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Oligomerization of nitrophorins

Attila Ambrus [a,*], Kenneth Friedrich [b], Árpád Somogyi [c]

[a] Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721, USA
[b] Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA
[c] Department of Chemistry, Mass Spectrometry Facility, University of Arizona, Tucson, AZ 85721, USA

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Abstract

Rhodnius prolixus is a blood-sucking insect that uses a mixture of nitrophorin (NP) proteins to deliver nitric oxide (NO) from the insect saliva to the hosts via a ferric heme coordinated to the protein, causing vasodilatation and anticoagulation to support their feeding. *R. prolixus* NPs 1–4 are very similar proteins (~20 kDa) with different NO affinities for stepwise NO release triggered by pH increase and histamine binding in hosts. Ultra-high-resolution X-ray structures of native and mutant NPs and their kinetic analysis already have revealed the fundamental steps of NO binding and release. In this study, we found that NPs can exist in multiple oligomerization states at higher concentrations. The oligomers are characterized by a combination of multiple biophysical methods. The intrinsic features of the oligomerization revealed here led us to propose that this intensive, moderately pH- and ligand-dependent oligomerization of NPs has physiological implications in the facilitation of the efficient storage and release of the highly reactive NO in the insect saliva and the victim, respectively.

Keywords: Nitrophorin; Oligomerization; MALDI–TOF; Nanospray ESI; DOSY–NMR; Analytical ultracentrifugation

Nitrophorins 1–4 (NP1–4)[1] are 20-kDa ferriheme proteins found in nearly millimolar concentrations in the saliva of the blood-sucking insect *Rhodnius prolixus*. They transfer nitric oxide (NO) to victims, inducing vasodilatation. They also sequester histamine and inhibit blood coagulation for supporting the feeding process [1–5]. The insect saliva is most abundant in NP1, but all of the other NPs bind NO and histamine in the same binding site. NP molecules are very similar proteins, having 38% overall sequence identity; NP1 and NP4 display 90%, whereas NP2 and NP3 share 79% identity. NPs bind NO by a ferric heme coordinated to the protein, and the dissociation constants of the NO complexes are in the millimolar range. Crystal structures have been determined recently for three of the four NPs (NP1, NP2, and NP4), all showing a lipocalin type of folding [6–8]. When NO binds to the protein, two flexible loops move around and bury the NO molecule, exposing a hydrophobic surface [7,9,10]. Kinetic studies have shown biphasic NO binding and release that can be in relation to the loop reorientations [11–14]. NO release is 10-fold faster at pH 8 (victim) than at pH 5 (saliva), facilitating NO release in victims. Efficient but reversible NO binding and storage capability of NPs in the saliva is essential for their readiness to function when the feeding starts.

In vivo, most of the physiologically important processes involve proteins and/or nucleic acids permanently complexed to one another, such as multienzyme complexes, or having stronger or weaker contacts in transition states while they perform their physiological functions. The interaction among such biomacromolecules that form short half-life or transitional complexes sometimes is relatively

* Corresponding author. Fax: +1 520 626 2466.
E-mail address: ambrus@pharmacy.arizona.edu (A. Ambrus).

[1] *Abbreviations used:* NP1–4, *Rhodnius prolixus* nitrophorins 1–4; NO, nitric oxide; DOSY–NMR, diffusion-ordered nuclear magnetic resonance spectroscopy; MW, molecular weight; MALDI–TOF, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; MS, mass spectrometry; NPC, NP from another species, *Cimex lectoralius*; TFA, trifluoroacetic acid; *m/z*, mass-to-charge ratio; FPLC, fast protein liquid chromatography.

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weak, providing the basis for rather rapid processes such as electron transfers [15], metabolic cycles [16], and DNA processing [17]. Because of the low stability of these complexes, they generally dissociate in vitro and their investigation by conventional biochemical/biophysical methods generally is very difficult [17] and, until recently, had received relatively little attention in biochemical research.

Analytical ultracentrifugation is a widely used technique for determining oligomerization order of macromolecules and providing the corresponding dissociation constants as well [18]. However, the intrinsic limitations of the method permit the identification of a maximum of three or four different molecular species present at the same time in the sample solution. Analysis of a higher number of species provides ambiguous results. Probably the greatest advantage of the method is that it is matrix free and the possibility of artificially generated associations can be ruled out.

Diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY–NMR) [19] is another useful method for the identification of different molecular weight (MW) macromolecules present abreast in solution; however, the successful acquisition of the signal needs sufficient concentration of the component in question (i.e., a millimolar concentration range that is approximately two orders of magnitude higher than that which is analyzable by analytical ultracentrifugation). The method uses a special NMR pulse sequence working with field gradients and is capable of determining diffusion coefficients of molecules without any concentration gradient. The intensity of the detected proton signal belonging to a particular molecular entity is dependent on the rate of diffusion of that molecule as the gradient eliminates more signal intensity of molecules in faster motion. The number of identifiable components in solution is limited only by resolution (if species exchange slowly on an NMR time scale). Comparing the diffusion coefficients of molecules in question with that of a reference, the MW can be calculated by formula [19]. The technique does not use any matrix, and the measurement can be performed directly in a physiological buffer solution. The possibility of external referencing provides an opportunity to examine molecules in their physiologically relevant conditions.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF), electrospray ionization (ESI), and nanospray ESI mass spectrometry (MS) methods are now fundamental sources of information in proteomics. The identification of higher MWs is possible using each of these methods with the recent developments of ionization sources and analyzers. ESI can destroy higher order oligomers but is widely used for diverse analyses of noncovalent interactions of smaller complexes. MALDI, as a soft ionization method, can easily identify higher molecular complex formations; its disadvantage is the necessary addition of artificial matrices to the sample to achieve ionization. Consequently, artificially (matrix-) facilitated formation of higher MW clusters on the sample plate cannot be ruled out. Nanospray ESI is also capable of analyzing oligomers in a matrix-deficient way (directly spraying the sample solution into the ion source) from very low amounts of samples, and the method can be fine-tuned to preserve higher order oligomers.

In this study, we analyzed the oligomerization of three NPs by FPLC gel filtration, MALDI, ESI, nanospray ESI-MS techniques, analytical ultracentrifugation, and DOSY–NMR. We propose that higher order oligomers of NPs found here might have physiological roles in NO binding and storage in the insect saliva.

Materials and methods

Chemicals

All chemicals were purchased from Sigma if not designated otherwise.

Protein expression and purification

NP4 was overexpressed in *Escherichia coli*, isolated from inclusion bodies, and renatured as described previously [8,9,20]. Point mutations of NP4 were produced, and the mutant proteins were purified as wild-type NP4 [12]. Three mutants of NP4 were generated: D129AL130A, T121V, and L123VL133V. NP1 and NP2 were expressed and purified as described previously [7,20], NP from another species, *Cimex lectoralius* (NPC), was expressed and purified as before [21], and NP4-NO complexation was carried out as published previously [11].

Mass spectrometry

Mass spectrometry analyses were performed on a Thermoquest LCQ Classic and a Bruker Reflex III MALDI–TOF instrument. Samples measured by MALDI–TOF were mixed with the saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in H_2O:acetonitrile (70:30) containing 0.1% of trifluoroacetic acid (TFA) in such a way that the total amounts of proteins deposited were in the picomolar range. Higher protein concentrations were achieved by using increasing concentration aliquots of the proteins that were deposited in the same final volume of 1 μl. The final protein concentrations were adjusted and calculated accordingly, also taking the volume of the matrix (1 μl) into account. In general, a laser attenuation of 80% was used. ESI experiments were performed on the Thermoquest LCQ Classic instrument by direct injection of the protein solutions into MeOH:H_2O (1:1) containing 2% of AcOH. Protein samples were prepared in 20 mM Tris and 100 mM NaCl (pH 7.5).

Nanospray ESI-MS

Nanospray mass spectra were recorded on a TOF mass spectrometer (LCT, Micromass, Manchester, UK). Nanospray ESI capillaries were prepared in-house from