ppm (**A**). Two 1M signals are at ~57 and 56.5 ppm, and one has a chemical exchange partner at 61.7 ppm, (larger chemical shift) and belongs to isomer **B**, while the other chemical exchange partner is at 55.3 ppm and belongs to isomer **A**. Then we see that two peaks for 5M and 8M are at 61.6 ppm with strong chemical exchange partners at ~53.9 ppm. One of these is thus at 61.6 ppm with its chemical exchange partner at 53.9 ppm, while the other is at 53.9 ppm with its chemical exchange partner at 61.6 ppm.

Thus, the two chemical exchange resonances for 5M and 8M of **A** must be the other two pairs of peaks, with 8 and 8' being at 64.6 and 66 ppm and 5 and 5' being at 63 and 62 ppm. So we have for **A** the order $8>5>1>3$, which means that the His59 imidazole plane must have an angle of ~138°, and for **A'** we have the same order, $8>5>3>1$, which means that the His59 imidazole plane angle changes to a much larger angle of about 162°, and thus a

change in seating of the **A** heme of 24° . For **B** we have the order $8>1>5>3$, suggesting a His59 imidazole plane orientation of $\sim 140^\circ$ if we allow $1>5$ (which we have seen several times), while for **B'** the order is $5\sim 1>8>3$, which is consistent with the His59 imidazole plane being at $\sim 123^\circ$ for **B'**, a total change in heme seating angle from the fixed His59 orientation of 17° . The only thing wrong with this scenario is that **B** should have $5>8>1>3$.

In terms of a change in heme seating, the heme in **A** has rotated about 3° clockwise (as we look at the heme from the distal side, with His59 behind the heme), to move 2-vinyl of **A** away from the F44 and F107 phenyl side chains as a way of relieving the congestion in that region, and depending on the orientation of the 2-vinyl group (see Fig. 5, left), it must rotate clockwise quite a bit more, consistent with a His imidazole plane angle of $\sim 162^\circ$, and the heme with orientation **A'** has to change its seating by quite a bit (24°) in order to move 2-vinyl away from F107 and F44. At the same time, the heme in orientation **B** has changed its seating by rotating counterclockwise by 5° , so that the His imidazole plane is at 140° , and the only way it can remove the conflict between the two phenyl side chains and 4-vinyl (Fig. 5, right) is to change its seating to produce a His imidazole plane angle of 123° , a clockwise rotation for **B'** of 17° . In fact, even for wild-type NP4 and NP2 (Fig. 5, top left and right, respectively), some rotation of the heme seating is required, which may explain why the wild-type proteins have $8>5>1>3$ for **A** of NP4 (which has F107 above the heme plane). This would mean that the heme seating produces a His59 imidazole plane angle with respect to the heme which is greater than 135° , while the **B** isomer of NP4 has $5>1>8>3$, which means that the heme seating produces a His59 imidazole plane angle less than 135° . This angle would remove the steric conflict of F107 with the 4-vinyl group. On the other hand, for NP2 (Fig. 5, top right), the major **B** isomer has $5=8>1>3$, which means that the His57 imidazole plane is oriented at exactly 135° , and 4-vinyl is nicely nestled against the phenyl ring. In comparison, the minor **A** isomer has $8>5>1>3$, which means that the angle is less than 135° and is strongly disfavored, because F42 would strongly interfere with having the 2-vinyl group so close to it. The chemical shifts of the methyl groups of **A** and **B** are given in Table 2a for this scenario.

There is a second scenario with regard to the assignment of the heme resonances which are undergoing chemical exchange, one which switches the 5 and 8 resonances for **A** and **B**. This means that for **A**, 5,5' are the widely spaced NOESY/EXSY peaks at 53.9 and 61.6 ppm while 8,8' are the closely spaced NOESY/EXSY peaks at 66 and 64.6 ppm. In this scenario, for **B**, 5,5' are the closely spaced NOESY/EXSY peaks at 63 and 62 ppm, while 8,8' are the widely spaced NOESY/EXSY peaks at 53.9 and 61.6 ppm. In this case the order of methyl resonances is $8>1>5>3$, with His57 oriented at $141\text{--}144^\circ$ and $8>5>1>3$, with His57 oriented at $136\text{--}137^\circ$ for the **A** and **A'** isomers, and for **B** and **B'** the orders become $5>1>8>3$, with His57 oriented at $120\text{--}123^\circ$ and $5>8>1>3$, with His 57 oriented at $132\text{--}134^\circ$. This scenario has the weakness that the $1>5$ order for isomer **A** is not that given by the angle plot for high-spin ferrihemes, but we have seen other violations of the $5>1$ order, as mentioned for the first scenario. However, it has the advantage that it allows both of the widely-spaced chemical exchange peaks that overlap at 61.5 ppm to vary in the *same* direction. This nicely leads to the very tall peak observed in the 1D spectrum at 25°C (Fig. 4). In this case the change in heme seating is smaller for both heme orientations, a change from a fixed His59 angle for **A** of $141\text{--}144^\circ$ with respect to the heme substituents to $136\text{--}137^\circ$ for **A'**, a total change of $4\text{--}8^\circ$, rather than the much larger change of 24° . For **B** this means a change from a fixed His59 angle of $120\text{--}123^\circ$ with respect to the heme substituents to an angle of $132\text{--}134^\circ$ for **B'**, a total change of $9\text{--}14^\circ$, rather than the larger $\sim 17^\circ$ angle of total change in heme seating of the first model. In this scenario, for both **A** and **B**, 5 and 8 do not change symmetrically by similar chemical shift differences, while in the angle plot for **A** they do, but for **B** they indeed do not. The chemical shifts of the methyl groups of **A** and **B** are given in Table 2b for this scenario. Additional mutants, currently in preparation in the

NP2(D1A) construct, may allow a final decision between these two scenarios. The NOESY/EXSY spectrum of the low-spin NP1(L44F)-ImH complex, where the same changes in heme seating are required, is shown in Supporting Information Fig. S6.

3.5. NMR Characterization of the Low-Spin Lewis Base Ligand Complexes of NP2 Distal Pocket Mutants

Proton NMR spectra of the low-spin form of NP2(L122T,L132V) complexed to ImH, recorded at 15 °C, are shown in Fig. 7, and the chemical shifts of the protons of the heme substituents are presented in Supporting Information Table S5, along with those of NP2(L122V,L132T) complexed with ImH. In Fig. 7 it can be seen that based on the WEFTNOESY spectra of NP2(L122T,L132V)-ImH, it is possible to achieve an almost complete assignment of the heme ^1H resonances of this complex, as summarized in Supporting Information Table S5. The order of heme methyl resonances and their chemical shifts are similar to those that were observed for the NP2 L→V mutants reported previously [25]. One difference, however, is that for NP2(L122T,L132V)-ImH, the order of heme methyl resonances changes with temperature over the range 35, 25, 15 °C from 1>5>3>8 ($\phi \sim 165^\circ$) to 1>5>3~8 ($\phi \sim 174^\circ$) to 1>5>8>3 ($\phi \sim 178^\circ$), with a very small spread of heme methyl resonances. (The Shift Patterns “angle plot” [65] for low-spin ferriheme proteins, shown in Supporting Information Fig. S7, is helpful in estimating these angles.) It is almost as though two sets of heme methyl resonances are exchanging rapidly to give averages with very small differences in methyl chemical shifts, but which vary with temperature. In the high-spin complex (Fig. 2), the His57 angle can be estimated to be about 137° at 25 °C, yet the binding angle for ImH appears to change from 165° at 35 °C (dihedral angle between His57 imidazole plane and ImH plane of 28°) to ϕ nearly 180° (dihedral angle of $\sim 43^\circ$), with some changes in heme nonplanar symmetry for this mutant as well (1>5). We note that 1M and 5M have nearly the same chemical shift. This has to mean a change in electron-withdrawing effect that makes a vinyl look like a propionate, probably due to a strong twisting of the vinyls out of the mean plane of the heme so that they have no conjugation with the porphyrin ring and thus no electron-withdrawing effect. However, no second orientation (“perpendicular”) of the ImH ligand was observed. The NP2(L122V,L132T)-ImH also shows temperature-dependent changes in the methyl peak order and spacing: at 35 °C the order is 5>1>3>8 (164° , dihedral angle of $\sim 29^\circ$), at 30 °C it is 3>5>1>8 (157° , dihedral angle of 22°); in this complex a second orientation of the ImH plane was found, which has heme methyl resonance order 8>5>3>1, yielding $\phi \sim 74^\circ$ and a dihedral angle to His57 imidazole plane of $\sim 61^\circ$, a “perpendicular” orientation. EPR spectra of some of these complexes were measured at 4.2 K many years ago, but were not included when [25] was published. They show that most of the L→V mutants have two orientations of the axial ImH at very low temperatures, one with “parallel” ImH planes (rhombic), the other with “perpendicular” ligand planes (“large g_{max} ”), but unfortunately the L→V,T and L→A mutants were not included in that study. The existing EPR spectra are shown in Supporting Information Fig. S8, where it is interesting to see that the rhombic EPR signal has the exact same g -values (± 0.01) for wt NP2 and the three L→V mutants ($g_1 = 3.03$, $g_2 = 2.26$, $g_3 = 1.31$), while the “large g_{max} ” signal shifts to larger g -value with increased perceived production of excess space in the distal pocket (L122 < L132 < L122,L132).

Proton NMR spectra of the low-spin form of NP2(L122A) and NP2(L132A), each complexed to ImH are shown in Supporting Information Fig(s). S9 and S10, respectively, where it can be seen that based on the HMQC and WEFT-NOESY spectra it is possible to achieve an almost complete assignment of the heme ^1H as well as some of the ^{13}C resonances of this complex, as summarized in Table 3, along with those of a number of other distal pocket mutants. The order of heme methyl resonances and their chemical shifts are similar to those observed for the NP2 L→V mutants reported previously. Both

NP2(L122A)-ImH and NP2(L132A)-ImH exhibit two orientations for the exogenous imidazole plane, with chemical exchange between them. (For the NP2 L→V mutants, only NP2(L122V)-ImH exhibited significant enough chemical exchange to allow calculation of the rates of ligand orientation exchange, as reported previously [25].) One orientation, with the order of heme methyl resonances 5>3>1>8, is close to parallel to the plane of His57, with $\varphi = 161^\circ$, a dihedral angle $\Delta\varphi = 27^\circ$ between the histidine imidazole and the exogenous imidazole ligand planes; the other orientation, with order of heme resonances 8>5>1>3 has $\varphi = 49^\circ$, which represents a large angle of rotation from the orientation of His57 (φ close to 135°), $\Delta\varphi = 86^\circ$. In these two cases, measuring from the N_p-Fe-N_p axis of pyrrole rings II and IV toward pyrrole rings I and III (a clockwise rotation for isomer **B**), $\Delta\varphi$ is the acute angle between ligand planes, but the same is not true for the 2MeImH complex for which the NOESY/EXSY spectrum is shown in Supporting Information Fig. S3, where the angle is 43° , as discussed below. The chemical exchange between these two orientations of ImH is very obvious at ambient temperatures for both of these L→A mutants, as is evidenced by intense chemical exchange cross peaks in the WEFT-NOESY map between methyl resonances for the two orientations, one of which shows no resonances in the 1D spectrum (Supporting Information Fig(s). S9 and S10). The rate constants for this chemical exchange at 30 °C are $k_f \sim 8.9 \text{ s}^{-1}$ and $k_r \sim 0.09 \text{ s}^{-1}$; these values were estimated from the “Two Site Chemical Exchange” program [66], as described above. Their ratio, $k_f/k_r = K_{eq}$ is about 100 for the equilibrium between the parallel and perpendicular orientations of the ImH ligand for the L132A mutant, as was also found for the L122V mutant. As for the L122V mutant, in the case of the L→A mutants of NP2 the substitution inside the heme pocket of a bulky residue for a smaller one changes the distal pocket structure, which changes the noncovalent interactions, and the ligand binding ability, and thereby causes two axial ligand orientations (parallel and perpendicular), with exchange between them which is slow on the NMR timescale (i.e., the resonances of both species are observed). Besides this, the possibility of hydrogen-bond donation from the imidazole N-H to the Gly130 carbonyl when ImH is oriented perpendicular to H57, as found in the structure of the double mutant NP2(L122V,L132V)-ImH (PDB file 1PM1) [14], may be the other reason for stabilization of a perpendicular ligand orientation, especially at low temperatures (for example, the temperatures of EPR experiments, 4.2 K [25]). Wild-type NP2-ImH shows no chemical exchange between two ImH orientations at pH 7.5, although it does show this chemical exchange at lower pH values, i.e., 6.5, 5.5, 4.5 [31].

We have previously shown that 2MeImH is a bulky ligand which can bind to a 5-coordinate heme protein only in a plane perpendicular to the protein-provided histidine [25], and indeed, with NP2(L132A)-2MeImH this ligand shows only one orientation for the imidazole plane in the WEFT-NOESY map, Supporting Information Fig. S3, for which the heme methyl shifts indicate the orientation is $\varphi = 43^\circ$, or an angle of rotation of the exogenous ligand from that of H57 (which has a H57 angle $\varphi = 133^\circ$, Table 1), $\Delta\varphi = 90^\circ$. However, very strong chemical exchange was also observed, but in this case it was between free and bound 2MeImH (chemical exchange cross peaks in the WEFT-NOESY spectrum at 2.5, 12.2 ppm between the methyl groups of the free and bound ligand, respectively). This indicates that 2MeImH is a weakly-binding ligand, with such a small binding constant that its midpoint potential cannot be measured by spectroelectrochemistry because the complex cannot be fully formed (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted), yet this ligand complex is very useful for saturation transfer experiments used to assign the methyl group order of the high-spin NP2 mutant at 25 °C (Supporting Information Fig. S3). The same was not true, however, for the NP2(L122A) mutant, which gave such intense chemical exchange cross peaks that the assignments of the 2MeImH complexes could not be made reliably. The chemical shifts of the NP2(L132A)-2MeImH are presented in Supporting

Information Table S1. The EPR spectrum of this complex is that of a “large $g_{\max}$ ” center, with $g_{\max} = 3.47$ and a very broad feature at $g = 1.95$ (Supporting Information Fig. S8).

The 1D, HMQC and WEFT-NOESY spectra of NP2(I120T)-ImH are shown in Fig. 8, for a freshly-prepared sample, for which the **A**:**B** ratio had not yet reached the equilibrium 2:3 ratio. The chemical shifts and assignments are presented in Table 4. The rather similar spectra of NP2(I120V)-ImH are shown in Supporting Information Fig. S11 and the chemical shifts of the heme resonances are presented in Supporting Information Table S4. In the case of the **B** heme rotational isomer, the H57 orientation angle is close to 135° , the angle observed for wild-type NP2. The observed methyl resonances of the **A** isomer of NP2(I120T) are shifted to larger chemical shifts than for the wild-type NP2 protein bound to ImH, but the spread of the methyl resonances and their average chemical shift is not significantly changed. Two orientations of the exogenous ImH plane are observed for each isomer: For the **A** isomer the “parallel” orientation has $\phi = 126^\circ$, with dihedral angle to H57 (at 134° , Table S2), $\Delta\phi \sim 7^\circ$, and the “perpendicular” orientation has $\phi = 50^\circ$, with dihedral angle $\Delta\phi \sim 84^\circ$. For the **B** isomer the “parallel” orientation has $\phi = 153^\circ$, with dihedral angle $\Delta\phi \sim 19^\circ$, while the “perpendicular” orientation has $\phi = 28^\circ$, with dihedral angle $\Delta\phi \sim 73^\circ$. It should be noted that all mutants of NP2 studied herein exhibit two orientations for the exogenous imidazole plane, with chemical exchange between them; occasionally it has been possible to measure the rates of exchange between parallel and perpendicular orientations of the axial imidazole plane, but often the amount of the minor perpendicular species is too small to allow measurement of the rate constants, as is the case of NP2(I120T).

The effect of the I120T mutation on the **A**:**B** ratio can be explained by comparison of the structure of wild-type NP2-ImH with wild-type NP4-ImH. In NP2 the distance between carbons 3M and the δ -CH₃ of Ile is only 3.28 Å [14], PDB file 1PEE, which is perhaps not large enough to allow a vinyl group to be stable at this position for the **A** isomer. (The same distance for NP2(L122V,L132V)-ImH is 3.72 Å,¹⁴ PDB file 1PM1.) In NP4 the distance 2(V)C α ---(Thr)Me is 4.57 Å, 1Me---(Thr)Me is 4.25 Å, and 1Me---(Thr)OH is 4.10 Å; Leu132 CD2 is 3.66 Å above the heme *meso*- α and 3.92 Å above 2-V α ; Leu132 CD1 is much further above the heme (reference 14, PDB file 2HYS). Leu122 CD1 is 3.75 Å from 5Me and CD2 is 3.99 Å from 6 β -C (reference 14, PDB file 2HYS), but these positions appear to be common to both heme isomers. Thus, there is no space limitation for heme binding as a result of these residues for the **A** isomer of NP4, but the bulky Ile-120 makes the **A** orientation much less favorable in NP2. Removal of this bulky residue by the mutation I120T makes the **A** isomer of NP2 much more favorable.

The 2D NMR spectra of NP2(S40A)-ImH are shown in Supporting Information Fig. S12. These 2D spectra were recorded on a fresh sample of the S40A mutant, before the protein had reached its stable 1:8 ratio shown for the high-spin form in Fig. 2b. Therefore it is easy to see both **A** and **B** heme resonances of the ImH complex, and the spectra of both isomers were assigned, as listed in Supporting Information Table S6. As can be seen in Fig. 2b, the *equilibrium* **A**:**B** ratio of the high-spin form is the same as that of the wild-type protein, which suggests that S40 is not very important in determining the **A**:**B** heme orientation ratio. In addition, the spread of the heme methyls for the high-spin form of the protein is close to the same as that of the wild-type protein, which suggests that removal of the hydroxyl group of S40 does not change the hydrophobicity or noncovalent interactions of the protein as much as does the introduction of hydroxyl groups in the distal pocket. However, the pattern of α -CH₂ propionate resonances of the high-spin complex is different from that of wt NP2 (Fig. 2b).

The WEFT-NOESY spectrum for the cyanide complex of NP2(L122V,L132T) is shown in Supporting Information Fig. S13, where it can be seen that the **B** isomer shows chemical

exchange with a minor isomer, **B'**; this minor isomer behaves similarly to those discussed below for NP1 and its mutants, in that the angular change in this case is the ruffling ridge rotation angle α [27], which is relatively small ($8\text{--}10^\circ$), rather than a parallel to perpendicular switch, as in the case of ImH binding to other NP2 mutants.

3.6. NMR Characterization of the Low-Spin Ligand Complexes of NP1 Distal Pocket Mutants

Proton NMR spectra of the low-spin form of NP1(T121I) complexed to ImH are shown in Fig. 9; similar spectra for the ImH and CN^- complexes of NP1(T121S) are shown in Fig. 10. In Fig. 9 it can be seen that based on the WEFT-NOESY and HMQC spectra of NP1(T121I)-ImH it is possible to achieve an almost complete assignment of the heme ^1H resonances of this complex, as summarized in Table 5. The order of heme methyl resonances and their chemical shifts are similar to those that were observed for wt NP1 previously [26]. The **A**:**B** ratio is about 1.5:1, so the **B** isomer is less stable compared to that of wt NP1. The major difference observed in NP1 mutants as compared to NP2 mutants is the heme complex dynamics. Considerable dynamics were observed, especially for the 3M resonance in the wt NP1-ImH complex [27] and to the symmetrical heme derivative of it [28], which were ascribed to changes in the ruffling dynamics of the heme itself [30]. The T121 mutations to I, L and S give the heme complex progressively more motional freedom. In Fig. 9 chemical exchange between two environments of the 3-methyl heme protons can be seen for both isomers, whereas for wt NP1-ImH only the **A** isomer showed this chemical dynamics [27, 28, 30]. However, when the stronger-binding ligand CN^- was bound to the heme, no chemical exchange was observed for the NP1(T121I) mutant, even at higher temperatures (not shown). This chemical exchange, when observed [27, 28, 30], is due to changes in heme ruffling, and the observation of no chemical exchange thus suggests that the dynamic changes in the ruffling deformation, as has been concluded previously for the symmetrical heme complexes of NP1 and NP4 [30], are not as great in the presence of the largest side chain (T121I). The chemical shifts of NP1(T121I)-ImH are listed in Table 5.

In Fig. 10 the proton NMR spectra of NP1(T121S) mutant bound to ImH (left) and CN^- (right) are presented. These spectra show very intense chemical exchange between **A** and **A'** and between **B** and **B'** forms, and the concentrations of the **A** and **A'** forms are much higher than observed for wt NP1 [30]. The effect of mutation on the heme complex dynamics is larger compared to that of the NP1(T121I) mutant (Fig. 9). Even when the stronger binding ligand CN^- is added, the complex still appears to have considerable freedom, and strong chemical exchange between **A** and **A'** forms is observed (Fig. 10, right).

4. Discussion

In this work we have shown that the ^1H NMR spectra of the high-spin forms of the distal pocket mutants of nitrophorins 2 and 1 are quite sensitive to changes in the shape and hydrophobicity of the heme binding cavity. In terms of distal pocket residues, the shortening of the leucines 122 and 132 of NP2 to valines has been discussed previously [25]; in this work further shortening of the leucines to alanines and changing of the hydrophobicity of the distal heme pocket by preparation of the two L $\rightarrow$ T,V mutants is reported, as discussed further below. A simple one or two residue mutation in the heme cavity from a larger to a smaller residue such as L $\rightarrow$ V $\rightarrow$ A leads to observable and somewhat complicated and unexpected changes in the heme center structure and properties, as analyzed by NMR spectroscopy herein, and thermodynamic measurements to be reported elsewhere (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted). For example, the L to V and A mutations lead to opposite changes in properties of the ligand complexes, with the ImH complexes of NP2(L122V), NP2(L132V) and NP2(L122V,L132V) being more stable than those of

NP2(L122A) and NP2(L132A) mutants. Likewise the L→V mutants show an increase in average methyl shift and spread of the methyl resonances over that of wt NP2, which suggests greater electronic asymmetry of the heme, while the L→A mutants show decreases in both of these properties, which suggests less electronic asymmetry of the heme.

However, the mutation of I120 of NP2 to any smaller residue (or T121 of NP1 to any larger residue) has major effects on heme **A:B** orientation ratio, but not heme seating, as can be seen in Fig(s). 2 and 4. Within the NP2 wild-type series, the I120T and I120V mutants have an **A:B** heme orientation ratio of about 1:1.5 and 1:2, respectively, as compared to wt NP2, 1:8. Thus, shortening the side chain of Ile by one carbon is enough to strongly decrease the favoring of the **B** heme orientation. We see that the I120T mutation allows further closure of the A-B loop and changes the heme seating for both the **A** and **B** heme orientation by ~1 degree.

In terms of distal pocket residues, shortening the side chains of NP2 residues L122 and L132 by one CH₂ group favors the **B** heme orientation with respect to wt NP2 slightly for the L122V mutant, while the L132V and double mutant, NP2(L122V,L132V) have identical **A:B** heme orientation ratio to the wild-type protein. However, the heme seating is slightly changed (from that represented by a His57 imidazole plane orientation relative to the heme ring of 135° to about 137°). For the L→A mutants, NP2(L132A) is well-behaved as the high-spin complex, with **A:B** ratio very similar to that of NP2(L132V), but a heme seating that is about 2° rotated in the opposite direction than the L→V mutant, yielding an overall ~4° difference in heme seating between the two L132 mutants. The other two L→A mutants also appear to favor the **B** heme orientation, but there are additional species present that cannot be identified, especially for the double L→A mutant, and hence we have not considered it further. The L→T,V variants, prepared to see how the T side chain might change the hydrophobicity, showed that while the spread of the heme methyl resonances is smaller, in all other respects the NMR spectra are very similar to those of the L→V double mutant. They show similar **A:B** ratios to the L→V double mutant, ~1:6 as compared to 1:8 for wt NP2. The striking difference for these two mutants is that their ligand binding ability is low (*K_{eq}* decreased by about one order of magnitude), and the chemical exchange between two ligand positions is weak in the ImH complexes. But the stronger-binding CN⁻ ligand shows strong chemical exchange cross peaks which are a result of changes in heme plane ruffling symmetry, as discussed in detail previously [30].

In addition to I120, the other residue in NP2 which may be important for determining **A:B** heme orientation ratio is F42, which, for the **B** heme orientation is near 3M and 4V, while for the **A** heme orientation it would be very close to 2V. Considering the residues which differ between NP2 and NP1/NP4, we note that this F42 is L44 for NP1, and we prepared the NP1 mutant L44F, and found that this mutant affects the heme seating quite a lot, and differently for the **A** and **B** heme orientations, in both cases making the “normal” seating with the His59 imidazole plane close to 135° unstable. Our estimations of the heme positions in this mutant show that the heme is moving from one seating to another, but cannot take on the “normal” seating, as discussed above and shown in Fig(s). 5 and 6 and Supporting Information Fig. S6. Additional studies of related mutants are in progress.

In terms of the ligand complexes of NP2, based on this work and that in which ligand binding constants and midpoint potentials of these mutants are measured (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted), we have found that there is a correlation between the imidazole binding constants and the existence of chemical exchange between the two exogenous imidazole ligand orientations (parallel and perpendicular), which is often observed in the NMR spectra of the complexes, particularly in the NOESY/EXSY spectra.

For those cases in which the imidazole binding constant is smaller for the mutant than that for wild-type NP2, chemical exchange between the two imidazole orientations is observed in the NMR spectra of the complexes, particularly in the NOESY/EXSY spectra. Some mutations severely decrease the exogenous ligand binding constants, by as much as one order of magnitude, for example, for the case of the NP2(L122V,L132T) and NP2(L122T,L132V) double mutants, for which the small ligand binding constants allowed chemical exchange to be observed between the high-spin (ligand free) form and the low-spin (ImH complex) at 25 °C, pH 7, which has not been observed for any other NP2 mutant with ImH. The significantly weaker binding of ImH allowed the assignment of high-spin resonances from NOESY/EXSY spectra, as shown in Fig. 7 for NP2(L122T,L132V). The same was not true of the imidazole complex of NP2(L122V,L132V), even though the side chains of Thr and Val are very similar in size ($-\text{OH}$ vs. $-\text{CH}_3$). Significant chemical exchange was also seen between two different species in terms of the 3M, 2V α and 8M, but not 1M resonances of NP2(L122V,L132T)-CN in the NOESY/EXSY spectrum in Supporting Information Fig. S12.

NP1 and NP4 have very different heme complex dynamics as compared to NP2. These dynamics can be seen by NMR spectroscopy as peak broadening for the high-spin forms, and as intense chemical exchange between two 3M groups in different environments of heme rotational isomer **A** [30] for the ImH and cyanide complexes. Similar quality NMR spectra were observed for all NP1 mutants of this study. The low-spin mutant NP1 ImH complexes show more information concerning complex dynamics than do the high-spin complexes. In NP1(T121I)-ImH the chemical exchange is somewhat less pronounced than for the NP1(T121S)-ImH complex at any given temperature, and the cyanide complex of NP1(T121I) does not show chemical exchange, at least at room temperature at pH 7.0. But for the NP1(T121S)-ImH complex there is significant chemical exchange for both the **A** and **B** heme orientational isomers (Fig. 10), even though in wt NP1 only the **A** heme orientation isomer shows this chemical exchange. Even for the tighter-binding ligand cyanide, the **A** isomer has intense chemical exchange for the 3M substituent. However, this chemical exchange is entirely different than that seen for several NP2 mutants, since it does not involve the exogenous ligand plane orientation, i.e., parallel and perpendicular orientations; rather, the observed change in ligand orientation is much, much smaller, of the order of $\sim 5-7^\circ$, and arises from a change in ruffling of the heme plane, from more ruffled to more planar for NP1, but not for NP2, as discussed in detail elsewhere [30].

We have observed for model heme systems, that when the porphyrin ring is planar, the axial ligands (imidazoles or pyridines) prefer parallel orientation, but when the porphyrin ring is ruffled by insertion of bulky substituents on the porphyrin ring the same ligands prefer perpendicular orientation [67]. The insertion of different substituents on the periphery of porphyrins is one of the ways to model the protein environmental effect on heme structure. In the case of the nitrophorins, the heme cavity (size, shape, hydrophobicity, etc.) affects the heme structure by making it more or less nonplanar, with the major contribution being ruffling, and in this way provides the possibility of perpendicular or parallel orientation for axial ligands. In solution the protein structure is 'breathing,' or undergoing numerous types of vibrations, which cause conformational changes in the heme cavity, as well as changes in heme plane distortion.

Thus, at ambient temperatures the heme may be undergoing rapid changes in the degree of ruffling and/or other nonplanar distortions on the timescale of vibrations (10^{-15} s^{-1}), as was observed for the symmetrical hemin complexes of NP1 and NP4 [30]. ADF calculations on OECFeL₂ (OEC = octaethylchlorin) indicated that the ruffle-planar and saddle-planar vibrations are extremely low in frequency, of the order of 22 and 17 cm^{-1} , respectively [68], and thus even within the protein pocket it is likely that iron(III) protoporphyrin is

undergoing macrocycle plane distortion vibrations that could allow a change in the orientation of the exogenous ligand [27, 30, 31]. In fact, nuclear inelastic scattering (NIS, also called nuclear vibrational resonance spectroscopy, NRVS [69, 70]) shows evidence of more than 100 low-energy vibrations, lower than 100 cm^{-1} , that involve motions of the ^{57}Fe nucleus of NP2 (A. Janoschka, B. Moeser, J. Wolny, H. Paulsen, I. Filippov, R. E. Berry, H. Zhang, A. I. Chumakov, F. A. Walker, V. Schünemann, to be submitted). The decrease in complex stability makes this process observable by NMR techniques. This effect was observed not only when the mutation provides more space for the ligand in the heme cavity, but also for all other types of mutation, when the binding ability becomes lower as a result of the mutation, probably due to a major change in the amount of ruffling, or, in the case of the L→V,T mutants, possibly due to a change in the number of water molecules in the distal pocket. Thus the observation of chemical exchange between two ligand orientations in the NMR spectra is an indirect observation of heme plane distortion dynamics.

When the hydrophobicity at certain points is changed, the hydrophobic interactions in the protein are disturbed with purpose. But at the same time, in ways that we cannot control, the electrostatic interactions and protein hydrogen-bonding network are also changed, and these changes can increase or decrease the possibility of creating hydrogen-bonds. But this is not the only way the hydrogen-bonding network is changed. By mutation the cavity shape and the available space is also changed. Thus, after mutation, certain hydrogen-bonds became impossible (or possible) because the distance between protons and their partners changes, and on the other hand, insertion of a hydrophilic residue makes the vicinity more attractive for water molecules, which increase the possibilities for hydrogen-bonding. All of this was not so clear to us before we began the work reported herein, and we thus now understand that an attempt to change cavity size or hydrophobicity by one simple mutation has more consequences than we expected. As observed, especially for the L→A mutants, even without changing the hydrophobicity, but just providing space for one or more water molecules is enough to change the hydrogen-bonding network inside the heme cavity. Thus the effect of mutations that involve changes in hydrophobicity is complicated, because there are no 'pure cases'. Thus we should discuss these changes in terms of noncovalent interactions, which include electrostatic, hydrogen-bonding and hydrophobic interactions; the hydrophobic interactions are probably the weakest. In spite of this, there are some correlations between changing hydrophobicity and complex electronic structure, that can be observed by NMR spectroscopy as differences in the spread and spacing of heme methyl resonances. Structure determination by X-ray crystallography of some of these mutants would be very helpful in understanding the reasons for the significant drop in binding constant of ImH and the decrease in spread of the heme methyl resonances of the high-spin complexes of NP2.

5. Summary and Conclusions

The difference in appearance of wt NP1 and wt NP2 spectra, observed by 1D and 2D NMR spectroscopy, is the result of the axial ligand orientation with respect to the heme molecule seating inside the heme binding pocket, the ratio (**A:B**) between the two heme orientational isomers, and heme ruffling or seating dynamics. Mutations can affect noncovalent interactions, which in turn affect the heme complex structure by changing the heme plane symmetry and distortion, the His57 orientation (slightly), as well as the distal ligand orientational stability, in terms of whether one or two distal ligand orientations are observed. All of these factors have impact on the behavior of the NO complexes of these mutant proteins, most of which have not been investigated. The paramagnetic complexes of the nitrophorins allow us to see the wide range of motions that the heme can undergo, which cannot be probed in the diamagnetic NP-NO complexes, because the NMR spectra of the

diamagnetic NP-NO complexes are not very sensitive to the dynamics of ruffling or seating changes of the heme.

We find that mutations affect more visibly NP2 than NP1, probably because of NP2's smaller and more crowded heme binding pocket due to the shortening of the A β -strand and the A-B loop by one amino acid each. Also, we find that mutation of the distal residues in NP1 changes the A:B ratio only a little, but shows some preference for the A orientation.

We find that a simple one or two residue mutation in the heme cavity from a larger to a smaller residue such as L-V-A leads to observable and somewhat complicated and unexpected changes in the heme center structure and properties, as analyzed by NMR spectroscopy herein and thermodynamic measurements to be reported elsewhere (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted). For example, the L to V and A mutations lead to opposite changes in properties of the ligand complexes, with ImH complexes of NP2(L122V) and NP2(L132V) being more stable than those of NP2(L122A) and NP2(L132A). Likewise, the L $\rightarrow$ V mutants show an increase in average methyl shift and spread of the methyl resonances over that of wt NP2, which suggests greater electronic asymmetry of the heme, while the L $\rightarrow$ A mutants show decreases in both of these properties, which suggests less electronic asymmetry of the heme.

We also find that changing the hydrophilic properties by mutations (I to T, I to S, S to A, or T to L) also affects the electronic asymmetry of the heme. In the first two cases the effect is the same as providing available space for insertion of water molecules by shortening the side chain (L to A).

The Ile120 side chain, especially methyl CD1, is very close to the heme, and together with the rest of the cavity structure, defines the heme orientation (A:B ratio) inside the heme cavity, so that the I120T and V mutants of NP2 provide much greater stability for the A orientation than does wt NP2. The L122 mutation does not affect the A:B ratio. Thus this residue does not make a significant difference in heme cavity structure. Mutation of the L132 residue to smaller side chains provides additional space in the heme cavity, large enough for insertion of additional water molecules (as shown in the crystal structures), and this makes a very large difference in heme properties, including chemical exchange between two distal ligand orientations (this work), ligand binding constants, and the midpoint potentials of the proteins in the absence of added ligand (R. E. Berry, A. Amoia, A. Weichsel, T. K. Shokhireva, M. N. Shokhirev, W. J. Golden, H. Zhang, W. R. Montfort, F. A. Walker, to be submitted). The exogenous imidazole complex parallel/perpendicular dynamics is under the control of the exogenous ligand binding properties and the protein noncovalent interactions, which are stronger in NP2 and weaker in NP1.

His57(59) sits at the same angle, with imidazole plane angle $\phi = 135 \pm 1-4^\circ$ in most complexes of NP2 and NP1. In spite of the similar orientation of the His imidazole plane in both orientational isomers, the β -CH which is perpendicular to the imidazole ring plane can have some differences in chemical shift, which shows some flexibility of the His57(59) rotamer conformation.

We also strengthen our previous conclusion [30] that the observed chemical exchange between two planar ligand orientations is due to heme plane dynamic motions, which change the heme plane symmetry and make it easier for an exogenous ligand to bind at a given angle, but more difficult at a different angle. Although easier to explain for a planar axial ligand, the same chemical exchange occurs when the cylindrical ligand cyanide is bound [27, 30], and we thus conclude that these heme plane dynamic motions also occur for the NO complexes of the nitrophorins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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6. Definitions

EXSY	(EXchange SpectroscopY)
HMQC	(Heteronuclear Multiple Quantum Correlation)
M0	(The methionine that is expressed as a result of the start codon of the gene (AT(U)G), and is in many cases removed by a methionine aminopeptidase enzyme of the expressing organism. If it is not removed, it will be present before the first expected amino acid of the protein, which in the case of the nitrophorins, can affect A-B and G-H loop conformation and dynamics.)
MALDI-TOF	(Matrix-Assisted Laser-Desorption/Ionization-Time of Flight) mass spectrometry
NOE	(Nuclear Overhauser Effect)
NOESY	(Nuclear Overhauser and Exchange SpectroscopY)
pH*	(the pH value measured, for a D ₂ O solution, with a pH meter having a glass electrode containing an H ₂ O reference solution)
PDB	(Protein DataBank, Research Collaboratory for Structural Bioinformatics, http://www.pdb.org/pdb/home/) “symmetrical hemin” (2,4-dimethyldeuterothemin)
WEFT	(Water-Eliminated Fourier Transform. A pulse presequence which applies a 180-degree pulse to all protons of the sample. This is followed by a delay which is optimized by trial and error to allow the signals from the protons which are far from the paramagnetic center (and hence have long relaxation times) to relax until their magnetization vector is in the xy plane, while the signals from the protons which are near the paramagnetic center relax back to their equilibrium magnetization along the +z axis. At this point the desired pulse, usually a 90° pulse, can be applied to all protons. This places the slow-relaxing protons, which are far from the paramagnetic center, along the -z axis, while the rapidly-relaxing protons which had completely relaxed to equilibrium, will have their magnetization vector placed in the xy plane, where it can evolve as desired, to be followed by other pulses to create a NOESY or COSY sequence for the protons of interest.)

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Synopsis

The high-spin Fe(III) ^1H NMR spectra of wild-type NP2 and NP1 and eighteen of their site-directed mutants, and the low-spin imidazole (ImH) and in some cases cyanide complexes of representative examples of these mutants have been investigated. It is found that among the distal pocket residues investigated, the residue most responsible for heme orientation and seating is I120(T121). F42(L44) and L106(F107) may also be important, but must be investigated in more detail.

Fig. 1.
Top, Scheme showing heme orientations **A** and **B**, with H57 behind the heme, and L122 and L132 shown in red and blue, respectively. *Bottom*, view of the heme of NP2 (PDB file 2EU7), showing residues Leu106 (yellow), Ile120 (green), and Phe42 (orange).

Fig. 2.

(a) ^1H NMR spectra of wild-type NP2 [26], NP2(L122V) [25], NP2(L132V) [25], NP2(L122V,L132V) [25], and the two NP2(L $\rightarrow$ V,T) double mutant high-spin complexes at 25 $^\circ\text{C}$, pH* 7.0. (b) ^1H NMR spectra of wild-type NP2 [26] NP2(I120V), NP2(I120T), NP2(S40A), NP2(L122A), and NP2(L132A) mutant high-spin complexes, recorded at pH* 7.0 and 25 $^\circ\text{C}$ (except for NP2(L132A) which was at 30 $^\circ\text{C}$). Two of the high-spin complexes of Fig. 2b (NP2(I120V) and NP2(L132A)) show evidence of sharper resonances from a small amount of low-spin complex in the 15–25 ppm region; the nature of the adventitious ligand is unknown. The L122A mutant shows much broader signals as compared to the corresponding L $\rightarrow$ V mutant. It can also be seen that the I120V and I120T mutants have nearly equal abundance of the **A** and **B** heme rotational isomers, which means that there are more resolved resonances as compared to the others. Samples were maintained at room temperature for 8 days to insure that the equilibrium **A**:**B** ratio had been reached, except for the NP2(L122A) and NP2(L132A) single mutants, which were not stable for extended periods of time.

Fig. 3.

NP2(L122T,L132V)-ImH spectra recorded with less than stoichiometric imidazole present in order to have a mixture of high-spin and low-spin species present at 35 °C, pH* 7.0 and 600 MHz. a) 1D ^1H and WEFT/NOESY map showing chemical exchange between the same heme protons in their HS and LS states; b) higher shielding region of WEFT/NOESY map showing NOE interactions between β -vinyl of the HS complex and chemical exchange between HS-LS complexes; c) WEFT/NOESY map showing NOEs between two $6\text{H}\alpha$ and also between two $7\text{H}\alpha$.

Fig. 4.

^1H NMR spectra of wild-type NP1 [26], NP1(Y28F) [26], NP1(L44F) [30], NP1(L44F,F107L), NP1(A42S), NP1(T121S), NP1(T121L), NP1(T121I) high-spin complexes, all at pH* 7.0 and 25 °C. Samples were maintained at room temperature for 8 days to insure that the equilibrium A:B ratio had been reached. It should be noted that several of the spectra have one or more sharp signals which are due to a small amount of a low-spin impurity complex which is produced by an adventitious ligand which was very difficult to remove; the Y28F, T121S and T121L single mutants and the L44F,F107L double mutant show this low-spin complex.

Fig. 5.

Space-filling models of the hemes of wt NP4 (with **A** heme orientation, PDB file 1D3S) and residues L44 below and F107 above the heme (top left)), and wt NP2 (with **B** heme orientation, PDB file 1EUO) and residues F42 below and L106 above the heme (top right)). With NP1,4's large F107 side chain (as shown, top left), it is natural to expect the wild-type proteins to rotate the heme slightly clockwise for the **A** isomer, and slightly counterclockwise for the **B** isomer. With NP2's large F42 side chain (as shown, top right), it is clear that the **B** heme rotational isomer fits nicely against the small L106 and large F42, but the **A** isomer would have serious difficulty fitting against the phenyl ring of F42, which is consistent with wt NP2 having **A**:**B** = 1:8. Two phenylalanine side chains, as for the model of the NP1(L44F) mutant (bottom left) or that of the NP2(L105F) mutant (bottom right), constructed using SWISS-PdbViewer, as described in the Experimental section, would make this region congested for both **A** and **B**, as shown below, left for NP1(L44F), or below, right for NP2(L106F). The latter mutant, and others related to it, have not yet been investigated.

Fig. 6. WEFT-NOESY/EXSY spectrum of high-spin NP1(L44F), recorded in D₂O at pH* 7.0 at 600 MHz and 35 °C. Chemical exchange connections between **A** and **A'** and between **B** and **B'** are marked.

Fig. 7. WEFT-NOESY spectrum of NP2(L122T,L132V)-ImH, recorded at 600 MHz at 15 °C, pH* 7.0. A large number of NOE connections are marked, including all four *meso*-H connections to neighboring substituents. All heme substituents except 4-vinyl- α -H have been assigned and are summarized in Supporting Information Table S5.

Fig. 8. WEFT-NOESY and HMQC maps of NP2(I120T)-ImH, recorded at 500 MHz, 25 °C, pH* 7.0. A fresh sample was used, rather than the equilibrated sample shown in Fig. 2b, in order to observe a majority of the resonances of both heme orientations. Two sets of heme methyl resonances (for the **A** and **B** orientations) can be seen clearly in the $^1\text{H}\{^{13}\text{C}\}$ HMQC map. EXSY cross peaks caused by chemical exchange between the parallel and perpendicular orientations for the two isomers are also shown.

Fig. 9.
 1D ^1H NMR and 2D WEFT-NOESY spectrum of NP1(T121I)-ImH, recorded at 30 °C, pH 7.0 at 600 MHz. Chemical exchange cross peaks between two species for the 3-methyl and 2-vinyl- α -CH for heme orientations **A** and **B** are observed.

Fig. 10.
 1D ^1H NMR and 2D WEFT-NOESY spectra of NP1(T121S)-ImH (left) and NP1(T121S)-CN (right) at 30 °C, pH 7.0 at 600 MHz. Chemical exchange cross peaks between two species for the 3-methyl and 2-vinyl- α -CH for heme orientations **A** and **B** are observed in each case.

Table 1

Chemical shifts of the heme resonances of the high-spin complexes of NP2-L→A mutants at 25 °C and pH* 7 wild-type NP2 and the L→V mutants. Also included are the L→V,T double mutants and the I120T, V mutants.

Isomer	NP2 WT B ^{a)}	NP2-(L122V) B ^{a)}	NP2-(L122A) B	NP2-(L132V) B ^{a),b)}	NP2-(L132A) B	NP2-(L122V, L132V) B ^{a)}	NP2-(L122T, L132V) B	NP2-(L120T) B	NP2-I120V B
to	1:8 ^{d)}	~1:12	~1:6	~1:8	~1:8	~1:10	1:6	~2:3	~1:2
n	58.6 ^{d)}	58.8 ^{a)}	54.0	60.0 ^{a)}	55.1	59.0 ^{a)}	58.0	54.8	60.5
n	49.5 ^{d)}	50.3 ^{a)}	50.4	52.8 ^{a)}	52.1	53.2 ^{a)}	53.2	53.2	51.8
n	64.1 ^{d)}	63.4 ^{a),b)}	59.0	67.1 ^{a)}	62.2 ^{b)}	65.8 ^{a),b)}	62.2 ^{d)}	58.3	65.0
n	64.1 ^{d)}	64.6 ^{a),b)}	59.0	67.7 ^{a)}	61.1 ^{b)}	67.6 ^{a),b)}	63.4 ^{d)}	59.2	65.5
Δ, ppm	18.2	17.2	15.7	16.8	-	15.2 ^{a)}	15.2	16.7	18.7
shift, ppm	8>5>1>3	8>5>1>3	8=5>1>3	8>5>1>3	5>8>1>3	8>5>1>3	8>5>1>3	8>5>1>3	8>5>1>3
ppm	59.1 ^{d)}	59.3 ^{a)}	55.6	61.9 ^{a)}	57.6	61.4 ^{a)}	59.0	55.2	60.8
	14.6 ^{d)}	14.3 ^{a)}	8.6	14.9 ^{a)}	10.1	14.4 ^{a)}	9.2	9.3	14.8
	135 ^{d)}	~136 ^{a)}	~135 ^o	~137 ^{o a)}	~133 ^o	138-139 ^o	136 ^o	137 ^o	~136 ^o

from [25].

ments proven by correlation to the low-spin 2MeImH complex via saturation transfer or NOESY experiments.

ments proven by correlation to the low-spin NMIm complex via saturation transfer or NOESY experiments

ments proven by correlation to the low-spin ImH complex via saturation transfer or NOESY experiments.

Heme Methyl Chemical Shifts for NP1(L44F), Scenario 1. The direction of movement of the primed methyls is listed as + or −.

Table 2a

	3	3'	1	1'	5	5'	8	8'
A	48.1	55.3+	57.0	55.0−	63	62−	64.6	66+
B	48.1	43.7−	56.5	61.7+	53.9	61.6+	61.6	53.9−

A: 8 > 5 > 1 > 3

64.6>63>57>48.1

φ ~ 138°, heme is rotated 3° cw from wt.

A': 8 > 5 > 1 > 3

66 > 62 > 55.3~55.0

φ ~162° Δ = 24°

chem ex is clockwise

B: 8 > 1 > 5 > 3

61.6>56.5>53.9 >48.1

φ ~ 140°, heme is rotated 5° ccw from wt.

B': 5 ~ 1 > 8 > 3

61.7~61.6>53.9>43.7

φ ~ 123° Δ = 17°

chem ex is clockwise

Heme Methyl Chemical Shifts for NP1(L44F), Scenario 2. The direction of movement of the primed methyls is listed as + or −.

Table 2b

	3	3'	1	1'	5	5'	8	8'
A	48.1	43.7−	55	56.5 +	53.9	61.6 +	66	64.6 −
B	48.1	55.3+	61.7	57.0 −	63.0	62.0 −	53.9	61.6 +

A: 8 > 1 > 5 > 3

66>55>53.9>48.1

φ~141–144°, heme is rotated 6–9° cw from wt.

A': 8 > 5 > 1 > 3

64.6>61.6>56.5>43.7

φ~136–137° Δ = 4–8°

chem ex is counterclockwise

B: 5 > 1 > 8 > 3

63>61.7>53.9>48.1

φ~120–123°, heme is rotated 13–15° ccw from wt.

B': 5' ~ 8' > 1' > 3'

62~61.6>57>55.3

φ~132–134° Δ = 9–14°

chem. ex is counterclockwise

Table 3
Assignments of Heme Resonances of the ImH Complexes of NP2 and its L→A and L→V mutants at 30 °C and pH* 7.0.

	NP2 wt. 30 °C (B)	NP2-(L122A) 30 °C (B)	NP2-(L122V) 30 °C (B)	NP2- (L122A) 30 °C (B')	NP2-(L122V) 30 °C (B')	NP2-(L132A) 30 °C (B)	NP2- (L132A) 13C 30 °C (B)	NP2-(L132V) 25 °C (B)	NP2-(L132A) 30 °C (B')	NP2- (L132V) 30 °C (B')
1M	11.5 ^{a)}	10.2	12.7 ^{a)}	22.1	19.1 ^{a)}	12.6	-21.7	14.0 ^{a)}	17.8	19.1
3M	15.6 ^{a)}	17.0	13.6 ^{a)}	Small	Small	13.2	-33.1	12.4 ^{a)}	Small	Small
5M	12.5 ^{a)}	10.7	13.6 ^{a)}	24.7	22.7 ^{a)}	14.5	-30.6	16.0 ^{a)}	20.9	21.9
8M	1.9 ^{b)}	3.7	2.9 ^{a)}	22.3	23.4 ^{a)}	5.3	-12.0	3.9 ^{a)}	22.9	24.3
7H _α	9.4, 4.9 ^{a)}	10.5, 5.8	8.4, 4.5			8.2, 4.3	-24.5	7.2, 3.5		
7H _β	-2.8, -3.3 ^{a)}	-3.1, -2.3	-2.6, -3.4			-2.2, -2.7	101.5	-2.7, -3.15		
6H _α	12.9, 11.8 ^{a)}	12.8, 10.7	13.0, 10.4			12.4, 10.5	-36.4	12.2, 11.4		
6H _β	-1.2, -2.8 ^{a)}	-1.2, -2.7	-0.8, -2.4			-0.5, -1.7	125.3	-0.7, -2.0		
2H _α	21.8 ^{b)} , c)	20.8	20.9			18.9	30.7	22.3		
2H _β	-5.9, -5.9 ^{b)} , c)	-4.8	-5.4			-5.6, -5.8	206.6	-4.5, -5.0		
4H _α	(6.1) ^{a)} , d)	6.4	6.7			6.3				
4H _β	(1.2, 0.7) ^{a)} , d)	0.3, 0.8	0.4, 0.0			-0.3, -0.7	139.2			
m-α	-1.9 ^{a)}	-1.7	-1.9			-2.1		-1.3		
m-β	7.7 ^{a)}	---	6.0		Chem. exch. ^{a)} $k_{BB} = 54 \text{ s}^{-1}$ $k_{BB'} = 0.5 \text{ s}^{-1}$ $K_{BB'} \sim 100$	4.9		6.1	Chem. exch. $k_{BB} = 8.9 \text{ s}^{-1}$ $k_{BB'} = 0.09 \text{ s}^{-1}$ $K_{BB'} \sim 100$	Chem. exch., but too weak to mens.
m-γ	-3.9 ^{a)}	-4.2	-3.3			-1.7		-3.5		
m-δ	9.9 ^{a)}		8.4			7.7		8.3		
M Order	3>5>1>8	3>5>1>8	3=5>1>8	5>8>1>3	8>5>1>3	5>3>1>8	3>5>1>8	5>1>3>8	8>5>1>3	8>5>1>3
M Av shift, ppm	10.4	10.4	10.7	---	---	11.4	24.4	11.6	---	---
Spread, ppm	13.7	13.3	10.7	---	---	9.2	21.1	12.1	---	---
ImH Angle	~152°	150°	158–159°	39–40°	51°	~161°	~157°	~165°	~49°	~49°

	NP2 w.t. 30 °C (B)	NP2-(L122A) 30 °C (B)	NP2-(L122V) 30 °C (B)	NP2-(L122A) 30 °C (B')	NP2-(L122V) 30 °C (B') ^{a)}	NP2-(L132A) 30 °C (B)	NP2-(L132V) 25 °C (B)	NP2(L132A) 30 °C (B')	NP2- (L132V) 30 °C (B')
Δ φ	~17°	14°	25–26°	83–84°	~82°	~27°	~23°	~33°	~86°
									~83°

- a) Taken from [25].
- b) Taken from Ref. **Error! Bookmark not defined.**
- c) Corrected assignments for the 2-vinyl group taken from [27].
- d) Assignment at 20 °C.
- e) Assigned at 15 °C
- f) ¹Hδ57 β-CH at 35 °C = 16.1 ppm.

Table 4

¹³C and ¹H Assignments of Heme resonances of the NP2(I120T)-ImH complexes.

NP2(I120T)-ImH 25 °C, pH* 7.0 NP2(I120T)-ImH 25 °C, pH* 7.0 NP2(I120T)-ImH 25 °C, pH* 7.0 (perpendicular orientation),					
Isomer A		Isomer B		isomer A'	isomer B'
¹ H	¹³ C	¹ H	¹³ C		
1M	1.9	-6.2	10.7	-18.7	24.0
3M	22.8	-51.1	17.0	-44.0	7.0?
5M	4.4	-9.1	11.6	-25.3	21.1
8M	9.0	-20.5	2.5	-6.6	24.9
6H α	12.1, 6.0	-34.9	13.4, 12.0	-44.3	
6H β	-2.4, -3.1	119.1	-1.5, -2.9	134.7	
7H α	13.0, 9.8	-41.5			
7H β	-1.9, -3.4	128.5			
2H α	18.4	41.0	20.2	25.1	
2H β	-5.1, -5.9	194.3	-5.7, -5.9	212.9	
4H α	6.9	86.9	6.2, 5.8	91.6	
4H β	0.7, 2.0	145.2	0.5, 1.2	130.4	
<i>m</i> - α	-3.8	52.5	-2.5	45.3	
<i>m</i> - β	9.3		8.0		
<i>m</i> - γ	-4.1	43.8	-3.8	44.1	
<i>m</i> - δ			10.0		
H57 β -CH	12.5	5.0	12.7		
H57 β -CH	6.5	5.0	6.5		
H57 α -CH	7.4		7.3		
M Order	3>8>5>1		3>5>1>8	8>1>5>3	5>1>8>3
M av. Shift	9.5	-21.7	10.5	-23.7	19.3
M spread	20.9	-44.9	14.0	-37.4	23.8
Angle	126°		153°	50°	41°
$\Delta \phi$	8°		19°	84°	73°

NP2(1120T)–ImH 25 °C, pH* 7.0 NP2(1120T)–ImH 25 °C, pH* 7.0 NP2(1120T)–ImH 25 °C, pH* 7.0 (perpendicular orientation),			
Isomer A	Isomer B	isomer A'	isomer B'
¹ H	¹ H	¹³ C	¹³ C
<i>k</i> _{B/B} , 35 °C		0.35 s ⁻¹	0.25 s ⁻¹
<i>k</i> _{B/B} , 35 °C		35.7 s ⁻¹	23.3 s ⁻¹
<i>K</i> _{eq} , 35 °C		103	95

Table 5

NMR chemical shift of NP1(T121I)–ImH mutant at 30 °C and pH 7.0.

	Isomer A		Isomer B	
	¹ H	¹³ C	¹ H	¹³ C
1M	−0.1	−0.6	8.3	−12.6
3M	25.3	−57.2	17.4	−44.0
5M	0.2	−0.6	12.2	−34.1
8M	8.1	−12.6	2.0	−5.6
6H α	11.4, 5.6		15.6, 7.1	−38.2
6H β	−2.2, −3.7	117.5	−2.0, −0	
7H α	16.5, 7.5	−39.4	12.3, 5.15	
7H β	0.0, −1.97	126.6	−2.3, −3.5	117.5
4H α	8.4		6.2	
4H β	2.4	125.0	2.2, 2.1	128.2
2H α	13.3	14.9	22.6	
2H β	−6.8, −6.6	187.2	−5.35	214.9
<i>meso</i> - α	−3.4		−2.95	
<i>meso</i> - β				
<i>meso</i> - γ	−3.7	45.3	−3.66	
<i>meso</i> - δ				
M order	3>8>5>1		3>5>1>8	
M av. shift	8.4	−17.8	10.0	−24.1
M spread	25.4	−56.6	15.4	−38.7
Angle φ	125		145	
H57 β -CH	12.6, 6.4	16.4		