Helip, a Heme Lipoprotein from the Hemolymph of the Cattle Tick, *Boophilus microplus*" 

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The main protein of the hemolymph of the cattle tick *Boophilus microplus* has been isolated and shown to be a heme lipoprotein (HeLp). HeLp has an apparent molecular mass of 354,000 and contains two apoproteins (103 and 92 kDa) found in equal amounts. HeLp presents a pI of 5.8 and a density of 1.28 g/ml and contains 33% lipids, containing both neutral lipids and phospholipids, and 3% of sugars. A remarkable feature of HeLp is the abundance of cholesterol ester (35% of total lipids), a lipid not previously reported in invertebrate lipoproteins. Western blot analysis showed HeLp in hemolymph from adult females and males, but not in eggs. Although HeLp contains 2 heme molecules, it is capable of binding 6 additional molecules of heme. *Boophilus* feeds large amount of blood, and we recently showed that this tick is unable to perform *de novo* synthesis of heme (Braz, G. R. C., Coelho, H. S. L., Masuda, H., and Oliveira, P. L. (1999) *Curr. Biol.* 9, 703–706). Injection of tick females with 55Fe-labeled heme-HeLp indicated that this protein transports heme from hemolymph to tissues. HeLp is suggested to be an essential adaptation to the loss of the heme synthesis pathway.

Heme is an ubiquitous molecule that takes part in several fundamental biochemical reactions such as respiration, oxygen transport, photosynthesis, and lipid desaturation (1). In contrast to all these beneficial features, heme is a powerful catalyst of the formation of reactive oxygen species, mainly by means of Fenton type reaction (2, 3). In fact, there are several reports of heme-induced oxidative damage to a broad range of biomolecules as lipoproteins, which are especially susceptible to lipid peroxidation driven by heme (4–6). Due to its potential toxicity, heme is always found associated with proteins, such as hemopexin and albumin, and several antioxidant mechanisms have been developed to protect cells (7–11). As described in the literature, heme transport and recycling does not occur in eukaryotic cells where the heme delivered to cells is degraded by heme oxygenase (1, 12).

Hematophagous arthropods usually ingest large amounts of vertebrate blood, comprising several times their own weight (13). The blood-sucking insect *Rhodnius prolixus* has a heme-binding protein in the hemolymph that protects the insect from heme induced lipid peroxidation (14, 15). In the case of cattle tick, *Boophilus microplus*, blood is the sole food source for all developmental stages and, during the last 48 h before dropping from the host, adult females may ingest as much as 100 times their weight in blood (16). We recently showed that *Boophilus* is an exception to the generally accepted statement that all eukaryotic cells synthesize their own heme (17). This tick does not have a functional heme synthesis pathway and thus must rely exclusively on the heme obtained from digestion of the vertebrate blood in order to make its own heme-proteins (18).

The heme biosynthetic pathway is also defective in some pathogenic bacteria that depend on host blood as a heme source, and proteins involved in heme transport have an essential role in the recovery of heme (19). The dependence of the tick on heme from its diet requires the development of mechanisms for heme absorption, transport, and recycling, which have not been described for any other multicellular organisms. In this article we describe the isolation and characterization of a major heme-lipoprotein (HeLp), from the hemolymph of the cattle tick, which is capable of binding additional heme and transport it to tick tissues.

**EXPERIMENTAL PROCEDURES**

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Fig. 1. HeLp purification. Hemolymph (1 ml) was applied to a DEAE-Toyopearl column (A) and eluted with a NaCl gradient (0–0.3 M). Fractions containing HeLp (indicated by a bar) were applied to IDA-Cu$^{2+}$ column (B) eluted with a glycine gradient (0–0.3 M). Crude hemolymph (lane 1), fractions of DEAE column (lane 2) and IDA-Cu$^{2+}$ fractions (lane 3) were applied to a native pore-limit PAGE (C) or to a SDS-PAGE (D). Molecular masses of markers are indicated in both panels at left. Native HeLp (mass 354 kDa) (C) and apolipoproteins (apoHeLp-A (mass 103 kDa) and apoHeLp-B (mass 92 kDa)) (D) are indicated at right.

fractions containing HeLp (identified by absorbance at 400 nm and by SDS-PAGE)$^1$ were pooled, diluted 10 times in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4), and applied to an agarose-iminodiacetic acid metal affinity column (Sigma) loaded with CuSO$_4$ (IDA-Cu$^{2+}$). After elution with a glycine gradient (0–300 mM), fractions (1.5 ml) containing HeLp were pooled and dialyzed against PBS. Protein concentrations were measured according to Lowry et al. (20) using bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis—SDS-PAGE was carried out in 5–22.5% acrylamide gradient gels (21). Gels were run at 120 V at room temperature for approximately 90 min, and stained with Coomassie Brilliant Blue G, according to Neuhoff et al. (22). The molecular mass of polypeptides in SDS-PAGE was determined using the following proteins as standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.2 kDa). Pore-limiting native PAGE was performed as described by Blanche et al. (23), using 5–22.5% acrylamide gradient gels, and the electrophoresis was performed for 20 h. The native molecular mass of was determined using the following proteins as standards: tigrogenulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa).

NH$_2$-terminal Sequence and Amino Acid Analysis—For NH$_2$-terminal sequencing, purified HeLp (0.5 nmol) was submitted to SDS-PAGE (7.5%) and transferred to a polyvinylidene difluoride membrane (24). The polypeptides in SDS-PAGE were determined using the following proteins as standards: tireoglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa).

Lipids—Purified HeLp (2–4 mg of protein) was dialyzed against water, dried in a vacuum centrifuge (Speed-Vac, Savant SC110) and weighted. Lipids were extracted with chloroform:methanol (1:1), containing 10 mM ammonium acetate and phosphoric acid (30%) and heating at 120 °C for 10–15 min (29). Quantification of different lipids was performed by computer scanning and analysis of stained HPTLC plates with the Quantiscan software (Biosoft, Cambridge, United Kingdom). Quantification of different lipids was performed by computer scanning and analysis of stained HPTLC plates with the Quantiscan software (Biosoft).

Carbohydrate Content—Total reducing sugar content was determined according to the method of Du Bois (30). To analyze carbohydrate composition, 2 mg of HeLp was submitted to acid hydrolysis (6 N trifluoroacetic acid, 100 °C for 5 h) and sugars were fractionated by paper chromatography using butanol/pyridine/water (3:2:1, v/v) as solvent (31).

Absorption Spectra and Heme Content—Light absorption spectra of native HeLp were obtained in PBS using a GBC-920 spectrophotometer (Victoria, Australia). Heme content was determined from the reduced minus oxidized spectra of the pyridine alkaline derivative (32).

Density Determination—Density of the HeLp particle was determined by KBr gradient (1.16–1.35 g/ml, 10 ml) ultracentrifugation using a 50Ti Beckman rotor (20 h at 4 °C, 45,000 rpm) using the methods described by Shipman et al. (33). Refraction index and absorbance at 280 nm of the gradient fractions were determined, and samples were submitted to a SDS-PAGE.

Isoelectric Focusing—Isoelectric point of HeLp was determined in a pre-cast isoelectric focusing gel (Ampholine PAGplate, pH 3.5–9.5, Amersham Pharmacia Biotech, Uppsala, Sweden).

Electrospray Mass Spectrometry (ES-MS)—Samples used for mass spectrometry analysis were total lipid extracts or lipids eluted from the HPTLC plate (with chloroform:methanol, 1:1). Lipids were dissolved in chloroform:methanol (1:1), containing 10 mM ammonium acetate and 0.1% formic acid. Spectra were obtained in a Finnigan LCQDuo ion trap mass spectrometer (Finnigan, ThermoQuest Inc., San Jose, CA). Samples were introduced into the electrospray source by injection through a fused silica capillary at a flow rate of 5 μl/min. ESI capillary voltage was set to 36–46 V, and temperature to 250 °C. Spectra were acquired at 3 s/scan over a mass range of m/z 150–2000. Collision-induced (ES-MS/MS) fragmentation of parent ions was carried at relative collisional energy from 25 to 35 V. Source parameters were optimized using cholesterol, cholesterol olate, triacylglycerol (triolein), phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-

$^1$The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphorylcholine; ES, electrospray; MS, mass spectrometry; HPTLC, high performance thin layer chromatography; IDA-Cu$^{2+}$ column, agarose-iminodiacetic acid metal ion affinity column loaded with CuSO$_4$.
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serine standards at 7–70 pmol/μl each, all purchased from Sigma.

Antibodies and Immunological Assays—HeLP (1 mg) was emulsified with an equal volume of Freund’s complete adjuvant and injected subcutaneously in the back of a rabbit. Boosters of 0.5–1.0 mg were injected at 2-month intervals, and blood was collected from an ear vein 3 months after the first injection. Western blots were performed according to Towbin et al. (34).

Heme Binding Assays—Binding of heme to HeLP (1 μM, 1 ml) was followed by adding a solution of 0.5 mM hemin in 0.01 M NaOH and following the absorbance at 411 nm, the hemin isosbestic point (35). Hemin bound to HeLP has a higher molar extinction coefficient than heme in aqueous buffer. Plotting the absorbance against the amount of added heme reveals binding as a steeper initial part of the curve, and saturation of binding sites is determined by the intersection with a second component of the curve, which is parallel to hemin added to buffer. Alternatively, HeLP can be observed in a native PAGE gel without staining, due to its heme content. This allowed direct visualization of binding by adding 1 mM hemin in 0.1 M NaOH to purified HeLP (50 μg) or crude hemolymph (1 μl), applying the samples to a pore-limiting PAGE as described above and recording the gels with a computer scan.

Heme Transport—Radiolabeled 55Fe-heme was synthesized as described by Galbraith et al. (36). HeLP was incubated with 55Fe-heme at a 1:1 molar ratio and passed through spin columns (37). HeLP bound 55Fe-heme (5 μl, 9000 CPM) was injected into the hemocoel of female ticks, and blood was collected 2-month intervals, and radioactivity was determined by adding 1 mM hemin in 0.01 M NaOH and following the absorbance at 411 nm, the hemin isosbestic point (35). The higher molecular mass proteins in lanes 1 and 5, fully engaged female hemolymph (lanes 2 and 6), partially engaged adult female hemolymph (lanes 3 and 7), and egg homogenate (lanes 4 and 8). HeLP apoproteins are indicated at left.

RESULTS AND DISCUSSION

HeLP Purification—Isolation of HeLP was achieved by means of a DEAE-Toyopearl column, followed by chromatography in an IDA-Cu²⁺ column (Fig. 1, A and B). The native molecular mass of HeLP, determined by pore-limit PAGE, was 342 kDa (Fig. 1C), a value close to the 365 kDa obtained by gel filtration fast protein liquid chromatography (data not shown). Therefore, an average molecular mass of 354 kDa is assumed hereafter. HeLP was composed by two apoproteins of 103 kDa (apoHeLP-A) and 92 kDa (apoHeLP-B), as judged by SDS-PAGE of the purified lipoprotein (Fig. 1D). The native PAGE profile showed that HeLP is the main hemolymphatic protein (Fig. 1C), and estimation of its concentration in the hemolymph by radial immunodiffusion showed titers around 50 mg/ml throughout adult development (data not shown). The apoproteins were found in a stoichiometry of 1:1, as judged by densitometry of the gel. Fig. 2 shows the NH₂-terminal amino acid sequence of 103- and 92-kDa apoproteins, obtained by Edman degradation. Homology searches did not indicate significant similarity to known proteins. The higher molecular mass protein bands in the SDS-PAGE gel (Fig. 1D) were shown to be aggregates of both polypeptides, as protein sequencing of its NH₂-terminal portion resulted in a combination of both apoprotein sequences.

Western blot using antiserum against purified HeLP showed that HeLP was not sex-specific (Fig. 3), being found in hemolymph from males (lane 1), fully engorged females (lane 2), and partially engorged adult females (collected before dropping from cattle) (lane 3). Plasma lipoproteins from both insects and vertebrates frequently are incorporated into yolk of developing oocytes, as it is the case of avian low density lipoprotein (38) or lipophorin, the main lipoprotein of insect hemolymph (39).
However, HeLp was not found in mature Boophilus eggs by Western blot (Fig. 3, lane 4), despite the high titer present in the hemolymph.

Physical Properties and Chemical Composition—The amino acid composition of HeLp is very similar to an eukaryotic average protein (40), except for a rather low isoleucine content (Table I). HeLp isoelectric point was pH 5.8. HeLp carbohydrate content was 3% (w/w), and mannose was the most abundant component (>90%; data not shown), a feature shared with most arthropod glycoproteins, where high mannose type oligosaccharides with a low degree of trimming are the general pattern (41). Purified HeLp has approximately 33% of lipids (55% neutral lipids and 44% phospholipids) (Table II and Fig. 4A). The most unusual trait of HeLp, concerning its lipid moiety, was the presence of high amounts of cholesterol ester (34.7% of the total lipid content). Therefore, a more detailed characterization of HeLp lipid composition was performed by ES-MS and ES-MS/MS. Pseudomolecular ion species showing higher relative intensity in the positive-ion mode spectrum of ES-MS total lipid extract were assigned as cholesterol oleate (m/z 686.5 = [M + 2NH₄]+), phosphatidylcholine (m/z 780.5 = 16:2/20:4-PC, [M⁺]; m/z 782.5 = 16:0/20:4-PC, [M⁺]; m/z 788.5 = 18:0/18:1-PC, [M⁺]; m/z 808.5, 18:1/20:4-PC, [M⁺]; 810.5 = 18:0/20:4-PC, [M⁺]), phosphatidylethanolamine (m/z 748.5 = 18:0/18:0-phosphatidylethanolamine; m/z 796.4 = 18:0/22:4-phosphatidylethanolamine) and triacylglycerol (m/z 907.5 = 18:0/18:0/18:1-triacylglycerol, [M + NH₄⁺]; m/z 909.6 = 18:0/18:0/18:0-triacylglycerol, [M + NH₄⁺]; and m/z 937.3 = 18:0/18:0/20:0-triacylglycerol, [M + NH₄⁺]) (Fig. 4B). Based on previous data (42–44) and on the collision-induced dissociation daughter-ion spectra (data not shown) of the pseudomolecular ion species at m/z 668.5 and 650.3, we were unable to assign as a precisely defined oxysterol ester. A true sample of cholesterol oleate (from Sigma, 1 µl = 651.1; m/z 668.5 = [M + NH₄⁺]) was partially converted to m/z 663.4 ion species when submitted to an air stream for 2 h (data not shown). The pseudomolecular ion species at m/z 663.5 was also found in lipid extracts from crude hemolymph, and its relative intensity increased upon handling of samples during protein isolation and lipid extraction (data not shown). Therefore, the fraction isolated from HPTLC spot migrating as cholesterol ester showed only the m/z 663.4 ion species (Fig. 4B, inset). In Fig. 4C (inset), we show that the pseudomolecular ion species at m/z 668.5 ([M + NH₄⁺]) is formed from the ion species m/z 668.5 (M + 2NH₄⁺). Fragmentation of the ion species at m/z 668.5 generated cholesterol-derived daughter ion species at m/z 368.3 ([M - C₁₈H₃₆O₂ + H⁺] and 386.1 ([M - C₁₉H₃₇O₃ + H⁺]), corresponding to the cleavage at C3-O1 and C1-O1 of cholesterol, respectively (44). A lower intensity ion species corresponding to the ammonium adduct of oleic acid (m/z 300.3; [C₁₈H₃₆O₂ + NH₄⁺]) was observed, providing definitive identification of cholesterol ester in HeLp. Two other major observed fragments (m/z 467.5 = [M - C₁₈H₃₆ + NH₄⁺] and 485.5 = [M - C₁₉H₃₇ + 2NH₄⁺]) were derived from the cleavage of the oleic acid moiety, resulting in the loss of 183 mass units from the parent ions at m/z 668.3 and 650.3 (Fig. 4C). To our knowledge, this is the first report of a cholesterol ester-rich...
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A density of 1.29 g/ml was determined by KBr gradient ultracentrifugation, a value that is higher than those reported for the more dense forms of high density lipoprotein, the mammalian cholesterol ester-rich lipoprotein, which are about 1.21 g/ml and have a lipid content of about 50% (45). This observed high density would fall into the very high density lipoprotein class, according to the classification of mammalian lipoproteins.

Analysis of HeLp secondary structure by circular dichroism (data not shown) showed a spectrum that suggests a reduced amount of α-helix when compared with mammalian high density lipoprotein apoproteins (46). The HeLp CD spectrum resembles those of insect lipophorins, which are characterized by a high proportion of β-sheet (47, 48). However, despite this common feature, other aspects suggest that HeLp is a distinct particle, such as the absence of cholesterol ester in lipophorins, where diacylglycerol is the most abundant neutral lipid (49). Furthermore, lipophorins from all insect species have a very conserved quaternary structure, with one apoprotein of 220–240 kDa and another with 70–80 kDa (49), distinct from HeLp-A and HeLp-B. Nevertheless, a more detailed structural characterization of HeLp, including complete apoprotein sequence, will be necessary to exclude similarity to insect or vertebrate lipoproteins.

**Heme Content and Binding**—The most remarkable characteristic of HeLp when compared with all other known lipoproteins is the presence of about 2 mol of heme/mol of HeLp (Table II). As can be seen in Fig. 4A, there is some amount of heme that is recovered together with lipids, and is separated by the HPTLC system, but the majority remains at the aqueous phase. HeLp visible absorption spectrum was clearly that of a hemeprotein, showing a characteristic peak at 398 nm (Fig. 5). However, the HeLp Soret bandwidth (about 80 nm) was unusually large when compared with other hemeproteins such as cytochromes and hemoglobin, which are typically in the range of 40–50 nm, thus suggesting the existence of multiple quantum states of the heme molecules in HeLp. Spectral profiles similar to that of HeLp were reported for albumin-bound heme, for heme bound to phospholipid membranes, and for heme in organic solvents such as Me2SO (50, 51). Heme has been implicated in oxidative damage to membrane phospholipids and to lipoproteins such as vertebrate low density lipoprotein (4–6). Lipophorin from the blood-sucking insect *Rhodnius prolixus* also was shown to be damaged by heme-promoted oxidation (10), and, in this case, one of the antioxidant mechanisms described was a heme-binding protein that renders the heme molecule redox inactive, thus avoiding the production of free radicals.

![Fig. 6. Binding of he to HeLp.](image)

Besides having two heme molecules bound, purified HeLp was capable of binding additional heme. This can be directly observed by the intensification of the HeLp band in native page without staining when it was incubated with heme (Fig. 6A). The same experiment was carried out with crude hemolymph, showing that the binding of heme by HeLp was not an artifact generated during isolation of the protein (Fig. 6A). Moreover, only HeLp showed up in the gel after addition of heme, indicating that it is the main heme-binding protein in the hemolymph of the tick. Spectrophotometric titration of the heme-binding sites of purified HeLp revealed that each molecule of HeLp can bind to six additional molecules of heme (Fig. 6B). Binding of these extra heme molecules did not alter HeLp far UV circular dichroism spectrum (data not shown), similar to results found for binding of heme by vertebrate albumin (52). The interactions of HeLp heme with its lipid and protein moieties, as well as its possible antioxidant role, are currently under study in our laboratory.

**Heme Transport**—In mammals, the intracellular pool of heme is controlled in order to keep equilibrium between synthesis and degradation (1). However, *Boophilus microplus* is not able to synthesize its own heme; therefore, we suggest that an obligatory counterpart of tick dependence on heme from its host should be the development of efficient ways to absorb heme from the midgut and transport this molecule to tissues. In mammals, there are two proteins involved in extracellular heme transport: hemopexin and albumin (12). As HeLp is the main heme-protein in the hemolymph, it would be a candidate to act as a vehicle in transport of heme to tissues. Oogenesis is the main event occurring in *B. microplus* adult females and tick eggs contain a large amount of heme, which gives then their characteristic deep brown color (13). Therefore, HeLp labeled with 55Fe-heme was injected into the hemocoel of female ticks, and radioactivity at hemolymph and ovaries were accompanied
(Fig. 7). In panel A we observed that radioactivity quickly decreased in the hemolymph after injection, in parallel to incorporation by the ovariies (panel B). Ticks maintained at 4°C after injection did not show either ovarian uptake nor hemolymph clearance, suggesting dependence on active metabolism. Taken together, our data indicate that HeLp plays a key role in the transport and recycling of heme in the tick, featuring as a major biochemical adaptation to blood feeding. We are currently looking for intracellular proteins involved in the uptake and reutilization of heme by tick tissues. Besides elucidating an important aspect in the biology of ticks, heme reutilization mechanisms could be targets in the development of control methods specifically directed toward hematophagous animals.

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