

Heme crystallization in the midgut of triatomine insects[☆]

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Received 13 April 2006; received in revised form 26 October 2006; accepted 8 December 2006

Available online 19 December 2006

Dedicated to the memory of Carmelita Mendes de Oliveira (1913–2006)

Abstract

Hemozoin (Hz) is a heme crystal produced by several blood-feeding organisms in order to detoxify free heme released upon hemoglobin (Hb) digestion. Here we show that heme crystallization also occurs in three species of triatomine insects. Ultraviolet-visible and infrared light absorption spectra of insoluble pigments isolated from the midgut of three triatomine species *Triatoma infestans*, *Dipetalogaster maximus* and *Panstrongylus megistus* indicated that all produce Hz. Morphological analysis of *T. infestans* and *D. maximus* midguts revealed the close association of Hz crystals to perimicrovillar membranes and also as multicrystalline assemblies, forming nearly spherical structures. Heme crystallization was promoted by isolated perimicrovillar membranes from all three species of triatomine bugs *in vitro* in heat-sensitive reactions. In conclusion, the data presented here indicate that Hz formation is an ancestral adaptation of Triatominae to a blood-sucking habit and that the presence of perimicrovillar membranes plays a central role in this process.

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Keywords: Triatominae; Heme; Hemozoin; Perimicrovillar membranes; Midgut

1. Introduction

A great variety of organisms, from protozoa to mammals, uses the vertebrate blood as the main food source. Hemoglobin (Hb) is a major blood component and during its digestion, in the

digestive tract of blood-feeding organisms, an intense release of peptides, amino acids and heme takes place (Francis et al., 1997; Brindley et al., 1997). Despite the obvious biological role, once in a free state heme acts as potent cytotoxic agent, promoting the lysis of many cell types (Chou and Fitch, 1980; Orjih et al., 1981) and also the generation of reactive oxygen species (ROS) through catalytic decomposition of organic hydroperoxides (Davies, 1988; Van der Zee et al., 1996). Heme-mediated-ROS generation leads to oxidative stress which could result in damage of biomolecules such as proteins, carbohydrates and lipids (Tappel, 1955; Vincent, 1989). Furthermore, at millimolar concentrations, heme can associate with cellular phospholipid membranes, reducing its degree of order and increasing its permeability (Schmitt et al., 1993). Thus, the way in which blood-feeding organisms deal with free heme is of central importance to their physiologies.

[☆] This paper is part of the 4th special issue of CBP dedicated to The Face of Latin American Comparative Biochemistry and Physiology organized by Marcelo Hermes-Lima (Brazil) and co-edited by Carlos Navas (Brazil), Rene Belebony (Brazil), Rodrigo Stabeli (Brazil), Tania Zenteno-Savín (Mexico) and the editors of CBP. This issue is dedicated to the memory of two exceptional men, Peter L. Lutz, one of the pioneers of comparative and integrative physiology, and Cicero Lima, journalist, science lover and Hermes-Lima's dad.

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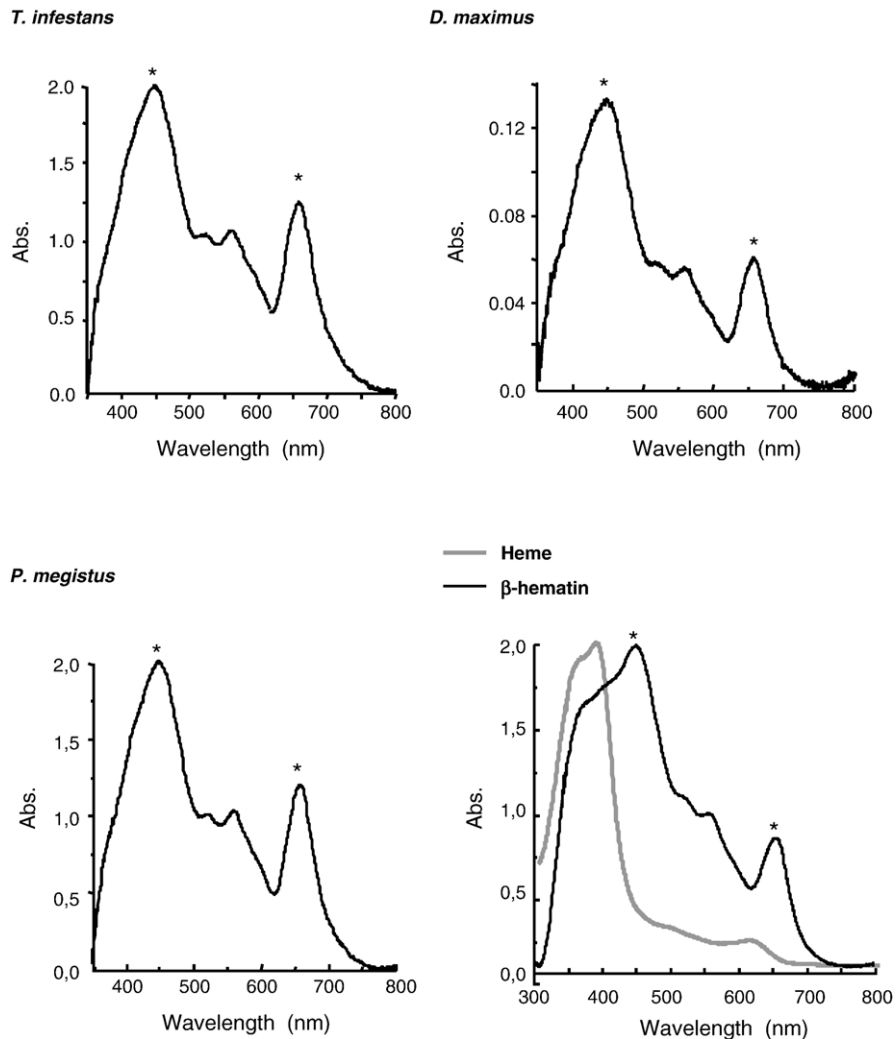


Fig. 1. UV-visible spectroscopic analysis of heme pigments in the midgut of three triatomine species. The midgut of blood-fed *Triatoma infestans*, *Dipetalogaster maximus* and *Panstrongylus megistus* insects were subjected to the Hz extraction protocol as described in the Materials and methods section. The obtained pigments were resuspended in 0.1 M NaHCO₃, pH 9.1 and analyzed by UV-visible spectrophotometry. Analysis of commercial hemin (gray line) and *in vitro* synthesized β -hematin were also carried out. The distinctive Hz peaks in all spectra were indicated by asterisks.

In order to overcome heme toxicity, very efficient mechanisms of detoxification have evolved in such organisms (Graça-Souza et al., 2006), including the enzyme heme oxygenase (Paiva-Silva et al., 2006) and heme binding proteins from the hemolymph of the cattle tick *Boophilus microplus* (Maya-Monteiro et al., 2004) and from *Rhodnius prolixus*, a vector of Chagas' disease (Dansa-Petretski et al., 1995). In malaria parasites, Hb digestion occurs inside the digestive vacuole, followed by crystallization of free heme into a dark brown pigment named hemozoin (Hz) (Slater et al., 1991; Pagola et al., 2000). Besides malaria parasites, heme crystallization was also reported in *R. prolixus* and in the helminth *Schistosoma mansoni*, representing one of the major heme detoxification pathways in these organisms (Oliveira et al., 1999, 2000a,b, 2002, 2004). Moreover, Hz was also found in both the avian protozoan *Haemoproteus columbae* and in the rediae of the trematode *Echinostoma trivolvis* (Chen et al., 2001; Pisciotto et al., 2005). Concerning the mechanism of heme crystallization, in *R. prolixus* we showed that the perimicrovillar membranes, which are phospholipid bilayer membranes that cover the

epithelial midgut cells (Lane and Harrison, 1979; Gutierrez and Burgos, 1986), were capable to promote heme crystallization *in vitro* (Oliveira et al., 2000a). Moreover, Hz crystals were found both associated to vesicles derived from perimicrovillar membranes and also free in the midgut lumen or even in multicrystalline assemblies, suggesting that hydrophobic environments and/or protein components of these membranes played an essential role in heme crystallization (Oliveira et al., 2005). Thus, since the presence of perimicrovillar membranes is a characteristic feature of all hemipterans, here we investigated whether heme crystallization could also occur in different species of the triatomine vectors of Chagas' disease.

2. Materials and methods

2.1. Chemicals and reagents

Hemin chloride was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

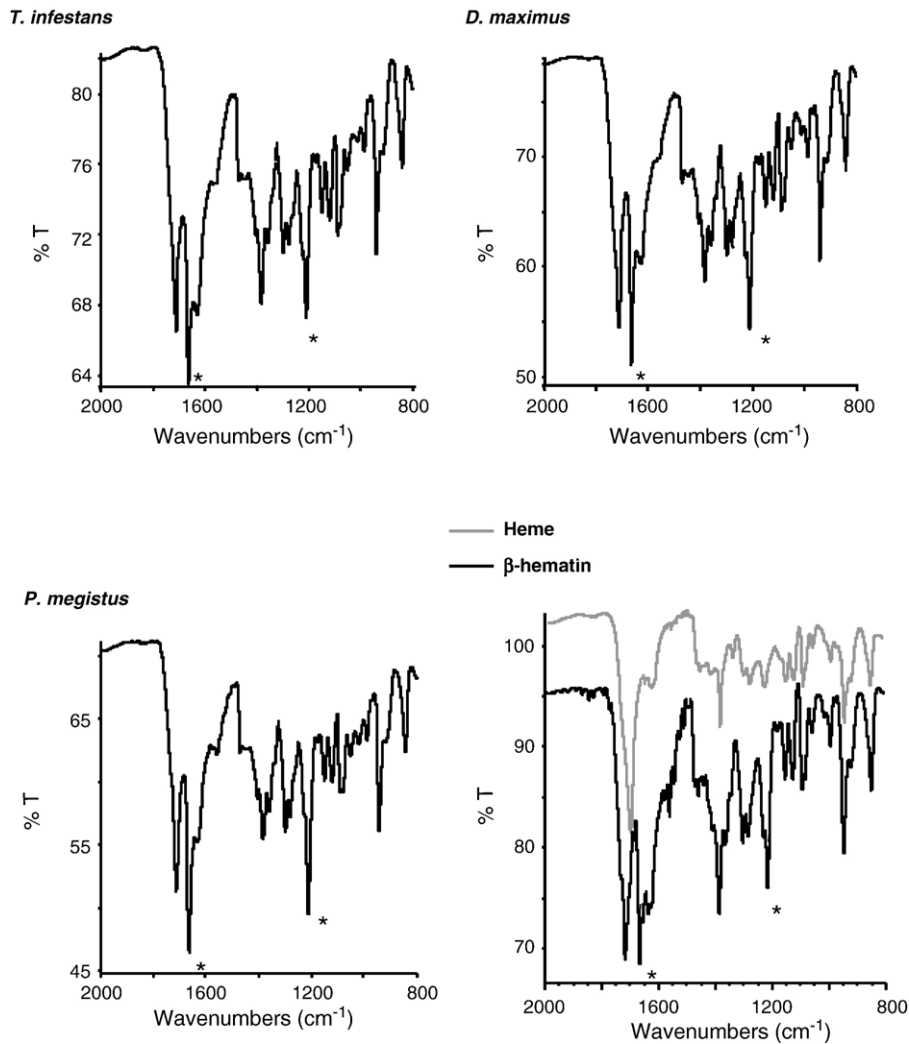


Fig. 2. FTIR spectroscopic analysis of heme pigments in the triatomine midgut. The midgut of blood-fed *Triatoma infestans*, *Dipetalogaster maximus* and *Panstrongylus megistus* insects were subjected to the Hz extraction protocol as described in the materials and methods section. KBr pellets of the obtained material were analyzed by Fourier-transformed infrared spectroscopy (FTIR). Analysis of commercial hemin (gray line) and *in vitro* synthesized β -hematin were also carried out. The distinctive Hz peaks in FTIR spectra are indicated by asterisks.

2.2. Animals

Blood-fed adult females of *Triatoma infestans*, *Dipetalogaster maximus* and *Panstrongylus megistus* were obtained from Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos at Departamento de Entomologia, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. *R. prolixus* adult females were reared with rabbit blood and kept for four days at 28 °C and 80% relative humidity.

2.3. Hz extraction and spectroscopic analysis

Hz was extracted from *T. infestans*, *D. maximus* and *P. megistus* based on a method previously described for *R. prolixus* (Oliveira et al., 1999). Midgut contents from these bugs were obtained by gently shaking the dissected midguts in 0,15 M NaCl. Tissue was discarded and the suspension was centrifuged at 15,000 $\times g$ for 20 min. The insoluble pigment was further purified by three washes with 0.1 M NaHCO_3 +

2.5% SDS, pH 9,1. The remaining solids were washed twice with water and kept at 4 °C until analysis. Spectrophotometric determinations were carried out by resuspending the pellets in 0.1 M NaHCO_3 pH 9.1 in GBC/UV-920 spectrophotometer. For infrared absorption experiments, KBr pellets were prepared from dried samples of each pigment and spectra were acquired for 32 cycles with a FTIR spectrometer (Nicolet, Magna 550).

2.4. Heme crystallization *in vitro*

Midgut samples from blood-fed insects were obtained and stored in 0.15 M NaCl containing 0.5 mM benzamidine, 50 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor, 0.02 mg/mL antipain, 10 μM pepstatin, 0.1 mM Zn acetate and 2 mM dithiothreitol. After homogenization, samples were kept at -70 °C until use. Later this material was centrifuged at 15,000 $\times g$ for 20 min at 5 °C and the amount of protein in the pellet was measured (Lowry et al., 1951). The capacity of these fractions to promote heme crystallization was evaluated as previously described (Oliveira et al., 2000a).

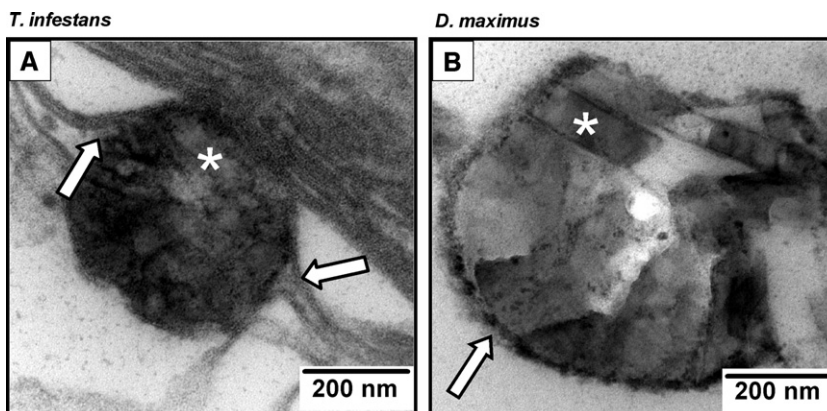


Fig. 3. Transmission electron microscopy of heme pigments in the triatomine midgut. Panel A shows a high magnification of a multicrystalline assembly in *Triatoma infestans* midgut in association with perimicrovillar membranes (arrows). The asterisk indicate a single crystal of Hz. Bar=200 nm. Panel B shows a multicrystalline assembly in *Dipetalogaster maximus* midgut and its association with perimicrovillar membranes (arrow). The asterisk indicate a single crystal of Hz. Bar=200 nm.

Briefly, samples of 20 μg of protein were incubated for 24 h at 28 °C in 0.5 M sodium acetate, pH 4.8, in the presence of 100 μM hemin. After incubation, the reaction mixture was centrifuged 15,000 $\times g$ for 20 min at 25 °C. The pellet was washed three times with 1 mL of 0.1 M NaHCO_3 + 2.5% SDS, pH 9.1, and twice with deionized water. The final pellet was solubilized in 0.1 M NaOH and the amount of heme determined spectrophotometrically at 400 nm in a GBC-UV/Vis-920 spectrophotometer.

2.5. Transmission electron microscopy (TEM)

Midguts from blood-fed *T. infestans* and *D. maximus* were fixed overnight at room temperature in 1% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. They were then rinsed and postfixed in 1% OsO_4 , 0.8% $\text{K}_3\text{Fe}(\text{CN})_6$ and 5 mM CaCl_2 in the same buffer for 1 h. Samples were dehydrated in acetone and embedded in epoxy Polybed resin (Polyscience). Ultrathin sections were stained with uranyl acetate, lead citrate and were observed in a Zeiss 109 electron microscope. Images were acquired using a Megaview II digital system.

3. Results

Since we identified Hz in the midgut of the triatomine insect *R. prolixus* our first approach was to investigate the presence of similar pigments in three other species of triatomine insects. So, using methods previously described for isolation of *R. prolixus* Hz (Oliveira et al., 1999), we obtained dark-brown insoluble pellets from midgut samples of blood-fed *T. infestans*, *D. maximus* and *P. megistus*. Fig. 1 shows the UV-visible absorption spectra of the intact pigments, which were markedly distinct from the absorption of heme (gray line) and very similar to that of β -hematin, *Plasmodium* Hz (Fitch and Kanjanangulpan, 1987), *R. prolixus* Hz (Oliveira et al., 2000a) and *S. mansoni* Hz (Oliveira et al., 2000b). These spectra share some characteristic features such as a broad Soret absorbance band centered near 450 nm and also another peak near 660 nm. These pigments were insoluble in neutral and slightly alkaline (pH 9.1) solutions but were readily dissolved in 0.1 M sodium hydroxide, a condition that changed their light absorption

spectra to that typical of monomeric heme (data not shown). Fourier transform infrared spectroscopy (FTIR) of intact pigments also revealed spectra similar to what was described for Hz from *Plasmodium*, *R. prolixus* and *S. mansoni*. Specific transmittance peaks near 1210 cm^{-1} and 1663 cm^{-1} indicate the presence of iron-carboxylate bonds responsible for Hz structure (Fig. 2 and Slater et al., 1991). Taken together, these results undoubtedly demonstrate that the triatomines *T. infestans*, *D. maximus* and *P. megistus* produce Hz in their midguts as an efficient way to detoxify heme.

We have recently shown heme crystals produced in *Schistosoma* and *Rhodnius* guts assemble themselves as multicrystalline structures composed of long regular brick-shaped crystals of approximately 200 nm length, forming large spherical structures (Oliveira et al., 2005). In contrast, Hz crystals in malaria parasites are arranged in a distinct pattern without the formation of

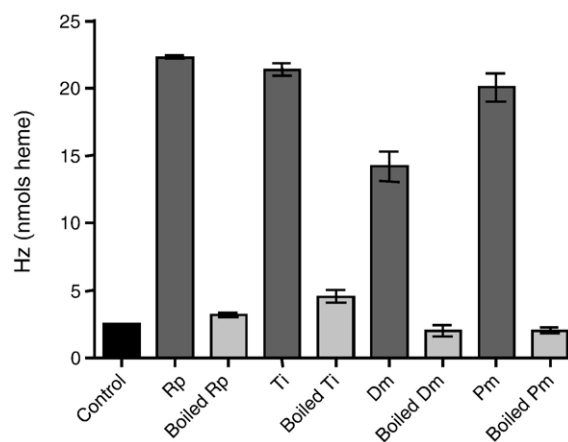


Fig. 4. Heme crystallization activity of triatomine perimicrovillar membranes. Dark grey bars represent samples of midgut membranes (20 μg of protein) from blood-fed *Rhodnius prolixus* (Rp), *Triatoma infestans* (Ti), *Dipetalogaster maximus* (Dm) and *Panstrongylus megistus* (Pm) were assayed for heme crystallization activity as described under methods. Light grey bars represent samples of midgut membranes (20 μg of protein) pre-incubated at 100 °C for 30 min, then assayed for heme crystallization activity. The black bar represents the product of heme crystallization in the absence of the triatomine midgut membranes. After incubation Hz was extracted following methods described in the literature. All results shown are expressed as mean \pm S.E.M. ($n=4$).

regular multicrystalline assemblies. The ultrastructural investigation of *T. infestans* and *D. maximus* midguts by transmission electron microscopy (TEM) revealed the presence of these multicrystalline assemblies of Hz in these two triatomine insects, just like those found in *R. prolixus* and in *S. mansoni* (Fig. 3, asterisks). Moreover, a close association of the multicrystalline assemblies to perimicrovillar membranes was also observed in both triatomine insects (Fig. 3, arrows). This prompted us to determine whether the perimicrovillar membranes of these three species of triatomine species promote heme crystallization, as we described in *Rhodnius*. In fact, preparations enriched in perimicrovillar membranes isolated from *T. infestans*, *D. maximus* and *P. megistus* midguts were all capable to promote Hz formation *in vitro* (Fig. 4). Interestingly, heme crystallization activities, induced by these membranes, were all sensitive to previous heating to 100 °C indicating that this activity is similar to that of *Rhodnius* (Oliveira et al., 2000a) and distinct from *Plasmodium* and *Schistosoma*, where boiling caused no changes in their abilities to promote Hz formation (Dorn et al., 1995; Oliveira et al., unpublished data).

4. Discussion

Increased levels of free heme inside the gut of blood feeders poses a challenge to these organisms, which usually ingest large amounts of Hb, due to the intrinsic heme toxicity (Ryter and Tyrrel, 2000). To avoid this, blood feeders developed several adaptations (Graça-Souza et al., 2006) such as precipitation of free heme inside their digestive tract through its crystallization into hemozoin (Hz), a process that represents the quantitatively most important heme detoxification pathway of these organisms (Slater et al., 1991; Pagola et al., 2000; Egan et al., 1994; Oliveira et al., 1999, 2000a,b, 2002, 2004; Graça-Souza et al., 2006). This mechanism is essential for *Plasmodium* survival as more than 95% of the heme iron released from host Hb is incorporated into Hz in food vacuoles (Egan et al., 2002). Inhibition of Hz formation has been shown to induce biological damage in both *Rhodnius* (Oliveira et al., 2000a) and *Schistosoma* (Oliveira et al., 2004). In the present work, we demonstrate that true Hz is found in the midgut of three species of triatomine insects – that, together with *Rhodnius*, are representative of the most important genera of this subfamily for Chagas disease transmission – and showed that perimicrovillar membranes play a key role in Hz formation in these insects.

Concerning evolution of hematophagy, there is a dispute about how old is the adaptation to a blood-feeding way of life in the subfamily Triatominae. Some authors have suggested that hematophagy is a monophyletic trait that appeared early in the origin of triatomine reduvids in the American continent in the late cretaceous, about 65 MYA (Gaunt and Miles, 2000). In contrast, Schofield (2000) has advocated the hypothesis that – in spite of the antiquity of the origin of the group – hematophagy is a comparatively recent behaviour, initiated less than 5 MYA and having evolved independently in the tribes Rhodniini and Triatomini. The fact that the capacity to promote Hz formation is widespread across the subfamily adds evidence in favour of a monophyletic origin of blood feeding. Hz formation represents

therefore an important adaptation that allows digestion of large amounts of blood and our evidence indicates a role for perimicrovillar membranes in heme crystallization in the midgut of Triatominae. As all hemipteran insects have perimicrovillar membranes, its presence in the midgut of the non-hematophagous reduvid ancestor could be regarded as a pre-adaptation of those insects that would have alleviated heme deleterious effects and opened the way to develop in the direction of hematophagy, a common feature of all present day triatomine bugs.

The ultrastructural organization of Hz crystallites in these three triatomine species is quite similar to that of *S. mansoni* and *R. prolixus* Hz but distinct from those found in *Plasmodium* Hz (Fig. 3; Hempelmann et al., 2003; Oliveira et al., 2005). Likewise, the heat-sensitivity of heme crystallization activity of triatomine perimicrovillar membranes shown here (Fig. 4) resembles the activity found in *Rhodnius* (Oliveira et al., 2000a) and differ from that present in *Schistosoma* (Oliveira et al., unpublished data), probably due to intrinsic features of the structures involved in Hz formation in the guts of these organisms. In *S. mansoni*, Hz is produced at the surface of extracellular lipid droplets present in their gut lumen (Oliveira et al., 2005) showing a heat-resistant heme crystallization activity, as would be expected for lipid-driven Hz formation (Oliveira MF, unpublished data). Mechanistically, the heat sensitivity of heme crystallization activity associated to perimicrovillar membranes suggests the participation of a protein in this process (Slater and Cerami, 1992). The only known protein described to date that would be involved in heme crystallization is the histidine-rich protein-II (HRP-II) in *Plasmodium* (Sullivan et al., 1996). However, parasites lacking the HRP-II or III genes are still capable to produce Hz (Sullivan, 2002). Therefore, the role of a specific protein as a physiological promoter of Hz formation remains elusive. As Hz formation takes place in close association with hydrophobic structures, such as the food vacuole membranes in *Plasmodium* (Hempelmann et al., 2003), the perimicrovillar membranes in triatomines (Figs. 3 and 4; Oliveira et al., 2000a) and lipid droplets in *Schistosoma* (Oliveira et al., 2005), it is tempting to propose that lipids by themselves would be capable to promote heme crystallization into Hz. In line with this possibility, it has been shown that incubation of heme with several compounds such as acetate (Egan et al., 2001), ethanol (Blauer and Akkawi, 2002), benzoic acid (Egan, 2002) and even dimethylsulfoxide (Oliveira MF, unpublished data) increased heme crystallization through solubilization of heme in acidic environments. The increment of heme solubility would occur through displacement of the axial water molecule bound to the central heme iron, thus by-passing the rate-limiting step of the process that is the rapid precipitation of heme (Egan et al., 2001). This is followed by slow conversion to crystalline Hz, in a process induced by phase transfer catalysts, a role that could be performed by lipids in *Plasmodium*, *Schistosoma* and in triatomines as well (Bendrat et al., 1995; Dorn et al., 1998; Fitch et al., 1999; Hempelmann et al., 2003; Jackson et al., 2004; Oliveira et al., 2000a, 2004, 2005). In fact, recent data from the literature demonstrated that under physiological conditions, heme crystallization was induced rapidly and spontaneously *in vitro* near long chain alcohol/water and lipid/water interfaces (Egan et al., 2006). Pisciotta and colleagues have also shown that neutral lipids

wrapping Hz crystals in *Plasmodium* food vacuoles play an important catalytic role in heme crystallization process (Pisciotta et al., in press).

In conclusion, heme crystallization has appeared independently in several groups of blood-feeding organisms that have no common ancestor. Among triatomine bugs, however, the ability to make Hz crystals seems to be generalized and can be regarded as the hallmark of the adaptation of the Triatominae to deal with heme intrinsic toxicity.

Acknowledgements

We would like to thank Prof. José Jurberg, from Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos at Dept. Entomologia, Fiocruz, Rio de Janeiro for kindly supplying blood-fed triatomines utilized in the present study. We are also indebted to Mr. Cláudio Figueiras, Mr. José de S. L. Junior and Mrs. Litiane M. Rodrigues for the excellent technical support. Supported by TWAS, CNPq, FAPERJ, FUJB, FAPESB, FINEP, PRONEX, PADCT and Capes. MFO and PLO are research scholars of CNPq.

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