Lipopolysaccharide interaction with hemolin, an insect member of the Ig-superfamily

Sirlei Daffre[a], Ingrid Faye[b,*]

[a]*Departamento de Parasitologia, Instituto Ciencias Biomedicas, Universidade Sao Paulo, CEP 05508-900 Cidade Universitaria, Sao Paolo, Brazil*
[b]*Department of Genetics, Stockholm University, S-10691 Stockholm, Sweden*

Received 18 February 1997

Abstract This study is an attempt to reach some understanding of how insects recognize intruding microorganisms and foreign entities while executing an immune response. We used lipopolysaccharide (LPS) from *Escherichia coli*, bound to a radiolabeled iodinated crosslinker, to identify hemolymph proteins from the *Hyalophora cecropia* moth that have the capacity to bind LPS. High amounts of radioactivity were conferred to hemolin, an immunoglobulin and NCAM-related protein, the concentration of which increases in insect hemolymph upon bacterial infection. We could demonstrate a concentration-dependant binding of hemolin to LPS. In addition we could show that Lipid A can compete for this binding, whereas KDO has no effect, indicating that hemolin interacts specifically with the Lipid A moiety of LPS.

Key words: Hemolin; Lipopolysaccharide; Insect immunity; Photoaffinity labeling

1. Introduction

It is a common notion that insects exhibit a potent antibacterial defence against bacteria that involves the induction of a number of genes, typically encoding bactericidal and bacteriostatic proteins and factors regulating these proteins [1]. However, the recognition events triggering the onset of this response are basically unknown. *Hemolin* is so far the only Ig-superfamily gene that is up-regulated during the immune response and has been an interesting candidate for studies of different recognition events [2].

Hemolin has been identified and cloned in two moths, *Hyalophora cecropia* and *Manduca sexta* [2,3]. It is present in low amounts in the hemolymph of naive insects and after bacterial infection the concentration increases 18-fold in *H. cecropia* pupae and 30–45-fold in *M. sexta* larvae, indicating a functional requirement during the course of an infection [3,4]. However, hemolin has no direct antibacterial activity [5]. The intriguing fact that hemolin belongs to the Ig-superfamily and is up-regulated by infection has encouraged many attempts to understand its functions. Sun et al. [2] demonstrated by in vitro studies that hemolin binds to bacteria on its own and in complex with a 125 kDa hemolymph protein and is thus regarded a natural candidate for recognition of foreign entities. Moreover, Ladendorff et al. [3] found that hemolin from *Manduca sexta* larvae attaches to hemocytes and that this binding is accompanied by inhibition of hemocyte aggregation [3]. Recently, Lanz-Mendoza et al. [6] developed an assay to study this hemocyte aggregation and showed that the aggregation, stimulated by PMA or LPS, is prevented by hemolin in a dose-dependent fashion.

In this work we first investigate the binding of proteins from a 0–40% ammonium sulfate fraction of cell-free hemolymph to bacterial LPS. One of the proteins binding was partially purified and shown to be hemolin. Recombinant hemolin was used in the further experiments and clearly shown to bind to LPS in a concentration-dependent manner. The binding is conferred through the Lipid A moiety of the LPS molecule, as indicated by competition experiments.

2. Materials and methods

2.1. Insects and hemolymph fraction

Diapausing pupae of the giant silk moth *Hyalophora cecropia* were obtained commercially from North American dealers. The pupae were injected with *Enterobacter cloacae* β12 [7] and the hemolymph was collected directly into a polystyrene tube containing few crystals of phenylthiourea. Hemocytes were removed from the hemolymph by centrifugation at 200×*g* for 15 min. Saturated ammonium sulfate was added to the hemolymph plasma to 40% of saturation and the solution was kept on ice for 1 h. The supernatant (hemolymph fraction) was recovered by centrifugation at 14000×*g* for 10 min at 0°C, desalted over a PD10 column (Pharmacia), and sodium phosphate buffer (pH 7.4) was added to a final concentration of 21 mM.

2.2. Purification of hemolin

Hemolin was obtained by chromatography using FPLC (Pharmacia). Hemolymph was collected in anticoagulant Ringer [8], dialyzed against 10 mM phosphate buffer (pH 6.5) containing 0.15 M NaCl, and applied to a gel filtration column (Superose 12) equilibrated with the same buffer. The fractions containing hemolin were pooled, dialyzed against 10 mM phosphate buffer (pH 6.5) and applied to an ion exchange column (Mono S), equilibrated with the same buffer. Elution was performed with a gradient from 0 to 1 M NaCl. The hemolin was eluted between 0.15 and 0.18 M. The presence of the hemolin was detected by autoradiography after SDS-PAGE electrophoresis of the eluted proteins incubated with [^{125}I]ASD-LPS (see below).

2.3. Recombinant hemolin

Recombinant hemolin was produced using the baculovirus expression system and purified as described (Gunne et al., unpublished data), using a column C 10/20 packed with CM-Sepharose CL-6B (Pharmacia).

2.4. Photoactivatable iodinated LPS

Photoactivatable iodinated *E. coli* 055:B5 LPS (Sigma; ([^{125}I]ASD-LPS) was prepared as described by Wollenweber et al. [9] with some modifications. The LPS solution in water (2 mg/ml) was incubated in a water bath at 37°C for 60 min, with intermittent vortexing, followed by sonication for 3 min (model MS 200, Thornton). Sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3′-dithiopropionate (SASD; Pierce Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO; 100 μg/μl) and 4 μl was added to 0.5 ml of LPS solution, followed immediately by borate buffer (500 μl, 0.1 M, pH 8.5). The reaction was sonicated for 3 min and incubated at room temperature for 20 min. An additional 4 ml of SASD solution was added and the reaction was kept for 30 min more. LPS coupled to SASD was dialyzed against PBS (20 mM phosphate buffer (pH 7.2), containing 0.15 M NaCl)

*Corresponding author. Fax: (46) 8-164315.
E-mail: Ingrid.Faye@genetics.su.se

PII S0014-5793(97)00397-9

overnight at 4°C and stored aliquoted (100 μl) at −20°C. Iodination of LPS-SASD (100 μl) was carried out using iodo-beads (Pierce Chemical Co.) according to the manufacturer's instructions. The radioactivity was counted in a γ-spectrometer and the approximate specific activity of [^{125}I]ASD-LPS in PBS obtained was between 2.8×10^8 and 1.8×10^9 cpm/mg. All reactions with photosensitive compounds were carried out under reduced light using a red-light source.

2.5. Detection of LPS-binding protein

For the reactions, hemolymph fraction, partial purified hemolin, or recombinant hemolin samples were mixed with [^{125}I]ASD-LPS, incubated for 30 min at room temperature, then irradiated with long-wave length UV light (366 nm) for 10 min. After photo-crosslinking, the samples were reduced with 2.5× SDS-PAGE sample buffer [9] and applied to SDS-PAGE. SDS-PAGE was performed as described [10]. Gels were stained with 0.12% Coomassie Blue R-250, 50% methanol, and 10% acetic acid and destained with 30% methanol and 7% acetic acid. The gels were dried and autoradiographed to detect radio-iodinated LPS-binding proteins. The autoradiographs were carried out using Kodak X-Omat XK1 film and one Du Pont Cronex Lightning Plus intensifying screen, at −70°C. The densitometry of the labeled hemolin in the autoradiographs was done in Flying-spot Densitometer.

2.6. Western blot analysis

The Western blotting was performed as described [11] using anti-rabbit IgG-alkaline phosphatase conjugate. Proteins applied to SDS-PAGE were electrophoretically transferred to nitrocellulose membranes and hemolin was detected by the incubation of the membranes with anti-hemolin rabbit serum at a dilution of 1:5000.

3. Results and discussion

The photoaffinity labeling can be used to determine target molecules, which can be cross-linked to specific ligands under physiological conditions in the presence of suitable light. We have chosen the hetero-bifunctional crosslinker 'SASD' [9] which is reactive only when exposed to ultraviolet or visible radiation. It can be ^{125}I-iodinated with high specific activity and has a disulfide bond near to the end to which the molecular probe of interest, in this case LPS, is coupled. By photoactivation and cleavage by a reducing reagent, the target protein(s) interacting with the probe end up with the radioactive label attached. The labeled protein can be easily identified in a SDS-PAGE by autoradiography. Wollenweber et al. [9] have shown that [^{125}I]ASD-LPS selectively binds to bovine serum albumin from a mixture of biologically relevant macromolecules. This method has since been proven useful in the characterization of LPS- and peptidoglycan-binding proteins on the murine lymphoid cell populations. Several groups have demonstrated that LPS binds selectively to the S2 subunit of Pertussis toxin using the same crosslinker [12–15].

To test the binding of LPS to hemolin or other hemolymph proteins from *Hyalophora cecropia*, we first tested the 40% ammonium-sulfate-soluble fraction of the hemolymph for binding to LPS by the above method. The Coomassie stained SDS-PAGE and its subsequent autoradiography analysis is shown in Fig. 1A. The proteins with ^{125}I-label were observed in three main bands in the gel, one near the beginning of the running gel one at 66 kDa and one at 50 kDa. In the absence of hemolymph (Fig. 1A, lane 3), some photo-cross-linking of LPS aggregates can be seen. The higher molecular mass band and the band at 66 kDa were judged to be subunits of the lipophorin [16]. We could confirm this by purifying lipophorin by ultracentrifugation in potassium bromide gradient [16] followed by incubation with [^{125}I]ASD-LPS (result not shown). We did not test the specificity of binding of lipophorin to LPS. It is likely, however, that the LPS binding to apoliphorin is due to its properties as a lipid carrier [17].

Kato et al. have studied the apolipophorin binding of LPS in the silkworm *Bombyx mori* and have shown lipohorin forms a complex with LPS. They suggested that the complex formation inactivates LPS, since it also reduces cecropin inducibility by LPS. It is known that a similar serum lipoprotein–LPS complex occurs in mammals. Perhaps this complex is a common pathway for LPS detoxification in both insects and mammals [18].

The major label by ^{125}I was detected, however, in the polypeptide of around 50 kDa (Fig. 1A, lane 2) which we suspected to be hemolin. To test this hypothesis, this polypeptide was partially purified (to ca. 80%) by chromatography, incubated with [^{125}I]ASD-LPS and submitted to Western blot using an anti-hemolin antiserum (Fig. 1B, lanes 1 and 2). The

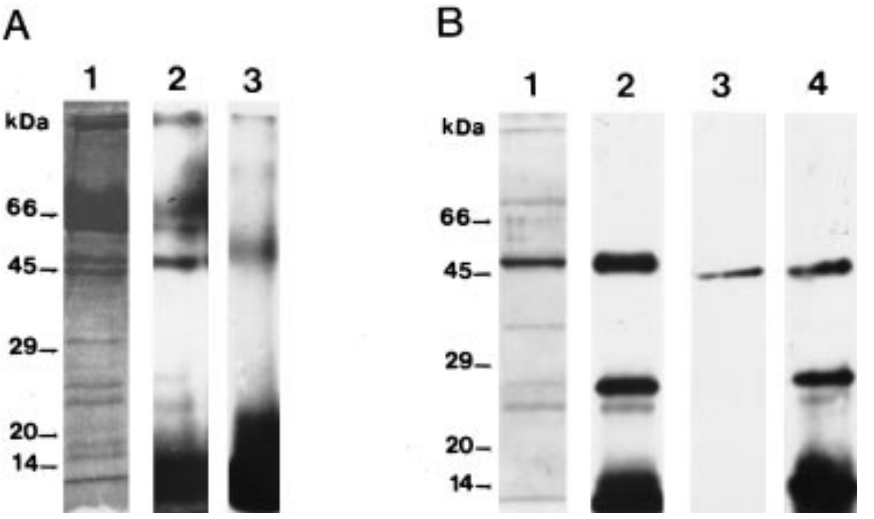

Fig. 1. Incubation of soluble fraction at 40% ammonium sulfate of the hemolymph (A) and enriched hemolin (B) with [^{125}I]LPS-ASD. The proteins were submitted to SDS-PAGE (10% acrylamide), stained with Coomassie Blue R (lanes 1A and 1B), and autoradiographed (lanes 2A and 2B). The autoradiography of [^{125}I]LPS-ASD is shown in lane 3A. Western blot of enriched hemolin fraction incubated with [^{125}I]LPS-ASD using serum against hemolin (lane 3B). The nitrocellulose filter shown in lane 3B was autoradiographed (lane 4B).

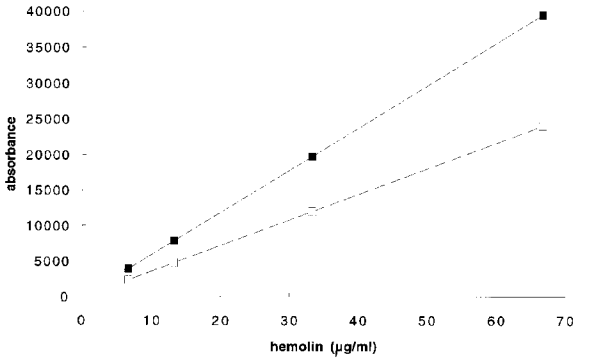

Fig. 2. Interaction of the recombinant hemolin with [^{125}I]LPS-ASD and inhibition of binding by underivatized LPS. [^{125}I]LPS-ASD (7.7 mg/ml) was added to hemolin (6.7–67 mg/ml) in a final volume of 30 μl, in an absence (filled square) or presence (open square) of underivatized LPS (187× excess). The samples were submitted to SDS-PAGE (12% acrylamide) and autoradiography. The densitometry of the hemolin band in the autoradiography was determined.

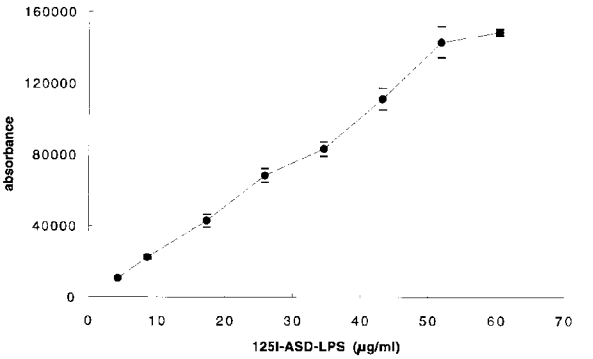

Fig. 3. Dose dependence of the interaction of recombinant hemolin with [^{125}I]LPS-ASD. Hemolin (25 μg/ml) was added to [^{125}I]LPS-ASD (4.3–60.5 μg/ml) in a final volume of 40 μl PBS. After UV light irradiation and PAGE-SDS, the densitometry of the hemolin band in the autoradiograph was determined. Each plotted value represents the mean of three separate experiments and the error bars indicate 1 SEM.

results in Fig. 1B, lane 3, show that hemolin antiserum recognizes the polypeptide around 50 kDa (Fig. 1, lane 2). The nitrocellulose filter was autoradiographed after the detection of hemolin by antiserum and ^{125}I-labeling observed in the same position as hemolin (lane 4). Through the purification process, a 26 kDa protein was also enriched, that gave a strong signal for LPS binding (Fig. 1A,B, lane 2). We decided to investigate if this polypeptide could be attacin, another *H. cecropia* immune protein, of which the molecular mass is estimated at 23 kDa. This idea came from the fact that Carlsson et al. [19] have indications that attacin, inhibiting the synthesis of certain outer membrane proteins in *Escherichia coli*, can interact with LPS. In addition, our purification schedule is similar to the one used for purification of attacin [20]. Our results, however, excludes this possibility since attacin antiserum could not recognize the 26 kDa polypeptide that bound to [^{125}I]ASD-LPS.

As the major radioactivity could be conferred to hemolin (Fig. 1A, lane 2) we decided to study its specificity of binding. For this purpose we used a recombinant form hemolin, produced in a Baculovirus system, in different competition experiments. Various amounts of hemolin were mixed with [^{125}I]LPS-ASD in the absence or presence of underivatized LPS in excess. After an incubation and photo-crosslinking procedure, the final samples were subjected to SDS-PAGE and analysed after Coomassie staining and/or autoradiography. The densitometry (absorbance units) of the labeled hemolin were plotted against the underivatized LPS added (μg/μl). The results in Fig. 2 demonstrate that the binding of [^{125}I]LPS-ASD to hemolin can be inhibited with underivatized LPS in a dose-dependent manner. However, even at 187× excess of underivatized LPS, only around 40% inhibition could be achieved. This could be due to the fact that LPS easily forms aggregates and it is therefore difficult to get the expected effect of large amounts. We also demonstrated that the binding is specific for the LPS molecule rather than the ASD cross-linking group, since the binding of [^{125}I]LPS-ASD to hemolin can be inhibited in a dose-dependent manner with underivatized LPS as well as purified Lipid A (see below).

In the next series of experiments we used increasing amounts of [^{125}I]LPS-ASD in our assays. Fig. 3 shows a concentration dependence in the interaction of 25 μg/μl hemolin mixed with various amounts of [^{125}I]LPS-ASD. The reaction mixture was incubated and handled as described above. The densitometry (absorbance units) of the labeled hemolin was plotted against [^{125}I]ASD-LPS (μg/ml) The results show that the binding of [^{125}I]ASD-LPS to hemolin is directly proportional to the amount of the LPS added; therefore, the binding is concentration dependent. The curve shows a tendency of saturation. The points fit well to a logarithm curve, considering statistical parameters. Also, the rate of binding within the two amounts of LPS is different; in higher amounts of LPS, the binding rate is lower, which also indicates the tendency of saturation.

The initial studies on hemolin function pointed to its affinity for bacterial surfaces [2,3]. These studies were extended and hemolin was found to bind to *E. coli* mutants that lack the carbohydrate core of LPS [21]. Recently, Zhao and Kanost [22] showed that the binding of *Manduca sexta* hemolin to bacteria is inhibited by competition with unlabeled hemolin, suggesting that this binding is specific.

In the next experiment we decided to investigate which LPS component interacts with hemolin. Based on the results from experiments with LPS mutants of *E. coli* [21], we tested the ability of unlabeled Lipid A and KDO to compete with LPS for the binding to hemolin. [^{125}I]LPS-ASD was mixed with

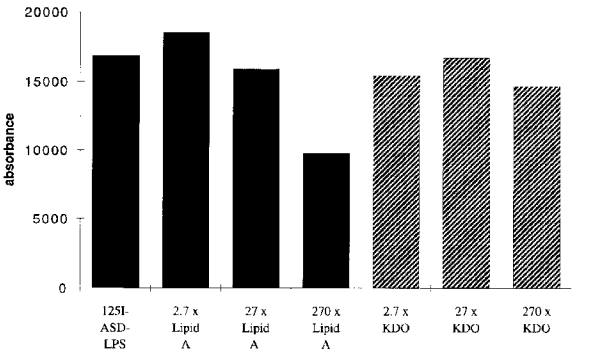

Fig. 4. Competition of the binding of [^{125}I]LPS-ASD to recombinant hemolin by two components of the LPS molecule, Lipid A, and KDO. Hemolin (25 μg/ml) was added to [^{125}I]LPS-ASD (8.6 μg/ml) containing Lipid A (2.7–270× excess), KDO (2.7–270× excess), or the probe alone ([^{125}I]LPS-ASD).

recombinant hemolin and excess Lipid A or KDO. The reaction mixture was incubated and handled as described above. The results show that purified Lipid A inhibits [^{125}I]LPS-ASD from binding to hemolin (≈42% of inhibition), suggesting that the binding of LPS to hemolin is mediated through the Lipid A moiety of the LPS molecule (Fig. 4). Although we cannot exclude the possibility that others bacterial-surface-like components, such as carbohydrates or peptidoglycans, are important for the binding of hemolin, our results indicate that hemolin may be a putative LPS recognition molecule participating in some step of the immune response in the moth *Hyalophora cecropia*.

Acknowledgements: This work was supported by grants from Fundacao de Amparo à Pesquisa do Estado de Sao Paulo (FASESP) and Conselho Nacional de Desenvolvimento Cientifico e Technológico (CNPq) to S.D., and by grants from the Swedish Natural Science Research Council, B-AA/BU 03773-316 to I.F.

References

[1] Faye, I. and Hultmark, D. (1993) in Parasites and pathogens of insects, pp. 25-53, Academic Press, San Diego, CA.
[2] S.-C. Sun, I. Lindström, H.G. Boman, I. Faye, O. Schmidt, Science 250 (1990) 1729–1732.
[3] N.E. Ladendorff, M.R. Kanost, Arch. Insect. Biochem. Physiol. 18 (1991) 285–300.
[4] K. Andersson, H. Steiner, Insect. Biochem. 17 (1987) 133–140.
[5] T. Rasmuson, H.G. Boman, Insect. Biochem. 9 (1979) 259–264.
[6] H. Lanz-Mendoza, R. Bettencourt, M. Fabbri, I. Faye, Cell. Immunol. 169 (1996) 47–54.
[7] I. Faye, A. Pye, T. Rasmuson, H.G. Boman, I.A. Boman, Infect. Immun. 12 (1975) 1426–1438.
[8] T. Trenczek, I. Faye, Insect. Biochem. 18 (1988) 299–312.
[9] H.-W. Wollenweber, D.C. Morrison, J. Biol. Chem. 260 (1985) 15068–15070.
[10] U.K. Laemmli, Nature 227 (1970) 680–685.
[11] Harlow, E. and Lane, D. (1988) Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, USA.
[12] M.-G. Lei, D.C. Morrison, J. Immunol. 141 (1988) 996–1005.
[13] R. Dziarski, J. Biol. Chem. 266 (1991) 4713–4719.
[14] R. Dziarski, J. Biol. Chem. 266 (1991) 4719–4725.
[15] M.-G. Lei, D.C. Morrison, J. Biol. Chem. 268 (1993) 1488–1493.
[16] J.P. Shapiro, P.S. Keim, J.H. Law, J. Biol. Chem. 259 (1984) 3680–3685.
[17] B.J. Blacklock, R.O. Ryan, Insect. Biochem. Mol. Biol. 24 (1994) 855–873.
[18] Y. Kato, Y. Motoi, K. Taniai, K. Kadono-Okuda, M. Yamamoto, Y. Higashino, M. Shimabukuro, S. Chowdhury, J. Xu, M. Sugiyama, M. Hiramatsu, M. Yamakawa, Insect. Biochem. Mol. Biol. 24 (1994) 547–555.
[19] A. Carlsson, P. Engström, E.T. Palva, H. Bennich, Infect. Immun. 59 (1991) 3040–3045.
[20] D. Hultmark, A. Engström, K. Andersson, H. Steiner, H. Bennich, H.G. Boman, EMBO J. 2 (1983) 571–576.
[21] O. Schmidt, I. Faye, I. Lindström-Dinnetz, S.-C. Sun, Dev. Comp. Immunol. 17 (1993) 195–200.
[22] L. Zhao, M.R. Kanost, J. Insect Physiol. 42 (1995) 73–79.