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On the mechanisms involved in biological heme crystallization

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Abstract Blood-feeding organisms digest hemoglobin, releasing large quantities of heme inside their digestive tracts. Free heme is very toxic, and these organisms have evolved several mechanisms to protect against its deleterious effects. One of these adaptations is the crystallization of heme into the dark-brown pigment hemozoin (Hz). Here we review the process of Hz formation, focusing on organisms other than *Plasmodium* that have contributed to a better understanding of heme crystallization. Hemozoin has been found in several distinct classes of organisms including protozoa, helminths and insects and Hz formation is the predominant form of heme detoxification. The available evidence indicates that amphiphilic structures such as phospholipid membranes and lipid droplets accompanied by specific proteins play a major role in heme crystallization. Because this process is specific to a number of blood-feeding organisms and absent in their hosts, Hz formation is an attractive target for the development of novel drugs to control illnesses associated with these hematophagous organisms.

Keywords Malaria · Schistosomiasis · Triatomine · Biocrystallization · Porphyrin

The Yin and Yang of heme

Heme is a key molecule in living cells because it plays the physiologically significant role of catalyzing most oxidative

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biological processes (Ponka 1999). The enormous advantages of heme utilization also present a challenge to aerobic life. Due to its chemical nature, heme is an amphiphilic molecule that associates with biological membranes and generates free radicals by reactions with the central iron atom. Although heme promotes the production of reactive oxygen species (ROS) when bound to the globin polypeptide chain (Sadrzadeh et al. 1984), its pro-oxidant effects are dramatically increased when heme is free rather than associated with any molecule (Dansa-Petretski et al. 1995; Ryter and Tyrrell 2000). The pro-oxidant toxic effects of heme may result from the inhibition of multiple enzymes (Aft and Mueller 1984), damage to biological membranes through lipoperoxide generation (Schmitt et al. 1993), cell lysis (Meshnick et al. 1977; Chou and Fitch 1981) or nucleic acid oxidation (Aft and Mueller 1983). Heme can also associate with phospholipid membranes, reducing their degree of order and permeability (Schmitt et al. 1993). At millimolar concentrations, free heme associates with cellular phospholipid membranes, disrupting their physical integrity (Schmitt et al. 1993) by a mechanism that is independent of heme's pro-oxidant effects.

Blood feeding occurs in distinct classes of organisms, from protozoa to mammals, and a hemoglobin-based diet demands special adaptive mechanisms to manage the enormous amount of heme released during the digestive process (Oliveira and Oliveira 2002; Graca-Sousa et al. 2006). This point was not previously well understood, and heme precipitates found as a by-product of digestion in hematophagous animals were once described by the generic name "hematin", inaccurately suggesting that they were nonspecific deposits of heme (Wigglesworth 1943; Homewood et al. 1972). Here we focus on a specific protective mechanism based on the precipitation of heme into crystals called hemozoin (Hz).

Hemozoin: the gemstone of blood-feeding organisms

An association between malaria and the presence of small dark-brown pigments in the internal organs of acute malaria patients was first made by Lancisi in 1717 and was referred to as the "malaria pigment" years before the discovery of its etiological agent, *Plasmodium* parasites. It was long believed that malaria pigment, or as it is recognized today, Hz, was a hemeprotein. However, in 1987 Fitch and Kanjananggulpan demonstrated that Hz did not contain protein and that the resulting product resembled β-hematin (βH), a synthetic heme product described by Hamsik in 1936, which is extremely insoluble in mildly basic solutions compared to heme. In seminal work in 1991, Slater and colleagues determined that the heme molecules in malaria pigment associate through iron-carboxylate bonds, and they were the first to structurally characterize, utilizing many spectroscopic tools, both Hz and βH (Slater et al. 1991). They showed that the Fourier-transformed infrared spectra of Hz and βH exhibited sharp peaks around 1,664 cm^{-1} and 1,211 cm^{-1}, indicating that heme monomers were attached through coordinated iron-carboxylate bonds. However, it was not known whether βH and Hz had the same molecular structure. In 1997, Bohle and colleagues showed by synchrotron radiation X-ray powder diffraction (SR-XRD) of *Plasmodium*-infected red cells and chemically synthesized βH that both materials exhibited the same diffraction pattern and therefore the same structure (Bohle et al. 1997). Finally, the complex structure of Hz was solved by SR-XRD in 2000 by Pagola and coworkers, who showed that it consisted of single heme molecules linked by reciprocal iron-carboxylate bonds, producing heme dimers interacting with other dimers through hydrogen bonds between the propionate side chains of the porphyrin ring (Pagola et al. 2000). Morphologically, Hz crystals from *Plasmodium* are remarkably regular in shape and size when viewed by transmission or scanning electron microscopy, resembling "small chocolate bars" (Noland et al. 2003).

Hemozoin formation as a key adaptation for hematophagy

We know today that Hz is the main protective mechanism against heme toxicity in malaria parasites (Egan et al. 2002). Heme crystallization occurs inside the digestive vacuole of malaria parasites and seems to be a special biomineralization process essential for *Plasmodium* survival, as more than 95% of the total heme iron released from hemoglobin digestion is incorporated into Hz (Egan et al. 2002; Egan et al. 2001). Hz was long considered unique to malaria parasites. However, it has also been identified in other blood-feeding organisms such as triatomine insects and the helminth *Schistosoma mansoni*, where it is also one of the major routes of heme detoxification (Oliveira et al. 1999; Oliveira et al. 2000a; Oliveira et al. 2000b; Oliveira et al. 2002; Oliveira et al. 2004; Oliveira et al. 2007). In addition, Hz has been found in the avian protozoan *Haemoproteus columbae* and in the rediae of the trematode *Echinostoma trivolvis* (Chen et al. 2001; Pisciotta et al. 2005). Heme crystallization reduces the pro-oxidant effects of heme with respect to three different parameters: cell damage, molecular damage and direct observation of free-radical generation (Oliveira et al. 2002). Thus, heme crystallization into Hz is recurrent in nature, and several hematophagous organisms make use of this strategy as an efficient means of disposing of large amounts of heme.

Because heme crystallization is unique to several blood-feeding organisms of direct medical interest but absent in

their hosts, Hz formation is an attractive drug target, especially against malaria. Interfering with Hz formation by exposing non-*Plasmodium* organisms to quinoline drugs has strong effects in both insect and helminth models (Oliveira et al. 2000a; Oliveira et al. 2004; Corrêa Soares et al. 2009). Feeding blood and chloroquine to *Rhodnius* caused not only a significant reduction in Hz content in the midgut but also an increase in the hemolymphatic levels of heme as well as lipid-peroxidation products (Oliveira et al. 2000a). Using the same rationale, our group demonstrated that chloroquine treatment of *Schistosoma*-harboring mice promoted a drastic reduction in several infection parameters including total worm protein and Hz content, worm viability, and parasitemia and deposition of eggs in the liver (Oliveira et al. 2004). Other quinoline methanols such as quinine, quinidine, and quinacrine also exhibit potent schistosomicidal activity, which correlates with their ability to inhibit Hz formation *in vivo* (Corrêa Soares et al. 2009). Quinidine was particularly effective against *Schistosoma* and is a promising lead compound for further development of new schistosomicidal agents. These data strongly indicate that heme crystallization in multicellular blood-feeding organisms is a key process for heme detoxification.

Heme crystallization: an unsolved process for an insoluble product

Although the precise mechanism by which heme is crystallized into Hz has been disputed over the years (Ridley 1996; Sullivan 2002; Hempelmann et al. 2003), a number of recent studies have yielded new perspectives on the detoxification of heme molecules into Hz. Formation of Hz is favored by acidic pH values near the pKa of the propionic side chains of the porphyrin ring (4.8–5.0), a condition close to the pH of the digestive vacuole of *Plasmodium* and of the digestive tracts of *Rhodnius* and *Schistosoma* (Slater and Cerami 1992; Bogitsh and Davenport 1991; Terra et al. 1988; Stiebler et al. 2010a). Hydrophobicity is also an important physicochemical requirement for spontaneous heme crystallization (Egan et al. 2006; Huy et al. 2007; Hoang et al. 2010; Stiebler et al. 2010b). Together, the available data point to the strong involvement of amphiphilic structures such as lipid droplets and phospholipid membranes in Hz formation (Bendrat et al. 1995; Oliveira et al. 2000a; Corrêa Soares et al. 2007; Stiebler et al. 2010a).

Amphiphilic structure-mediated heme crystallization

Bendrat and colleagues were the first to report that lipid fractions purified from *Plasmodium* food vacuoles promote efficient Hz formation *in vitro* (Bendrat et al. 1995). Confirming this, purified lipids such as arachidonic, linoleic, oleic, and palmitoleic acids and even detergents promote efficient heme crystallization *in vitro* (Dorn et al. 1998; Fitch et al. 1999). Nevertheless, trioleoylglycerol, cholesterol, dioleoylphosphatidylethanolamine, stearic and palmitic acids have no effect on heme crystallization (Dorn et al. 1998; Fitch et al. 1999). In fact, Fitch and colleagues demonstrated that about 70% of heme crystallization activity was recovered from *Plasmodium*-infected red blood cells by chloroform extraction (Fitch et al. 1999). Egan and colleagues showed that monoacylglycerol is the most efficient lipid for interface-mediated heme crystallization (Egan et al. 2006). Because Hz formation takes place in close association with amphiphilic structures such as the lipid droplets in *Plasmodium* food vacuoles (Hempelmann et al. 2003), the double-layered phospholipid membranes in triatomine insects (Oliveira et al. 2000a; Oliveira et al. 2007; Silva et al. 2007; Stiebler et al. 2010a) and the lipid droplets in *Schistosoma* (Oliveira et al. 2005; Corrêa Soares et al. 2007), it is tempting to propose that lipids play a major role in biological heme crystallization.

Extracellular phospholipid membranes in the midgut of Triatomine insects

The digestive tracts of insects belonging to the Triatominae subfamily, popularly known as “kissing bugs”, can be functionally divided into two distinct regions: the crop, where the ingested blood is stored, and the midgut, where the blood is digested and the products are absorbed. In the midgut, the epithelium is covered by a double-layered phospholipid membrane known as the perimicrovillar membrane (PMVM) (Terra et al. 1996), which has few integral proteins and extends toward the midgut lumen with dead ends (Lane and Harrison 1979; Gutiérrez and Burgos 1986; Terra et al. 1996). Figure 1 shows the general architecture of the midgut of the triatomine *Dipetalogaster maximus*.

The reported physiological roles of this structure include the compartmentalization of the digestive process (Houseman and Downe 1983), immobilization of digestive enzymes to avoid excretion (Cristofolletti et al. 2003) and amino acid absorption (Terra et al. 1988). The PMVM has been implicated in *Trypanosoma cruzi* epimastigote proliferation and development in triatomines (Garcia et al. 1989), and it also acts as a physical and chemical barrier against the toxic products of blood digestion (Oliveira et al. 1999). We previously demonstrated the intimate association between Hz crystals and the PMVM in four distinct triatomine species (Oliveira et al. 1999; Oliveira et al. 2000a; Oliveira et al. 2005; Oliveira et al. 2007; Silva et al. 2007) and that Hz formation seems to begin at specific sites within the PMVM, as indicated by the spotted sites of heme peroxidase activity staining (Silva et al. 2007). Nascent Hz crystals were also

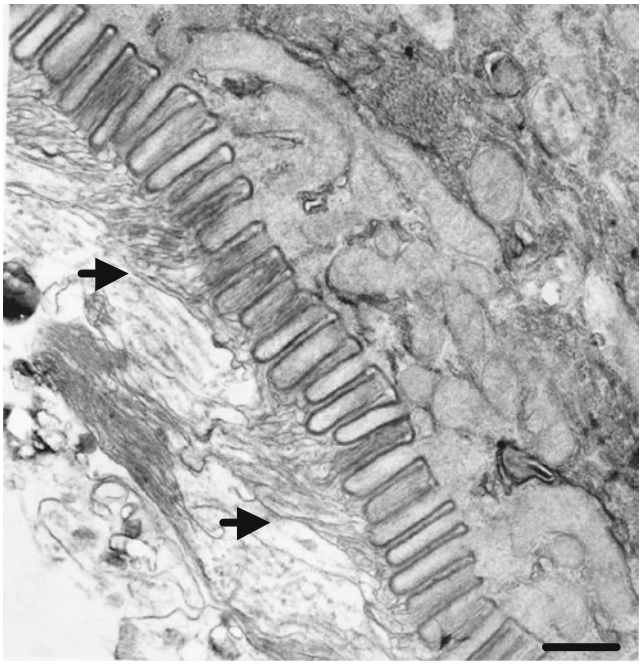

Fig. 1 Ultrastructural analysis of the midgut cells from the triatomine *Dipetalogaster maximus*, revealing the presence of numerous sheaths of double-layered phospholipid membranes (PMVM, black arrows), which are released into the midgut lumen. Note that the PMVM cover the microvilli of midgut cells. Bar=0.6 μm. Image obtained by the Dansa-Petretski laboratory

observed in the *Rhodnius* PMVM (Oliveira et al. 2005). Heme crystallization could be induced *in vitro* with isolated PMVM in temperature-sensitive reactions (Oliveira et al. 2000a). Fractionation of lipid and proteins from isolated PMVM showed that both were able to induce Hz formation *in vitro*, and the content of PMVM within the midgut, as estimated by the activity of the biomarker α-glucosidase, was correlated with midgut Hz content (Silva et al. 2007). We also recently demonstrated that Hz is by far the dominant iron species in the *Rhodnius* midgut, representing at least 97% of all iron content 4 days after blood feeding (Stiebler et al. 2010a). Interestingly, lipids extracted from the PMVM of blood or plasma-fed insects were highly efficient catalysts of heme crystallization *in vitro* (Stiebler et al. 2010a).

Extracellular lipid droplets in the Schistosoma gut

The *S. mansoni* gut contains extracellular "lipid-like droplets" in both the gut lumen and the gastrodermis (Morris 1968). These lipid droplets were hypothesized to represent different degrees of the breakdown of blood components because they were associated with "dense caps" at the droplet surfaces. In fact, Halton later demonstrated that hematin was present only in the gut lumen of both sexes of *S. mansoni* but was absent from gut epithelial cells (Halton 1967). Our group demonstrated the presence of heme multicrystalline assemblies that are always associated with the surface of extracellular lipid droplets in the *S. mansoni* gut (Corrêa Soares et al. 2007). This suggests that Hz formation is initiated at the hydrophilic–hydrophobic interface provided by the lipid droplets and then proceeds toward the particle's core, which is supported by recent findings that Hz formation occurs rapidly and efficiently at organic–water and lipid–water interfaces (Egan et al. 2006; Hoang et al. 2010). We have also demonstrated that lipids present in the extracellular lipid droplets of the *S. mansoni* gut play a key catalytic role in Hz formation, possibly by providing a hydrophilic–hydrophobic interface for heme dehydration, solubilization and crystallization into Hz. The rate of Hz formation mediated by lipid droplets is inversely related to the number of pre-existing Hz crystals on the droplet surfaces, suggesting that autocatalysis is less efficient than interface-mediated crystallization. Supporting these data, we recently showed that the reduction of medium polarity results in early heme solubilization, which then provides suitable physicochemical conditions for spontaneous heme crystallization *in vitro* (Stiebler et al. 2010b). Hydrophilic–hydrophobic interfaces and organic solvents could conceivably share a common mechanism for driving heme crystallization by reducing the medium water activity.

Protein-mediated heme crystallization

Plasmodium food vacuoles concentrate the activities responsible for both hemoglobin degradation (Goldberg et al. 1990) and heme crystallization (Slater and Cerami 1992). Heme-crystallization activity in *Plasmodium* food-vacuole extracts is sensitive to antimalarial quinoline drugs and heat, indicating that a protein with a " heme *polymerase*" activity is involved. This hypothesis is supported by reports of the involvement of specific proteins in Hz formation in *Plasmodium* and *Rhodnius prolixus* (Jani et al. 2008; Silva et al. 2007; Mury et al. 2009).

Protein-mediated Hz formation in Plasmodium

The first proteins demonstrated to be involved in Hz formation in *Plasmodium* were the histidine-rich proteins (HRPs), which are localized in the parasite digestive food vacuole (Sullivan et al. 1996). HRP II binds most of the heme molecules at acidic pH through aspartic acid residues rather than histidine residues, which was expected because the imidazole groups of the histidines are not dissociated at the pH of the digestive vacuole and cannot interact with heme (Lynn et al. 1999). However, parasites lacking the HRP II or III genes are still capable of producing Hz (Sullivan 2002). HRP II is likely not involved in Hz formation because 97% of HRP II is exported to the

erythrocyte cytosol, while the remaining 3% is located in the *Plasmodium* food vacuole (Akompong et al. 2002). Recently, a novel protein named HDP (heme-detoxification protein) was clearly shown to be involved in heme crystallization in *Plasmodium* (Jani et al. 2008). Because HDP is sufficient for Hz production *in vitro* and is localized in the digestive food vacuole, this would explain why HRP II knockout parasites can still successfully produce Hz.

Alpha-glucosidase-mediated heme crystallization in Rhodnius prolixus

Two major components present in the PMVM of triatomine insects have been implicated in heme crystallization: lipids (Silva et al. 2007; Stiebler et al. 2010a) and the biochemical marker of the PMVM, the enzyme α-glucosidase (Ferreira et al. 1988; Silva et al. 1995; Silva et al. 2007; Mury et al. 2009). Knockdown of the α-glucosidase gene or pharmacological inhibition of its activity by diethylpyrocarbonate and castanospermine cause profound effects on Hz formation *in vivo* (Mury et al. 2009). The component responsible for Hz formation in the *R. prolixus* midgut is clearly located at the PMVM (Oliveira et al. 2000a; Oliveira et al. 2005; Oliveira et al. 2007; Silva et al. 2007; Stiebler et al. 2010a), is heat sensitive (Oliveira et al. 2000a), is correlated with α-glucosidase activity (Silva et al. 2007; Mury et al. 2009) and can be mediated by total lipids extracted from the PMVM (Stiebler et al. 2010a). Furthermore, Hz formation mediated by the PMVM occurs under physicochemical and physiological conditions that are present in both the male and female midgut throughout the stages of development (Stiebler et al. 2010a). We have shown that heme crystallization induced by the *R. prolixus* PMVM proceeds in three kinetically different stages: the first occurs over the first 6 h of crystallization reaction, the second between 6 h and 24 h and the last between 24 h and 72 h. Based on these data, we can propose a model for the events that culminate in Hz formation (Fig. 2). Because its action is observed at the beginning of the process, α-glucosidase likely promotes nucleation of the crystal in the first step, forming the first seeds of Hz. The second step is mediated by membrane lipids, where the lipid-water interface facilitates crystal growth (Hoang et al. 2010; Stiebler et al. 2010a). Then nascent Hz crystals are released from hydrophilic-hydrophobic interfaces and, finally, the crystal is extended by means of autocatalysis because the amount of preformed Hz may be sufficient to induce crystallization at a reduced rate. Diethylpyrocarbonate and castanospermine, classical inhibitors of α-glucosidase activity, inhibit Hz formation only when added at the beginning of the process, further implicating the involvement of α-glucosidase in Hz formation only in the nucleation step (Mury et al. 2009).

Concluding remarks

Herein, we reviewed recent advances in our understanding of the process by which heme is crystallized into hemozoin (Hz). Malaria parasites use heme crystallization to detoxify

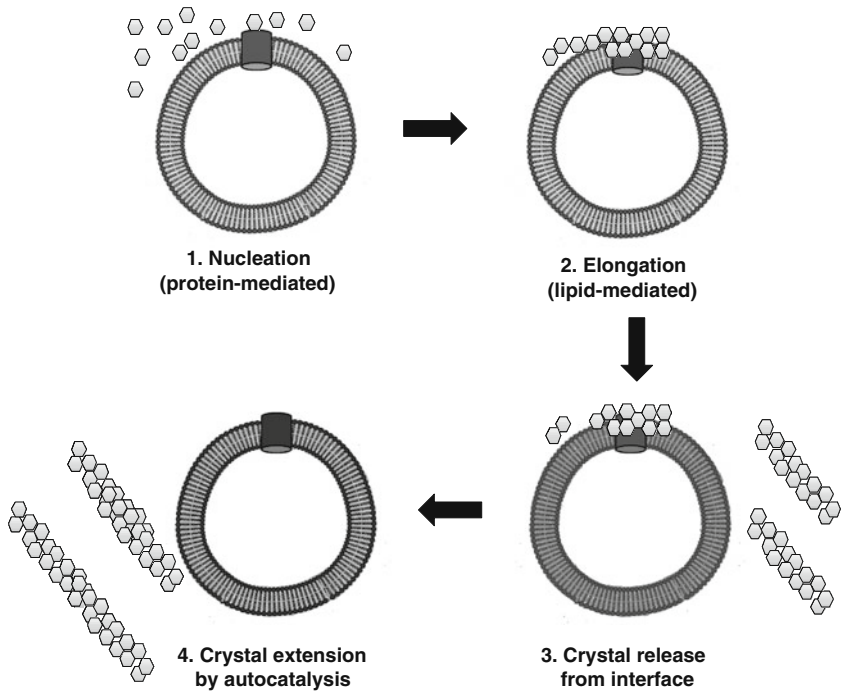

Fig. 2 Proposed model for biological crystallization of heme molecules into Hz. 1. Nucleation step mediated by proteins; 2. Elongation step induced by lipids at the surface of hydrophilic-hydrophobic interfaces; 3. Release of nascent Hz crystals from hydrophilic-hydrophobic interfaces; 4. Extension of Hz crystals by autocatalysis

at least 95% of the iron obtained from red blood cells, and quinoline compounds exert their antimalarial effects by targeting this process. Hz is not unique to malaria parasites; it has been identified in other eukaryotes from protozoa to helminths and insects, and disruption of this process by quinoline antimalarials causes some degree of damage to these organisms. Future research will reveal the details of the mechanisms involved in Hz formation, particularly how heme molecules released from hemoglobin digestion are sequestered as regular crystal phases by amphiphilic structures and specific proteins. The mechanisms by which the impairment of heme crystallization by quinoline antimalarials leads to cell damage also remain to be unraveled.

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