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C11449. Native N-Terminus Nitrophorin 2 from the Kissing Bug: Similarities to and Differences from NP2(D1A)

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Abstract

The first amino acid of mature native nitrophorin 2 is aspartic acid, and when expressed in *E. coli* the wild-type gene of the mature protein retains the methionine-0 which is produced by translation of the start codon. This form of NP2, (M0)NP2, has been found to have different properties from its D1A mutant, for which the Met0 is cleaved by the methionine aminopeptidase of *E. coli* [R. E. Berry, T. K. Shokhireva, I. Filippov, M. N. Shokhirev, H. Zhang, F. A. Walker, *Biochemistry* **2007**, *46*, 6830]. Native N-terminus nitrophorin 2 ((ΔM0)NP2) has been prepared by employing periplasmic expression of NP2 in *E. coli* using the pelB leader sequence from *Erwinia carotovora*, which is present in the pET-26b expression plasmid (Novagen). This paper details the similarities and differences between the three different N-terminal forms of nitrophorin 2, (M0)NP2, NP2(D1A), and (ΔM0)NP2. It is found that the NMR spectra of high- and low-spin (ΔM0)NP2 are essentially identical to those of NP2(D1A), but the rate and equilibrium constants for histamine and NO dissociation/association of the two are different.

Introduction

The nitrophorins are a group of nitric oxide-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; its binding to the nitrophorins thus prevents the insect’s detection for a period of time [7]. These properties of the nitrophorins of the adult insect, *Rhodnius prolixus*, named NP1-4 (in order of their decreasing abundances in the adult insect saliva), assist the bug in obtaining a sufficient blood meal. NP2 is unique, for in addition to 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 – 13], NP2 [14, 15] and NP4 [16 – 22]. 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 more

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commonly have α -helical globin or 4-helix bundle folds [23, 24]. The ferriheme prosthetic group is bound to the protein *via* a histidine ligand (His59 for NP1 and NP4, His57 for NP2 and NP3), and the sixth coordination site is available to bind NO or other ligands. In the absence of added exogenous ligands, the sixth coordination site is filled by a water molecule, which yields a weak ligand field that produces a high-spin, $S = 5/2$ state. Upon addition of ammonia, histamine, various imidazoles, hydroxide or cyanide ion to the solution, this added ligand binds, generally with a very large binding constant K_{eq} of 10^8 M^{-1} or larger (or small K_d of tens of nM or smaller) [25, 26, 33], all of which are low-spin, $S = 1/2$. And unlike most other heme proteins, the nitrophorins form stable complexes in both Fe^{III} and Fe^{II} oxidation states with NO, the most stable of which are the $Fe^{II}NO$ complexes, which are paramagnetic, $S = 1/2$, and have stabilities which make NO binding irreversible ($K_d \sim pM$ to tens of fM) [12, 25, 26]. In comparison, the $Fe^{III}NO$ complexes, which are diamagnetic, $S = 0$, have stabilities that facilitate release upon dilution ($K_d \sim$ tens of nM to μM [25]). Ligand binding has been investigated by a number of techniques [12, 25 – 38], including by NMR spectroscopy [12, 29 – 38].

Early in the investigation of the nitrophorins a cDNA library was produced from the salivary glands of the adult insect, and the genes for the four nitrophorins were cloned and sequenced [39, 40]. It was found that the genes of each of the four proteins contain a leader sequence which codes for export to the salivary glands, which is cleaved when the protein reaches its destination to produce the mature native proteins. For expression in *E. coli*, the leader sequences were thus deleted, leaving only the DNA sequences that code for the mature forms of the proteins. Three of the four nitrophorins of the adult kissing bug, NP1, NP2 and NP3 have a charged amino acid at the start of this native protein sequence, as shown in Figure 1. The Met0 which results from translation of the start codon is not cleaved from these three, thus yielding (M0)NP1, (M0)NP2, and (M0)NP3, as compared to NP4, which has its native N-terminal residue, A1, because the Met0 is easily cleaved by the methionine aminopeptidase of *E. coli* when the N-terminal residue has a small size and no charge [42]. The presence of Met0 in recombinant NP1, NP2 and NP3 likely causes a major difference in the conformation of the A-B and G-H loops of the nitrophorins, as is known to be the case for wt NP2 [34]. For NP4 these loops are known to change their conformations as a function of pH, and are believed to be the major hindrance to the escape of NO [17]. The presence of M0 may possibly also change the size and shape of the heme binding pocket. Thus we investigated this major difference further by preparing first the D1A mutant of NP2, which, like NP4, lacks Met0 [34], and in this work, we have prepared native N-terminus NP2.

We start our explanation of the results of this work by pointing out that protohemin IX is unsymmetrical because of the placement of the 2,4-vinyl groups, and thus any protein which contains protohemin IX (also called heme *b*) consists of two isomers, which we have called **A** and **B**, as shown in Scheme 1. In these two isomers the individual environments of each of the eight heme substituents are different. The **A:B** ratios of (M0)NP2, NP2(D1A) and native N-terminus NP2 at pH* 7.0 (which, from this point forward will be called (Δ M0)NP2), are each different, as will be discussed further below. From our study of the D1A mutant of NP2 we found indeed that the rates of NO and histamine binding and release, the equilibrium K_d s, the heme **A:B** ratio and the half-life for attainment of that **A:B** ratio were all significantly affected by removal of Met0 [34]. However, because we were not convinced that NP2(D1A) was identical in all its properties to (Δ M0)NP2, in this work we have prepared (Δ M0)NP2, which has its native first residue, D1. Herein we describe how we were able to remove Met0, and the properties of the resulting (Δ M0)NP2, as compared to those of NP2(D1A) and (M0)NP2.

Experimental

Native N-terminus NP2 expression

Previously [30, 34, 41] the method used for (M0)NP2 production utilized BL21(DE3) *E. coli* cells (Novagen) transformed with a pET-24a (or pET-19a) T7-based expression plasmid (Novagen) which was used to express (M0)NP2 as inclusion bodies. These inclusion bodies were solubilized and refolded to produce soluble *apo*-(M0)NP2, which, following purification, was titrated with its cofactor, iron protoporphyrin IX. (M0)NP2 produced this way has a non-native methionine on the N-terminus of the nitrophorin which is not cleaved by *E. coli*'s methionine aminopeptidase during expression in the cytoplasm [42]. To remove this Met0, we employed periplasmic expression of NP2 using the pelB leader sequence from *Erwinia carotovora* [43], which is present in the pET-26b expression plasmid (Novagen). The oxidizing environment of the periplasm facilitates the correct formation of the nitrophorin's two disulfide bonds, and the cleavage *in vivo* of the pelB N-terminal signal peptide during translocation to the periplasm [44] results in soluble expression of the native sequence of NP2 with an authentic N-terminus (D1 as the first residue). The highest protein yield was obtained when the protein was expressed at 12.5°C using *Arctic Express*(DE3) *E. coli* cells (Stratagene) that express the cold-adapted chaperones to facilitate low temperature growth. In addition, the final growth medium needed to be supplemented with 5-aminolevulinic acid to promote protohemin IX biosynthesis.

Except where indicated, materials were obtained from Sigma-Aldrich and used without further purification. Using standard genetic engineering methods, the DNA sequence coding for the expression of NP2 was inserted into a pET-26b expression plasmid (Novagen) between the restriction sites NcoI and XhoI, followed by site-directed mutagenesis to delete the bases ATG from the NcoI restriction site. This resulted in an expression plasmid encoding for a pelB leader sequence placed N-terminal to the native NP2 sequence. All protein expressions were done from freshly transformed cells using DNA-sequenced stocks of expression plasmid that had been stored at -80°C. The NP2 expression plasmid was transformed into *ArcticExpress*(DE3) *E. coli* cells (Stratagene) using standard protocols, as described with the *ArcticExpress* competent cell instruction manual. Kanamycin sulfate (IBI Scientific) was used for plasmid selection on LB agar plates, and the overnight culture (37 °C) used LB broth containing 100 µg/mL (172 µM) kanamycin sulfate and 20 µg/mL (42 µM) gentamicin (Invitrogen) for selection. The overnight culture was used to inoculate the LB growth medium (20 mL per L) which was grown at 30 °C in a shaker-incubator (235 rpm) until an OD_{600nm} of 0.8 was reached (~3 hours). The incubator then was set to 12.5 °C, and after 15 minutes, expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). In addition, 0.1 mM 5-aminolevulinic acid (ALA) was added, and after both 2 and 4 days of growth, an additional 0.1 mM 5-ALA was added. After 6 days the cells were harvested by centrifugation and the cell paste was stored at -80 °C.

Native N-Terminus NP2 Purification

Purification was performed on ice, in a cold box, or employed water-jacketed columns kept cold with chilled water. Cells were suspended with homogenization into 100 mM sodium phosphate buffer at pH 7.5 containing 5 mM magnesium chloride, 25 µg/mL Dnase (Roche), and 200 µg/mL lysozyme (EMD Chemicals) (5 mL per gram wet cells). The cells were subjected to brief sonication, and the insoluble cell debris was removed by centrifugation (40 minutes at 22,000 g). Ammonium sulfate was added to 50% saturation (2 M), and centrifugation was again used to remove the precipitated proteins. The solution was then loaded onto a hydrophobic interaction column (Pharmacia XK50/30 column packed with 100 mL Phenyl-sepharose 6 Fast Flow, Sigma, equilibrated with 1 M ammonium sulfate) and eluted with water (all rosy red NH₃-bound nitrophorin fractions were collected).

The sample was further concentrated to less than 10 mL using an Amicon stirred cell concentrator with a 10 kDa cutoff membrane and loaded onto a gel filtration column (a 5 mL pre-packed HiTrap desalting guard column connected in series with a Pharmacia C26/40 column packed with 32 cm of Sephacryl S-100 high resolution, GE Healthcare, equilibrated with 100 mM tris(hydroxymethyl)amino-methane (Tris) buffer at pH 8), and eluted with the same Tris buffer. The colored fractions were collected and diluted with DI water 1:4 to a final Tris buffer concentration of 20 mM, and loaded onto an anion exchange column (two 5 mL pre-packed HiTrap Q HP columns connected in series, GE Healthcare, and equilibrated with 20 mM Tris buffer at pH 8), washed with 20 mM Tris buffer, and eluted with 50 mM Tris buffer. The eluted sample was diluted with an equal volume of DI water (to a final Tris buffer concentration of 25 mM) and reloaded onto a cleaned and equilibrated HiTrap Q HP column. Before eluting the bound sample, the Q column was connected in series with a trypsin affinity column (1 mL pre-packed HiTrap benzamidine FF column, GE Healthcare), and then eluted with 50 mM sodium phosphate buffer at pH 7.5 with 0.5 M NaCl.

The sample (about 3 mL from the previous step) was then loaded onto a gel filtration column (a 5 mL pre-packed HiTrap desalting guard column connected in series with a HiPrep 26/60 Sephacryl S-100 HR column, GE Healthcare, equilibrated with 100 mM sodium acetate buffer at pH 5 with 100 mM NaCl). The peak fraction eluted from the gel chromatography was dialyzed against 30 mM sodium acetate buffer at pH 4.7 before being further purified by cation exchange chromatography. In no more than 5-mg batches, the dialyzed nitrophorin was loaded onto a cation exchange column (three 5 mL HiTrap SP HP columns connected in series), washed with 20 mL of the 30 mM sodium acetate buffer at pH 4.7 and eluted with a salt gradient (1 M NaCl in 30 mM sodium acetate buffer). Pure native N-terminus NP2, (Δ M0)NP2, eluted as a single peak at an ionic strength of $\sim$ 4 mS/cm. Each batch of native N-terminus NP2 was preliminarily characterized by UV-visible and by mass spectrometry using MALDI-TOF (Matrix Assisted Laser Desorption-Time-of-Flight) which gave values of 19,923 to 19,917 Da, which confirmed the expected absorbance spectrum and mass for (Δ M0)NP2 of 19,922 Da (average isotopic mass). The protein was stored in lyophilized form at -80°C until use.

NP2(D1A) was expressed, isolated and purified as described elsewhere [34], and was also stored in lyophilized form at -80°C until use.

NMR spectroscopy

NMR samples were prepared in D_2O containing 50 mM phosphate buffer at $\text{pH}^* = 7.0$ and 6.5 (pH^* indicates that the pH meter reading was not corrected for the deuterium isotope effect), and in a mixture of phosphate and acetate buffer for $\text{pH}^* 6.0$, and 50 mM sodium acetate buffer (prepared using acetic acid- d_4 (Cambridge Isotope Laboratories) and sodium deuterioxide (30 wt.% solution in D_2O)) at lower pH values (5.5 and 5.0). NMR spectra were collected over the temperature range 25 to 35 $^{\circ}\text{C}$ with the proton chemical shifts referenced to residual water. NOESY and HMQC spectra were collected at 30 $^{\circ}\text{C}$ with the proton chemical shifts referenced to residual water, and obtained on a Bruker DRX-600 spectrometer operating at 600.130 MHz proton Larmor frequency. The $^1\text{H}\{^{13}\text{C}\}$ HMQC experiments were recorded at a proton Larmor frequency of 600.130 MHz using a 5 mm inverse-detection probe with decoupling during acquisition. A relaxation delay of 200 ms and refocusing time of 2.5 ms ($J = 200$ Hz) were used. The WEFT-NOESY experiments, recorded at 600.13 MHz, utilized 80 ms relaxation delay and 125 ms recovery-delay. The mixing time for the NOESY experiments was 12 ms. All 2D spectra were collected with 1918 or 3072 data points in t_2 and with 512–540 blocks in t_1 with 80–512 scans/block.

Ligand Dissociation Constant Measurements for the Fe(III) Complexes

These measurements were carried out as described previously [34], by adding aliquots of the chosen axial ligand to solutions of the air-stable Fe(III) nitrophorin (1 to 10 μM) and recording the optical spectra from 300 to 700 nm on a Perkin-Elmer UV-vis Lambda 19 at 27°C. Because of the high ligand affinities, even under these dilute solution conditions, a significant fraction of the added ligand was bound, and therefore a mutually depleting model [45, 46] was used to analyze the data

$$(\Delta A/\Delta A_{\max}) = (P_t + L_t + K_d) / (2P_t) - \{[(P_t + L_t + K_d)^2 - (4L_t P_t)]^{0.5} / (2P_t)\} \quad (1)$$

where ΔA is the change in absorbance at a given wavelength for a specific total ligand concentration L_t , $\Delta A_{\max}$ is the change in absorbance of the fully complexed species at the same wavelength determined by extrapolation, P_t is the total protein concentration, and K_d is the dissociation constant for ligand L from the Fe(III) protein. Nitric oxide dissociation constant measurements were performed using aliquots of a 200 μM solution of *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, at 98% purity, obtained from World Precision Instruments) in ultrapure water (18 m Ω) containing EDTA (50 μM) prepared freshly on the day of use. The protein solutions were prepared in degassed buffer containing CuCl (~1 mM, Aldrich) and titrated anaerobically with SNAP, a fairly stable compound that decomposes stoichiometrically and rapidly in the presence of Cu(I) to generate NO and a disulfide product, allowing a much more accurate titration than that from working with gaseous NO [47].

Kinetics of Ligand Binding and Release

Methods used for stopped-flow measurements have been described elsewhere, and have been reported for (M0)NP2 at pH 5 and 8 [25]. Histamine (Hm) kinetics have been reported for (M0)NP2 and NP2(D1A) at pH 5.5 and 7.5 [34]. Stopped-flow measurements of histamine binding were performed on an Olis Stopped-Flow RSM 1000 instrument. Protein solutions (5 to 10 μM) in 100 mM sodium phosphate buffer at pH 7.5 and 5.5 were rapidly mixed with an equal volume of the same buffer containing various concentrations of histamine. Using the Olis GlobalWorks 3D Analysis software to fit the multiple wavelength data, the absorbance changes were fit with a single exponential to obtain the observed rate constants ($k_{\text{obs}}^{\text{Hm}}$ in s^{-1}) at different histamine concentrations (3 to 160 μM) for a large number of repeated measurements. According to the histamine binding reaction described in Scheme 2, plotting $k_{\text{obs}}^{\text{Hm}}$ against histamine concentration will give a slope of $k_{\text{on}}^{\text{Hm}}$ (second order rate constant for histamine binding in $\text{M}^{-1} \text{s}^{-1}$) according to the equation

$$k_{\text{obs}}^{\text{Hm}} = k_{\text{on}}^{\text{Hm}} [\text{Hm}] + k_{\text{off}}^{\text{Hm}} \quad (2)$$

where [Hm] is the histamine concentration, and the intercept, $k_{\text{off}}^{\text{Hm}}$ is the reverse rate constant (or dissociation rate constant) which is very small ($<1 \text{ s}^{-1}$) and can more accurately be determined from the relationship $K_d = k_{\text{off}}/k_{\text{on}}$.

For nitric oxide binding the $k_{\text{on}}^{\text{NO}}$ rate at ambient temperature is too large to be reliably measured by this method, especially at low pH, since the maximum k_{obs} rate that can be observed is limited by the mixing time of the apparatus. Thus, the much slower dissociation rate constant ($k_{\text{off}}^{\text{NO}}$) for the release of nitric oxide (NO) from the Fe-NO complex was determined by measuring the rate of the displacement reaction by histamine. Protein solutions (5 to 10 μM) in 100 mM sodium phosphate buffer at pH 7.5, or 100mM sodium acetate buffer (with 172 mM NaCl) at pH 5.0, were titrated with a minimum amount of

nitric oxide (using the NO donor SNAP as described previously for nitric oxide dissociation constant measurements) until fully complexed (as observed by UV/*vis*, ~15 μ M), and rapidly mixed with an equal volume of the same buffer containing various high concentrations of histamine (10 to 100 mM) to displace the nitric oxide. As with the association measurements described above the absorbance changes were fit with a single exponential to obtain the observed rate constants (k_{obs}^{NO} in s^{-1}). The displacement reaction shown in Scheme 3 can be described by the following equation

$$k_{obs}^{NO} = k_{off}^{NO} / [1 + (k_{on}^{NO} [NO] / k_{on}^{Hm} [Hm])] \quad (3)$$

where k_{obs}^{NO} is the observed first order displacement rate constant, k_{off}^{NO} is the nitric oxide dissociation rate constant, k_{on}^{NO} is the bimolecular rate constant for nitric oxide binding, [Hm] is the histamine concentration, k_{on}^{Hm} is the bimolecular rate constant for histamine binding (eq 2), and [NO] is nitric oxide concentration. In this experiment, [NO] $\ll$ [Hm]; thus, nitric oxide displacement is the rate-determining step, and eq 3 simplifies to $k_{obs}^{NO} = k_{off}^{NO}$ (since $k_{on}^{NO} [NO] / k_{on}^{Hm} [Hm] < \sim 0.01$). The measurements were repeated a number of times and averaged. All measurements were made at 27 $^{\circ}$ C.

Results

Comparison of the 1D 1H NMR spectra of (M0)NP2, NP2(D1A) and (Δ M0)NP2

In Figure 2 are shown the 1D 1H NMR spectra of the high-spin Fe(III) forms of (M0)NP2, NP2(D1A) and (Δ M0)NP2 over the chemical shift range of 75 to 15 ppm at 25 $^{\circ}$ C in D_2O , recorded after each protein had reached its equilibrium **A:B** ratio at pH* 7.0. It should be noted that although the spectra of these three forms of NP2 are grossly similar to each other, the chemical shifts of (M0)NP2 are not the same as those of NP2(D1A) and (Δ M0)NP2. One of the most noticeable differences is the spacing between the two α -propionate proton resonances, for $6\alpha'$ and $7\alpha'$ at 31.6 and 28.0 ppm, respectively, for (M0)NP2, but at 28.7 and 26.5 ppm, respectively, for NP2(D1A) and exactly the same chemical shifts for (Δ M0)NP2. In all three proteins, the chemical shifts of all resonances are dependent on temperature, because of the paramagnetism of the heme, here with $S = 5/2$, as shown in Supporting Information Figure S1. The chemical shifts of all resonances are expected to follow the Curie law,⁴⁸ although it is difficult to verify this over the very limited temperature ranges available for aqueous protein samples. The differences in the chemical shifts of these resonances and their 6α and 7α partners between the NP2(D1A) / (Δ M0)NP2 pair and (M0)NP2 are undoubtedly due to different equilibrium positions of the 6- and 7-propionates, with the carboxylates above or below the mean plane of the heme, on average, and how much freedom of motion those carboxylates have (i.e., whether one or the other is involved in a hydrogen-bond). Whether or not the carboxylates are above or below the mean plane of the heme is not a well-conserved feature of the crystal structures of the P2₁2₁2 unit cell, with the 1EUO, 2EU7, 1PM1, and 2ALL structures having both of the carboxylates below the mean plane of the heme, meaning that the protons of the α -carbons are pointing toward the distal side of the heme, while the 1T68 and 2ACP structures have the carboxylates above the mean plane of the heme, meaning that the protons of the α -carbons are pointing toward the proximal side of the heme, and structure 2A3F has the 6-propionate carboxylate pointing downward (protons of the α -carbon pointing toward the distal side of the heme) and the 7-propionate carboxylate pointing upward (protons of the α -carbon pointing toward the proximal side of the heme). In two structures one of the oxygens of the 6-propionate carboxyl is within hydrogen-bonding distance of the Tyr85-OH (2.55 $\text{\AA}$, 1T68; 2.47 $\text{\AA}$, 2ACP), and in one structure one of the oxygens of the 7-propionate carboxyl is within hydrogen-bonding distance of the Tyr38-OH (2.78 $\text{\AA}$, 1EUO). In all other structures both heme carboxylates are too far from either of these tyrosines to be able to form a

hydrogen-bond that could stabilize the orientation of the propionate. However, a variety of positions are observed for both tyrosines among the seven structures available on the web (Protein Databank, PDB) having the P2₁2₁2 unit cell. It is thus clear that they could adjust the distance between their phenol O-H proton and the propionate carboxylate, mainly by changing the χ^1 and χ^2 angles, to form a hydrogen-bond at higher pH values in solution, where the propionates are deprotonated (the pK_as of the propionates have not been measured, but they are certainly at least partially deprotonated at pH* 7.0), if that hydrogen-bond is energetically favorable to the molecule. Whether it is favorable or not is difficult to determine, but should be different for (M0)NP2 than for the other two proteins because the presence of M0 prevents closure of the A-B loop, and Tyr38 is the last residue of that A-B loop; all structures except that of 2EU7, NP2(D1A)-NH₃, are of (M0)NP2. The chemical shifts of three of the four 6- and 7-H α and H α' resonances of all three proteins change quite a bit with pH, as shown for (Δ M0)NP2 in Supporting Information Figures S2 and S3.

An important difference among the three N-terminal proteins is the rate of equilibration of the **A:B** heme orientational ratio. (M0)NP2 reaches its equilibrium **A:B** ratio of ~1:25[‡] within about 10 days at room temperature at pH* = 7.0, while NP2(D1A) requires significantly more than one month to reach its equilibrium **A:B** ratio of 1:14 at pH* 7.0 (the spectrum shown in Figure 2 was recorded after three months' equilibration time). In contrast, while the spectra of NP2(D1A) and (Δ M0)NP2 look extremely similar, (Δ M0)NP2 had almost reached its equilibrium **A:B** ratio (1:12 at pH* 7.0) by the time the protein had been through the last chromatography column of its purification (about 10 days after the collection of cells from the expression (for which the (Δ M0)NP2 protein was already loaded with protohemin which was synthesized by the bacteria during cell growth)). Very little further change occurred over the next three weeks, as shown in Figure 2. Hence, although the proton NMR spectra of NP2(D1A) and (Δ M0)NP2 are nearly identical, the amount of time required to reach this equilibrium ratio is shorter for (Δ M0)NP2 than for NP2(D1A).

We have found that the heme orientational ratio changes with pH, as is shown in several examples of how the equilibrium ratios of **A:B** were measured, in Supporting Information Figure S4. Thus, at pH* 5.0, (Δ M0)NP2 has an **A:B** ratio of ~1:5, and NP2(D1A) has a similar **A:B** ratio at equilibrium at that same pH* value. (M0)NP2 also changes **A:B** ratio as a function of pH, from ~1:25 at pH* 7.0[‡] to ~1:11 at pH* 5.0. The reason for this change in **A:B** ratio may be related to a group of four amino acids, E53, Y81, Y104 and F42, which are found inside the β -barrel of the lipocalin very near the 3-methyl and 4-vinyl side chains of the heme, as shown in Figure 3 for PDB file 2EU7, the structure of NP2(D1A) bound to NH₃, obtained at pH* 6.5 [15], where we expect E53 to be mainly deprotonated (estimated pK_a = 5.8 $\pm$ 0.6 [38]). The distance between Y81 OH and E53 OE1 is 2.46 Å, and the distance between Y104 OH and E53 OE2 is 2.44 Å, which suggests strong hydrogen-bonds to the E53 side chain carboxyl by both tyrosines. Mutation of E53 to any other residue produces a protein that cannot be folded for (M0)NP2, and is difficult to fold for NP2(D1A) [38]. The mutation Y104F in either (M0)NP2 or NP2(D1A) and the corresponding NP4(Y105F) is equally difficult to fold [R. E. Berry, D. Muthu, T. K. Shokhireva, S. A. Garrett, A. M. Goren, H. Zhang, F. A. Walker, manuscript in preparation]. With an estimated pK_a for E53 of 5.8 [38] for NP2(D1A)-NO, as this carboxyl group is protonated as the pH* is lowered to 5.0, the hydrogen-bonds between Y81 and Y104 and the E53 carboxyl group would be weakened significantly, or broken entirely. Thus we hypothesize that in all N-terminal forms of NP2, the weakening at low pH, or loss by mutation of the Tyr104-Glu hydrogen-bond destabilizes the structure observed in the pH 6.5 crystal structure of

[‡]Previously, the equilibrium **A:B** heme orientational ratio of (M0)NP2 has been reported to be 1:8 [31 – 34, 36], but in this study we have measured this ratio more accurately after longer equilibration times of 8 days or more, instead of 2–3 days, and we now find that this equilibrium ratio is actually ~1:25 at pH* 7.0 (this work).

NP2(D1A)-NH₃ [15], and allows Y104 and E53 to move in ways that may make additional space available in the region of these four amino acids for the 2-vinyl side chain of the **A** heme orientational isomer, thus increasing the stability of the **A** isomer and increasing the **A**:**B** heme ratio at low pH*. It would be interesting to obtain the crystal structure of (ΔM0)NP2 at low pH (5.0) to test this hypothesis.

Along with this Y-E-Y triad is the nearby F42, whose mutation to A in NP2(D1A) causes the **A** heme orientation to be favored [R. E. Berry, D. Muthu, T. K. Shokhireva, S. A. Garrett, A. M. Goren, H. Zhang, F. A. Walker, manuscript in preparation]. Thus F42 appears to be largely responsible for the preference of the **B** heme orientation of NP2(D1A). Mutants with larger aliphatic side chains, including Leu (the residue present in the corresponding site in (M0)NP1), are also tolerated at this position, as demonstrated by the fact that the F42L mutant of (ΔM0)NP2 folds well [S. A. Garrett, unpublished results].

Another interesting difference among the three proteins is that only (ΔM0)NP2 shows clear evidence of the small His57 β-CH resonance of the **A** heme orientation at 19.8 ppm, which is seen in addition to the major peak for the **B** heme orientation, at 18.3 ppm (Figure 2). At least part of the reason this was observed for (ΔM0)NP2 but not for the others is that the proton resonances are somewhat sharper for this protein than for the other two.

In Figure 4 are shown the 1D ¹H, the ¹H{¹³C} HMQC and the WEFT-NOESY spectra of the low-spin (*S* = ½) NMeIm complex of (ΔM0)NP2, recorded at pH* 7.0 in D₂O at 30°C. The same spectra, recorded at pH* = 7.0 and 32 °C in D₂O, published previously for NP2(D1A)-NMeIm [34], are shown in the Supporting Information, Figure S5, with many more labels for heme resonances provided. The chemical shifts of the heme substituents for both of these protein complexes, as well as those of (M0)NP2-NMeIm, are summarized in Table 1, where it can be seen that the heme substituents of (ΔM0)NP2-NMeIm and NP2(D1A)-NMeIm have extremely similar chemical shifts at pH* 7.0. As for the high-spin forms of these proteins, the chemical shifts of the NMeIm complexes of these NP2 constructs vary with pH*. Thus, the chemical shifts for the heme substituents of (ΔM0)NP2-NMeIm at pH* 5.0 are included in Table 1. As can be seen, all proton chemical shifts are different at the lower pH, and even the order of heme methyl resonances has changed, from 5 > 3 > 1 > 8 at pH* 7.0 to 3 > 5 > 1 > 8 at pH* 5.0, which suggests that the NMeIm ligand has changed its orientation somewhat, from about 161.5° at pH* 7.0 to about 155° at pH* = 5.0, although the 5 and 1 methyls are much closer together at pH* 5 than is usually observed [49]. Nevertheless, as shown in Figure 5, the assignment of all four methyl groups is confirmed by the 1D saturation transfer experiments.

Ligand Binding Constants and Kinetics of Histamine and NO Binding Measurements

An example of the data obtained for NO *K_d*^{III} measurement at pH 7.5 is shown in Figure 6. The results of the equilibrium dissociation constant measurements for NP2-D1A and (ΔM0)NP2 complexes are summarized in Table 2. As can be seen, the histamine dissociation constants at both pH 5.5 and 7.5 are the same for (M0)NP2 and NP2(D1A), and somewhat larger for (ΔM0)NP2, while the NO dissociation constants are too small to be measured at pH 5.5, but at pH 7.5 the *K_d*^{NO} values for (M0)NP2 and NP2(D1A) are the same (5 nM), while that of (ΔM0)NP2 are five times larger (25 nM), indicating that the presumably protonated D1 weakens the NO complex significantly at a pH close to that of the tissues of the victim.

An example of the stopped-flow kinetics of histamine binding is shown in Figure 7, where histamine binding to (M0)NP2, NP2(D1A) and (ΔM0)NP2 at pH 7.5 can each be fit with a single exponential, and the observed histamine binding rates at various histamine concentrations can be plotted to yield a straight line and the histamine binding rate constant.

The histamine kinetics results are summarized in Table 2, and as observed previously [34], show NP2(D1A) to have slower overall kinetics by a factor of 1.6 than (M0)NP2 at pH 5.5, and factors of 2.5 and 5.1 at pH 7.5, for $k_{\text{on}}^{\text{Hm}}$ and $k_{\text{off}}^{\text{Hm}}$, respectively. This slower kinetics was attributed to removal of M0, which allows loops to close over the opening to the heme pocket, and thus hinders entry and exit of the histamine molecule [34]. For (Δ M0)NP2, with a native N-terminus, we also observe slower kinetics, by a factor of 1.4 and 1.2 at pH 7.5, for $k_{\text{on}}^{\text{Hm}}$ and $k_{\text{off}}^{\text{Hm}}$, respectively, but by a much more significant factor of 10.6 and 8.4 at pH 5.5, for $k_{\text{on}}^{\text{Hm}}$ and $k_{\text{off}}^{\text{Hm}}$, respectively. This much more significant pH dependence indicates that in the absence of M0 the N-terminal aspartate plays a role in the mechanism of histamine binding and release.

For the NO equilibria and kinetics, the K_{d}^{NO} and $k_{\text{on}}^{\text{NO}}$ were both too small and large, respectively, to be measured reliably at low pH at 27 °C, but the $k_{\text{off}}^{\text{NO}}$ could be measured. As with the larger Hm ligand the $k_{\text{off}}^{\text{NO}}$ was found to be smaller, by a factor of 3.3 and 1.6, for NP2(D1A) and (Δ M0)NP2, respectively, at pH 5.0. At pH 7.5 the $k_{\text{off}}^{\text{NO}}$ values for NP2(D1A) and (Δ M0)NP2 are similar to each other and about half the value found for (M0)NP2. However, more interesting is the fact that $k_{\text{on}}^{\text{NO}}$ for NP2(D1A) is about half the value found for (M0)NP2, while k_{on} for (Δ M0)NP2 is 1/10 the value found for (M0)NP2, and thus 1/5 the value found for NP2(D1A). As a consequence, the equilibrium K_{d}^{NO} values are the same for (M0)NP2 and NP2(D1A), but *five times larger* for (Δ M0)NP2. This may suggest that after NO escapes, the A-B and G-H loops close, to prevent re-entry of NO. Thus, the two N-terminal forms of NP2, with D1 or A1 as the first amino acid, are not the same in their effect on the kinetics and thermodynamics of NP2.

Discussion

The NMR studies of the three N-terminus forms of NP2, and in particular NP2(D1A) and (Δ M0)NP2, have shown that the ^1H chemical shifts are extremely similar to each other in both the high-spin and the low-spin NMeIm complex forms, although the rate of equilibration of the **A** and **B** heme orientations are not the same (slower for NP2(D1A)). However, with regard to the thermodynamics and kinetics, the three proteins are different from each other in ways that are not apparent from the similar NMR spectra. It is likely that the A-B loop (the protein loop between the A and B β -strands) is able to close over the heme binding pocket when the N-terminal methionine is not present to hinder it, as is the case for NP4-NO [16, 17], and that this slows passage of ligands in and out of the pocket, but to different extents for the native N-terminus with D1 than for A1. It should also be noted that X-ray crystallography shows that the loop conformation of NP2(D1A)-NH₃ at pH 7.5 is not the same as that of NP4(NO) at pH 5.6 [16, 17]. (There is no hydrogen-bond between the N-terminal amino group and the glutamate (E32) in the A-B loop of NP2(D1A) because in NP2, that residue is an aspartate (D31), and the side chain is too short to form the H-bond.) Likewise, the A-B loop conformation of NP2(D1A) is very likely not the same as that of (Δ M0)NP2, but at present we do not know how the conformation of the latter looks. Nevertheless, it is clear that the opening to the heme pocket is similarly closed compared to that of NP4(NO) and significantly more closed than that of (M0)NP2, which would clearly slow the passage of constituents (histamine and also NO) in and out of the heme pocket.

The two ligands, histamine and NO, behave entirely differently at pH 7.5, where NO is released and histamine is bound. Histamine binds at pH 7.5 with a K_{d} that is 50% larger for (Δ M0)NP2 than for NP2(D1A), and with a k_{on} that is about a factor of 2 larger for (Δ M0)NP2 than for NP2(D1A) and a k_{off} that is about a factor of 4 larger for (Δ M0)NP2 than for NP2(D1A). In contrast, at pH 7.5 NO dissociates from (Δ M0)NP2 at the same rate as it does from NP2(D1A), but (Δ M0)NP2 binds (or re-binds) NO at pH 7.5 significantly more *slowly* than do both (M0)NP2 and NP2(D1A). This results in weaker NO equilibrium

binding (larger K_d) for ($\Delta M0$)NP2 at pH 7.5. Thus, one way of looking at NO release and re-binding is that ($\Delta M0$)NP2 seems to be able to fully close the A-B and G-H loops after NO is released, which prevents it from re-binding. Thus we see that although ($\Delta M0$)NP2 and NP2(D1A) are alike in several ways, in the important area of kinetics and thermodynamics, they were not the same. This indicates that D1, especially in its deprotonated form (pH 7.5), is involved in some special interactions that cause the A-B loop to close after NO escapes. Thus, obtaining 3-dimensional structural information about ($\Delta M0$)NP2 and its complexes is a high-priority goal for the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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	1	_____α ₁ _____	_____β _A _____	--A-B loop--	_____β _B _____	
NP1	KCTKNALAQT	GFNKDKYFNG	DVWYVTDYLD	LEPDDVPKRY	CAALAAGTAS	
NP4	ACTKNAIAQT	GFNKDKYFNG	DVWYVTDYLD	LEPDDVPKRY	CAALAAGTAS	
NP2	DCSTNISPKQ	GLDKAKYFSG	.KWYVTHFLD	KDP.QVTDQY	CSSFTPRES	D
NP3	DCSTNISPKK	GLDKAKYFSG	.TWYVTHYLD	KDP.QVTDQY	CSSFTPKESG	
	51	_____β _C _____	_____β _D _____	_____β _E _____		
NP1	GKLKEALYHY	DPKTQDTFYD	VSELQEEESPG	.KYTANFKKV	EKNGNVKVDV	
NP4	GKLKEALYHY	DPKTQDTFYD	VSELQVESLG	.KYTANFKKV	DKNGNVKVAV	
NP2	GTVKEALYHY	NANKKTSFYD	IGEGKLESSG	LQYTAKYKTV	DKKKAVLKEA	
NP3	GTVKEALYHF	NSKKKTSFYD	IGEGKLGSLG	VQYTAKYNTV	DKKRKEIEPA	
	101	_____β _F _____	_____β _G _____	_____β _H _____		
NP1	TSGNYTFTV	MYADDSSALI	HTCLHKGNKD	LGDLIYAVLNR	NKDTNAGDKV	
NP4	TAGNYTFTV	MYADDSSALI	HTCLHKGNKD	LGDLIYAVLNR	NKDAAAGDKV	
NP2	DEKNSYTLTV	LEADDSSALV	HICLREGSKD	LGDLIYVLTH	QKDAEPSAKV	
NP3	DPKDSYTLTV	LEADDSSALV	HICLREGPKD	LGDLIYVLTH	QKTGEPSATV	
	151	_____α ₂ _____	_____α ₃ _____			
NP1	KGAVTAASLK	FSDFISTKDN	KCEYDNVSLK	SLLTK		89% identity
NP4	KSAVSAATLE	FSKFISTKEN	NCAVDNDSLK	SLLTK		"
NP2	KSAVTQAGLQ	LSQFVGTKDL	GCQYD.DQFT	SL~~~		78% identity
NP3	KNPVAQAGLK	LNDFVDTKTL	SCTYD.DQFT	SM~~~		"
						overall 40% identity

Figure 1.

Protein sequences of the mature native nitrophorin proteins of the adult *R. prolixus* insect (with the leader sequences, discussed in the text, deleted). As expressed previously [11, 12, 14 – 22, 25 – 33, 35 – 41], only NP4 has its native A1 as first amino acid, while the other three proteins have Met0 still present as the first amino acid of the isolated recombinant protein.

Figure 2.

NMR spectra of the high-spin forms of (M0)NP2, NP2(D1A) and (ΔM0)NP2, recorded at the indicated pH* in D₂O at 25 °C.

Figure 3.

The heme of NP2(D1A) (PDB file 2EU7), showing the residues closest to it. In particular, note E53 (black) with H-bonds from Y81 (orange) and Y104 (green), as well as the pair F42 (yellow) and L106 (deep blue), both behind and very close to the heme 4-vinyl.

Figure 4.

NMR spectra of the low-spin form of ($\Delta M0$)NP2-NMeIm, recorded at pH* 7.0 in D₂O at 30°C. *Top*, 1D ¹H spectrum, *middle*, ¹H{¹³C} HMQC, and *bottom*, WEFT-NOESY with selected assigned peaks labeled.

Figure 5. 1D Saturation transfer difference spectra of (ΔM0)NP2 at pH* 5.0, 30 °C, in the presence of about 0.2 equivalents of NMeIm, showing the order of heme methyl resonances for both high- and low-spin forms of the protein.

Figure 6.

Nitric oxide titration of ($\Delta M0$)NP2 at pH 7.5, 27 °C, showing the fit to Eq. 1 to determine K_d^{NO} , and an inset showing the UV/visible spectra at various NO concentrations.

Figure 7.

Histamine k_{on} measurement for ($\Delta M0$)NP2 at pH 7.5 and 5.5, at 27 °C. *Inset:* A kinetic fit for a single data point using the Olis GlobalWorks 3D Analysis software to fit the multiple wavelength data. The absorbance changes were fit with a single exponential to obtain the observed rate constants (k_{obs}^{Hm} in s^{-1}) at different histamine concentrations.

Scheme 1.

Heme orientations **A** and **B** for NP2, with His57 behind the heme, Ile120, Leu122 and Leu132 in front of the heme. The His57 imidazole plane is shown as a line which makes an angle ϕ to the defined x-axis.

Scheme 2.

Scheme 3.

Table 1

NP2 Construct Heme Substituent Assignments for the **B** Heme Orientation, Complexed to NMeIm, in D₂O at pH 7.0.

Substituent	(M0)NP2 ^a	NP2(D1A) ^a	(ΔM0)NP2 ^c	(ΔM0)NP2 ^c
	¹ H (32 °C)	¹ H (32 °C)	¹ H (30 °C)	¹ H (30 °C, pH 5.0)
1M	13.8	13.6	13.5	11.3
3M	13.4	13.8	13.7	16.5
5M	15.4	15.0	15.0	12.2
8M	1.0	1.65	1.5	2.0
2Hα	22.9	22.6	22.6	22.1
2Hβ	−5.6, −5.7	−5.3, −5.3	−5.4, −5.5	−4.7, −4.8
4Hα	6.1	6.4	–	–
4Hβ	1.0, 0.4	1.2, 0.6	1.2, 0.7	1.6, 1.0
6Hα	13.8, 12.5	13.5, 13.0	13.7, 12.6	14.3, 11.9
6Hβ	−0.9, −2.4	−0.6, −2.0	−0.8, −2.2	−1.2, −2.6
7Hα	8.0, 4.1	8.7, 4.4	8.6, 4.2	9.2, 4.2
7Hβ	−3.0, −3.7	−2.5, −3.1	−2.5, −3.2	−1.9, −2.9
<i>m-α</i>	−1.5	−1.6 ^b	−1.7	−2.0
<i>m-β</i>	6.7	7.3	7.0	–
<i>m-γ</i>	−3.8	−3.1	−3.3	−4.2
<i>m-δ</i>	9.5	9.9	9.8	10.3

^aFrom [34].

^bShokhireva TK, Walker FA. J Biol Inorg Chem. 2012;17:911.

^cThis work.

Table 2Histamine and nitric oxide equilibrium binding (K_d) and kinetics (k_{on} and k_{off}) measurements.

N-terminus:	(M0)NP2 MDC...	NP2(D1A) AC...	(ΔM0)NP2 DC...
Histamine:			
K_d^{Hm} (nM) at pH 5.5	250 ± 50^c	250 ± 50^b	317 ± 42^{TW}
k_{on}^{Hm} ($\mu M^{-1}s^{-1}$) at pH 5.5	0.7 ± 0.1^a	0.4 ± 0.1^a	0.061 ± 0.001^{TW}
k_{off}^{Hm} (s^{-1}) at pH 5.5	0.163 ± 0.011^b	0.101 ± 0.011^b	0.019 ± 0.003^a
K_d^{Hm} (nM) at pH 7.5	10 ± 2^c	10 ± 4^b	15 ± 2^{TW}
k_{on}^{Hm} ($\mu M^{-1}s^{-1}$) at pH 7.5	4.5 ± 0.1^b	1.8 ± 0.1^b	3.2 ± 0.1^{TW}
k_{off}^{Hm} (s^{-1}) at pH 7.5	0.056 ± 0.003^b	0.011 ± 0.001^b	0.046 ± 0.005^a
Nitric Oxide:			
K_d^{NO} at pH 5.0	Too small to meas.	Too small to meas.	Too small to meas.
k_{on}^{NO} at pH 5.0	Too large to meas.	Too large to meas.	Too large to meas.
k_{off}^{NO} (s^{-1}) at pH 5.0	0.049 ± 0.002^{TW}	0.015 ± 0.005^{TW}	0.030 ± 0.002^{TW}
K_d^{NO} (nM) at pH 7.5	5 ± 1^b	5 ± 1^b	25 ± 3^{TW}
k_{on}^{NO} ($\mu M^{-1}s^{-1}$) at pH 7.5	32.3 ± 6.5^a	17.8 ± 3.6^a	3.7 ± 0.5^a
k_{off}^{NO} (s^{-1}) at pH 7.5	0.161 ± 0.005^{TW}	0.089 ± 0.002^{TW}	0.093 ± 0.002^{TW}

TW This work,

^a calculated from the relationship $K_d = k_{off}/k_{on}$,^b from [34],^c from [26].