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Lipoprotein-induced phenoloxidase-activity in tarantula hemocyanin

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ABSTRACT

Phenoloxidases play vital roles in invertebrate innate immune reactions, wound closure and sclerotization processes in arthropods. In chelicerates, where phenoloxidases are lacking, phenoloxidase-activity can be induced in the oxygen carrier hemocyanin *in vitro* by proteolytic cleavage, incubation with the artificial inducer SDS, or lipids. The role of protein–protein interaction has up to now received little attention. This is remarkable, as lipoproteins — complexes of proteins and lipids — are present at high concentrations in arthropod hemolymph. We characterized the three lipoproteins present in tarantula hemolymph, two high-density lipoproteins and one very high-density lipoprotein, and show that the two high-density lipoproteins have distinct structures: the more abundant high-density lipoprotein is an ellipsoid particle with axes of ~22.5 nm and ~16.8 nm, respectively. The second high-density lipoprotein, present only in trace amount, is a large discoidal lipoprotein with a diameter of ~38.4 nm and an on-edge thickness of ~7.1 nm. We further demonstrate that the interaction between lipoproteins and hemocyanin induces phenoloxidase activity in hemocyanin, and propose that this activation is due to protein–protein interaction rather than protein–lipid interaction, as neither lipid micelles nor lipid monomers were found to be activating. Activation was strongest in the presence of high-density lipoproteins; very high-density lipoproteins were found to be non-activating. This is the first time that the ability of lipoproteins to induce phenoloxidase activity of hemocyanin has been demonstrated, thus adding novel aspects to the function of lipoproteins apart from their known role in nutrient supply.

1. Introduction

The major proteins in chelicerate hemolymph are the oxygen carrier hemocyanin and the so called non-respiratory proteins which are thought to be lipoproteins. Hemocyanins, together with tyrosinases and catecholoxidases (commonly subsumed as phenoloxidases) belong to the family of type 3 copper proteins [1]. This protein family is characterized by the presence of two Cu^{+}-ions per functional unit, each one being complexed to three histidine residues. Upon oxygenation these Cu^{+}-ions change their valencies to Cu^{2+} and reversibly bind molecular oxygen as peroxide in a μ–η^2: η^2-coordination [2–7]. Tyrosinases (E.C. 1.14.18.1), catalyze the *ortho*-hydroxylation of mono-phenols to *ortho*-diphenols and the subsequent oxidation to *ortho*-chinons. Catecholoxidases (E.C. 1.10.3.1) catalyze only the oxidation of *ortho*-diphenols to *ortho*-chinons [8,9]. Both enzymes are involved in melanization processes in bacteria, plants and animals [8,10–14] and in immune defense reactions and cuticle hardening in many invertebrates [5,10,15–18]. Hemocyanins, on the other hand, are the oxygen-carriers in many mollusks and arthropods. In arthropods, they exist as multimers of hexamers of ~72 kDa subunits [as deduced from the cDNA sequence of tarantula hemocyanin, 19], e.g. 1×6, 2×6, 4×6, 6×6 or 8×6 [20–23]. Due to their vital functions for either oxygen transport or defense reactions and for post-molt sclerotization of the cuticle in arthropods, both phenoloxidases and hemocyanins are present in these animals [15,23–25]. Oddly, in the most basal arthropods, the chelicerates, phenoloxidases have not been found; neither in the hemolymph, nor on transcript level. However, as phenoloxidases and hemocyanins are structurally related and the latter have been shown to possess an intrinsic phenoloxidase-activity, the hypothesis that chelicerates utilize hemocyanins as endogenous phenoloxidases was proposed [16,18,22,23].

Activation of hemocyanin phenoloxidase activity *in vitro* is commonly induced by incubation with the anionic detergent sodium-dodecylsulfate [SDS, 26–32]. However, activity could also be induced by the presence of free fatty acids and phospholipids [26,33]. In the chelicerate and so-called 'living fossil' *Limulus polyphemus* the anionic phospholipid phosphatidylserine was found to be the most potent activator [17].

Furthermore, hemocyanin-derived phenoloxidase-activity can be induced by limited proteolysis [34] or incubation with components of the blood coagulation cascade, which consists of several serine proteinases [35], thus resembling the proposed activation of crayfish prophenoloxidases to the active phenoloxidases [15]. Interestingly, enzymatic activity of hemocyanin in *Limulus* could even be induced when the serine proteinase activity of the coagulation-cascade factors was

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specifically blocked, thus hinting that protein–protein interactions might be sufficient to induce phenoloxidase-activity in *Limulus* hemocyanin [35]. However, the mode of *in vivo* activation of hemocyanin to a phenoloxidase remains enigmatic.

Prompted by our long-standing interest in tarantula hemocyanin and its enzymatic properties, as well as by some old reports on high fatty acid contents in tarantula lipoproteins [36,37] we investigated the possible role of hemolymph lipoproteins in the activation of hemocyanin to a phenoloxidase in the tarantula *Eurypelma californicum*[1]. We have undertaken in-depth biochemical and biophysical characterization of the lipoproteins present in the hemolymph of *Eurypelma californicum* and are adding substantial new data to the hitherto existing data from previous, partial characterizations [36,37,39]; thus filling gaps in the picture of lipoproteins in a basal chelicerate. We further demonstrate that hemolymph lipoproteins may represent endogenous activators of hemocyanins towards phenoloxidase-activity, and that this activation is most likely due to the interaction of the apolipoproteins with the hemocyanin. Hence, we establish a new role for this class of proteins in *Eurypelma californicum*, additional to its role in lipid transport.

2. Materials and method

2.1. Animals and hemolymph collection

Tarantulas (*Eurypelma californicum*) were obtained through a commercial provider (Carolina Biological Supply). They were kept individually at 20–25 °C with a 16/8 h light/dark cycle and were fed crickets every other week. The hemolymph was drawn by puncturing the pericard, collecting the emerging hemolymph with a glass capillary and immediate dilution in ice-cold stabilization buffer (100 mM Tris-HCl, pH 7.8, 5 mM $CaCl_2$, 5 mM $MgCl_2$) to prevent the native 4 × 6-mer hemocyanin from dissociation. Hemocytes and cellular debris were removed by centrifugation (15,000×g, 15 min, 4 °C) to yield cell-free hemolymph.

2.2. Lipoprotein purification

Hemolymph samples were adjusted to a density $\rho = 1{,}350$ kg/m^3 by the addition of solid KBr, overlaid with an equal volume stabilizing buffer and subjected to density gradient ultracentrifugation for 3 h and 417,000×g as described [40]. After centrifugation the individual lipoproteins, high-density lipoprotein (HDL)-1, HDL-2 and very high-density lipoprotein (VHDL), were collected individually and re-adjusted to a density of $\rho = 1{,}350$ kg/m^3 with solid KBr. The VHDL-fraction was then overlaid with five VHDL-fraction volumes stabilization buffer + KBr ($\rho = 1{,}250$ kg/m^3) followed by 2.5 VHDL-fraction volumes stabilization buffer + KBr ($\rho = 1{,}160$ kg/m^3) and subjected to a second ultracentrifugation as above for further purification. The two HDL fractions were overlaid with equal volumes of stabilization buffer adjusted to densities of $\rho = 1{,}300$ kg/m^3, 1,250 kg/m^3, 1,200 kg/m^3, 1,150 kg/m^3, 1,100 kg/m^3, 1,050 kg/m^3 with solid KBr, and stabilization buffer alone. The samples were then centrifuged at 200,000×g overnight, as described [41]. In some cases, the HDL were stained with 1 μM of the fluorescent fatty acid analog Bodipy FL_{12} (Molecular Probes, Invitrogen) for better visualization. After ultracentrifugation the purified lipoproteins were dialyzed against stabilization buffer or phosphate buffered saline (PBS, 137 mM NaCl, 8 mM Na_2HPO_4, 3 mM KCl, 2 mM KH_2PO_4, 1 mM Na_2-EDTA, pH 7.4) and stored at 4 °C.

[1] According to newer studies, *Eurypelma californicum* is a *nomen dubium* and is most likely identical with *Aphonopelma hentzi* [38]. For sakes of clarity and consistence with the literature, however, we will use the common term *Eurypelma californicum* throughout this study.

2.3. Hemocyanin purification

4 × 6-mer tarantula hemocyanin was isolated from clarified hemolymph (see above) by gel filtration on a Sephacryl S300 16/60 (Pharmacia, GE Healthcare) equilibrated in stabilization buffer with a flow rate of 0.6 mL/min and a detection wavelength of 280 nm essentially as previously described [30].

2.4. SDS-PAGE

Denaturing SDS-PAGE was carried out as described [42]. Gels were stained with Coomassie G250 and analyzed with the software ImageJ (http://rsb.info.nih.gov/ij) after digitalization [40].

2.5. Analytical ultracentrifugation

Sedimentation velocity experiments were carried out at 30,000 rpm at 20 °C in a Beckman XL-I Analytical Ultracentrifuge in 3 mm Epon double sector cells and detection at 220 nm (HDL) and 230 nm (VHDL). Scans were taken every minute, beginning five minutes after reaching the desired speed. The obtained sedimentation profiles were analyzed by an extended van Holde–Weischet algorithm implemented in the program, UltraScan II (http://www.ultrascan.uthscsa.edu).

2.6. Dynamic light scattering

Diffusion coefficients of lipoproteins and the presence/absence of lipid micelles were determined with by dynamic light scattering (DLS) on a Zetasizer NanoS instrument (Malvern) equipped with a 633 nm He/Ne-laser as light source. The measurements were taken in triplicates at 20 °C after an equilibration time of one minute and corrected for the intrinsic viscosity of the used buffer (PBS, $\eta = 1.0102$ mNs/m^2 or stabilization buffer, $\eta = 1.0313$ mNs/m^2).

2.7. Spectroscopy

UV/Vis-spectroscopy was carried out on a Hitachi U3000 instrument in the range of 200–800 nm with a scan speed of 120 nm/min. All spectra were run in triplicate and buffer corrected.

Fluorescence spectra were recorded on a Hitachi F4500 instrument with an excitation wavelength (λ_{Ex}) of either 280 nm (tryptophan and tyrosine fluorescence) or 290 nm (tryptophan fluorescence) at 20 °C. The emission was recorded in the range between 300 and 450 nm with a scan speed of 240 nm/min. The slit-width was 5 nm. Denaturation of the lipoproteins was monitored with excitation at 280 nm and 290 nm in the range of 300–450 nm at 20 °C after denaturing for ten minutes at room temperature in 2.0 M, 4.0 M and 6.0 M guanidin hydrochloride (GuHCl). All spectra were run in triplicate and buffer corrected.

2.8. Lipid analysis

Lipoproteins were dried down *in vacuo* and subsequently extracted twice with 100 μL chloroform/methanol 2:1 (v/v) for five minutes at room temperature. The combined extracts were then dried down *in vacuo* and either reconstituted in hexane (for neutral lipid analysis) or, in chloroform/methanol 98:2 (v/v) for phospholipid analysis. The lipid classes were separated by normal-phase chromatography on either a Spherisorb CN-column (3 × 250 mm, 5 μm particle size, MZ-Analysentechnik) with a hexane/methyl-*tert*-butyl-ether gradient for neutral lipids [43], or on a Spherisorb Si-column (3 × 150 mm, 3 μm particle size, MZ-Analysentechnik) using a chloroform/methanol/acetic acid/H_2O gradient for phospholipid analysis [40]. The eluting lipids were detected and quantified by evaporative light scattering detection using a SEDEX 85 (Sedere) with N_2 as carrier gas, as described [40,43].