Apicomplexa micropore: history, function and formation

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Abstract

The micropore, a mysterious structure found in apicomplexan species, was recently shown to be essential for nutrient acquisition in *Plasmodium falciparum* and *Toxoplasma gondii*. However, the differences between the micropores of these two parasites questions the nature of a general apicomplexan micropore structure and whether the formation process model from *Plasmodium* can be applied to other apicomplexans. We analyzed the literature on different apicomplexan micropores and found that *T. gondii* probably harbors a more representative micropore type than the more widely studied ones in *Plasmodium*. Using recent knowledge of the Kelch 13 protein interactome and gene depletion phenotypes in the *T. gondii* micropore, we propose a model of micropore formation. Thus enriching our wider understanding of micropore protein function.

The discovery of the micropore

All known members of the Apicomplexa are parasitic with most of them (including *Plasmodium*, *Toxoplasma* and *Eimeria*) being pathogens of human, domestic and aquatic animals and responsible for causing severe public health problems and huge economic loss. These organisms are eukaryotic with typical organelles such as the nucleus, the Golgi, the mitochondrion, the endoplasmic reticulum, but also possess unique organelles such as the apicoplast, the conoid and the rhoptries. Among the unique ones, a structure with a plasma membrane invagination surrounded by **electron dense rings (EDRs)** which is located on a pore within the **inner membrane complex** (**IMC**, see Glossary), was first reported in sporozoites of *P. falciparum* and termed the “micropyle” [1] (Type I micropore, Fig. 1). This was later found in both the bradyzoites and tachyzoites of *T. gondii,* and called “micropore”, based on its possible function as a feeding pore [2]. Subsequently, micropores with a similar morphology were found in many sporozoa and related organisms, such as the dinoflagellates *Hematodinium* and *Colpodella* [3-6].

Based on observations of uptake of host cytoplasm in the *Plasmodium* erythrocytic stages, Aikawa *et al.* [7] recommended renaming the erythrocytic stage micropyle as the "cytostome"[7]. Meanwhile, the older term “micropyle” was thought to be inaccurate, and possibly misleading, and has been renamed as the micropore [7-10]. The term cytostome in Apicomplexa is now restricted to describe the erythrocytic stage of *Plasmodium* parasites only. In this paper, we suggest the use of micropore and classify “micropores” into two distinct types. We describe the term “cytostome” as a specialized form present during the erythrocytic stage of *Plasmodium*, which is identified by an invagination of the **parasitophorous vacuole membrane (PVM)** without the limitations imposed by the IMC, as Type II depicted in **Figure 1**).

Structure and type of micropore

In order to reveal any consensus characteristics of the micropore in typical members of the Apicomplexa, we re-analyzed micropore morphology data from the literature, in the context of a 18s-rRNA based phylogenetic tree (**Figure 2**). Data indicates that *Plasmodium* exhibits stage-specific morphological conversion of the micropore (**Figure 1 and 2**). Interestingly, however, the micropore (Type II) in the trophozoite of this parasite within the red blood cells is well characterized with two layers of membrane invagination - the parasite plasma membrane (PM) and the PVM. However, in the early stage of the trophozoite, the micropore (Type II variant) of *Plasmodium* spp. may just begin to invaginate without the PVM invagination[7]. As the *Plasmodium* develops into the merozoite in the vertebrate host or sporozoite in the mosquito vector, the inner membrane complex (IMC) is formed, presenting the micropore within the IMC pore with only an invaginated monolayer of the parasite plasma membrane visible (Type I). At these stages, the size of the micropore has shrunk (with sizes refering to both the diameter of EDRs “OD” and depth of invagination, see also below).

In the order Piroplasmorida, which includes parasites such as *Babesia* and *Theileria*, the trophozoites reside inside erythrocytes and their micropores are similar to the ones found in *Plasmodium* trophozoites [11, 12]. However, since the PVM in *Babesia* and *Theileria* is dissolved soon after invasion, their micropores are the type II variant that lacks both the IMC and PVM invagination [11-13]. When *Babesia* develops into sporozoites in the vector salivary glands, the micropore is also converted into a type I structure [14]. In the bovine parasite *Neospora* spp., type I micropores are observed [15].

In the rest of the members of the Apicomplexa, we barely found evidence for any type II

75 micropores. For example, the tachyzoite of *T. gondii*, an active proliferating stage within
76 mammalian cells, normally harbors 1 to 2 type I micropores with a plasma membrane
77 invagination (like a cup of about 100 nm in diameter) within an IMC pore, surrounded by
78 two **EDRs** [16]. Therefore, we consider that the type I micropores are present in major
79 members of the Apicomplexa (**Figure 2**), while the type II micropores have, so far, only been
80 observed in the parasite stages found inside the erythrocytes. This is supported by observing
81 similar structures within avian *Plasmodium* species [7]. We propose that the type II
82 micropores, including the variants, are an adaptation to parasitism in the erythrocytes
83 where the parasite IMC is dissembled and enables the presence of an enlarged micropore,
84 resulting in the formation of the type II structure.
85 It is noteworthy that there are significant differences between the two types of micropores
86 in the Aconoidasida. 1) The IMC is only found in the type I but not in the type II, as the IMC
87 degrades at the ring stage, and is absent in the trophozoite and then is rebuilt in the
88 merozoite. 2) The type II may represent an active form of the micropore, as the trophozoite
89 engages with active uptake of host nutrients using 3 to 4 micropores per trophozoite [17],
90 while the type I may represent a less active form. 3) The depth of the EDRs in the
91 erythrocytic stage appears shallower and shaped like two dots in the longitudinal section of
92 the type I micropore rather than two sticks in the type II. This is not true, as they are actually
93 in similar shape and size (data from **Figure 2** and schematic diagram in **Figure 1**).
94 Therefore, we consider that the Type I is the ubiquitous type of micropore in apicomplexans
95 both in terms of size and structure. In this case, the *T. gondii* micropore is the best
96 characterized model organelle, being more representative, for studying structure and
97 function.
98

The function of the micropore

It should be noted that the active uptake of host cytoplasm by the type II microspore or cytostome of the *Plasmodium* parasite, is characterized by observations of the dense materials from the red blood cell, accompanied by the invagination of the PVM [18]. However, in mature merozoites, this uptake probably ceases, invagination of the PVM cannot be observed anymore and the size of the cytostome is reduced to nearly a size comparable to the micropore type I size. Therefore, the size changes seem to be correlated with the activity of the uptake function. Based on this, some studies proposed that the micropore might be non-functional or dormant [7].

With the advances in electron microscopy, the discovery of clathrin-like bristle coat and budding vesicles at the base of the micropore in *T. gondii* bradyzoites suggests that the micropore is not quiescent but rather dynamic [16]. Using the electron tomography (ET) data from Wan *et al.* [19], the budding vehicle was observed in the *T. gondii* tachyzoite, supporting its potential role in endocytosis. This observation also implies that the endocytosis occurring at the micropore may be receptor-mediated [16, 19]. 3D reconstruction of micropores in the general state (**Figure 3A**) and undergoing endocytosis (**Figure 3B**) highlights the significant differences between these two stages.

Another strong piece of evidence supporting the role of the micropore in endocytosis is the presence of the **Kelch 13 (K13)** protein at the EDRs. *K13* was initially identified as a gene associated with artemisinin resistance in *P. falciparum* [20, 21]. Later, K13 was found to be located in the EDRs of the cytostome [22] and, therefore, was suggested to have a function related to endocytosis restriction in the ring stage [23]. Further studies on K13 in *Toxoplasma* micropores using immuno-electron microscopy [19, 24] confirmed the involvement of the micropore and the key role of protein K13 in endocytosis. Furthermore,

this is also supported by an imbalance in plasma membrane homeostasis. This is a secondary phenotype of defective endocytosis and is characterized by the accumulation of excess SAG1-positive plasma membranes in K13-depleted *Toxoplasma* [24]. Significantly, knockdown of K13 or its interacting proteins results in an abnormal morphology of the *T. gondii* micropore [19]. This was the first demonstration of a link between K13 and its interacting proteins as well as the micropore structure and function.

The protein components of the micropore in *Plasmodium* and *Toxoplasma* and potential mechanisms

Although it has been 60 years since the discovery of the micropore, the components of micropore have been unknown until recently. As mentioned above, K13 is probably the first protein identified in the micropore. After the discovery of K13, other components of the complex were identified using the Bio-ID technique in *P. falciparum* [23], while a similar set of core proteins were also identified in *Toxoplasma* (**Figure 4**) (Wan et al., 2023; Koreny et al., 2023). These studies confirmed a shortlist of 4 conserved micropore proteins including K13, **epidermal growth factor receptor substrate-15** (**EPS15**), **ubiquitin binding protein-1** (**UBP1**), **dynamin related protein C** (**DrpC**) and **adaptor protein 2** (**AP2**). The precise biological characteristics and functions of these proteins requires further studies to confirm. K13 is the most famous protein, as it is related to the artemisinin resistance found in *P. falciparum* [20, 21], which was identified due to its functional nature in nutrient salvaging [23]. K13 probably works as an adaptor, as it contains a Kelch domain and a Broad complex Tramtrack Bric-a-brac/Pox virus Zinc finger (BTB/POZ) domain. Such domain-containing proteins have been shown to be involved in presenting proteins, due for degradation, to ubiquitination in other eukaryotes [25, 26], suggesting a potentially similar mechanism in

147 K13 function [27].

148 A second protein is UBP1, which acts as a key K13-interacting partner [19, 23, 24] and is also
149 related to the artemisinin resistance [28], contains a GTPase domain, which may hydrolyze
150 GTP to release C-terminal adducts of ubiquitin [27]. Such activity may help to de-
151 ubiquitinate nearby proteins, i.e. other micropore components. Since ubiquitination has role
152 in regulating endocytic recycling, UBP1 may promote endocytic recycling, a process which
153 has been proposed to occur in yeast [29].

154 The third protein, EPS15, may function as a scaffolding adaptor protein and contains two
155 EPS15 homology (EH) domains and a coiled-coil region. EPS15 in other eukaryotes has been
156 shown to bind ubiquitin [30], and play important roles in controlling endocytosis [31]. While
157 the EPS15 from other eukaryotes have a ubiquitin-interacting motif and an AP2 binding
158 domain [19], the apicomplexan EPS15 lacks both of them, suggesting possible differences in
159 EPS15 binding partners or degradation targets. However, the AP2 binding studies and the
160 ubiquitinated interactome shows that the apicomplexan EPS15 retains both activities [19,
161 24]. Therefore, either there are two divergent motifs/domains which remain to be
162 recognized or indirect interactions may be operating. Additional evidence lies in the
163 localization of the micropore at the boundaries of the IMC membrane plate sutures (ISCs) as
164 indicated by the ISC3 signals [24]. As components of the IMC (IMC1, IMC4, IMC10, IMC14,
165 GAPM2B and GAPM1a) and the sutures (ISC1) were found in both the micropore
166 interactome and the ubiquitination proteome of *Toxoplasma* [19, 24, 32], the activity of
167 EPS15 in ubiquitin-interactions would be expected. Additionally, the binding of AP2 may be
168 involved in the trafficking of the ubiquitin modified receptors and is involved both in
169 secretion and endocytosis, as found in other eukaryotes [30] [33].

170 The fourth protein, AP2 is consists of four subunits (α, β, μ, σ) and is responsible for the

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171 endocytosis of cargoes from the cell surface [34]. Subunits μ and σ form a steric blockade
172 which is released when phosphorylated by the adaptor-associated kinase 1 [35], resulting in
173 the binding of μ to cargo proteins and to phosphatidylinositol-4,5-bisphosphate in proximity
174 to the membrane [36]. Importantly, the subunit α was found to interact with EPS15 in other
175 eukaryotes [37], suggesting a similar role in the Apicomplexa. Interestingly, results indicated
176 that the β subunit could interact with clathrin in other eukaryotes [38], while *Plasmodium*
177 experiments provided evidence of an interaction between AP2β and clathrin [23]. However,
178 clathrin was thought not to be involved in *Toxoplasma* micropore function [23, 39]. To
179 resolve this discrepancy, we would like to clarify if AP2β is also a shared subunit of AP2 and
180 Adaptor Protein 1 (AP1), and where the latter is located to the Golgi network, as this is the
181 common situation in many eukaryotes[34]. Thus, it is probable that AP1 is clathrin-
182 dependent in the Golgi network as in other eukaryotic organisms, while AP2 is micropore
183 specific and has lost the ability to bind clathrin. Since AP2β is likely to be the subunit
184 corresponding to the clathrin-binding activity, it might have distinct versions due to, as yet
185 undetermined, alternative translation initiation or splicing. This explanation is supported by
186 the differences in localization of AP2β compared with the other micropore proteins [19, 24].
187 The final protein, DrpC contains only a recognizable GTPase domain, but as an apicomplexan
188 specific form, it functions in vesicle trafficking and division in *T. gondii* [40]. It was suggested
189 that dynamin could act cooperatively with Bin-Amphiphysin-Rvs (BAR) domain proteins and
190 actin, and stimulate GTP-dependent membrane scission [41, 42]. For instance, in mice, actin-
191 nucleating proteins, actin and BAR domain proteins accumulate at the base of arrested
192 endocytic clathrin-coated pits, where they support the growth of dynamic long tubular
193 necks which are finally terminated by dynamin [43]. Such a situation has been partially
194 observed in *Plasmodium*, in which hemoglobin containing vacuoles required a dynamin-actin

system that localizes at the base of the micropore [44]. In *Toxoplasma,* electron tomography revealed that DrpC exhibits a ring-like localization feature, close to the basal end of the micropore [24], where the budding occurred [19]. Therefore, DrpC may also cooperate with actin and BAR proteins as dynamin and is responsible for generating budding vesicles at the base of the micropores and for pinching off vesicles, before transporting them to the cytoplasm. However, two BAR domain proteins have been identified in *Toxoplasma* but neither localized to the micropore [19], nor interacted with DrpC based on the interactome studies [24]. Therefore, the involvement of the BAR domain protein in apicomplexan micropore endocytosis remains questionable.

Using *Plasmodium* and *Toxoplasma* as queries, **Supplementary Table S1** shows the identified homologs across other apicomplexan and the relevant Phylum including Perkinsids, Dinoflagellates and Ciliates based on the genome blast of the core micropore proteins mentioned above. These molecular clues back up the notion that the micropore exists broadly across the Apicomplexa. Also, it is reasonable to expect that *Plasmodium*, with two types of micropore at the present, has more unique micropore proteins.

Models for the formation of the micropore in *Plasmodium* and *Toxoplasma*

Over many years, several formation models have been proposed for the micropore. A pioneering study by Volkmann *et al.* [45], proposed a tri-stage development scheme for the *Klossia helicina* micropore based on morphological observations with limited resolution. In the last two decades, two much clearer models from the cytostome studies in *P. falciparum* trophozoites have been proposed which provide clues to the formation and workings of the micropore (**Figure 4**). Model 1 suggests that the *Plasmodium* plasma membrane invagination is the first stage of micropore formation, followed by the PVM, and then the emergence of

the EDRs which surround the neck of the invagination, resulting in the maturation of the micropore [17]. Later, an updated model was proposed based on the evidence which showed the emergence of independent EDRs prior to any membrane invagination [44]. However, these models of formation are solely reliant on TEM observations on normal cells but unfortunately lack quantitative data. It might be ambiguous to judge these models based on a rarely visualized structure since the observations might be captured at an intermediate stage of the micropore formation. The stage at which the PM and PVM invagination occurs (i.e. **Figure 4B**) has been considered as an organelle for pinocytosis, a process occurring independently of the micropore development, as supported by Elliottet *et al.*[46]. However, the main point of contention, between these models, is whether the invagination of the plasma membrane is a transition state which occurs before formation of the EDRs of the micropore or whether this is a new mechanism of endocytosis.

Despite the models of cytostome mentioned above, no model is available for the type I micropore, yet. Based on recent knowledge of micropore components and structural defects in micropore-associated protein-depleted parasites, we have managed to propose an updated micropore formation model (**Figure 5**). Recently generated data from ourselves and others suggests that the sequential key events should be as follows: first, the inner membrane complex forms a pore (**Figure 5A-B**); second, the plasma membrane forms a regular wine glass invagination inside the pore (**Figure 5C**); third, the formation of an EDRs structure is completed (**Figure 5D**).

The evidence supporting this formation order relies on the TEM observations in parasites depleted with K13 or EPS15. In parasites depleted with EPS15, cells with defective micropores① (**Figure 5**) in which no pitting in the PM but abnormal pitting in the IMC instead of a notch and no EDR were observed [19]. We may conclude that the notch in the

IMC is a prerequisite for PM invagination and EDRs formation. While the lack of an IMC pore and the abnormal pitting in the IMC could be attributed to the failure of the membrane invagination and EDRs formation, e.g. by related protein mis-positioning. Therefore, EPS15 may be involved in the process of opening the IMC pore. In-situ tagging fluorescence microscopy revealed the possible localization of EPS15 as a ring decorating the IMC pore [24]. Since IMC was not observed in the type II micropore[23], EPS15 depletion may not result in similar phenotype.

When K13 is depleted, type ③ and ④ defective micropores were formed. These defective micropores have a notched IMC but no EDR, with either no PM invagination, or an abnormally enlarged PM invagination. These observations suggest K13 is a core component of the micropore EDRs and this is supported by the interactome studies from Wan *et al.* [19] and Koreny *et al.* [24]. Furthermore, the EDRs are required for stable PM invagination.

The knock-down of UBP1 results in defective micropores of type ② with a larger opening size of the micropore. This observation suggests that UBP1 may be responsible for restricting the size of the PM invagination. It has been long known that yeast UBP1 contains transmembrane domains [29], which is also true for *Toxoplasma* UBP1. The EDRs may form during the initiation of the PM invagination, but might be torn apart if the PM invagination is over extended. Fluorescence microscopy from Koreny *et al.* [24] also support our opinion that UBP1 is located right next to the PM invagination. Since UBP1 depleted cells retain at least a partial EDR, the integration of UBP1 in the EDRs may be a non-essential step.

The knock-down of **proteophosphoglycan 1 (PPG1)** results in about a 50% more chance of observing micropores with normal morphology [19]. When conducting a detailed comparison of morphology, we found that parasites depleted with PPG1 had micropores that have an ~20% oversize in their outer diameter (~60% increased volume) than the ones

in the parental cells. Therefore, there is a higher chance of observation by TEM than expected and PPG1 likely restricts the size of micropores. However, as the inner diameter of the micropore remains unchanged, compared to the PM invagination, the PPG1 depletion may result in a loose connection between two layers of the EDRs. This is supported by data from Koreny *et al.* [24] that PPG1 (CGAR) locates near K13 at the IMC pore proximal to UBP1.

Concluding remarks

The micropore was first described in *Plasmodium* more than 60 years ago, and subsequently in various species of apicomplexans. During their complex developmental stages, two types of the micropores have been observed. Compared to the *Plasmodium* cytostome (type II micropore), *Toxoplasma* hosts a type I micropore which has recently been demonstrated to be a functional nutrient uptake organelle and is commonly found in other apicomplexan members. Both types of micropores share a common set of protein components, represented by key components K13 and EPS15. Based on EM and precise component studies, our new knowledge on micropore will certainly enable us to better understanding of its formation process. Obviously, more are needed to be be carried out on the new model we proposed. Furthermore, important questions remain as to how these components interact with each other and how these two types of micropore switch in the stage development during the life-cycle and adapted endocytosis activity (see Outstanding Questions). As a key tunnel of nutrient (and possibly drug) uptake, it is important to illuminate the functioning of the micropore to guide us to new chemotherapeutic strategies.

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Declaration of Interests

The authors declare no competing interests.

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432
433 **Figure legends**
434 **Figure 1. Two types of micropores are found in the life stages of *Plasmodium*, while only**

type I micropores are found in *Toxoplasma*. There are type I micropores in the sporozoite and merozoite stages and type II micropores in the trophozoite stage of *Plasmodium falciparum*. Type I is characterized by the presence of electron dense rings (EDR) on an inner membrane complex (IMC) pore and a plasma membrane (PM) invagination. The type II is characterized by the additional invagination of the parasitophorous vacuole membrane (PVM) but without the IMC. Note that in some cases, type II variants are observed, for instance, when there is the dissolution of the PVM or where there are low uptake properties at the early stage of infection. In these cases, only the plasma membrane invagination may be present. A parameter diagram for morphonology data is also provided which can be used in conjunction with Figure 2..

Figure 2. The morphonology data of the micropore in the members of the Apicomplexa. Note: ID, inner diameter. OD, outer diameter. ED, electron dense ring depth, also see diagram in Figure 1 to show the measurable features of the micropores. M, merozoite. T, trophozoite. MaG, macrogametes. MiG, microgametocytes. CM, cyst merozoites. OS, oocyst sporozoite. EM, Erythrocytic merozoite. ExM, Exo-erythrocytic merozoite. References: a, *E. i* data from [3]; b, *E. t* re-measured from [47]; c, *I. s*[3, 48]; d, *E. a*[3, 49]; e, *S. t*[3]; f, *T. g*; g, *B. b*[50]; h, *B. j*[51]; i, *K. h*[45]; j, *H. s*[52]; j *K. s*[53]; k, *H. s*[54]; l, *S. p*[55]; m, *S. h*[3]; n, *P. f*[17]; o, *P. k*[3]; p, *P. v*[56]; q, *B. o*[57]; r, *T. a*[58]; s, *T. p*[3]; t, *C. v*[59, 60].

Figure 3. Three-dimensional reconstruction models of the middle sections of the micropores. The micropores were imaged and reconstructed using electron tomography. (A) the micropore model in the general state. (B) the micropore model with a budding vesicle. Green, pellicle. Orange and red, IMC. Yellow, dense ring. Bars, 100 nm. Adapted from [19].

Table S1. Conservation of possible micropore components in major apicomplexans and relative organisms. Components of micropores in *T. gondii* and *P. falciparum* revealed by Bio-ID and other techniques as indicated below. A, DiQ-BioID. B, TurboID. C, classical Bio-ID. D, Fluorescence microscopy with endogenous GFP/mCherry-tagging. E, CLEM (correlative light and electron microscopy). F, immuno-EM. G, STORM (stochastic optical reconstruction microscopy). H, AID-TEM. I, IFA (Immuno-fluorescence assays). J, 3D-SIM (3D structured illumination microscopy)

Figure 4. Two models of type II micropore formation in *Plasmodium*. Model 1, the PM invaginates first (A), the parasitophorous vacuole membrane (PVM) invaginates subsequently (B), then the electron-dense rings (EDRs) surround the parasite plasma membrane (PM) invagination (C). Model 2, the EDRs are located under the PM (D), then the PM and PVM invaginates through the EDRs (E). Adapted from [17] and [44].

Figure 5. The model of type I micropore formation and corresponding defects found in micropore component defective *Toxoplasma* cells. A) Parasite plasma membrane (PM) and inner membrane complex (IMC) that have not yet formed the micropore. B) a pore is formed in IMC. C) PM invaginates inside the IMC pore. D) Electron-dense rings (EDRs) form between the PM invagination and IMC. TEM of abnormal micropore in defective cells:①, IMC pore defects and PM is unable to invaginate, and no EDR, instead the IMC invaginates; ②, EDR defects and PM invagination defects, with a clear IMC pore but no PM invagination, nor EDR; ③, EDR defects, no EDR and no neck with expanding PM invagination； ④, Opening defect with large IMC pore and large EDRs;

483