A missing metabolic pathway in the cattle tick *Boophilus microplus*

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Heme proteins are involved in a wide variety of biological reactions, including respiration, oxygen transport and oxygen metabolism [1]. The heme prosthetic group is synthesized in almost all living organisms except for a few pathogenic bacteria and trypanosomatids that use blood as food [2,3]. There is a general belief that all nucleated animal cells synthesize heme [1,4]. However, blood-feeding arthropods ingest enormous amounts of vertebrate blood in a single meal and the heme pathway has not been studied in these animals. We have examined heme synthesis in two hematophagous arthropods – the blood-sucking bug *Rhodnius prolixus* and the cattle tick *Boophilus microplus*. We show that *R. prolixus* makes heme and has a fully operative heme biosynthetic pathway, while *B. microplus* does not. To our knowledge, this is the first report of an animal that does not synthesize its own heme and relies solely on the recovery of heme present in the diet. Because of the inability of *Boophilus* to synthesize heme and its ability to deal efficiently with large amounts of free heme, we propose this organism as a good model for studying heme transport and reutilization in animal cells.

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Received: 19 March 1999
Revised: 13 April 1999
Accepted: 17 May 1999

Published: 21 June 1999

Current Biology 1999, 9:703–706
http://biomednet.com/elecref/0960982200900703

 ISSN 0960-9822

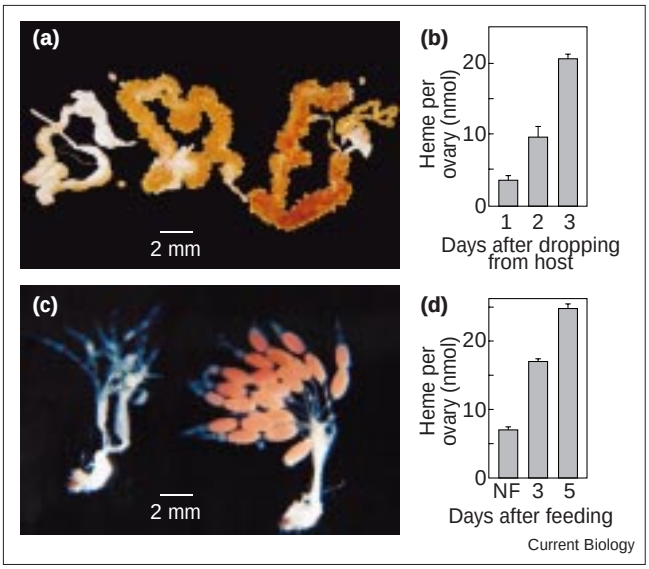
Results and discussion

A special feature of the digestion of vertebrate blood in hematophagous arthropods is the generation of enormous amounts of free heme. This led us to question how the heme biosynthetic pathway operates in these animals.

A critical metabolic stage for studying the heme biosynthetic pathway in these two arthropods is the triggering of oocyte growth (vitellogenesis) that follows a blood meal. In *R. prolixus*, adult females feed several times, with intervals set to three weeks under controlled laboratory conditions. *B. microplus* females feed only once during their life cycle. Once the adult females of both species stop feeding, they drop from the host and start oocyte development.

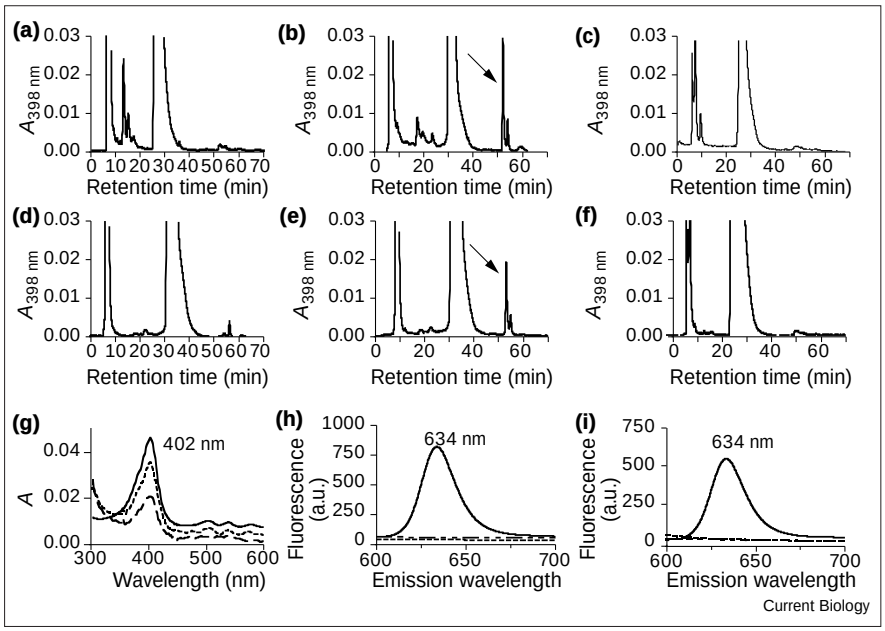
Figure 1a,c shows the striking ovarian development that occurs in each species over a period of a few days. The color of the oocytes is due to the large amounts of accumulated heme proteins. The main storage protein of tick eggs, vitellin, is a high-molecular-weight heme protein that makes up more than 80% of the protein of ovaries and eggs [5]. In *Rhodnius*, the heme is bound to a small 15 kDa polypeptide, the *Rhodnius* heme-binding protein (RHBP) [6]. Oogenesis in both species therefore depends on the synthesis of heme proteins [6,7]. Over a 3-day period the heme content of *B. microplus* ovaries reaches six to seven times its initial value (Figure 1b). A *Boophilus* female lays eggs continuously for two weeks, and before its death approximately 50% of body weight has been converted

Figure 1



Heme content and ovarian development in *Boophilus* and *Rhodnius*. In the first days of the vitellogenic phase there is a dramatic increase in the size and heme protein content of the ovaries. (a) *Boophilus* ovaries 1, 2 and 3 days (from left to right) after the female has dropped from the host. The dark brown color of oocytes is due to accumulated heme proteins. (c) *Rhodnius* ovaries from unfed females (left) and 5 days after a blood meal (right). The pink color of oocytes is due to heme protein. Histograms show the heme content of ovaries from *Boophilus* (b) and *Rhodnius* (d). NF, not fed.

Figure 2



Protoporphyrin accumulates in *Rhodnius*, but not in *Boophilus*, after ALA injection. Comparison of HPLC elution profiles of tissue extracts. In (a–f) the absorbance (*A*) scale was chosen to show the protoporphyrin peak, if present. The very high heme peak (30–32 min) is thus truncated. (a–c) Hemolymph of (a) *Rhodnius* control, (b) *Rhodnius* injected with ALA and (c) *Boophilus* injected with ALA. (d–f) Ovaries from (d) *Rhodnius* control, (e) *Rhodnius* injected with ALA and (f) *Boophilus* injected with ALA. The arrows indicate protoporphyrin peaks (52.5 min). (g) Absorbance spectra of standard protoporphyrin (solid line), the protoporphyrin peak of *Rhodnius* hemolymph extract (dotted line) and *Rhodnius* ovaries (dashed line). (h,i) Fluorescence emission spectra of the protoporphyrin peak (52.5 min) or, in the absence of a peak, the material collected at 50–55 min from hemolymph (h) and ovaries (i). Solid line, *Rhodnius* injected with ALA; dashed line, *Rhodnius* control; dotted and dashed line, *Boophilus* injected with ALA. The results shown are typical of five experiments.

into a batch of eggs containing up to 250 nmol of heme (data not shown).

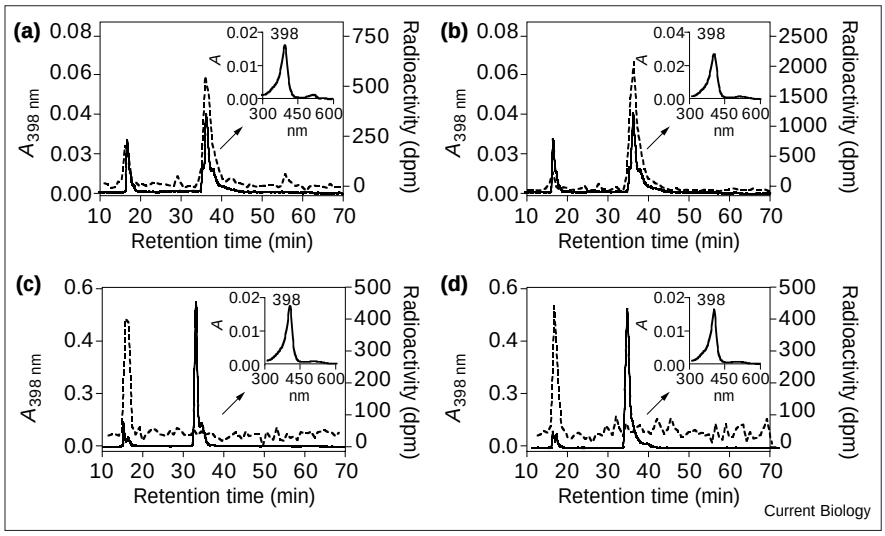
To study the heme biosynthetic pathway *in vivo* we injected females of both species with large amounts of δ-aminolevulinic acid (ALA), a heme precursor. If the heme biosynthetic pathway is active, this injection should result in accumulation of porphyrin [8], as the synthesis of ALA is the rate-limiting reaction in the pathway [4,9]. The presence of porphyrin in hemolymph and tissue extracts fractionated by high-performance liquid chromatography (HPLC) was analyzed spectrophotometrically and by fluorescence (Figure 2), because all porphyrins are fluorescent. In the chromatograms of tissue extracts from injected *Rhodnius* females, there is an additional peak at a retention time corresponding to that of protoporphyrin (52.5 min, Figure 2b,e). This peak is positively identified as protoporphyrin by its characteristic absorbance and fluorescence spectra [10] (Figure 2g,h,i). The protoporphyrin peak is absent in *Boophilus* tissue extracts (Figure 2c,f) and no fluorescent material is found in this region (50–55 min) of the chromatograms (Figure 2h,i). In other organisms, other porphyrins accumulate upon ALA administration, but in *Rhodnius* no other intermediates of the heme pathway accumulated after ALA injection.

These results show that *Rhodnius* accumulates protoporphyrin in its tissues upon ALA injection while *Boophilus* does not. From this we conclude that porphyrin biosynthesis occurs *in vivo* in *Rhodnius*. The negative result obtained with *Boophilus* means either that the pathway is not operative *in vivo* or that this animal has unusually high levels of ferrochelatase and therefore all additional porphyrin synthesized from injected ALA is converted to heme.

To distinguish between these two possibilities, we injected females of both species with tracer amounts of [^{14}C]ALA. Figure 3a,b shows that in extracts of *Rhodnius* tissues (hemolymph and ovaries respectively) most of the label is recovered in the heme peak, identifiable by its retention time (30–32 min) and absorbance spectra (Figure 3, insets). Some radioactivity is eluted from the column together with other polar materials that have the same retention time (7 min) as standard ALA. When the same experiment is carried out with *Boophilus* tissues (Figure 3c,d), no radioactivity is associated with the heme peak and the label is recovered as ALA, showing that the injected heme precursor is taken up by tissues but is not converted to porphyrin. Even when eggs laid several days after injection were collected, all radioactivity was found in the ALA peak (data not shown). These results indicate that heme biosynthesis, a ubiquitous pathway found in all other animal cells, is not active in *B. microplus*. Another possibility is that the metabolic flux in the pathway is below the detection limits of the methods used. This is not likely because it would not explain the huge amounts of heme found in developing ovaries when massive synthesis of storage heme protein is taking place (Figure 1a,b).

To determine whether the inability to synthesize heme from ALA is due to the absence of one of the enzymes of

Figure 3



Fate of [^{14}C]ALA in *Rhodnius* and *Boophilus*. Absorbance (*A*, solid line) and radiolabel distribution (dpm, dashed line) in HPLC elution profiles of tissue extracts. (a) *Rhodnius* hemolymph; (b) *Rhodnius* ovaries; (c) *Boophilus* hemolymph; (d) *Boophilus* ovaries. The inset in each panel is a wavelength scan of the heme-containing peak (arrow). The radioactive peak at 7 min corresponds to unused ALA. The figure shows the results of a typical experiment from the five carried out.

the pathway, we measured the activity of δ-aminolevulinate dehydratase (ALA-D; EC 4.2.1.24) in several tissues of both species. ALA-D was chosen because it is typically the most abundant enzyme of the pathway [11]. ALA-D activity was high in all *Rhodnius* tissues tested (Table 1), while no activity was detected in *Boophilus*. The optimum pH for ALA-D activity in *Rhodnius* was 7.5, a value in the usual range for animals. To ensure that the failure to detect enzyme activity in *Boophilus* was not due to an inappropriate pH, it was assayed at eight different pH values (pH 6–9.5), with the same results. ALA-D activity is abolished in other animals *in vivo* and *in vitro* by the specific inhibitor succinyl acetone [12]; in *Rhodnius* this compound effectively inhibits ALA-D *in vitro* (data not shown). Injection of 2.5 μmol succinyl acetone completely inhibited oviposition in *Rhodnius* but had no effect in *Boophilus* (Table 2). These results are consistent with the hypothesis that the heme biosynthesis pathway is not operative in *B. microplus*.

Table 1

ALA-D activity in *Rhodnius* and *Boophilus*.

Tissue	*Rhodnius prolixus* (units)*	*Boophilus microplus* (units)*
Crop	2.62 ± 0.09	NT
Accessory gland	1.56 ± 0.05	NT
Fat body	3.37 ± 0.11	ND
Ovaries	1.19 ± 0.06	ND

*1 unit = 1 nmol porphobilinogen (PBG)/h/mg protein. Values are means of five experiments ± standard error of the mean. ND, not detectable; the lower limit for detection under conditions of the assay was 0.2 nmol PBG/h. *Rhodnius* tissues were from adult females on the 4th day after feeding. *Boophilus* tissues were from fully engorged females on the 3rd day after dropping from the host. NT, not tested.

It is not likely that the absence of heme biosynthesis in *Boophilus* is a consequence of a simple feedback mechanism. Heme biosynthesis is controlled by intracellular heme levels [1,11], and large amounts of heme are indeed released upon hemoglobin digestion in hematophagous arthropods. In *B. microplus* the released heme must be transported throughout the body to be incorporated into the heme proteins required for cellular function. A high load of heme might be responsible for reducing ALA synthase activity [2], but it would not explain why *Boophilus* cannot convert preformed ALA to heme (Figures 2,3). As we cannot detect ALA-D activity in *Boophilus*, and there is no published evidence that ALA-D levels might be controlled by heme, we suggest that ALA-D is missing.

The situation in *Boophilus*, where the heme biosynthetic pathway does not operate and where ingested heme is extensively reutilized, has not been found in any other animal species [1,4]. In vertebrates, heme reutilization, if it occurs at all, is physiologically marginal. The evolution of an efficient means of recovering heme from the host's hemoglobin and distributing it safely to the tissues may be the most striking aspect of *Boophilus*'s adaptation to hematophagy. This species has apparently

Table 2

Effect of succinyl acetone (SA) injection in *Rhodnius* and *Boophilus*.

Treatment	*Rhodnius prolixus* (number of eggs)*	*Boophilus microplus* (mg of eggs)†
Control	29.4 ± 1.74	141.3 ± 2.55‡
2.5 μmol SA	0	134.0 ± 4.03‡

*Mean of 15 animals. †Mean of 10 animals. ‡*t*-test: $P > 0.1$.

developed mechanisms for heme absorption, hemolymphatic transport and cellular uptake that provide heme to all the cells of the body. It will be our aim to clarify the details of this process.

Heme biosynthesis was conclusively demonstrated in *Rhodnius*. However, this should not be taken as a demonstration that the insect does not use the heme it ingests. In previous work with *Rhodnius*, we found that heme from the meal reaches the hemolymph [13] and that the amount of heme in the diet influences ovarian development [14]. The possibility that heme absorption from the host and *de novo* porphyrin synthesis both occur in *Rhodnius* requires further investigation. Blood-sucking animals are vectors of important human and animal diseases. The specific mechanisms of heme absorption and reutilization that must exist in *Boophilus* could be targets for control methods specifically directed against blood-feeding ectoparasites.

Materials and methods

Animals

The *R. prolixus* colony was maintained at 28°C and 70% humidity, as described [5]. *B. microplus* (Porto Alegre strain) free of *Babesia* were reared at the Universidade Federal do Rio Grande do Sul, Porto Alegre, on calves obtained from a tick-free area. Ticks were kept at 28°C and 80% humidity until use.

ALA injections

Injections in *Rhodnius* were carried out as described [14]. [4-^{14}C]ALA (47.6 mCi/mmol, Du Pont; 500,000 cpm per female) and succinyl acetone (2.5 μmol) were injected 24 h after feeding, and tissues collected 24 h later. Unlabelled ALA (2.5 μmol; Sigma) was injected 3 days after feeding, and tissues collected 48 h later. Controls were injected with saline. *Boophilus* were injected with the same amounts of these compounds in the ventral cuticle. Engorged females were injected 1 day after dropping from the bovine host and tissues were collected 48 h later.

Organs and hemolymph

Animals were dissected and organs were collected in 0.15 M NaCl containing 0.5 mM benzamidine, 50 μg/ml soybean trypsin inhibitor, 0.02 mg/ml antipain, 10 μM pepstatin, 0.1 mM Zn acetate and 2 mM dithiothreitol (DTT). Hemolymph was collected 24–48 h after ALA injections, in the presence of phenylthiourea in 0.15 M NaCl containing the same protease inhibitors.

Tissue extractions and HPLC fractionation

Ovaries were homogenized in 0.15 M NaCl. Heme content was measured by reduced minus oxidized spectra of the pyridine alkaline derivative [13]. For HPLC, a Shimadzu LC-10AT HPLC device and a diode array detector (model SPD-M10A) were used, with a Shimadzu 15 cm CLC-ODS column. Porphyrin and heme were extracted twice by adding tissue homogenate from 2–4 animals to an equal volume of HCl:acetone (2.5:97.5) [15]. The pooled supernatants were fractionated by reverse-phase HPLC. Solvent A was a mixture of a 0.1 M $(NH_4)H_2PO_4$ (pH 3.5) and methanol (55:45, v/v). Solvent B was methanol. A 40 min gradient at a flow rate of 0.5 ml/min increased the proportion of solvent B from 60 to 100%. Solvent B was maintained at 100% for 30 min. A mixture of standard porphyrins (Porphyrin Products, Logan, UT), hemin and ALA (Sigma) was fractionated in this system and the retention times were: ALA, 6.3 min; uroporphyrin, 15 min, coproporphyrin, 25.8 min; protoporphyrin, 52.5 min; hemin, 32 min. Fluorescence spectra were determined using a Hitachi model F4500 spectrofluorometer. The excitation wavelength was 398 nm.

ALA-D assays

The reaction mixtures contained 100 μl 0.1 M Tris buffer pH 7.5, 2 mM DTT, 0.1 mM Zn acetate and 50–90 μl enzyme preparation in a final volume of 0.18 ml. For determination of the optimum pH, Tris was replaced with 100 μl of a buffer containing Na acetate, Na citrate, Tris and glycine (0.2 M each) adjusted to the desired pH. After a 10 min preincubation at 37°C, 20 μl 0.1 M ALA (neutralized immediately before the assay) was added. After 2 h incubation the reaction was stopped with 60 μl 10% (w/v) trichloroacetic acid containing 0.1 M $HgCl_2$. The supernatant was mixed with an equal volume of freshly prepared modified Ehrlich's reagent and the colored complex formed with porphobilinogen (PBG) was measured as described [16]. Protein concentrations were estimated [17] using bovine albumin as standard.

Acknowledgements

We thank Martha Sorenson for a critical review of the manuscript and Itabajara Vaz for providing *B. microplus*. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (Finep), Programa de Núcleos de Excelência (PRONEX) and Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT).

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