Crystalloids: fascinating parasite organelles essential for malaria transmission

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Abstract

Crystalloids are malaria parasite organelles exclusive to the ookinete and young oocyst life stages that infect the mosquito. The organelles have key roles in sporozoite development and infectivity, but the way this is facilitated on a molecular level remains poorly understood. Recent discoveries have shed new light on these processes.

Malaria and transmission

Malaria remains a serious global health problem that affects millions and kills over 400,000 people annually. The disease is caused by infection with apicomplexan parasites of the genus *Plasmodium*, with *P. falciparum* the deadliest among several human malaria parasite species. Malaria parasites are spread by mosquitoes and a large part of the *Plasmodium* life cycle takes place in the insect. This begins with the uptake of male and female gametocytes with the blood meal of an anopheline mosquito, and ends several weeks later with the injection of sporozoites by mosquito bite to initiate new infections in the human host (**Figure 1A**). The main developmental steps that take place in between are: (*i*) gametogenesis and fertilisation in the midgut lumen (hour 1); (*ii*) transformation of the zygotes into elongated motile forms termed ookinetes (day 1); (*iii*) crossing of the midgut epithelium by the ookinetes, followed by their transformation into young oocysts (day 2); (*iv*) growth and division of the oocysts, known as sporogony, to generate thousands of sporozoites (weeks 1-2); (*v*) sporozoite egress from the oocyst and colonisation of the insect's salivary glands (weeks 2-3) (**Figure 1A**). Use of insecticides continues to be a key intervention to limit malaria transmission and disease, but this vector control approach is under threat from increasing insecticide resistance and alternative transmission control measures are needed. These include interventions based on blocking parasite development in the insect with antimalarial drugs or vaccines that are administered to humans and taken up by the mosquito with its *Plasmodium*-infected blood meal [1].

Sporogony and crystalloids

Malaria parasites suffer severe population losses in the mosquito midgut and for this reason sporogony constitutes a vital parasite multiplication step to ensure successful transmission

from the insect back to the vertebrate. Sporogony remains a poorly studied part of the *Plasmodium* life cycle, but an important advance came with the discovery that an enigmatic parasite organelle called the crystalloid, which forms in ookinetes within hours of parasite uptake by the mosquito, is critically involved (reviewed in [2]) (**Figure 1B**). This finding raised new interest in the crystalloids from a parasite cell biology perspective, but also as a potential route to targeting sporogony at a more accessible, early stage of transmission when the parasite resides in the midgut lumen.

First described in 1962, crystalloids are parasite subcellular structures that have long been implicated in malaria transmission by virtue of their exclusive presence in ookinetes and early oocysts [2]. Electron microscopy shows that crystalloids are clusters of tightly packed small spherical units (**Figure 2A**), but experimental evidence regarding their origins and functions remained elusive until studies in the mouse malaria parasite *P. berghei* provided proof of a spatial, temporal and functional link with a group of *Plasmodium* proteins that are essential for sporogony [2]. The six proteins in question, named LCCL lectin adhesive proteins (LAPs), are highly conserved and possess a unique modular architecture of domains implicated in protein, lipid and carbohydrate binding, including the LCCL domain (pfam03815, named after its founding proteins *Limulus* clotting factor C, Coch-5b2, Lgl1) [2]. Using *P. berghei* parasites that stably express LAPs fused to a green fluorescent protein (GFP) tag, it was shown that LAPs 1-3 co-localise with an endoplasmic reticulum (ER) marker in female gametocytes and re-localise during ookinete development to the crystalloids [2, 3] (**Figure 2B**). LAPs 4-6 also localise in crystalloids, but translational repression in female gametocytes results in their protein not being expressed until the early zygote stage when translational silencing is lifted [4]. Knockout of any of the six LAPs in *P. berghei*, either individually or in pairs, gives rise to a similar loss-of-function phenotype characterized by a failure of the oocyst to complete

differentiation and produce sporozoites [2, 3] (**Figure 2C**). The shared loss-of-function phenotypes and crystalloid localisations of the LAPs, as well as their conformational co-dependence [5], indicated that these molecules operate in concert as a protein complex, which was indeed experimentally demonstrated in a later study [6].

Several studies of LAP mutants demonstrated a role in crystalloid biogenesis. First, it was shown that knockout of LAP1 or LAP3 in *P. berghei* abolished crystalloid formation altogether [2, 3] (**Figure 1B**, **Figure 2D**). Second, a mutant parasite line expressing LAP3 lacking its LCCL domain exhibited a marked delay in crystalloid formation in ookinetes, which helped reveal that organelle formation occurs through microtubule-dependent transport and assembly of ER-derived vesicles [3]. Third, carboxy-terminal GFP tagging of LAP4, but not the other LAPs, unexpectedly produced a mutant phenotype with regards to crystalloid biogenesis giving rise to abnormally formed crystalloids [7]. These crystalloid defects affect sporogony in different ways: whilst LAP null mutants without crystalloids give rise to oocysts that fail to sporulate and reach a larger than normal size, the LAP4::GFP-expressing mutant with abnormal crystalloids produced smaller oocysts that sporulated earlier than normal giving rise to non-infectious sporozoites [7]. On a cellular level, oocyst growth and mitosis in LAP mutants is indistinguishable from wildtype oocysts during the first week of oocyst development leading up to cytokinesis [3, 7]. By contrast, on a molecular level LAP null mutant oocysts display markedly lower expression levels of sporozoite genes and their transcription factors that is already apparent before cytokinesis would normally occur, indicating that events leading up to the sporulation defect could happen early in, or even upstream of sporogony [8].

Other crystalloid proteins

93 More noteworthy advances in our understanding of crystalloid molecular biology came with
94 recent discoveries of two enzymes that are localised in *P. berghei* crystalloids. The first of
95 these is a palmitoyl-S-acyl transferase (PAT), named DHHC10, that like the LAPs was shown
96 to be required for crystalloid biogenesis and sporozoite development [9]. PATs catalyse S-
97 palmitoylation, a widespread post-translational lipid modification of proteins. PATs have a
98 highly conserved Asp-His-His-Cys (DHHC) motif within a cysteine-rich domain, as well as four
99 membrane-spanning domains that direct their localization to a variety of cellular membranes
100 and compartments. The identification of DHHC10 as an essential crystalloid protein suggests
101 that S-palmitoylation plays a key role in the biogenesis and/or function of the organelle, and
102 that the crystalloid accommodates substrates of this enzyme that require palmitoylation to
103 facilitate successful sporogony.

104 More recently, a second crystalloid-resident enzyme was identified and characterised in *P.*
105 *berghei*: NAD(P) transhydrogenase (NTH), a multi-pass transmembrane protein that
106 generates NADPH [10]. The study showed that NTH null mutant parasites are unable to form
107 crystalloids and do not support sporozoite formation in the oocyst, like null mutants of LAPs
108 and DHHC10. Parasites expressing structurally intact NTH that was rendered enzymatically
109 inactive through a point mutation were able to form crystalloids, but again did not support
110 sporozoite formation [10], demonstrating that NTH has a structural role in crystalloid
111 biogenesis and an enzymatic role in sporogony. The apparent functional dependence of the
112 crystalloids on NADPH produced by NTH forms the basis for the hypothesis that the organelle
113 harbours NADPH-dependent enzymatic activity. NTH null mutants are not impeded in their
114 ability to form ookinetes and oocysts [10], and thus it seems unlikely that this source of
115 NADPH is required for neutralising oxidative stress encountered by the parasites in the

mosquito midgut. Instead, NADPH production by NTH more likely reflects the presence of anabolic processes in the organelle.

Most recently, using GFP affinity purification and mass spectrometry, it was shown that the LAP complex is part of an extended protein interaction network that is enriched in known and novel crystalloid proteins [11]. These include members of a family containing 'CPW-WPC' domains (pfam09717) [12]; a novel family of proteins with pleckstrin homology-like domains [11, 13]; and a membrane protein with a TPM domain (pfam04536, named after its founding proteins TLP18.3, Psb32, MOLO-1), of which a structural paralogue was previously reported to reside in the organelle [14]. These results point to a diverse and intricate organelle contents, and indicate that proteins destined for the crystalloid interact in the ER creating a 'crystalloid protein complex' that enables both crystalloid targeting and formation. This model supports the reported structural role of NTH in crystalloid biogenesis [10] and, by analogy, explains how structurally and functionally diverse crystalloid proteins such as the LAPs, DHHC10 and NTH can generate similar loss-of-function phenotypes.

Future perspectives

Given the structure of the crystalloid organelle it is tempting to speculate that it constitutes a specialized adaptation of the vesicular transport system of the cell, transporting critical cargo from the ER to other cell compartments, or the extracellular environment, during sporogonic development. Many questions remain about its specific modes of action, but recent advances in our understanding of its formation and molecular composition, in particular the identification of two essential membrane-bound enzymes and the suggestion of additional NADPH-dependent enzymatic activity in the organelle [10], are fascinating and form a useful basis for further studies. It also increases the likelihood that specific inhibitors

of crystalloid biogenesis or function can be developed to target sporogony and sporozoite transmission. Antimalarial compounds were recently shown to be effectively absorbed into the mosquito after short exposure to a treated surface [15]. This important discovery has opened new paths for drug delivery to malaria vectors, making the search for compounds that impede development of the mosquito stages of the parasite, including the sporogonic stages, more imperative. This adds greater value and urgency to our collective efforts to uncover the molecular processes that underlie *Plasmodium* biology in the mosquito.

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Declaration of Interests

The authors declare no competing interests.

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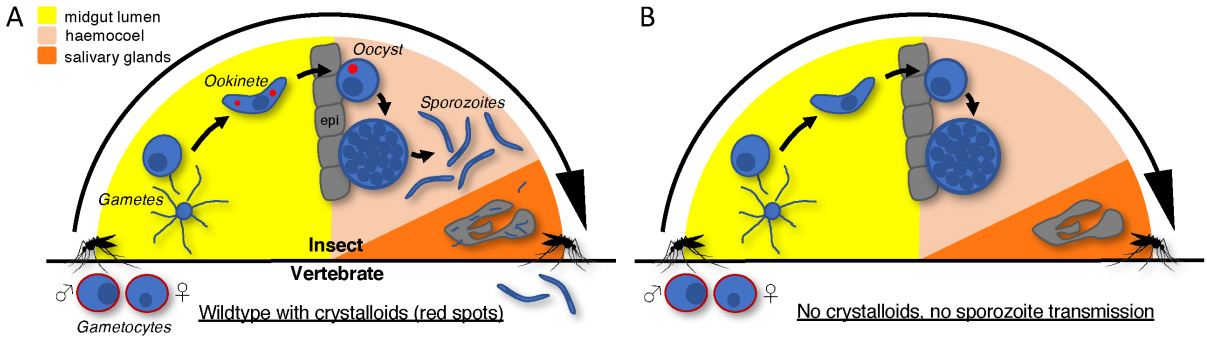
Figure 1. Development of malaria parasites in the mosquito vector. (A) Wildtype parasites. Gametocytes entering the midgut lumen during blood feeding undergo rapid gametogenesis followed by fertilization. The resulting zygotes transform into motile ookinetes that possess crystalloids (red spots). Ookinetes cross the midgut epithelium (epi) and transform into oocysts on the haemocoel side. Oocyst grow and divide to produce sporozoites that colonise the salivary glands and are transmitted back to the vertebrate host via mosquito bite. Parasites are depicted blue, mosquito tissues grey. (B) Parasites carrying mutations that abolish crystalloid biogenesis. In the absence of crystalloids, oocysts undergo growth and mitosis, but fail to produce sporozoites.

Figure 2. Crystalloids have an essential role in sporogony. (A) Ultrastructure of crystalloids in a *P. berghei* ookinete section. The crystalloids appear as clusters of tightly packed small spherical units. Scale bar = 500nm. (B) Live fluorescence images of early zygotes, an ookinete and a young oocyst of a *P. berghei* line expressing the crystalloid protein LAP3 fused to green fluorescent protein (LAP3::GFP). LAP3 resides in the endoplasmic reticulum in early zygotes and relocates to the crystalloids during ookinete development. Ookinetes typically have two crystalloids that merge during oocyst transition. Scale bar = 5μm. (C) LAP3::GFP expressing oocysts develop normally and produce hundreds of sporozoites (containing narrow elongated nuclei in blue), while oocysts of a *P. berghei* LAP3 knockout line (LAP3-KO) undergo growth and mitosis, but fail to produce sporozoites. DNA is stained blue. Scale bar = 10μm. (D) Knockout of LAP3 prevents crystalloid biogenesis: Crystalloids are absent in *P. berghei* LAP3-KO ookinetes, while LAP3::GFP-expressing ookinetes possess normal crystalloids (arrows). Scale bar = 1μm. Images adapted from [3].

213 Figure 1



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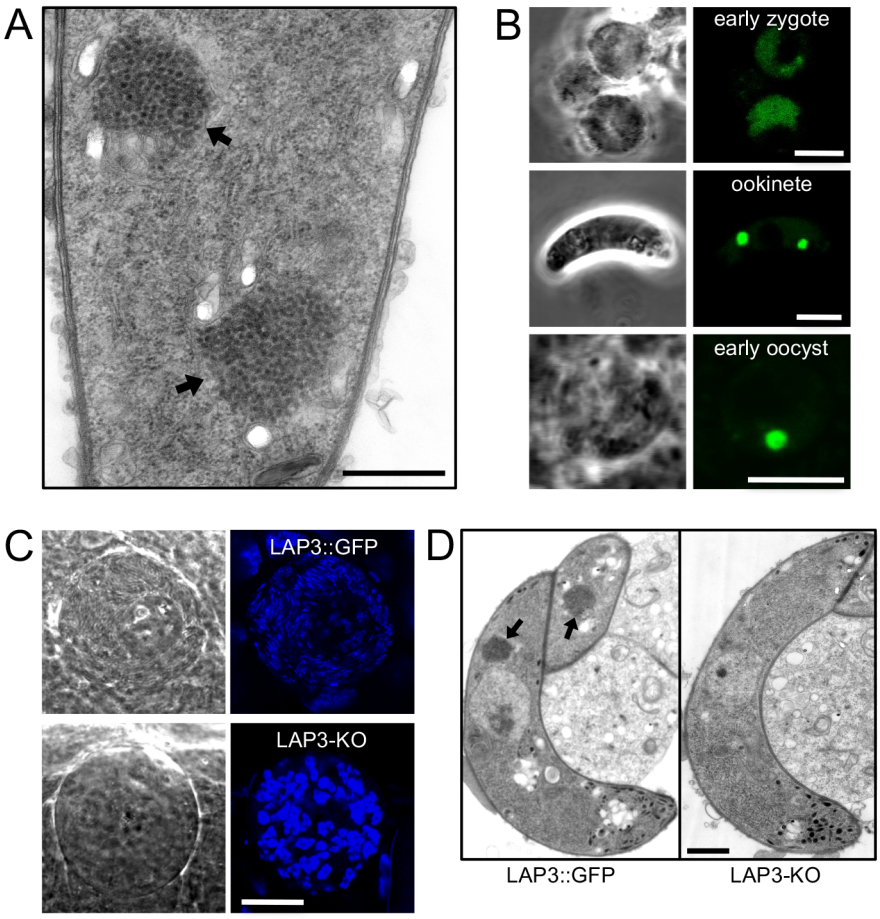
215 Figure 2



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