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Overexpression in *Escherichia coli* and functional reconstitution of the liposome binding ferriheme protein nitrophorin 7 (NP7) from the blood sucking bug *Rhodnius prolixus*

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Abstract

A number of ferriheme proteins, termed nitrophorins (NPs), occur in the saliva of the bloodsucking insect *Rhodnius prolixus* ('kissing bug'), which is a vector for Chagas' disease. Nitrophorins bind the heme *b* cofactor in the β -barrel of their lipocalin fold, which is further anchored through a proximal histidine–Fe^{III} bond. The distal Fe^{III} coordination site then binds nitric oxide (NO) for delivery into a host's tissues during blood feeding, where, upon NO release, the distal Fe^{III} site acts as a histamine trap to delays the victim's immune response. Previously, four nitrophorins from *Rhodnius prolixus*, NP1 to NP4, have been extensively characterized. Recently, another nitrophorin, NP7, was discovered in a cDNA library derived from the same insect. Among the *R. prolixus* nitrophorins, NP7 was found to be unique in its ability to bind to negatively charged cell surfaces. However, the yield of functional recombinant NP7 was rather low when the established protocol for NP1–4 was followed. Here, we report on a novel expression and reconstitution method for NP7 that yields sufficient amounts of pure protein for extensive characterization (28-fold increase). This method may prove useful for the reconstitution of other proteins with a lipocalin fold.

Keywords

bloodsucking insects; heme protein reconstitution; lipocalin refolding; liposome-protein interaction; nitrophorin; phosphatidylserine; *Rhodnius prolixus*

Introduction

The nitrophorins (NPs)¹ are a group of nitric oxide (NO)-carrying heme proteins found in the saliva of bloodsucking insects. One such insect is *Rhodnius prolixus* (the 'kissing bug'), which in its adult phase has at least four salivary NPs, designated NP1–4, in order of their abundances

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¹*Abbreviations used:* cyt *c*, cytochrome *c*; DEA/NO, diethylammonium (Z)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate; DTT, 1,4-dithio-D,L-threitol; GdmCl, guanidinium chloride; GSSG, oxidized glutathione; Hm, histamine; ICP, inductively coupled plasma; IPTG, isopropyl β -D-1-thiogalactopyranoside; LB broth, Luria-Bertani broth; LUV, large unilamellar vesicles; lysoPC, L- α -lysophosphatidylcholine; MALDI, matrix assisted laser desorption ionization; Mops, 3-(*N*-morpholino)propanesulfonic acid; NOS, nitric oxide synthase; NP, nitrophorin; OES, optical emission spectrometry; PA, L- α -phosphatidic acid; PC, L- α -phosphatidylcholine; PE, L- α -phosphatidylethanolamine; PG, L- α -phosphatidyl-D,L-glycerol; PI, L- α -phosphatidylinositol; PS, L- α -phosphatidyl-L-serine; SM, sphingomyelin; TCEP, tris(2-carboxyethyl)phosphine-HCl; Tricine, *N*-tris(hydroxymethyl)methylglycine; TOF, time-of-flight.

in the insect saliva [1]. Two additional nitrophorins, designated NP5 and NP6, have been mainly found in the five instar nymphal stages of the insect development [2]. Another nitrophorin (NP7) has recently been discovered from a cDNA library created from salivary glands of Vth instar nymphs [3]. These interesting heme proteins sequester NO, produced by a nitric oxide synthase (NOS) present in the salivary gland epithelium [4], keeping it stable for long periods of time in the acidic saliva (pH 5 – 6) [5] by binding it as an axial ligand to a ferriheme center [6]. As depicted in Fig. 1a, upon injection into the tissues of the insect's victim, NO dissociates and diffuses through the tissues to the nearby capillaries [5] to cause vasodilatation and thereby allow more blood to be transported to the site of the wound. At the same time, histamine (Hm), whose role is to cause swelling, itching, and the beginning of the immune response, is released by mast cells and platelets of the victim [7]. Hm binds to the distal heme sites of NPs, hence preventing the insect's detection during feeding [8]. These two properties of the NPs of *R. prolixus* contribute to the transmission of the protozoan *Trypanosoma cruzi*, the vector of Chagas' disease [9], to the victim via the feces of the insect, left behind at the site of the bite [1,9]. Chagas' disease is a serious health problem in tropical Latin America [9], and both the insect and the disease have now reached the United States [10].

NP1-4, have been investigated by a number of spectroscopic techniques, spectroelectrochemistry, and stopped-flow kinetics, and the solid-state structures of one or more ligand complexes of NP1, NP2, and NP4 have been determined by X-ray crystallography [11,12]. All structures are very similar and the structure of NP2 is presented as an example in Fig. 1b. The structures are unique for heme proteins, in that the heme *b* is located at the open end of a β -barrel [12], rather than in the more commonly observed largely α -helical globin or four-helix bundle folds. The nitrophorins belong to the lipocalin fold, a family of proteins with very low sequence identity. The lipocalins consist of an eight-stranded antiparallel β -barrel forming a central hydrophobic cavity [13]. The loops connecting the strands of the β -barrel and forming the entrance to the ligand binding cavity are flexible. Lipocalins are found in all types of living organisms and serve a variety of functions from hormone and lipid transport to coloration [13]. It is typical for them to present a number of disulfide forming Cys residues (Fig. 1b). Normally, lipocalins act by binding a small-molecule ligand, but they can also act by binding proteins in solution or receptors [14]. In the case of the nitrophorins the ferriheme prosthetic group is bound to the protein via a His ligand, leaving the sixth coordination site available to bind NO or other ligands.

Of all the *R. prolixus* NPs discovered, NP7 is especially interesting since it was found to bind to L- α -phosphatidyl-L-serine (PS)-bearing phospholipid membranes, which NP1-4 cannot do [3,15]. In platelets and mast cells, the loss of membrane asymmetry, which leads to the display of PS on the outer surface, is rapid and tightly coupled to other activation events [16]. NP7 would thus be attracted to the negatively-charged surface of the activated platelets, while NP1-4 would remain in solution, diffusing away from the feeding site while releasing NO over a larger area. Besides its action as an NO delivery system, NP2 can also inhibit the intrinsic factor Xase complex, through binding with factor IX and IXa. NP7, however, was shown to inhibit prothrombinase activity through interaction with the prothrombinase activating PS [3]. The binding to PS is accomplished through charge $\leftrightarrow$ charge interactions with the positively charged NP7 surface on the protein surface opposite to the heme-binding site.

Once bound on an activated platelet, NP7 can release NO to inhibit platelet aggregation and act as an anticoagulant by blocking coagulation-factor binding sites. NO is highly reactive ($t_{1/2} < 1$ s in a biological environment), but is protected from oxidation when bound to the heme iron of the NPs [17]. Targeted delivery to activated surfaces at the point of feeding may enhance the activity of NP7 as a platelet aggregation inhibitor by delivering NO in a protected form to its site of action and preventing its removal from the feeding area by diffusion and blood flow.

The amino acid sequence identities between NP7 and the other *R. prolixus* nitrophorins amount to 41% (NP1), 60% (NP2), 61% (NP3), and 42% (NP4), respectively. In Fig. 1c the amino acid sequence alignment between NP2 and NP7 is presented, which shows how accurately the sequences align; thus, a very similar protein fold is expected. Although NP1-4 have been extensively studied and are structurally well-characterized, it remains still a matter of debate why *R. prolixus* uses a whole bundle of NPs rather than just one [15], as seems to be the case with *Cimex lectularius* (the bedbug) [18]. In addition, the six life stages of *R. prolixus* (five instar nymphs and the adult stage) use different expression patterns of NP1-NP6 [2], but it is not clear why. The detailed understanding of the similarities and differences of the isoforms on the molecular level is a prerequisite to answering these questions. Therefore, we initiated the careful characterization of the specific chemical and physical properties of NP7. Here we report on a novel method for producing sufficient amounts of NP7 necessary for the successful accomplishment of the project.

Materials and methods

Materials

1- α -phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine, PC) from egg yolk, 1- α -phosphatidyl-L-serine (1,2-diacyl-*sn*-glycero-3-phospho-L-serine; PS) from bovine brain, 1- α -phosphatidylinositol (1,2-diacyl-*sn*-glycero-3-phospho-(1-D-*myo*-inositol); PI) from soybean, 1- α -phosphatidic acid (sodium 3-*sn*-phosphatide; PA) from egg yolk, 1- α -phosphatidyl-D,L-glycerol (ammonium salt of 1-(3-*sn*-phosphatidyl)-*rac*-glycerol; PG) from egg yolk, 1- α -lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphocholine; lysoPC) from egg yolk, sphingomyelin (*N*-acyl-D-sphingosine-1-phosphocholine; SM) from bovine brain, and cardiolipin (diphosphatidylglycerol) from bovine heart were purchased from Sigma. All other chemicals were of the highest grade available. Recombinant NP1-4 from *R. prolixus* were expressed and purified as was previously described [19].

Preparation of the expression plasmid

The cDNA for NP7 (GenBank accession no. **AY585746**) subcloned into pET-17b (Novagen) was a generous gift of Drs. J. F. Andersen and J. M. C. Ribeiro (NIH, Maryland) [3]. Standard PCR was applied using the primer pair 5'-cat gcc ATG GAA TGT AGC GTA AAC GTA ATT-3' and 3'-AGA AGC ACC GTT TTT ATC ATT gag ctc gcc-5' (coding residues in upper case) to amplify the gene leaving out the first three amino acids and generating *Nco* I (5' end) and *Xho* I (3' end) restriction sites (underlined). The cDNA was ligated into pET-28a(+) (Novagen) and the plasmid designated pNP7 Δ 1-3^{Kan}. The characterization of this mutant is currently in progress. To get *wt* NP7, pNP7 Δ 1-3^{Kan} was amplified with the primer pair 5'-tta aga agg aga tat agc ATG CTG CCT GGT GAG TGT AGC GTA-3' and 3'-TAC GCT ACA CTC ACC AGG CAG CAT gct ata tct cct tct taa-5' to insert the first three amino acids LeuProGly (underlined; see Fig. 1c) by quick change mutagenesis [20] using *Pfu* DNA polymerase (Stratagene). This plasmid was designated pNP7^{Kan}.

Expression and purification of nitrophorin 7

Prior to expression, pNP7^{Kan} was transformed into competent *Escherichia coli*, strain BL21 (DE3) (Novagen). The protein was expressed in 16 L of LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) containing 30 μ g L⁻¹ kanamycine A and 0.005% (v/v) Antifoam 289 (Sigma) at 37 °C in O₂-bubbled glass bottles. Expression was induced at a cell density of OD_{600 nm} $\approx$ 0.8 with 1 mM IPTG and cells were grown for another 5 h. Upon harvest, cells were resuspended in 160 mL of 100 mM Tris/HCl (pH 7.4), 20 mM EDTA, 10% (w/v) sucrose, 0.2 mg mL⁻¹ lysozyme, 1 mM DTT and stirred for 30 min at ambient temperature. After centrifugation (16,000 $\times$ g, 4 °C, 40 min) the pellet was resuspended in 160 mL 100 mM Tris/HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT and sonified on ice for 5 cycles of 1 min (Branson

Sonifier 250, 50% duty cycle, output = 5) with resting intervals of 1 min. Inclusion bodies were harvested by centrifugation (16,000×g, 4 °C, 40 min) and then subjected to 6 washing steps: 2 times with 160 mL 30 mM Tris/HCl (pH 7.4), 30 mM NaCl, 1 mM EDTA, 2% (v/v) Tween-20, 1 mM DTT, 2 times with 160 mL 30 mM Tris/HCl (pH 7.4), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 2 times with 160 mL 30 mM Tris/HCl (pH 7.4), 30 mM NaCl, 1 mM EDTA, 1 mM DTT.

Inclusion bodies were suspended in 120 mL of 1.5 M GdmCl, 10 mM Mops/NaOH (pH 7.0), 1 mM EDTA, 10 mM DTT on ice and then centrifuged. The pellet was then solubilized in 120 mL of 3.5 M GdmCl, 10 mM Mops/NaOH (pH 7.0), 1 mM EDTA, 2 mM tris(2-carboxyethyl) phosphine-Cl (TCEP) and incubated for 2 h at 4 °C before centrifugation (15,000×g, 4 °C, 20 min). The supernatant was rapidly diluted under vigorous stirring into 1.2 L of 10 mM Mops, 10% (w/v) sucrose, 500 mM L-arginine, 1 mM EDTA, 1 mM oxidized glutathione (GSSG) titrated with HCl to pH 6.6 at ambient temperature and centrifuged again. The supernatant was dialyzed (NMWL: 12 – 14 kDa) overnight at 4 °C against 12 L of 10 mM Mops, 10% (w/v) sucrose, 50 mM L-glutamate, 50 mM NaCl titrated with NaOH to pH 6.6. Precipitate was removed by centrifugation.

Heme reconstitution

NP7 was reconstituted by step-wise heme titration. A solution of 50 mM hemin-Cl in 100 mM KOH was diluted 1:50 with 100 mM NaH₂PO₄/NaOH (pH 7.5). 15 µL-aliquots of hemin solution were added to the vigorously stirred protein solution at ambient temperature and the insertion of heme into the protein was followed photometrically. Occasionally, aggregated heme was removed by centrifugation (15,000×g, 15 min, 4 °C) and then hemin titration continued until $A_{400\text{ nm}}/A_{280\text{ nm}} \approx 2$. Upon addition of 10% (v/v) 1 M NaOAc/HOAc (pH 5.0) the solution was centrifuged and concentrated by ultrafiltration (NMWL: 10 kDa) to 65 mL. The protein was purified in aliquots of 10 mL by size-exclusion chromatography on a Sephacryl S-100 HR 26/60 column (Amersham Biosciences) connected to a Sephacryl S-100 pre-column (2.6 × 15 cm) in 200 mM NaOAc/HOAc (pH 5.0), 2% (w/v) glycerol at 4 °C.

Determination of heme/NP7 ratio and extinction coefficients

Apo-NP7 was prepared on ice by decreasing the pH to ~2.5 using 2 M HCl and subsequent extraction with cold 2-butanone as described for cytochrome *c* peroxidase [21]. Upon dialysis against 25 mM Tricine/NaOH (pH 7.5) the extinction coefficients were determined by the method of Edelhoch [22] from the absorption spectra recorded in 20 mM NaH₂PO₄/KOH (pH 6.5), 6 M GdmCl in comparison to the spectrum in 50 mM Mops/NaOH (pH 7.5), 100 mM NaCl.

The iron and sulfur content of holo-NP7 was determined by ICP-OES (IRIS Advantage; Thermo Jarrell Ash Corp.) upon dialysis against 1 L 25 mM Tricine/NaOH (pH 7.5) containing Chelex 100 (one spatula; Sigma) in the dialysis solution. Prior to analysis an absorption spectrum was recorded and subsequently 1% HNO₃ was added. The dialysis solution was used as a blank.

Preparation of liposomes

3 mg of PC as a carrier phospholipid and 1 mg of another phospholipid were dissolved in 2.6 mL CHCl₃ (PS, PA, PG, and PC), 2:1 (v/v) CHCl₃/CH₃OH (PI), 4:1 (v/v) CHCl₃/CH₃OH (lysoPC and cardiolipin), or 9:1 (v/v) CHCl₃/CH₃OH (SM). Liposomes were prepared according to the evaporation method of Deamer and Bangham [23,24]. In brief, 4 mL of 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl was heated to 70 °C in a Liebig condenser closed with a septum on the bottom side. A continuous stream of argon was gently bubbled through the solution. The phospholipid solution was injected over a period of 10 min into the hot buffer

solution. The suspension was heated for another ~1 h and then temperature decreased to 25 °C within ~1 h. The liposome suspension was then passed through a 1.2 µm hydrophilic polyethersulfone filter (Pall Life Sciences) and then subjected to a 5 mL HiTrap Desalting column (Sephadex G-25; Amersham Biosciences) equilibrated in the same buffer. The liposomes were examined by phase contrast microscopy.

Binding of nitrophorin 7 to liposomes

50 µL of 2 µM NP7 or a mix containing 2 µM NP1-4 each in 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl was mixed with 50 µL of a 1 mg mL⁻¹ suspension of liposomes in the same buffer. The mixture was incubated for 30 min at 37 °C and then centrifuged for 30 min at 20,000×g. The supernatant was removed and the liposomes suspended in 15 µL of 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl. Then 15 µL of SDS-sample buffer containing 5% (v/v) 2-mercaptoethanol was added and heated in a boiling water bath for 5 min. Samples were centrifuged again and then subjected to SDS-PAGE. The gels were developed with 0.1% Coomassie Brilliant Blue G-250 in 10% HOAc, 40% CH₃OH.

Phase contrast microscopy of liposomes in the presence of nitrophorin 7

200 µL of 1 mg mL⁻¹ liposomes consisting of either PS or PC in 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl were mixed with 10 µL of 10 µM NP7, 10 µM NP7 and 100 µM histamine·HCl, or 10 µM NP7 pre-incubated with 2 µL of 10 mM diethylammonium (Z)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) dissolved in 10 mM NaOH. Incubation was performed for 30 min at 37 °C. The samples were then centrifuged for 30 min at 20,000×g and the pellet resuspended in 10 µL of 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl. Samples were examined by phase contrast microscopy at 100× magnification.

Results and discussion

Expression and purification of nitrophorin 7

We initiated the detailed characterization of NP7 from *R. prolixus* in comparison to the well-characterized NP1-4 using various spectroscopic techniques such as NMR, EPR, spectroelectrochemistry *etc.* All of these methods require high amounts of protein. The cDNA for NP7 was inserted into pET-17b expression vector and the expression in *E. coli* previously described [3]. However, this expression system did not yield sufficient amounts of protein to carry out the desired experiments (~0.2 mg per L⁻¹ expression culture in our hands). Previously, NP1-4 have also been expressed in *E. coli* using the pET-17b expression vector (ampicillin resistance) [19] but in recent experimental work (T. Kh. Shokhireva, R. E. Berry, I. Filippov, M. N. Shokhirev, H. Zhang, F. A. Walker, submitted for publication) higher yields were obtained with the pET-24a(+) and pET-28a(+) expression vectors (kanamycin resistance). By analogy, the NP7 cDNA was inserted into pET-28a(+) and the protein expressed and reconstituted similarly to NP1-4. However, although sufficient amounts of protein were obtained in inclusion bodies as judged by SDS-PAGE (Fig. 2), the refolding procedure did not improve the yield due to continuous protein precipitation throughout the refolding process. As a consequence, a new refolding and reconstitution method was developed.

The following properties of NP7 were observed to be different compared to NP1-4 and demanded significant changes in the preparation method. (i) It was found that phosphate at high pH strongly promoted the precipitation of NP7. It was therefore substituted with Mops buffer. (ii) Furthermore, high salt concentrations in general decreased NP7 solubility. Therefore, contrary to the other *R. prolixus* nitrophorins ion exchange chromatography was avoided. (iii) In addition, the apo-protein was found to be extremely unstable and it precipitated during size-exclusion chromatography. (iv) NP7 tends to precipitate at pH >7.0. Therefore, the pH during the preparation method was kept lower than for NP1-4.

An overview of the NP7 production method is summarized in Table 1. The SDS-PAGE presented in Fig. 2 shows that NP7 was highly overexpressed in *E. coli* cells (lane 1) and the majority of protein appeared in inclusion bodies after sonification (lane 2). The inclusion bodies were washed with detergent (2% Tween-20; lane 3), high salt (500 mM NaCl; lane 4), and low salt buffer (lane 5) which substantially improved the protein purity. Another washing step was performed with 1.5 M GdmCl, a concentration at which NP7 was not solubilized (lane 6). Finally, the inclusion bodies were solubilized in 3.5 M GdmCl (lane 7). At this point, 2 mM TCEP was added to accomplish full cysteine reduction.

The refolding was performed by rapid dilution into a buffered solution containing 1 mM of GSSG to support the formation of the necessary disulfide bridges (compare Fig. 1b and c). The addition of 500 mM L-arginine significantly stabilized NP7 at this step, and this arginine was slowly removed by dialysis. Subsequently, the protein was reconstituted with the heme cofactor which was added in aliquots. Heme aggregates were removed by centrifugation at several times during the titration, until a heme:protein ratio of 1 was reached (spectrophotometric verification; Fig. 2, lane 8). The protein was concentrated and purified by a final size-exclusion chromatography step; a representative chromatogram in Fig. S1 shows that the protein was already rather pure at this point and it eluted as a monomeric species with an apparent $M_r \approx 26$ kDa. The absence of additional major bands in the SDS-PAGE of the solution applied to size-exclusion chromatography (Fig. 2, lane 8) suggests that the additional peaks in the chromatogram originate from disulfide bridged dimers and multimers. This suggestion is supported by the apparent molecular mass of the species present in the additional peaks. Finally, the SDS-PAGE of the eluted protein (Fig. 2, lane 9) shows that the reconstituted NP7 is highly pure (~5.5 mg per L expression culture); this was further confirmed by MALDI-TOF MS (calculated for $[\text{NP7} + \text{H}]^+$ (including the initial Met): 20,969 Da, observed: 20,966 Da). Unlike NP1-4, NP7 was found unstable when lyophilized. Therefore, the protein was stored at -20°C in 200 mM NaOAc/HOAc (pH 5.0), 10% (v/v) glycerol until use.

Functionality of the nitrophorin 7 heme center

Properties of apo- and holo-NP7 are summarized in Table 2. Fig. 3a shows the absorption spectrum of the protein, which has a maximum at 275 nm, and the maximum of the Soret absorption is at 403 nm, in agreement with the previous report [3], and indicative of the high-spin Fe^{III} complex. Through titration with hemin a maximum ratio $A_{280\text{ nm}}/A_{403\text{ nm}} \approx 2$ could be reached which is similar to the other *R. prolixus* nitrophorins [25]. Elemental analysis of the S and Fe content by ICP-OES revealed a heme:protein ratio of 0.93. Based on this analysis, the molar extinction coefficients for NP7, NP7-NO, NP7-Hm and apo-NP7 were obtained (Table 2). The binding of NO to NP7 at pH 5.5 [5] was tested by incubation with the NO donor DEA/NO, yielding an absorption maximum at 417 nm. The binding of the native ligands NO (added as DEA/NO) and Hm was further tested at pH 7.4 (Fig. 3b) showing a Soret band at 417 nm and 410 nm, respectively, which is comparable to the previously reported values [3]. NO was blown out of the NP7 solution with argon overnight and the spectrum recorded again. The resulting spectrum was the same as observed before addition of NO, and is indicative of a high-spin Fe^{III} species (Fig. 3b) showing that the ferriheme was not reduced by excess NO. This is typical for the *R. prolixus* nitrophorins and a marked difference to other mono-His bound heme-proteins like hemoglobin and myoglobin [26,27].

Binding of nitrophorin 7 to negatively charged liposomes

The ability of the recombinantly prepared NP7 to bind to PS containing membrane surfaces was tested using large unilamellar vesicles (LUV) composed of PC and PC:PS (3:1). To gain deeper insight in the specificity of membrane recognition, the examination was expanded to include six other phospholipids PA, PG, PI, lysoPC, SM, and cardiolipin where PC was always used as a carrier phospholipid in a 3-fold mass excess. The vesicles were examined regarding

their shape and diameter by phase contrast microscopy (see below). The LUVs were then incubated with NP7 or, as a control, with a mixture of NP1-4. In agreement with the previous report [3], Table 3 shows that the recombinant NP7 did not bind to PC but did bind to PC:PS (3:1) vesicles. In addition, NP7 also interacted with PA, PG, PI, and cardiolipin, but not with lysoPC and SM-containing LUV. In contrast, none of the isoforms NP1-4 interacted with any of the eight phospholipids tested (see Fig. S2).

A comparison of the chemical structures of the eight phospholipids (Fig. S2) leads to the conclusion that a NP7-phospholipid complex is generally formed when the phospholipids carries a net negative charge, regardless of the headgroup attached as a second ester to the phosphate groups. The strong diversity of functionalities esterified to the phosphate group suggests that the negative charge on the latter is the major point of interaction between NP7 and the membrane surface. This is rather interesting, in view of the relatively large structural and/or charge differences, *e.g.*, when comparing the bulky *myo*-inositol ring on PI to the uncovered phosphate group of PA or with the +1 / -1 charged groups attached through ι -serine in the case of PS. However, the result shows that the molecular recognition is relatively nonspecific. Similar behavior has been reported for cytochrome *c* (cyt *c*), which binds to PS and PG, but not to the neutral PC and ι - α -phosphatidylethanolamine (PE) [28,29].

Recognition of PS exposure by proteins is important in biological processes such as the assembly of coagulation complexes [30] and the triggering of apoptosis through cyt *c* [28, 29]. Thus, NP7 recognizes PS bearing membrane surfaces as an indicator of activation and uses this as a means of targeting the surfaces of activated platelets [3,15]. Activated platelets undergo changes in the plasma membrane that promote assembly of coagulation complexes at the wound site and allow the formation of a fibrin clot. The membrane bilayer is mainly composed of the neutral PC, PE, SM, and the negatively charged PS [16]. Activation by collagen or thrombin leads to an increase in intracellular $[Ca^{2+}]$ and a rapid loss of membrane asymmetry [16]. Other cell types, including mast cells [31], also lose membrane asymmetry when activated. However, in erythrocytes, although they do not contain PS, the outer leaflet is composed of a substantial amount of PA, PI, and ι - α -phosphatidyl-D-*myo*-inositol-4,5-bisphosphate (PIP₂) (a total of 1 – 3%) [32-34]. Because NP7 does not distinguish between the anionic phospholipids a function on binding to other types of cells even in their resting states may be considered.

Microscopy of liposomes in the presence of nitrophorin 7

The quality of liposomes was examined by phase contrast microscopy using 100 $\times$ magnification. The liposomes are of spherical shape and their diameter was determined to be $1.2 \pm 0.3 \mu\text{m}$ (Fig. S3). Surprisingly, when PS composed liposomes were examined in the presence of NP7, they lead to vesicle aggregation (Fig. S3). This was found to be independent of whether the NP7 was coordinated to NO or Hm. As a control, liposomes composed of PC did not show aggregation in the presence of NP7 at all. We have further examined the other liposome types reported in this study (Table 3) and found that all of the net-negatively charged liposomes were aggregated by NP7 whereas the neutral ones were not. The observed effect is not necessarily of biological relevance, since liposomes are of course an oversimplified model for cellular membranes. However, the previously reported model structure of NP7 [3] suggested that the site opposite to the heme binding pocket is the only binding site for net negatively charged liposome surfaces due to its highly positive charge. For geometrical reasons, however, the connecting ('gluing') properties of NP7 can be best explained by the existence of a second site of interaction with liposomes. The previously described biphasic binding kinetics toward PC:PS (3:1) monolayers with low K_d values of 4.8 and $755 \times 10^{-9} \text{ M}$ [3] may support this hypothesis. Further research on the structural properties of NP7 will be

needed to answer these questions. Liposome aggregation was not observed with NP1-4 (data not shown).

Conclusions

The presented recombinant expression and reconstitution method for NP7 from *R. prolixus* substantially increased the yield of this ferriheme protein. The protein was fully functional in the sense that it binds the native ligands Hm and NO, the latter without causing Fe^{III} reduction, as is known for other heme proteins. In addition, the property of NP7 to bind to negatively charged phospholipid membranes was established. The data presented extend this behavior toward any type of net negatively charged liposome surface. Overall, the method presented might prove useful also for other nitrophorins or, in a broader sense, for other proteins with a lipocalin fold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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c

	10	20	30	40	50	60	
NP2	DCSTNIS	PKQGLDKAKY	FSGKWYVTHF	LDKDPQVTDQ	YCSSFPTPRES	DGTVKEALYH	(57)
NP7	LPGECSVNI	PKKNLDKAKF	FSGTWYETHY	LDMDPQATEK	FCFSFAPRES	GGTVKEALYH	(60)
	α_1		β_A	AB-loop	β_B	β_C	
	70	80	90	100	110	120	
NP2	YNANKKTSFY	NIGEGKLESS	GLQYTAKYKT	VDKKKAVLKE	ADEKNSYTLT	VLEADDSSAL	(117)
NP7	FNVDKVSFY	NTGTGPLESN	GAKYTAKFNT	VDKKGKEIKP	ADEKYSYTVT	VIEAAKQSAL	(120)
	β_D		β_E		β_F		
	130	140	150	160	170	180	
NP2	VHICLREGSK	DLGDLYTVLT	HQKDAEPSAK	VKSAVTQAGL	QLSQFVGTKD	LGCQYDDQFT	SL (179)
NP7	IHICLQEDGK	DIGDLYSVLN	RNKNALPNKK	IKKALNKVSL	VLTKFVVTKD	LDCKYDDKFL	SSWQK (185)
	β_G	GH-loop	β_H	α_2	α_3	α_4	

Fig.1.

(a) Schematic representation of the action of NO delivery through nitrophorins (NPs) from *Rhodnius prolixus*. NO loaded NPs are injected from the salivary gland (pH 5 – 6) into the victim's tissue. Increased pH (~7.4) and dilution lead to the release of NO in blood plasma which accounts for vasodilation and inhibits blood coagulation. The empty coordination site on the heme-iron of NP is ready to bind histamine (Hm) which helps suppressing the victim's immune response. (b) Ribbon diagram of the crystal structure of NP2 from *Rhodnius prolixus* with NH₃ as proximal heme ligand (PDB accession no. [1EUO](#)) [35]. The eight strands of the β -barrel are labeled A-H. Displayed are the heme cofactor with the distal His57 and the disulfide forming Cys2–Cys121 and Cys39–Cys170. (c) Amino acid sequence alignment of NP2 (Swiss-Prot accession no. [Q26241](#)) and NP7 (TrEMBL accession no. [Q6PQK2](#)) sharing a 60% sequence identity. Indicated are the α -helices and β strands present in the structure of NP2 [35] and the proximal His (outlined)).

Fig. 2.

Purification steps of recombinant NP7 documented by SDS-PAGE (4 – 20%) stained with Coomassie Brilliant Blue G-250 in 40% CH₃OH, 10% HOAc. Left side: Induction of an *E. coli* culture with IPTG reveals a strong expression of NP7. Right side: Samples of the various purification steps were collected and dialyzed (NMWL: 12 – 14 kDa) against 8 M urea prior to mixing with sample buffer containing 5% (v/v) 2-mercaptoethanol. Lane 1, resuspended *E. coli* cells; lane 2, *E. coli* cells after sonification; lane 3, inclusion bodies after washing with 2% Tween-20; lane 4, inclusion bodies after washing with 500 mM NaCl; lane 5, inclusion bodies after washing with low salt; lane 6, inclusion bodies after incubation in 1.5 M GdmCl; lane 7, protein after solubilization in 3.5 M GdmCl and reconstitution; lane 8, protein after heme-reconstitution; lane 9, after size exclusion chromatography.

Fig. 3.

Absorption spectra of ferriheme-NP7 species. (a) Spectra in 100 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$ (pH 5.5) without ligand added (---) and in the presence of 400 μM DEA/NO (—). (b) Spectra in 50 mM Mops/NaOH (pH 7.4), 100 mM NaCl in the presence of 1 mM histamine (Hm) (.....), 400 μM DEA/NO (—), and NO removed from the Fe^{III} by argon blown over the liquid surface over night (---).

Table 1Purification steps and enrichment of NP7 from *Rhodnius prolixus* expressed in *Escherichia coli*^a

Purification steps (numbers refer to lanes in Fig. 2):		Total protein mass (mg)	Volume (mL)	Enrichment (-fold)
1	Resuspended <i>E. coli</i> cells	- ^b	170	
2	Sonification	3,570 ^c	170	1
3	2 × washing with 2% Tween-20	- ^d	170	
4	2 × washing with 500 mM NaCl	491 ^c	170	7.3
5	2 × washing with low salt	247 ^c	170	14.5
6	Washing with 1.5 M GdmCl	298 ^c	120	12.0
7	Solubilization in 3.5 M GdmCl	324 ^c	120	11.0
8	Heme-reconstitution	138 ^e	65	25.9
9	Size-exclusion chromatography (see Fig. S1)	88 ^e	150	40.6

^aCorresponding to a preparation from 16 L of expression culture.^bWet mass: 34 g.^cEstimated from the absorption at 280 nm ($\epsilon_{280\text{ nm}} = 27,300\text{ M}^{-1}\text{ cm}^{-1}$; see Table 2) upon dialysis against 8 M urea.^dWet mass: 15.4 g.^eDetermined from the absorption at 403 nm ($\epsilon_{403\text{ nm}} = 81,000\text{ M}^{-1}\text{ cm}^{-1}$; see Table 2).

Table 2
Properties of recombinant apo-NP7 and ferri-heme-NP7 from *Rhodnius prolixus*.

Properties of apo-NP7:				
TrEMBL accession no.		O6PQK2		
GenBank accession no.		AY585746		
Molar extinction coefficients for apo-NP7: ^b		$M_r =$ $pI =$ $\epsilon_{274\text{ nm}} =$ $\epsilon_{280\text{ nm}} =$ 20,968.0 Da ^d 9.21 ^d 28,700 M ⁻¹ cm ⁻¹ c 27,300 M ⁻¹ cm ⁻¹		
Properties of ferriheme-NP7:				
heme b / NP7 ratio: ^d 93%				
f		Molar extinction coefficients of ferriheme complexes: ^e		
		NO		Hm
wavelength (nm)	ϵ (M ⁻¹ cm ⁻¹)	wavelength (nm)	ϵ (M ⁻¹ cm ⁻¹)	wavelength (nm) ϵ (M ⁻¹ cm ⁻¹)
275 ^g	47,600			275 ^g 43,600
280	46,800			280 42,900
403 ^g	81,000	417 ^g	92,100	410 ^g 72,900
470 ^g	9,940	531 ^g	12,300	530 ^g 9,770
627 ^g	3,660	565 ^g	14,400	565 ^g 6,400

^a Calculated for the apo-protein including the initial Met originating from the expression in *E. coli* and taking into account the presence of the 2 Cys–Cys disulfides (see Fig. 1b). Values were calculated at http://www.expasy.ch/tools/pi_tool.html/.

^b Apo-NP7 was prepared by extraction with 2-butanone [34] and extinction coefficients determined by the method of Edelhoch [35] for a spectrum recorded in 50 mM Mops/NaOH (pH 7.5), 100 mM NaCl.

^c Absorption maximum.

^d Fe and S were quantified by ICP-OES [36]. NP7 contains 6 S atoms.

^e Based on the quantification of Fe (heme absorption) and S (absorption at 275 and 280 nm).

^f No ligand added.

^g Absorption maxima (compare Fig. 3).

Table 3

Dependence of the binding of recombinant NP7 from *Rhodnius prolixus* to liposomes on the phospholipid charge^a.

Phospholipid ^b	Net charge per phospholipid	Phospholipid head group ^c	Binding of NP7 (+/-)
PC	0	choline	-
PS	-1	L-serine	+
PA	-2	-	+
PG	-1	glycerol	+
PI	-1	D-myo-inositol	+
lysoPC	0	choline	-
SM	0	choline	-
cardiolipin	-2	glycerol ^d	+

^a Compare Fig. S2.

^b Liposomes were composed of 3:1 (w/w) PC/phospholipid. 100 µg of liposomes were incubated with 2 µg of NP7 from *R. prolixus* for 30 min at 37 °C.

^c Functionalities esterified with the phosphato group of the phospholipid.

^d Cardiolipin is a di-phospholipid fused through the glycerol head-group.