The Crystal Structure of Nitrophorin 2

A TRIFUNCTIONAL ANTIHEMOSTATIC PROTEIN FROM THE SALIVA OF RHODNIUS PROLIXUS*

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John F. Andersen§ and William R. Montfort§

From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

Nitrophorin 2 (NP2) (also known as prolixin-S) is a salivary protein that transports nitric oxide, binds histamine, and acts as an anticoagulant during blood feeding by the insect Rhodnius prolixus. The 2.0-Å crystal structure of NP2 reveals an eight-stranded antiparallel β-barrel containing a ferric heme coordinated through His57, similar to the structures of NP1 and NP4. All four Rhodnius nitrophorins transport NO and sequester histamine through heme binding, but only NP2 acts as an anticoagulant. Here, we demonstrate that recombinant NP2, but not recombinant NP1 or NP4, is a potent anticoagulant; recombinant NP3 also displays minor activity. Comparison of the nitrophorin structures suggests that a surface region near the C terminus and the loops between β strands B-C and E-F is responsible for the anticoagulant activity. NP2 also displays larger NO association rates and smaller dissociation rates than NP1 and NP4, which may result from a more open and more hydrophobic distal pocket, allowing more rapid solvent and NO release rates when deprotonated, hydrogen bonds to invariant Tyr51. Surprisingly, this tyrosine lies on the protein surface in NP1 and NP4.

Blood feeding in insects has evolved independently multiple times, resulting in a diverse array of molecules designed to circumvent host hemostatic defenses (1–3). Among the most remarkable of these substances are the nitrophorins (NPs),1 a group of heme proteins found in the saliva of the bug Rhodnius prolixus (4–7). NPs are unique in being multifunctional, in that multiple anti-hemostatic activities are combined into a single protein. NPs possess a potent vasodilatory activity due to release and transport of nitric oxide (4). NO binding with NPs occurs in the salivary gland lumen, and release occurs at the site of feeding after being transported through the insect mouth parts. There, NO induces relaxation of the vascular endothelium (vasodilation) through activation of soluble guanylate cyclase. The NPs also bind histamine that is released from mast cells around the bite (5). The affinity of the proteins for histamine is extremely high, which may serve both to displace the NO and to provide a significant local antihistaminic activity. Finally, NPs possess potent anticoagulant activity via inhibition of the factor Xase complex of the intrinsic pathway (7–9).

There are four NPs in the R. prolixus saliva that are designated as NP1–NP4 and can be divided into two groups based on sequence relationships (3, 7, 10). NP1 and NP4 are 90% identical, and NP2 (also known as prolixin-S) and NP3 are 80% identical (Fig. 1). The two groups are more distantly related, with NP1 and NP2 being 47% identical. The NPs are all Fe(III) heme proteins, but the sequence groups differ in both their NO binding and anticoagulation properties. Although all four proteins bind nitric oxide and histamine, only NP2 possesses strong anticoagulation activity (7, 8). NP1 and NP4 bind NO with lower affinity (0.5–1 μM at pH 8.0) than do NP2 and NP3 (0.02 μM at pH 8.0) (11). The differences in affinity are due to both larger association rate constants and smaller dissociation rate constants in NP2 and NP3. The differences in ligand release rates may serve to extend the duration of the NO signal in the host or increase the effective radius of the signal around the bite.

NP4 binding to NO (but not to cyanide or ammonia) induces a large conformational change in the protein that results in burial of the NO ligand in the distal pocket and a substantial increase in NO binding affinity (12). The mechanism for this is not yet clear, but it appears to involve a change in distal pocket polarity. Interestingly, activation of the soluble guanylate cyclase catalytic domain is also thought to occur through a NO-induced conformational change, in this case through the Fe(II) heme center in the regulatory domain of the protein (13). The different NP forms provide naturally occurring variants that differ in the kinetics and thermodynamics of ligand binding and therefore provide clues to the structural basis for NO-induced conformational changes (11). NP2 has the highest affinity for NO, so structural comparisons with NP4, which binds NO less strongly, are of particular interest. The NP-N0 complexes are also resistant to the autoreductive reactions that are seen with NO complexes of Fe(III) globins (11, 14). The structural basis for stability of the Fe(III) form of the protein is not yet known.

NP2 has special importance in being the only anticoagulant among the nitrophorins. The protein acts by inhibiting the intrinsic factor Xase complex, and its activity is independent of the heme moiety (8). Zhang et al. (9) showed NP2 to be a hyperbolic mixed-type inhibitor that inhibits factor IXa-catalyzed cleavage of factor X in the presence of factor VIIIa or phospholipid or both. However, it has no effect against the basal proteolytic activity of factor IXa in the absence of both

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The atomic coordinates and structure factors (code 1EUO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ Current Address: NIAID, National Institutes of Health, Bldg. 4, 4 Center Dr. MSC 0425, Bethesda, MD 20892.

‡ To whom correspondence should be addressed: BSW 518, Dept. of Biochemistry, University of Arizona, Tucson, AZ 85721. E-mail: montfort@u.arizona.edu.

1 The abbreviations used are: NP, nitrophorin; RMSD, root mean square deviation.
factor VIIa and phospholipids, suggesting that complex formation is 
interfered with. NP2 also appears to bind more tightly with 
the enzyme-substrate complex than the substrate-free 
complex (9). These observations suggest that NP2 inhibits this 
specific conformation of factor IXa found in the factor 
Xase complex. Surface plasmon resonance analyses performed 
by Isawa et al. (15) showed specific binding of NP2 with factors 
IX and IXa and also showed that NP2 inhibits assembly of the 
factor Xase complex. Additionally, these studies revealed a 
previously uncharacterized inhibition of the factor VIIa-tissue 
factor complex by NP2, suggesting that NP2 also may inhibit 
activation of factor X via the extrinsic pathway.

The NPs have been placed in the lipocalin protein family 
based on the structures of NP1 and NP4 (16, 17). The NP 
structure is comprised of an eight-stranded anti-parallel β-bar 
reconstituting a large ligand-binding cavity that contains a 
single ferriheme molecule. The heme is bound to the protein 
through proximal coordination of a histidine side chain with 
the iron atom. A single NO molecule is coordinated with the 
ligand is replaced by water after release (12). Histamine also 
occupies the distal pocket, to the exclusion of NO, and is bound 
with the enzyme-substrate complex than the substrate-free 
solution. Both models gave the same solution to the rotation 
factor, but the correlation coefficients were low. Different translation 
solutions were obtained with the two models, and the solution obtained 
with the NP4 model was rejected on the basis of an unlikely crystal 
packing arrangement. A number of solutions having similar magnitudes 
of the translation function were obtained with the NP1 model, 
and the top solution (function value = 0.24) was subjected to rigid body 
refinement using data from 8.0–3.0 Å, resulting in an R factor of 0.54. 
A model of NP2 was constructed based on the NP1 structure in which 
side chains conserved between NP1 and NP2 were included, but non-
conserved residues other than glycine were modeled as alanine. 
This model was refined by simulated annealing using XPLOR and CNS (24, 
25), with data from 9.0 to 3.0 Å included, and the 
R factor dropped to 0.39. Additional cycles of simulated annealing and manual rebuilding 
were performed using 2Fo – Fe, Fo – Fe, and annealed Fo – Fe omit maps. 
Density for side chains that differed between NP1 and NP2 was 
observed, indicating that the molecular replacement solution was correct. 
The side chain of Ile on the distal heme pocket was not included in the 
model until the structure was near completion, in order to independ-
ently monitor the quality of electron density as the resolution was 
increased, indicating that the molecular replacement solution was correct. 
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**Experimental Procedures**

**Protein Preparation**—NP1–NP4 were prepared as described previ-
ously (11, 17, 18). Briefly, each of the four cDNAs was modified by 
polymerase chain reaction to remove the sequence encoding the signal 
peptide. In each case, an ATG codon was added immediately 5′ of the 
coding sequence of the mature protein in order to initiate translation. 
The modified cDNAs were cloned into the expression vector pET17b and 
expressed in the strain BL21(DE3). Inclusion bodies obtained from 
these cultures were denatured, refolded, and reconstituted with heme 
as described previously (11, 17, 18). Recombinant NP2 was then puri-
ified by chromatography on Q-Sepharose (Amersham Pharmacia Bio-
tech) and Sephacyr S-200 (Amersham Pharmacia Biotech).

**Anticoagulation Assay**—Each of the four NPs was assayed using the 
activated partial thromboplastin time assay. The activated partial 
thromboplastin time reagent was obtained from Sigma and contains 
rabbit brain cephalin in a buffered 0.1 M solution of elagic acid. NPs 
at various concentrations in either 100 mM sodium phosphate, pH 7.5, 
or water were added to human normal coagulation control serum (Sig-
mac). After incubation for 1 min at 37 °C, the activated partial throm-
boplastin time reagent was added to the serum-NP mixture and incubated 
at 30 °C for 3 min. Coagulation was initiated by adding 0.1 ml of 20 mM 
CaCl2. The formation of clots was determined visually.

**Crystalization and Data Collection**—Crystals were obtained by 
the hanging drop vapor diffusion method using 2.8 M ammonium phos-
phate, 0.1 M Tris-HCl, pH 7.7, as precipitant. Initially, small plate-like 
crystals were obtained using 2.8 M ammonium phosphate. These were 
then introduced by macroseeding into drops equilibrated with 2.6 M 
ammonium phosphate, 0.1 M Tris-HCl, pH 7.7. The plates grew slowly, 
eventually reaching a size of 0.8 × 0.2 × 0.04 mm, and diffracted to 2.0 Å nominal resolution.

Data were collected at room temperature using a FAST area detector 
and an Enraf-Nius rotating anode generator operated at 40 kV and 95 
mA. Images were collected and reflections were integrated and indexed using 
MADNES (19). The data were reduced using PROCOR (20) fol-
lowed by SCALA (21). The crystals were found to be orthorhombic with 
unit cell dimensions of a = 40.3 Å, b = 128.0 Å, and c = 33.7 Å. The pattern of systematic absences placed the crystal in the space group 
P2_1_2_1, with one NP2 molecule in the asymmetric unit (Table I).

Molecular Replacement—Molecular replacement was performed using 
xplor (22, 23). Two search models were employed: a model of NP4 
with protein side chains differing between NP4 and NP2 changed to 
alanine and containing heme, and a polyalanine model of NP1 with 
heme removed. Rotation solutions were subjected to Patterson correla-
tion refinement, and the best solutions were entered into the transla-
tion search. Both models gave the same solution to the rotation 
factor, but the correlation coefficients were low. Different translation 
solutions were obtained with the two models, and the solution obtained 
with the NP4 model was rejected on the basis of an unlikely crystal 
packing arrangement. A number of solutions having similar magnitudes 
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conserved residues other than glycine were modeled as alanine. 
This model was refined by simulated annealing using XPLOR and CNS (24, 
25), with data from 9.0 to 3.0 Å included, and the 
R factor dropped to 0.39. Additional cycles of simulated annealing and manual rebuilding 
were performed using 2F_0 – F_1, F_1 – F_2, and annealed F_0 – F_1 
omit maps. Density for side chains that differed between NP1 and NP2 was 
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**FIG. 1. Sequence comparison of NP 1–4.** The alignment was obtained using the PILEUP program of the GCG package (Genetics Computer 
Group, Madison, WI) and verified by structural superpositions of NP1, NP2, and NP4. The structural core used for superposition of NP1 and NP4 is 
indicated by asterisks above the alignment and consists of residues 21–30 (β-strand A), 39–46 (strand B), 52–59 (strand C), 65–71 (strand D), 
80–89 (strand E), 102–123 (strands F and G), and 129–140 (strand H). Numbering at the top is for NP2 and NP3, and numbering at the bottom is 
for NP1 and NP4.
RESULTS

The Structure of NP2—The NP2 structure was determined by molecular replacement using a polyalanine model of NP1 as a starting point. The final model displayed good refinement statistics (Table I) and exhibited good electron density for all but two of the 180 residues in the final model (Fig. 2). Like NP1 and NP4, NP2 has a lipocalin fold consisting of an eight-stranded antiparallel β-barrel containing a central ligand-binding cavity (Fig. 3). At the C-terminal end of the barrel, two α-helices are present, and a disulfide bond tethers the C terminus to the β-barrel. The N-terminal portion of the protein is also disulfide bonded to the barrel, and contains a single turn of α-helix prior to the first strand of the β-sheet. For expression in *Escherichia coli*, methionine was added to the N terminus of the protein, and this residue was found to be well ordered in the crystal and stabilized through interaction with an adjacent NP2 molecule. An N-terminal methionine was not visible in either the NP1 or NP4 crystal structures (16, 17), although Edman degradation indicated that it was present in NP1 but not in NP4 (17, 18).

A heme ligand is contained within the central cavity of NP2 and is bonded to the protein via coordination with the imidazole portion of His57 on the proximal side of the heme (Fig. 4). A distal ligand is also evident and is considered to be an ammonia molecule, based on previous results with NP1 and NP4 (16, 17). The Ca positions of the β-barrel are very similar in NP4 and NP2. When a core is defined containing residues from each of the eight strands of the barrel (Figs. 1 and 3), the RMSD for NP2 when a core is defined containing residues from each of the eight strands of the barrel (Figs. 1 and 3), the RMSD for NP2 (12, 16, 17).

| Data measurement and refinement statistics for NP2 |
|---------------------------------------------------|
| Resolution range (Å) | 9.2–2.0 |
| Total observations | 49,410 |
| Unique observations | 12,186 |
| Multiplicity | 4.2 (3.2) |
| \(R_{\text{free}}\) (%) | 10.6 (34.8) |
| \(I/\sigma(I)\) | 4.5 (1.9) |
| Structure refinement |
| \(R_{\text{crv1}}\) | 0.19 |
| Most favorable \(\phi/\psi\) (%) | 91.4 |
| allowed \(\phi/\psi\) (%) | 100 |
| RMSD bond lengths (Å) | 0.006 |
| RMSD bond angles (°) | 1.27 |
| Average B-factor |
| Main chain | 25.2 |
| Side chains | 27.8 |
| Heme | 22.3 |
| Solvent | 36.4 |

Values in parentheses are for the highest resolution shell: 2.1–2.0 Å.

| Table I |
|---------------------------------------------------|
| Data measurement |
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| Total observations | 49,410 |
| Unique observations | 12,186 |
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The loops surrounding the heme-binding pocket show a variety of conformations in the NP structures determined to date (16, 17). Unlike the case with NP4, the large α loop that lies between strands A and B of the β-barrel (Fig. 3, loop A-B) is well ordered in the NP2 crystals. This is probably due to stabilization of the region through contacts with an adjacent protein molecule. In previous studies with NP4, loops A-B and G-H were found to undergo a major conformational change on NO binding that buries the NO ligand within the distal pocket (12). This conformational change appears to be related to stabilization of the NO complex and to the biphasic association kinetics observed in ligand binding experiments with NO. In NP2 the position of loop A-B is similar to the “open” NO-free conformation seen in NP1 and NP4. As with other NPs, loop G-H (residues 123–128) shows disorder in the NP2 structure that is consistent with NO-induced movement, as previously observed with NP4 (12, 16, 17).

Heme Binding Environment—The heme moiety of NP4 has orientational disorder in that half of the hemes are “right-side-up,” and half are “upside-down” (12). With NP2, omit electron density maps calculated after simulated annealing of a model from which the vinyl methylene groups were removed show strong density for one heme orientation and no density for the other. This indicates that, unlike NP4, NP2 has a single heme orientation. The heme conformation in NP2, like that for NP1 and NP4, is severely distorted from planar. This distortion arises in large part from a rotation of the pyrrole rings about their Fe-N bonds, referred to as heme “ruffling,” which may serve to stabilize the ferric iron center with respect to reduction (30). The NP2 pyrroles are rotated to about the same or to a slightly greater degree than those of NP4 (12).

FIG. 3. Ribbon diagram of the NP2 structure. The eight strands of the β-barrel are labeled A–H, and the heme, bound in the central cavity of the protein, is shown in black.

FIG. 2. Representative 2Fo - Fc electron density. Shown are portions of β-strands A and H.
The distal pocket ligand-binding environment of NP2 is quite similar to that found in NP1 and NP4 (16, 17) (Fig. 4). The side chains of Leu122, Leu129, and Leu132 occupy positions similar to those of the corresponding residues in the previously determined NP structures. These hydrophobic residues surround the NO ligand in the NP4-NO complex (12) and sandwich the imidazole ring in the NP1-histamine complex (16). Also, the residues interacting with the alkylamino group of histamine are in similar positions, consistent with the near identity of kinetic and thermodynamic values for histamine binding to the three proteins. However, replacement of Thr121 of NP1 and NP4 with isoleucine (Ile120) adds a bulkier nonpolar group to the NP2 distal pocket that increases its hydrophobicity (Fig. 4). The conformational change associated with binding of NO appears to be mediated by hydrophobic interactions, because no hydrogen bonds are formed with the ligand. An increase in the hydrophobicity of the pocket may in part be responsible for the at least 10-fold greater affinity for NO in NP2 when compared with NP1 and NP4 (11). In NP4, the side chain of Lys125 projects between the propionates and forms electrostatic interactions with both groups. This interaction places one of the propionates on the distal side of the heme plane, which may restrict access to the distal pocket. In NP2, Glu124 (corresponding to Lys125 in NP4) does not interact with the propionate groups. Rather, the hydroxyl group of the conserved residue Tyr38 (corresponding to Tyr40 in NP4) forms a hydrogen bond with the propionate carboxyl, causing it to lie on the proximal side of the heme plane (Fig. 4). This increases the size of the observed solvent channel leading to the distal ligand-binding site relative to the NP1 and NP4 models. The second heme propionate group is stabilized by hydrogen bonding with the hydroxyl group of Tyr85 through an intervening water molecule (Fig. 4). An interaction of this type does not occur in NP1 or NP4, in which Tyr85 is replaced by phenylala-
nine, but the propionate group lies in a similar position in all three proteins.

Environment of Glu\textsuperscript{53}—Of particular interest in studies of NP function are the pH-dependent changes in NO affinity that occur in all of the NPs. This change in affinity is due to a reduction in the NO release rate at low pH and appears to be a mechanism for controlling the release and binding of NO in the insect (\textit{\textit{pH}} 6.0 (31)) and host (\textit{\textit{pH}} 7.4). Evidence for pH-dependent structural changes in the region of Glu\textsuperscript{53} (Glu\textsuperscript{55} in NP1 and NP4), near the back of the heme-binding pocket, were found in structures of NP4 obtained at pH 5.6 and 7.5 (12, 17) (Fig. 6). At the higher pH, 2–3 water molecules appear near the Glu\textsuperscript{55} carboxyl group, along with some rearrangement of side chains in that vicinity, suggesting that the Glu 55 carboxyl becomes deprotonated.

In the NP2 crystal obtained at pH 7.8, a different arrangement is seen (Fig. 6). Like NP1 and NP4, the carboxyl group of Glu\textsuperscript{53} lies approximately on the heme plane and is located 3.7 Å from the external methylene group of the B-ring vinyl. No water is present around Glu\textsuperscript{53}, but the side chain of Tyr\textsuperscript{81} is rotated into the protein interior, with respect to its position in NP4, where it forms hydrogen bonds with Glu\textsuperscript{53} and Tyr\textsuperscript{17} (Fig. 6A). Tyr\textsuperscript{81} functionally replaces the most ordered of the water molecules seen in NP4, in that the carboxyl group of Glu\textsuperscript{53} now forms a hydrogen bond with Tyr\textsuperscript{81} (2.7 Å) rather than water. Tyr\textsuperscript{81} is conserved in all four NPs (Fig. 1), so it was quite surprising to find it on the protein exterior in NP1 and NP4 and on the protein interior in NP2, where it takes on an entirely new role. Other changes in the vicinity of Glu\textsuperscript{53} include the exchange of Phe\textsuperscript{107} in NP4 for Leu\textsuperscript{106}, with the side chain of the latter occupying a position similar to Phe\textsuperscript{107} in NP4 at pH 5.6 (Fig. 6).

\textit{The Anticoagulation Activity of NP2—}The inhibitory activity of recombinant NP1–NP4 toward the intrinsic coagulation pathway was tested using the activated partial thromboplastin time assay. NP2 was clearly the most active of the four (Fig. 7), consistent with the results of Ribeiro \textit{et al.} (8) and Sun \textit{et al.} (7). When the results for NP2 were fit to a rectangular hyperbola, the IC\textsubscript{50} was estimated to be 1.9 \textmu M (Fig. 7). NP3 showed weak, possibly nonspecific, inhibitory activity and was approximately 15 times less potent than NP2. NP1 and NP4 showed no detectable activity at concentrations up to 20 \textmu M, clearly indicating that they do not interact with the intrinsic factor Xase complex in an inhibitory manner (Fig. 7). Apparently, NP3 does interact with the complex, but more weakly than NP2 (Fig. 7). This weak activity had not been noted previously but is not surprising given the high degree of amino acid sequence similarity between NP2 and NP3.

\textbf{DISCUSSION}

We have determined the 2.0 Å structure of NP2 and assessed the ability of all four \textit{Rhodnius} nitrophorins to inhibit blood coagulation. The general fold of NP2 is similar to those of NP1 and NP4, but a number of differences in the ligand-binding region and on the surface of the molecule are present. These differences are likely to account for the greater affinity of NO for NP2 and for the anticoagulatory activities found only in NP2. Below, we discuss the structural details likely to account for the unique NP2 functional properties.

\textit{Increased NO Affinity for NP2—}We have recently completed detailed kinetic and equilibria studies of ligand binding and release by all four nitrophorins (11). Histamine was found to bind with similar affinity to all four nitrophorins, but NO binding differed in that NP1 and NP4 bound less tightly to NO than NP2 and NP3. These differences in binding correlate with the differences in sequence identity among the four proteins.
NP1 and NP4 are 90% identical, and NP2 and NP3 are 80% identical, but overall, the four proteins display only 38% identity (Fig. 1). Both binding and release of NO was found to be multiphasic in all of the nitrophorins, consistent with Scheme 1, in which NP-NO represents an initial, quickly formed complex, and NP*-NO represents a more slowly formed, stabilized complex (11, 32). The crystal structure of NP4-NO (12) displays an extensive NO-induced conformational change that probably represents the NP*-NO complex in Scheme 1 and, based on kinetic analyses, apparently occurs in all four nitrophorins.

Kinetic and thermodynamic constants for NO binding to NP2 and NP4, representing the NP1/NP4 and NP2/NP3 pairs, are shown in Table II, as are the values for the ferric forms of elephant myoglobin, sperm whale myoglobin, cytochrome c, and catalase (33, 34). These proteins use the same heme but have very different protein environments. A comparison of NO association rates among these proteins is instructive because the rates are limited by access to the sixth heme ligation site (Fig. 1). Both binding and release of NO was found to be

\[
\text{NP} + \text{NO} \overset{k_1}{\longrightarrow} \text{NP} - \text{NO} \overset{k_2}{\longrightarrow} \text{NP}^* - \text{NO}
\]

**Scheme 1**

Comparison of dissociation rate constants among these proteins is more complicated, because thermal cleavage of the NO-heme bond is a rate-limiting factor. However, here again, the \(k_{-2}\) value for NP2 is about 5–15-fold larger than that for NP4. Examination of the equilibrium dissociation constants for the proteins (\(K_d\)) and comparing them with the value calculated from the association and dissociation rate constants for the first binding step (\(K_{1}\)) reveals the presence of additional NO binding steps in the nitrophorins, as indicated in Scheme 1, but not in the other proteins included in Table II. The overall binding affinity of NO for NP2 is considerably higher than for NP4, apparently due to factors influencing \(k_1\) and \(k_{-2}\), because their \(K_{-1}\) values are quite similar (Table II).

The structural basis for the tighter NO binding in the nitrophorins is apparently linked to the NO-induced conformational change revealed in the NP4-NO complex (12). For example, mutations to Asp 30 in NP4, which becomes buried in the closed conformation, lead to larger release rates. \(^2\) However, the mechanism by which this conformational change is induced and the means by which it restricts NO release are not clear. The key to this may involve a change in polarity in the distal pocket. On binding NO, nonpolar amino acids pack into the distal pocket, and at least five water molecules are squeezed out, leading to a more hydrophobic distal pocket. Although 13 new hydrogen bonds are formed in the closed conformation and 9 are lost, the only direct contact between the protein and NO is van der Waals contacts. Thus, the driving force for pocket closure may be the nonpolar nature of NO, which is 70 times more soluble in n-hexane than in water (37, 38).

The NP2 and NP4 structures are consistent with the hypothesis that solvent reorganization is rate-limiting for NO binding and release. NP2 is in the open conformation in the present structure but has fewer ordered solvent molecules in the distal pocket, due in part to the placement of both heme propionate groups below the heme and a change of Thr\(^{121}\) in NP4 for Ile\(^{120}\) in NP2 (Fig. 4). The larger, more hydrophobic NP2 distal pocket may facilitate the exiting of water molecules as NO binds, leading to the larger bimolecular rate constant found in comparison to NP4. Likewise, the more hydrophobic NP2 pocket may restrict the solvent reentry that accompanies NO release, leading to the smaller NO release rates. Other possible limiting factors, such as the formation of stronger than usual Fe-NO bonds in the closed conformation, have not yet been ruled out. However, the NO stretching frequency in NP1-N0 was found to be typical for ferric Fe-NO complexes (14), and the NP heme reduction potential is relatively pH-independent despite the increased affinity for NO exhibited at lower pH (11). Taken together, these factors suggest that there is nothing unusual about the NP-N0 iron bond and that it does not change as the distal pocket polarity is altered. Comparative studies directly assessing the Fe-NO bond strength in all four nitrophorins are needed to address whether the proteins differ in intrinsic ligand bond strength.

**Multiple Environments of Invariant Glu\(^{53}\) and Its Role in pH-dependent NO Release**—The pH variance in NO binding affinity is largely due to changes in \(k_{-2}\) (11) (Table II). This step also displays a biphasic behavior in its own right (11), which is not well understood at present and is not indicated in Scheme 1. The mechanism by which pH changes lead to altered NO release rates is not yet clear but appears to involve a buried carboxylate. The only clearly demonstrated pH-dependent structural changes in the NPs involve the region surrounding Glu\(^{55}\) of NP4 (12). At pH 7.5, the side chain oxygens of Glu\(^{55}\)...
Loop B-C (residues 47–51 in NP2 and 48–52 in NP4) is highlighted in yellow. The remaining atoms are shown in green cyan (residues 174–179 in NP2 and 175–184 in NP4) is highlighted in release rate, although some variance with pH remains. This release rates at all pHs that are similar to the wild type low pH (pH 5), where the value is \( k \). These hydrogen bonds are of likely importance. NP2 was found by Zhang et al. (9) to superimpose on the two water molecules near Glu55 in the NP4 structure determined at pH 5.6, these water molecules are gone, and Phe107 and Ser72 rotate to fill the space previously occupied by the waters. These structural changes appear to be driven by the need to solvate the deprotonated and charged Glu55 side chain at higher pH. Mutation of Glu55 to Gln in NP4 results in a protein with same purpose: solvation of the deprotonated carboxylate of the buried glutamate side chain at higher pH values. This result is clearly plays a different role in NP2 than in NP4.

Anticoagulation Properties of NP2—Potent anticoagulation activity is a property of NP2, but not NP1, NP3, or NP4 (8). When heme is removed from the protein by extraction, the activity remains. Because the anticoagulation activity of NP2 is almost certainly due to surface features of the protein, the significant topological differences between NP2 and NP1/NP4 are of likely importance. NP2 was found by Zhang et al. (9) to be hyperbolic mixed-type mechanism inhibit activation of factor X by factor IXa within a. Maximal inhibition by NP2 requires both factor VIIIa and phospholipids, although inhibition is detectable with either phospholipid or factor VIIIa in the presence of factor IXa. In the absence of both factor VIIIa and phospholipids, NP2 has little inhibitory activity against the basal level of amidolytic activity displayed by factor IXa (15). The kinetics of inhibition were characterized as hyperbolic-mixed type, whereby the inhibitor binds more tightly to the enzyme-substrate complex than to the substrate-free complex (9). This results in a decrease in both the apparent \( K_m \) and \( k_{cat} \) for factor X activation when compared with the uninhibited reaction. These studies suggest that NP2 interacts with a conformation of factor IXa that is stabilized in the factor IXa-membrane surface-factor VIIIa complex and that NP2 interacts more favorably with factor IXa when factor X is bound. Tighter binding of NP2 with the enzyme-substrate complex could be due to substrate- or surface-induced conformational effects or to direct

| Protein | pH | \( k_1 \) | \( k_{-1} \) | \( k_2 \) | \( k_{-2} \) | \( K_{1a} \) | \( K_d \) |
|---------|----|-----------|-----------|-----------|-----------|-------------|------------|
| NP4     | 5  | 2.1       | 3.4       | NM        | 0.04      | 1.6         | 0.05       |
| NP2     | 5  | 22        | 52        | ~51       | 0.02      | 2.4         | ~0.001     |
| NP4     | 8  | 2.3       | 6.3       | NM        | 1.4       | 2.7         | 0.54       |
| NP2     | 8  | 33        | 32        | ~42       | 0.12      | 1.0         | 0.02       |
| MbIII (el) | 7 | 22        | 74        | NA        | 1.8       | NA          | NA         |
| MbIII (sw) | 7 | 0.19      | 71        | NA        | 61        | 62          | NA         |
| CytIII  | ~7 | 0.0072    | 0.044     | NA        | NA        | 5.7         | 5.5        |
| CatIII  | ~7 | 30        | 170       | NA        | NA        | NA          | NA         |

*The values listed for \( K_{1a} \) are the ratios \( k_{-1}/k_1 \), whereas the values listed for \( K_d \) are measured equilibrium dissociation constants, except for NP2 (pH 5), where the value is \( k_{cat}/k_{on} \).

Values for NP2 and NP4 are taken from Ref. 11. The values listed for \( k_{-2} \) are the weighted averages of the reported biphasic rate constants. Reliable values for \( k_2 \) could not be measured (NM), and they could only be estimated for NP2. NA, not applicable.

Kinetic values for ferric elephant (el) myoglobin were taken from Ref. 33.

Values for the ferric forms of sperm whale (sw) myoglobin (Mb III), horse heart cytochrome c (Cyt III), and bovine liver catalase (Cat III) are taken from Ref. 34.

**Fig. 8. Comparison of NP2 and NP4 molecular surfaces.** Shown are NP2 (A) and NP4 (B) in a space-filling representation. The C terminus (residues 174–179 in NP2 and 175–184 in NP4) is highlighted in cyan. Loop E-F (residues 91–97 in NP2 and 92–98 in NP4) is highlighted in red. Loop B-C (residues 47–51 in NP2 and 48–52 in NP4) is highlighted in yellow. The remaining atoms are shown in green.
interaction of NP2 with the bound substrate. It appears that NP2 acts by interfering with assembly of the factor Xase complex by binding with factor IXa (9). Surface plasmon resonance results support this interpretation by showing that NP2 binds directly with factor IXa in the presence of calcium ions but not with free factor VIIIa (15). It was also shown that NP2 inhibits interaction of factor IXa with the phospholipid membrane (15).

NP2 and NP3 differ at only 37 of 180 amino acid positions (Fig. 1), but NP2 is much more potent as an anticoagulant. The amino acid nonidentities between NP2 and NP3 map to the surface of the NP2 structure but are not generally clustered, making it difficult to suggest that any particular area is involved in the quantitative difference in potency between NP2 and NP3.

Structural comparisons between NP2 and the inactive NP1 and NP4 show major differences in the molecular surface. In the NP2 structure, the C terminus contains four fewer residues than NP1 or NP4, resulting in the exposure of a face of the β-barrel containing portions of strands B, C, D, and E to the solvent (Fig. 8). Additionally, the conformation of loop B–C, which differs by nearly 10 Å at its apex between NP2 and NP4, results in more exposure of the surface of the β-barrel than in NP1 and NP4. On the boundary of the exposed surface of the β-barrel lies the large loop E–F, which forms a ridge with a number of prominently exposed amino acid side chains. This region contains a stretch of six consecutive amino acids (residues 93–98, Fig. 1) that differ between the highly active anticoagulant NP2 and the less active NP3 (Fig. 8). The highly different surfaces that NP2 and NP4 would present to the factor Xase complex are illustrated in Fig. 8, and experiments to determine whether this region is important for the anticoagulation function of NP2 are under way.

Biological Implications—The work presented here and elsewhere demonstrates that R. prolixus has adapted a common lipocalin fold to perform several functions to assist in the process of blood feeding. The proteins seem to be most similar to pigment proteins found in other insects, suggesting that R. prolixus NP5s may be derived from proteins such as these. Because blood feeding has evolved numerous times in insects, it is expected that the recruitment of existing protein folds to perform novel functions in blood feeding will be encountered frequently. Already, an NO transport protein that is functionally very similar to the NPs has been reported from R. prolixus (39). Considering sequence comparisons show that this protein bears no evolutionary relationship to the R. prolixus NPs. Also, a histamine-binding protein from the saliva of the tick Rhhipicephalus appendiculatus has been characterized and found to be a lipocalin that does not contain heme and has little sequence homology to the NPs (41).

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