Developmental and Comparative Immunology 35 (2011) 155–163

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Possible role of phosphatidylserine–hemocyanin interaction in the innate immune response of *Limulus polyphemus*

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ARTICLE INFO

Article history:
Received 23 July 2010
Received in revised form 25 August 2010
Accepted 27 August 2010
Available online 24 September 2010

Keywords:
Hemocyanin
Phenoloxidase
Phospholipid
Phosphatidylserine
Enzyme activation
Spectroscopy
Innate immunity

ABSTRACT

Phenoloxidase enzymes and the associated pro-phenoloxidase activation cascade play an essential role in the immune response of arthropods. Phenoloxidase activity can be elicited in the oxygen carrier, hemocyanin, by the addition of the artificial inducer, SDS. There is some evidence to support hemocyanin acting as a phenoloxidase *in vivo*; however, the identity of natural activators remains unclear. This study explores the role of the phospholipid, phosphatidylserine, as a possible natural activator of hemocyanin-derived phenoloxidase activity. Characterisation of the structural changes associated with activation of hemocyanin-derived phenoloxidase suggests that phosphatidylserine induces similar conformational changes to those caused by the artificial inducer, SDS. We propose that anionic phospholipids, in particular phosphatidylserine, may act as natural activators of hemocyanin-derived phenoloxidase.

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1. Introduction

It is now well documented that it is possible to change the function of hemocyanin, *in vitro*, from an oxygen carrier to an enzyme activity that plays a key role in invertebrate innate immunity, namely phenoloxidase (PO) (E.C. 1.10.3.1) (Decker et al., 2007). One of the most effective activators of hemocyanin-derived PO activity, *in vitro*, is the artificial inducer, SDS. We have recently shown that SDS-induced PO activation in hemocyanin is associated with conformational changes which enhance substrate access to the di-copper centre of this protein (Baird et al., 2007). It is assumed that the presence of SDS mimics the effects of putative natural activators present in arthropod hemolymph, such as small antimicrobial peptides (Nagai et al., 2001), fatty acids and phospholipids (Baird et al., 2007).

The phospholipid phosphatidylserine (PS) is normally located on the inner surface of the cell plasma membrane but in cells entering the apoptotic pathway, PS appears on the outer surface of the membrane. Exposure of PS on the outer membrane of the cell surface is considered a hallmark of programmed cell death in eukaryotes. It has been proposed that release of inner membrane phospholipids, including PS, during apoptosis leads to the activation of pro-phenoloxidase (ProPO) in insects and crustaceans (Bilda et al., 2009; Sugumaran and Nellaiappan, 1990, 1991). Our findings suggest PS may elicit PO activity in hemocyanin, a type 3 copper protein, which is structurally related to ProPO (Zlateva et al., 1996; Decker and Tuczek, 2000). This study presents an analysis of hemocyanin-derived PO activity, and associated conformational changes, in hemocyanin from *Limulus polyphemus*, associated with the addition of the putative natural activator of PO activity, phosphatidylserine (PS).

2. Methods

2.1. Purification of L. polyphemus hemocyanin

Hemolymph was obtained via cardiac puncture using sterile 16 gauge needles (BD Microlance 3). Extracted hemolymph was centrifuged immediately at 500 × *g* for 5 min at 4 °C to remove the cellular fraction. The supernatant was centrifuged at 400,000 × *g* at 4 °C for 90 min and Hc pellets were re-suspended in stabilisation buffer (5 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.5), yielding partially purified Hc. Partially purified Hc was applied to a Sephacryl S-500 HR (120 cm × 1.6 cm) gel filtration column (GE Healthcare), previously equilibrated with Stabilisation Buffer. The gel filtration column was calibrated using Blue Dextran (2 MDa), thyroglobulin (670 kDa) and apoferritin (450 kDa). The protein concentration was determined by UV absorbance mea-

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doi:10.1016/j.dci.2010.08.015

surements at 280 nm, using the value of 1.39 for the absorbance of a 1 mg/mL solution of Hc from *L. polyphemus*, in a quartz cuvette with pathlength 1 cm. Purified Hc was characterised by 280 nm: 350 nm absorption ratio values and by SDS-PAGE. Fractions with a 280 nm:350 nm absorption ratio value of 4.2, indicative of oxyhemocyanin, were pooled and analysed by SDS-PAGE (4–12% NuPAGE-Novex, Bis-Tris gels (Invitrogen)). Peptide mass fingerprinting (Fingerprints Proteomics Facility, University of Dundee) was used to confirm the identity of protein species separated by SDS-PAGE.

2.2. Preparation of phospholipids

L-α-Phosphatidyl-L-serine from Soybean (P0474), L-α-phosphatidylinositol (P0639) and 1-palmitoyl-sn-glycero-3-phosphocholine (L5254) were purchased from Sigma–Aldrich Chemical Company Ltd. Phospholipid stock solutions were prepared in 100 mM Tris–HCl, pH 7.5 at concentrations of 1 mg/mL. Re-suspended phospholipids were placed in a water bath and sonicated (Decon FS 200 frequency sweep) at room temperature for no more than 1 h. Sonicated phospholipids were stored at 4 °C under nitrogen gas. Phospholipid solutions were passed through 0.1 μm pore syringe filters (Anotop 10, Whatman) 6 times in order to remove large unilamellar vesicles. Phospholipids were added to PO assays to the following final concentrations: PS at 0–20 μg/mL (0–25.6 μM), PI at 10 μg/mL (11.7 μM) and LPC at 10 μg/mL (20.2 μM).

2.3. Dynamic light scattering

All dynamic light scattering measurements were recorded at 20 °C on a Malvern, Zetasizer Auto Plate Reader (50 mW 830 nm Laser). A 50 μl sample of 10 μg/mL phospholipid, in100 mM Tris–HCl, pH 7.5, was placed in a single unit on a 384 well plate. Similar conditions were used to analyse Hc at 1 mg/mL, in 100 mM Tris–HCl, pH 7.5, in the absence and presence of phospholipids (final concentration of 10 μg/mL). Particle size measurements were recorded using 13 scans of 10 s duration over a period of approximately 10 min. Equipment was cleaned using 6 M Guanidine HCl, 10% Decon, 0.1 M HCl and 0.1 M NaOH prior to use.

2.4. Phenoloxidase assay measurements

Spectrophotometric determination of phenoloxidase activity was carried out at 20 °C in a 96-well plate (MDS VERSA max microplate reader). Each assay (100 μl volume) consisted of 2 mM dopamine hydrochloride in 100 mM Tris–HCl, pH 7.5 and *Limulus* Hc at a final concentration of 1 mg/mL. Hc was pre-incubated for 10 min with phospholipids at concentrations ranging from 0 to 20 μg/mL or with the anionic detergent SDS at a concentration of 3.5 mM. PO activity was initiated by the addition of dopamine. PO activity was detected by observing an increase in absorbance at 475 nm arising from the formation of dopachrome and its derivatives. One unit is defined as 1 μmol of dopachrome formed per minute, with an absorption coefficient for dopachrome at this wavelength of 3600 M^{-1} cm^{-1}.

2.5. Circular dichroism

CD spectra were recorded on a Jasco J-810 spectropolarimeter at 20 °C. 1S-(+)-10-camphorsulphonic acid was used to calibrate the spectropolarimeter. All spectral measurements were carried out using 100 mM sodium phosphate buffer, pH 7.5. In all far and near UV experiments, each measurement was corrected by the subtraction of a spectrum of buffer alone.

Spectra in the far UV region (180–260 nm) were recorded in quartz cylindrical cells of pathlength 0.02 cm at a protein concentration of either 0.3 mg/mL, or 0.4 mg/mL. Data were analysed over the wavelength range 195–240 nm with DICHROWEB, using SELCON 3 and protein reference 3 to determine the secondary structure content. Samples of Hc (0.3 mg/mL), in the presence of 10 μg/mL phospholipids or 3.5 mM SDS, were pre-incubated for 10 min before CD measurements were recorded. In each case, 4 scans were recorded (and averaged) at a scan rate of 10 nm/min with a time constant of 2 s. Samples of Hc (0.4 mg/mL) were pre-incubated for 10 min in the presence of increasing concentrations of PS (0–20 μg/mL) before CD measurements were recorded. In each case, 4 scans were recorded (and averaged) at a scan rate of 50 nm/min with a time constant of 0.5 s.

Spectra in the near UV region (250–400 nm) were recorded in a quartz rectangular cell of pathlength 1 cm using a protein concentration of 0.4 mg/mL. Samples of Hc (0.4 mg/mL) were pre-incubated for 10 min in the presence of increasing concentrations of PS (0–20 μg/mL) prior to CD measurements. In each case 1 scan was recorded at a scan rate of 10 nm/min with a time constant of 2 s.

2.6. Fluorescence spectroscopy

All experiments were recorded on a Perkin Elmer LS50 spectrofluorimeter at 20 °C. Intrinsic tryptophan fluorescence was recorded using a quartz cuvette of 1 mL capacity at a protein concentration of 0.1 mg/mL in 100 mM sodium phosphate buffer, pH 7.5. The excitation wavelength used was 290 nm with a 5 nm bandwidth for the excitation and emission. Increasing concentrations of PS (0–20 μg/mL) or SDS (3.5 mM) were incubated with Hc for 10 min prior to fluorescence measurements. All scans were recorded at a rate of 50 nm/min and corrected by the subtraction of a spectrum of buffer alone. Control experiments indicated that PS and SDS made no contribution to the fluorescence signals.

2.7. Absorption spectroscopy

Absorption spectra of Hc samples were recorded on an Ultrospec 2100 *pro* UV/Visible spectrophotometer over the range of 240–380 nm. The properties of the copper binding site of Hc were monitored via the absorption peak at 340 nm which is characteristic of type three copper proteins. The effects of increasing concentrations of PS (0–20 μg/mL) and SDS (0–3.5 mM) on absorption spectra were determined by incubating 0.3 mg/mL Hc for 10 min prior to absorption spectra measurements.

2.8. Statistical analysis

All phenoloxidase enzyme assays were performed on three independent occasions. Results are expressed as the mean ± standard error. Assays were analysed via a single variance ANOVA test. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Purification of hemocyanin from L. polyphemus

The success of Hc purification was judged by the 280 nm:350 nm absorption ratio values (Fig. 1A) and by SDS-PAGE (Fig. 1B). A 280 nm:350 nm absorption ratio value of 4.2 was obtained, which is characteristic of type 3 copper proteins and reflects values previously obtained in similar studies (Zlateva et al., 1996). SDS-PAGE indicated the presence of Hc subunits with a molecular mass of 70 kDa and 72 kDa (identity confirmed by peptide mass fingerprinting), with little sign of degradation or presence of contaminants. Typically, we purified 200 mg of Hc from 10 mL of hemolymph. Dynamic light scattering experiments produced a calculated radius of gyration for the purified *L. polyphemus* Hc of 7.46 nm, suggesting

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