Haemozoin formation in the midgut of the blood-sucking insect *Rhodnius prolixus*

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Abstract Malaria parasites digest haemoglobin and detoxify the free haem by its sequestration into an insoluble dark-brown pigment known as haemozoin (Hz). Until recently, this pigment could be found only in *Plasmodium* parasites. However, we have shown that Hz is also present in the midgut of the blood-sucking insect *Rhodnius prolixus* [Oliveira et al. (1999) Nature 400, 517–518]. Here we show that Hz synthesis in the midgut of this insect is promoted by a particulate fraction from intestine lumen. Haem aggregation activity is heat-labile and is inhibited in vitro by chloroquine (CLQ). Inhibition of Hz formation in vivo by feeding insects with CLQ leads to increased levels of haem in the haemolymph of the insect, which resulted in increased lipid peroxidation. Taken together, these results indicate that a factor capable of promoting Hz crystallisation is present in *R. prolixus* midgut and that this activity represents an important physiological defence of this insect against haem toxicity.

Key words: Hemozoin; Heme; Chloroquine; *Rhodnius prolixus*

1. Introduction

Free haem is a powerful generator of reactive oxygen species that can damage many biomolecules [1]. Moreover, due to its amphiphilic nature, haem associates with phospholipid bilayers and interferes with their physical integrity, leading to membrane disruption [2]. Hence, many adaptations were developed during the course of evolution to protect organisms against the deleterious effects of free haem [3]. A special situation is found in parasites that feed on vertebrate blood such as the *Plasmodium* parasites that during its life-cycle digest up to 80% of host cell haemoglobin, thus producing large amounts of its prosthetic group haem [4]. Part of the resulting free haem molecules are sequestered into a dark-brown crystalline structure called malaria pigment or haemozoin (Hz) [5,6].

Counteracting haem toxicity is also a major problem to blood-sucking insects, which ingest several times their own weight on vertebrate blood and digest it within a few days after the meal [7]. In *Rhodnius prolixus* (Hemiptera, Reduviidae), haemoglobin breakdown occurs in the midgut through the action of lysosomal type proteolytic enzymes [8]. Protein digestion results in an intense release of free haem and, likewise intra-erythrocytic stages of *Plasmodium*, this insect must have effective defences against haem toxicity. In support to this view, a recent report from our group showed that Hz is also synthesised in the midgut of *R. prolixus* which is an important vector of the Chagas' disease parasite *Trypanosoma cruzi* [9]. This was the first description of Hz in an organism different from *Plasmodium* parasites. Here we studied the process of haem aggregation in the midgut of *R. prolixus* and characterised this process as an important mechanism to avoid haem toxicity in this insect.

2. Materials and methods

2.1. Chemicals and reagents

Haemin chloride, chloroquine (CLQ) diphosphate, thiobarbituric acid and butylated hydroxytoluene were purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Animals

Adult *R. prolixus* females were reared at 28°C and 80% relative humidity and fed on rabbit blood or plasma using artificial feeders [10].

2.3. Haemolymph

Four days after feeding, haemolymph was collected by cutting a leg and applying a gentle pressure to the insect abdomen. Haemolymph was added 130 μg/ml phenylthiourea and 150 μg/ml butylated hydroxytoluene, centrifuged at $12\,000 \times g$ for 10 min and the cell pellet was discarded. Haemolymph was kept at −70°C until analysis.

2.4. Electron microscopy

R. prolixus midguts or midgut luminal contents were fixed overnight in 2.5% glutaraldehyde and 4% *p*-formaldehyde in 0.1 M cacodylate buffer, pH 7.4. After fixation, samples were post-fixed with osmium tetroxide, dehydrated in acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss 900 TEM.

2.5. FTIR spectrometry

KBr pellets were prepared from dried samples. Spectra were acquired for 32 cycles with a FTIR spectrometer (Nicolet, Magna 550).

2.6. Haem aggregation assay

The intestinal contents from insects fed on rabbit plasma were obtained 4 days after feeding and stored in 0.15 M NaCl at −70°C. This material was centrifuged at $20\,000 \times g$ for 20 min at 5°C and the

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amount of protein in supernatant and pellet was measured following the method of Lowry [11]. These fractions were used to measure haem aggregation activity [12]. A sample corresponding to 12 μg of protein was incubated for 24 h at 28°C in 0.5 M sodium acetate, pH 4.8, in the presence of 100 μM haemin. After incubation, the reaction mixture was centrifuged at 15 000×*g* for 15 min at 25°C. The pellet was washed three times in 1 ml of 0.1 M $NaHCO_3$+2.5% sodium dodecyl sulfate, pH 9.1, and twice with deionised water. The final pellet was solubilised in 0.1 M NaOH and the amount of haem was determined spectrophotometrically at 400 nm in a GBC-UV/Vis-920 spectrophotometer. All experiments were repeated at least three times.

2.7. Culture of Rhodococcus rhodnii

Isolated colonies of *R. rhodnii* were obtained from *R. prolixus* crop content in LB-agar medium supplemented with 0.1% glucose and 0.1% lactic acid by successive plaquing of isolated colonies [13].

2.8. Haem quantification

Haem content of the haemolymph was quantified by measuring the alkaline pyridine-haemochrome, using the reduced minus oxidised spectra [14].

2.9. Thiobarbituric acid reactive substances (TBARS) assay

TBARS were measured as described by Graça-Souza et al. [15]. Haemolymph (15 μl) was collected and added to 1.2 ml polypropylene tubes containing 100 μl of 50 mM sodium phosphate buffer pH 7.4, 200 μl of TBA (1% in acetic acid). The tubes were incubated at 4°C for 1 h and at 97–98°C for 30 min. After this incubation, samples were mixed with 500 μl of *n*-butanol, and centrifuged at 20 000×*g* for 10 min. TBARS in the organic phase were measured by the absorbance at 532 nm.

3. Results and discussion

3.1. Induction of Hz synthesis by a particulate fraction of R. prolixus midgut

After a blood meal, *R. prolixus* midgut presents a huge amount of Hz granules (Fig. 1a and reference [9]). Midgut luminal preparations of blood-fed insects have high levels of haem and Hz, which could interfere with the assays of Hz synthesis in vitro. To avoid this problem, insects were fed with rabbit plasma using an artificial feeder [10]. This procedure results in clear gut extracts with less than 1% of haem or Hz content in their midguts when compared with blood-fed animals (data not shown). When analysed by transmission electron microscopy (TEM), rabbit plasma-fed insects do not show Hz granules in their midgut lumen (Fig. 1b). However, similar to blood-fed insects, their midgut cells are covered with a layer of perimicrovillar membranes (PMM) (arrowheads in Fig. 1a,b). These membranes are found in all hemipteran insects, covering the microvilli midgut and bleb to the intestinal lumen [16]. Midgut luminal content was collected and centrifugation of this material results in a particulate fraction comprising of PMM and a flocculent material (Fig. 1c) and a supernatant fraction containing soluble material. Only the particulate fraction is capable of promoting haem aggregation into a dark-brown pigment in vitro by incubation with haem under acidic conditions (Fig. 1d). This pigment displays a typical Hz FTIR spectrum, with its distinctive peaks at 1210 cm^{-1} and 1663 cm^{-1} (inset Fig. 1d). The haem aggregation activity into Hz is derived from *R. prolixus* tissues, as no activity is associated with rabbit plasma or with *R. prolixus* intestinal actinomycete endosymbiont, *R. rhodnii* (Fig. 2a). Therefore, Hz synthesis induced by this particulate fraction in *R. prolixus* may be analogous to the activity found in *Plasmodium* food vacuoles. However, the particulate fraction-derived factor responsible for promoting Hz synthesis in *R. prolixus* is heat-labile since prior heating of this fraction abolishes Hz formation in vitro (Fig. 2b). This suggests a mechanism different from that is described for *Plasmodium falciparum*, where boiling trophozoite extracts did not change the ability to induce Hz formation [17].

Despite all the recent attempts to characterise haem aggregation in *Plasmodium* parasites, this is still a controversial issue as many factors have been suggested to promote Hz synthesis. The presence of an enzyme was implied, as crude extract from trophozoites promotes Hz formation in vitro [18]. Also, a group of histidine-rich proteins (HRPs) present in the food vacuole of *P. falciparum* were able to induce haem aggregation [12]. Other reports have shown that pre-formed Hz can seed Hz production even in the absence of protein by

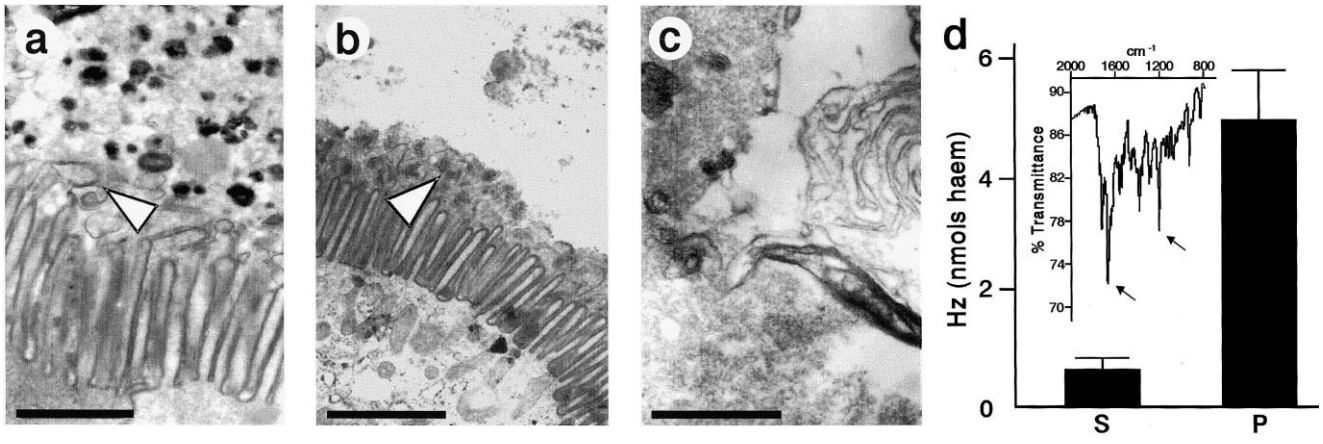

Fig. 1. TEM of plasma and blood-fed *R. prolixus* midgut and Hz synthesis in vitro. a: TEM of a stained cross section of a blood-fed *R. prolixus* midgut 4 days after feeding showing the midgut epithelial cells, the PMM (arrowhead) and many aggregates in the intestinal lumen. b: TEM of a plasma-fed *R. prolixus* midgut 4 days after feeding showing the presence of PMM (arrowhead) and the absence of luminal electron-dense aggregates. c: TEM of a particulate fraction of plasma-fed *R. prolixus* midgut luminal content, collected 4 days after feeding showing detached PMM and a flocculent material. Scale bars represent 1 μm (a), 1.7 μm (b) and 0.7 μm (c). d: Hz synthesis induced by midgut luminal particulate fraction. Midgut luminal content from insects fed with rabbit plasma was collected 4 days after feeding and centrifuged at 20 000×*g* for 15 min and the haem aggregation activity was assayed using 12 μg of proteins from the supernatant (S) or the pellet (P). Results shown are mean ± S.E.M. ($n = 3$). The inset shows the FTIR spectrum of the pigment produced in vitro by the particulate fraction. Hz peaks at 1210 cm^{-1} and 1663 cm^{-1} are indicated by arrows.

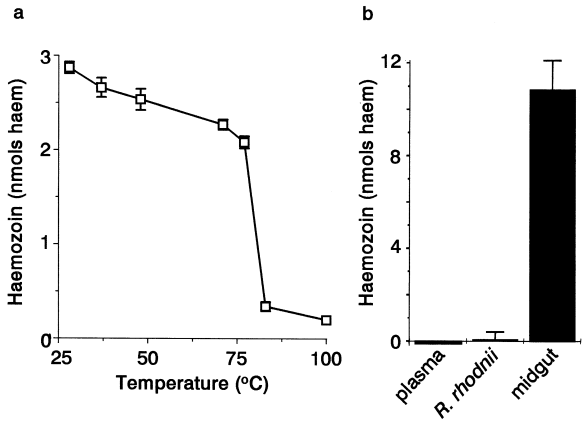

Fig. 2. Haem aggregation activity of midgut is heat-labile and is derived from *R. prolixus* cells. a: Samples of intestinal luminal content (12 μg of protein) from insects fed with rabbit plasma were pre-incubated at the indicated temperatures for 30 min, then assayed for haem aggregation activity for 12 h at 28°C. Results shown are mean ± S.E.M. ($n = 4$). b: Samples (12 μg of protein) of rabbit plasma (plasma); the endosymbiont *R. rhodnii* or *R. prolixus* midgut particulate fraction from insects fed with plasma (midgut) were assayed for haem aggregation activity. Results shown are mean ± S.E.M. ($n = 3$).

means of an autocatalytic process [17]. Furthermore, phospholipid-driven haem sequestration into Hz has also been reported [19–21]. Whether HRPs or phospholipids are true catalysts of Hz elongation in *Plasmodium* or they act instead by seeding haem aggregation is not clear yet. Regardless the biochemical characterisation of Hz synthesis in *Plasmodium*, the present work indicates that the haem aggregation activity in *R. prolixus* midgut resides in a midgut particulate fraction and that this factor behaves differently from that activity found in *P. falciparum* food vacuoles [17]. Interestingly, the fact that Hz crystallisation activity found in *R. prolixus* midgut is associated to a particulate fraction points to another difference to malarial HRPs which are soluble proteins [12]. Efforts to identify the component responsible for stimulating Hz production in *R. prolixus* midgut are in progress in our laboratory.

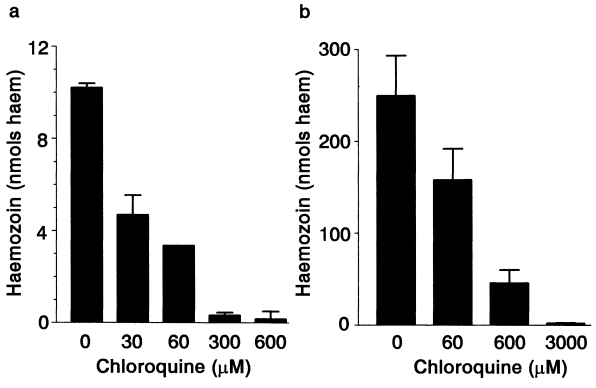

Fig. 3. Effect of CLQ on haem aggregation. a: Hz synthesis in vitro induced by midgut particulate fractions was assayed in the presence of different concentrations of CLQ. Results shown are mean ± S.E.M. ($n = 4$). b: Adult females of *R. prolixus* were fed with rabbit blood supplemented with different concentrations of CLQ. After 4 days, the insects were dissected and the amount of Hz present in the midgut was measured. Results shown are mean ± S.E.M. ($n = 5$).

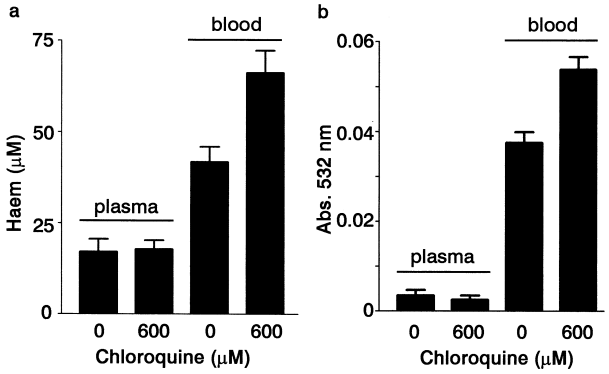

Fig. 4. Inhibition of Hz synthesis increases haem titres and lipid peroxidation in the haemolymph. Insects were fed with plasma or blood without CLQ or containing 600 μM CLQ. a: Total haem concentration in the haemolymph. Haem in the haemolymph was determined 4 days after feeding. Results shown are mean ± S.E.M. ($n = 5$). b: Measurement of lipid peroxidation in the haemolymph by TBARS assay. Haemolymph was collected 4 days after feeding and TBARS was determined as described in Section 2. Results shown are mean ± S.E.M. ($n = 7$).

3.2. Inhibition of Hz synthesis by CLQ

It is very well established in the literature that quinoline-derived drugs, such as quinine and CLQ, are potent inhibitors of *Plasmodium* growth [22]. It has been proposed that one of the mechanisms of CLQ action is that being a weak base it would accumulate in high concentrations inside the parasite's food vacuole. Although the mechanism of their anti-malarial action has not been completely elucidated, there is evidence that Hz formation is an important target [23]. Fig. 3a shows that haem aggregation in vitro induced by *R. prolixus* midgut particulate fraction is strongly inhibited by CLQ. In vivo, when insects are fed with blood supplemented with different concentrations of CLQ, Hz formation is also inhibited (Fig. 3b). Interestingly, feeding *R. prolixus* with blood and CLQ led to midgut swelling (data not shown), a feature that was also reported in *Plasmodium* digestive vacuole [24]. These results show that, likewise in *Plasmodium*, a quinoline compound can inhibit the synthesis of Hz in *R. prolixus*.

3.3. Physiological role of Hz synthesis

In order to determine the physiological relevance of Hz synthesis to *R. prolixus*, we fed adult females with blood supplemented with CLQ. Fig. 4a shows that prevention of Hz formation by feeding the insects with blood and 600 μM of CLQ results in an increased haem concentration in the haemolymph. This effect cannot be attributed to CLQ alone, as insects fed with plasma plus CLQ have no effect on haem concentration in the haemolymph. Since the deleterious effects of haem are well known, these findings strongly suggest that inhibition of Hz synthesis imposes a strong oxidative stress to the insect due to haem overload [1]. In fact, measurement of lipid peroxidation products in the haemolymph from insects fed with blood and CLQ shows increased TBARS levels compared to control blood-fed animals (Fig. 4b). However, differently from *Plasmodium*, CLQ treatment in *R. prolixus* does not lead to insect's death. The explanation for this is that the insect makes use of several other defences against the toxic effects of haem in order to circumvent this condition [15,25,26]. Beyond Hz synthesis, other adaptations were developed in order to prevent the deleterious effects of free haem

in *R. prolixus*. Earlier reports from our laboratory have shown that this insect has in its haemolymph a haem-binding protein (RHBP) with an antioxidant role [25], unusually high levels of urate [15] and glutathione peroxidase [26]. Therefore, the results presented here indicate that, together with the other mechanisms mentioned above, haem detoxification into Hz by midgut particulate fraction in *R. prolixus* seems to be an important defence of this animal to avoid haem deleterious effects.

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