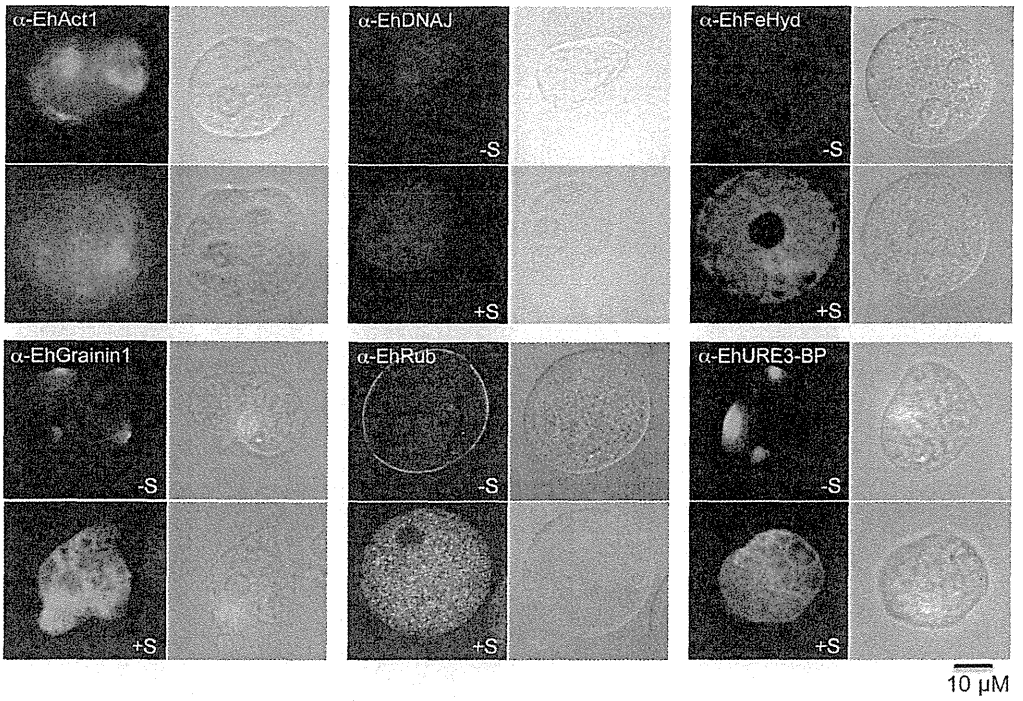


FIG. 7. **Localization of proteins identified as membrane-associated by immunofluorescence microscopy.** Amoebae were fixed with paraformaldehyde and treated with (+) or without (−) saponin. The trophozoites were incubated with specific antiserum (diluted 1:200 in PBS ± saponin), followed by AlexaFluor secondary antibody and Hoechst staining of nuclei. Localization was assessed by fluorescence microscopy.

viously reported to be localized to the ER in other organisms, such as Sec61 alpha subunit (AAU43735) (41), CAAX prenyl protease (XP_648770) (42), alpha-1,3 mannosyltransferase ALG2 (XP_653222) (43), and dolichol monophosphate mannose synthase (XP_651399) (44) (Table S2). Although many ER transmembrane proteins were identified, other ER membrane proteins were not identified in this study. For example, none of the highly expressed members of the cysteine protease binding family (CPBF), which are involved in the transport of cysteine proteases and carbohydrate digesting enzymes from the ER to downstream organelles was identified in the present study (45–47). In addition, only nineteen out of 102 Rab GTPases, most of which were expressed in the trophozoites and possessed the C-terminal isoprenylation motif were isolated from the cell surface (48). The subcellular localization of these 19 Rab GTPases is currently investigated to determine key Rab proteins that mediate the transport from the internal membrane to the cell surface.

A recent large-scale study analyzed the proteins in isolated *Entamoeba* uropods, which are formed and released from amoebae following the incubation of trophozoites with ConA (49). These uropods consist of folded membranes and therefore are composed primarily of surface-associated proteins with small amounts of cytoplasm. Similar to our findings, several of the proteins identified in the uropod proteome are not obviously plasma membrane associated, as they do not contain a transmembrane domain or signal sequence. Interestingly, ~75% of the uropod-associated proteins were also identified in the present study, including proteins involved in trafficking, several Rab family proteins and ribosomal proteins. The major difference between the two studies was the identification of several peptidases in the uropods, including four cysteine peptidases belonging to the C family, two dipeptidyl-peptidases and a serine carboxypeptidase, none of which were present in the surface-associated proteome (49). Most of the surface proteins present in the uropod proteome are likely not uropod specific, but may be localized in general to the outer plasma membrane of the trophozoite.

An analysis of the surface proteome of *Trichomonas vaginalis* identified a total of 411 proteins, of which 23% are present in other subcellular compartments, suggesting that these proteins are contaminants of membrane fractions (27, 49). These putative contaminants included the mentioned above ribosomal proteins as well as Rab proteins, all of which were present in the surface as well as in the uropod proteome of *E. histolytica* (27, 49).

To solve these uncertainties, we used immunofluorescence microscopy to analyze the surface expression on live trophozoites of 23 representative proteins. *In silico* analysis indicated that 12 of these proteins were not membrane associated. The remaining proteins were putatively prenylated or palmitoylated, were nonclassically secreted, or contained a signal peptide and/or transmembrane domain. Interestingly, immunofluorescence analyses of live trophozoites unequivo-

cally indicated that 17 of these 23 proteins were present on the cell surface. An additional three proteins showed surface association on paraformaldehyde-fixed but nonpermeabilized amoebae. Therefore, only three of these 23 proteins, a DnaJ family protein, iron hydrogenase, and a hypothetical protein XP_652420, can be classified as contaminants. In addition, four additional proteins, none of which were identified using our proteomic approach, were localized on the plasma membrane. Similar to EhRab7D and EhRab7E, which were identified as surface-associated proteins, EhRab 7G and Rab7H were found at the trophozoite surface. Rubrerythrin was also associated with the plasma membrane, whereas Fe-hydrogenase showed a cytoplasmic localization. Most of the proteins analyzed show no uniform surface stain. Instead, bead-like protrusion could be observed. One explanation may be that vesicles containing the respective proteins fuse at these areas with the plasma membrane. Nevertheless, this cannot explain, why a similar but less distinct staining was observed in biotin-labeled cells.

Immunelectron microscopy may help in future experiments to analyze if the proteins are localized on the inner or outer cell membrane. In summary, these results indicate that more proteins than suggested are surface-associated, including several molecules lacking an identifiable secretion signal or membrane binding domain. However, despite all controls included, it cannot be ruled out that the list of putative surface proteins contains also some contaminants.

Studies have shown that some proteins are indirectly associated with the extracellular surface. These peripheral membrane proteins bind temporarily, either to the lipid bilayer or to integral membrane proteins, through a combination of noncovalent interactions, including hydrophobic and electrostatic interactions. Among the proteins indirectly associated with the plasma membrane are disulfide isomerase, enolase, heat shock proteins, malate dehydrogenase, triosephosphate isomerase, thioredoxin and superoxide dismutase, all of which were also identified in the current study (25, 50–53). Interestingly, similar to *E. histolytica*, various ribosomal proteins of mycobacteria were identified as being surface-exposed (51).

Proteins lacking a signal sequence likely do not utilize the classical secretory pathway. These molecules of *Entamoeba* may reach the surface by undergoing unconventional secretion. For example, proteins may be secreted through translocation processes that convey these proteins across plasma membranes. These processes may be nonvesicular, or may involve the generation of exo- or ectosomes (54–57). The SNARE machinery, which is highly conserved in all eukaryotes and is present in the surface proteome of *E. histolytica*, may be involved. The SNARE machinery is responsible for membrane fusion as well as for the fusion of secretory vesicles with the plasma membrane. Using this pathway, secreted enzymes, membrane proteins and lipids can reach the cell surface (58, 59). In addition, the focal exocytosis of endomembranes including the ER may be crucial in compensating for the loss of cell surface area during phagocytosis (60, 61).

Studies in *Acanthamoeba* suggested that secreted peptidases may be trapped in vesicles that return membranes from the digestive vacuole to the plasma membrane (62). Some of these peptidases have a high affinity for membranes and are bound to the vesicle membrane. After fusion with the plasma membrane, the contents of these vesicles are released and their internal surface is exposed to a more alkaline pH, decreasing the affinity of these peptidases for the membrane and resulting in their release (63). Cysteine peptidases, along with additional proteins of *E. histolytica* detected at the plasma membrane, may be released by a similar mechanism.

Interestingly, six out of nine core glycolytic enzymes were found in the putative surface proteome (hexokinase, phosphofructokinase, fructose-1,6-biphosphate aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase (GAPDH), enolase). One can speculate that the lysis of trophozoites during the labeling process and therefore the release of these highly expressed glycolytic enzymes may be the reason for their occurrence in the membrane fraction. However, less than 2% of amoebae died during the biotin labeling procedure, which almost excludes this possibility. For enolase, fructose-1,6-biphosphate aldolase, triosephosphate isomerase and GAPDH a surface association was described in several organisms including *Paracoccidioides brasiliensis*, *Streptococcus suis, Trichomonas vaginalis, Lactobacillus plantarum* 299v, *Mycobacterium tuberculosis, Candida albicans,* and *Staphylococcus aureus* (64–70). Interestingly, like also seen for *E. histolytica* these proteins are anchorless and the mechanism by which they reach the cell surface is unknown. It was shown that the extracellular matrix components fibronectin, laminin as well as mucin act as ligands for aldolase, enolase, triosephosphate isomerase, and GAPDH (64–66, 71, 72). Furthermore, these glycolytic enzymes have been identified to act as plasminogen receptors (70, 73). A nine residue plasminogen binding motif, which was found within the enolase sequence of several organisms, including *P. brasiliensis* and *S. pneumoniaea* was also found within the enolase of *E. histolytica* (64, 74). It was shown that the interaction of the different microorganisms with plasminogen is important for invasion of the host and establishment of an infection. For *Trichomonas vaginalis* it was described, that the binding to plasminogen mediates the penetration of the basement membrane and the adhesion to extracellular matrix proteins, like laminin and fibronection (67, 72, 75). It is hypothesized that *T. vaginalis* can adhere to the basement membrane after disruption of the vaginal epithelium (72, 76). Among others, the interaction of extracellular components including fibronectin, laminin, and mucin are also known as important factors for adhesion of *E. histolytica* of the human intestine (77, 78). Therefore it can be postulated, that enolase, aldolase, triosephosphate isomerase, and GAPDH, which were identified in the surface proteome of *E. histolytica* may be important

for the colonization and establishment of the infection. In addition, it was shown that the surface-exposed GAPDH of *Streptococcus pneumoniae* can act as ligand for the complement component C1q. It is postulated that this interaction led to modulation and evasion of the immune system (79). Hexokinase, which was also identified in the surface proteome of *E. histolytica* is normally cytoplasmic. Nevertheless, a phosphotyrosine-containing hexokinase was found to be associated with the plasma membrane fraction of sperms (80, 81). Surface biotinylation of intact mouse sperm followed by precipitation with either hexokinase antiserum or with avidin-Sepharose suggests that this hexokinase possesses an extracellular domain (81). Phosphofructokinase was also found in the bull sperm surface proteome. However, it is not known if this molecule is localized at the surface. In general, an association of glycolytic enzymes with the *E. histolytica* membrane makes sense, because of the high membrane turnover and to drive membrane fusion events.

In conclusion, we have identified the almost complete surface proteome of *E. histolytica*. Most notably, about 50% of the identified proteins lacked the conventional characteristics associated with membrane localization. Nevertheless, the vast majority is indeed membrane-associated. This unexpected finding suggests that the plasma membrane is not a static cellular compartment, but rather part of a highly interconnected cellular machinery, in which intracellular membrane systems are in constant exchange with the plasma membrane. This can result in the presence of proteins outside of the cell that have no conventional signs of plasma membrane-association.

*Acknowledgments*—We thank Susann Ofori and Heike Stephanowitz for their skillful technical assistance.

* This work was supported by the Deutsche Forschungsgemeinschaft (DFG, BR 1744/11-2).

[S] This article contains supplemental Fig. S1 and Tables S1 and S2.

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Biochimie xxx (2013) 1–15

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Review

# Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa☆

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ARTICLE INFO

*Article history:*
Received 30 June 2013
Accepted 24 November 2013
Available online xxx

*Keywords:*
Mitochondria
Mitochondrion-related organelles
Mitosome
Hydrogenosome
Parasite
Evolution

ABSTRACT

The mitochondria have arisen as a consequence of endosymbiosis of an ancestral α-proteobacterium with a methane-producing archae. The main function of the canonical aerobic mitochondria include ATP generation via oxidative phosphorylation, heme and phospholipid synthesis, calcium homeostasis, programmed cell death, and the formation of iron–sulfur clusters. Under oxygen-restricted conditions, the mitochondrion has often undergone remarkable reductive alterations of its content and function, leading to the generation of mitochondrion-related organelles (MROs), such as mitosomes, hydrogenosomes, and mithochondrion-like organelles, which are found in a wide range of anaerobic/microaerophilic eukaryotes that include several medically important parasitic protists such as *Entamoeba histolytica*, *Giardia intestinalis*, *Trichomonas vaginalis*, *Cryptosporidium parvum*, *Blastocystis hominis*, and *Encephalitozoon cuniculi*, as well as free-living protists such as *Sawyeria marylandensis*, *Neocallimastix patriciarum*, and *Mastigamoeba balamuthi*. The transformation from canonical aerobic mitochondria to MROs apparently have occurred in independent lineages, and resulted in the diversity of their components and functions. Due to medical and veterinary importance of the MRO-possessing human- and animal-pathogenic protozoa, their genomic, transcriptomic, proteomic, and biochemical evidence has been accumulated. Detailed analyses of the constituents and functions of the MROs in such anaerobic pathogenic protozoa, which reside oxygen-deprived or oxygen-poor environments such as the mammalian intestine and the genital organs, should illuminate the current evolutionary status of the MROs in these organisms, and give insight to environmental constraints that drive the evolution of eukaryotes and their organelles. In this review, we summarize and discuss the diverse metabolic functions and protein transport systems of the MROs from anaerobic parasitic protozoa.


## 1. Introduction

The mitochondria are believed to have been generated by the endosymbiosis of an ancestral α-proteobacterium in a methane-producing archae or an ancestral eukaryote [1–4]. The main function of the canonical mitochondria is ATP production using oxygen as the final electron acceptor. The metazoan mitochondria also function for heme and phospholipid synthesis, calcium homeostasis, programmed cell death [5], and the formation of iron–sulfur (Fe–S) cluster [6]. ATP generation using oxidative phosphorylation and the electron transport chain (ETC) is the major selective force for the retention of the organelle among the diverse aerobic eukaryotes. However, under oxygen-restricted conditions, the mitochondrion has undergone or is still undergoing tremendous alterations, mostly reductive ones, of its content and function. During such reductive evolution in the organisms lacking oxygen-reliant respiration, Fe–S cluster biogenesis appears to be, with a few exceptions mentioned below, the most conserved among the various mitochondrial functions known in aerobic eukaryotes.

The reductive changes of mitochondria are observed in organisms that adapted to the anaerobic conditions, and such changes were most likely driven by the independence of ATP generation on oxidative phosphorylation in the mitochondria. Such reduced mitochondria are called mitochondrion-related organelles (MROs), which are found in a wide range of anaerobic/microaerophilic eukaryotic lineages that belong to distinct subgroups (Fig. 1). It



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http://dx.doi.org/10.1016/j.biochi.2013.11.018

Please cite this article in press as: T. Makiuchi, T. Nozaki, Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa, Biochimie (2013), http://dx.doi.org/10.1016/j.biochi.2013.11.018

should be noted, however, alterations of mitochondria are not necessarily reductive as seen in the case of fumarate and nitrate respiration in the mitochondria from the organisms reside in hypoxic environment (see Sec 3 below). Among medically and veterinarily important parasitic protists, which often reside in anaerobic niche in the body, there are a variety of organisms possessing MROs. MROs have been so far characterized in Amebozoa [7], Fornicata [8], Parabasalia [9], Alveolata [10], microsporidium (Fungi) [11], and Stramenopiles [12]. Clinical manifestations, epidemiology, and pathogenesis were previously reviewed elsewhere [13–17]. MROs were also demonstrated in free-living anaerobic unicellular eukaryotes [18–20]. Therefore, the transformation from aerobic mitochondria to MROs appears to have occurred in independent lineages as a consequence of convergent evolution under anaerobic/microaerophilic conditions. MROs have been recently demonstrated in Rhizaria [21]. However, MROs have not been discovered from the Plantae.

In this review, we summarize and discuss the contents and functions of the MROs mainly from anaerobic parasitic protists, because genomic, transcriptomic, proteomic, metabolic, and biochemical evidence has accumulated mainly from the MRO-possessing human- and animal-pathogenic protists due to their medical and veterinary importance. However, we also extend our discussions to free-living eukaryotes when the relevant data are available. Very little is known on the protein import system of the MROs. This is particularly the case for the most reduced or divergent MROs (e.g., *Entamoeba*), in which a very limited number of canonical components have been identified and characterized. Thus, in the later part of this review, we will summarize and discuss the protein import system of the MROs.

## 2. Discovery of MROs

It is generally believed that the mitochondria arose as a consequence of the endosymbiosis of an ancestral α-proteobacterium by an ancestral eukaryote. Thus, it was presumed, until the middle of the 1990s, that there is (or, at least, was), in theory, a eukaryote that has (or had) not gained the mitochondrion [22]. A group of parasitic protists that belong to the Amoebozoa, e.g., *Entamoeba histolytica*, the Fornicata, e.g., *Giardia intestinalis* (*Giardia lamblia*), the Parabasalia, e.g., *Trichomonas vaginalis*, and the Microsporidia, were presumed to be such primitive eukaryotes that lack the mitochondrion and were named accordingly as the Archezoa by Cavalier-Smith [22]. To support a premise that the Archezoa are the most primitive eukaryotes, it was shown that prokaryote-like ribosomes (70S) are conserved in the genome of microsporidia and many archezoan species are positioned near the root of a eukaryotic kingdom in the phylogenetic tree of small subunit ribosomal RNA sequence (SSU rRNA) [23,24] and translational elongation factor 1α (EF1α) [25]. However, *Entamoeba* was positioned among mitochondrion-possessing eukaryotes in SSU rRNA and EF1α trees [23–25].

Regardless of the inference obtained by phylogenetic analyses, these protists apparently lack, based on biochemical assays, the tricarboxylic acid cycle, the cytochrome-mediated ETC, and oxidative phosphorylation, suggesting lack of functional mitochondria [26]. As the phylogenetic analyses based on ribosomal genes and representative central genes have proven controversial, the conundrums such as whether the Archezoa are the premitochondrial eukaryotes and whether the lack of machinery for aerobic energy generation in the Archezoa is not due to primitive absence, but the result of secondary loss, were not properly addressed.

In 1995, genes encoding Cpn60 and pyridine nucleotide transhydrogenase (PNT) were found in the *E. histolytica* genome by Clark and Roger [27]. Cpn60 and PNT were believed to be localized in canonical mitochondria, and phylogenetic analysis showed that *Entamoeba* Cpn60 forms a monophyletic clade with Cpn60 from mitochondriate eukaryotes. These data are consistent with the hypothesis that *E. histolytica* possessed mitochondria in the past

| | Groups | Representative species | Class | Genomic DNA | Cristae | Number / cell | Respiratory chain[e] | Alternative oxidase | TCA cycle | Fe-S cluster biosynthesis | Metabolism: Amino acids | Metabolism: Lipid & fatty acids |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| unikont | Amebozoa | *Dictyostelium*[a] | 1 | +204 | +205 | ~ 50[206] | +207 | +207 | +207 | ISC[41, 103] | +207 | +207 |
| | | ***Entamoeba***[a] | 5 | - | - | 25 ~150[41] or 6500[38] | -208 | -208 | -208 | NIF[37, i] | -64 | ?[64, j] |
| | | *Mastigamoeba*[b] | 3 or 4[k] | -19 | -19 | > 1[19] | I[19] | ? | +19 | NIF[19, 126] | +19 | +19 |
| bikont | Plants | *Arabidopsis*[a] | 1 | + | + | 700 ~ 2000[209] | +210 | +211 | +210 | ISC[212] | +210 | +210 |
| | Euglenozoa | ***Trypanosoma***[a] | 1 | +42 | +213 | 1[214] | +215 | +94 | +215 | ISC[216] | +217 | +218 |
| | | *Euglena*[b] | 2 | +42 | +219 | 1[220] | +221 | +222 | +223 | ? | +217 | +224 |
| Excavata | Heterolobosea | *Sawyeria*[b] | 3 or 4[18] | -18 | -18 | ≥ 1[18] | I[18] | ? | SCS[18] | ISC[18] | +18 | +18 |
| | Parabasalia | ***Trichomonas***[a] | 4 | -42 | -60 | 200 ~ 400[226] | I[72] | -227 | SCS[42] | ISC[85] | +105 | - |
| | Fornicata | ***Giardia***[a] | 5 | -42 | -225 | 25 ~150[41] | -228 | -226 | -223 | ISC[8] | - | ?[63, 120] |
| Chromalveolata | Alveolata | ***Plasmodium***[a] | 1 | + | +116, l | 1[229] | +230, m | +230 | +230 | ISC[230] | +230 | -230 |
| | | ***Cryptosporidium parvum***[a] | 5 | -231 | - | 1[41] | I[80, m] | +80 | -80 | ISC[124] | -232 | -232 |
| | | ***Cryptosporidium muris***[a] | 1 or 2[n] | -80 | +233 | ≥ 1[233] | I, II[80, m] | +80 | +80 | ISC[80] | ? | ? |
| | Stramenopiles | ***Blastocystis***[a] | 3 | +42 | +58 | ≥ 1[52] | I[42], II[54] | +54 | +52, 54, n | ISC[70] | +54 | +54 |

**Fig. 1.** Characteristics of mitochondria and MROs in eukaryotes. Classification of MROs is based on Müller *et al.* [42]. Parasitic species are shown in bold. Anaerobic/microaerobic parasitic protists are highlighted with gray background. Abbreviations are: SCS, succinyl-CoA, synthetase; Arg, two mitochondrial enzymes involved in arginine biosynthesis, carbamoylphosphate synthase and ornithine transcarbamoylase. Notes: [a]Genomic data are available. [b]Only EST data are available. [c]A genome sequencing project undergoing. [d]A crista-like structure. [e]"+" indicates that all complexes are present, while the number of present complex(es) are shown in Roman. [f]"?" indicates that the presence or absence is unknown. [g]The localization of succinyl-CoA synthetase to hydrogenosomes was only predicted. [h]Partial (only some enzymes are present). [i]The NIF localization in the MRO is under debate. [j]Enzymes involved in the metabolisms were detected by proteome analyses, but the localization of enzymes in the MRO was not individulally confirmed. [k]Hydrogen production in the MRO has not been biochemically confirmed. [l]The presence of the cristae is stage dependent. [m]In *Plasmodium* and *Cryptosporidium*, H+ -translocating NADH dehydrogenase complex (NDH1, Complex I) is substituted by a single-subunit NADH dehydrogenase (NDH2). [n]It is not known whether the electron receptor in *Cryptosporidium muris* is exclusively oxygen and/or other organic compounds [193–233].

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and has secondarily lost them. This discovery in *E. histolytica* prompted a survey of mitochondrion-related genes in other archezoan organisms. By the late 1990s, genes encoding a 70-kD mitochondrial molecular chaperon (Hsp70), Cpn10, and Cpn60 were identified from the genome of diplomonads (*G. intestinalis*), parabasalids (*T. vaginalis*), and microsporidia (*Encephalitozoon cuniculi*, *Nosema locustae*, and *Varimorpha necatrix*) [28–35]. Moreover, electron microscopic examination of these archezoan organisms revealed the existence of homogeneous double membrane-bound organelles containing the above-mentioned mitochondrial-type chaperones [10,36–40]. These organelles which were found from the archezoa, at present, became to be called mitochondrion-related organelles (MROs) generically, although functions and components are different between MROs in individual species [41,42].

## 3. Classification of MROs

Mitochondria are categorized into five classes according to the classifications by Müller *et al.* [42] (Table 1). First, mitochondria are grouped into classes 1–4 (ATP-producing) and class 5 (ATP-non-producing) based on the ability to generate ATP. Class 1 mitochondria are defined as "**aerobic mitochondria**" that exclusively use oxygen as the terminal electron acceptor. Class 2–4 mitochondria are anaerobic-type mitochondria that use an endogenous organic or environmental compounds such as fumarate or nitrate as the electron acceptor. Class 5 mitochondria are most reduced ones, and referred as "**mitosomes**" which lack ETC, produce neither ATP nor $H_2$, and do not use electron acceptors [42–48]. Furthermore, class 2–4 mitochondria are classified into class 2 "**anaerobic mitochondria**" (which produce no $H_2$ and possess ETC), class 3 "**$H_2$-producing mitochondria**" (which produce $H_2$ and possess ETC), and class 4 "**hydrogenosomes**" (which produce $H_2$ and lack ETC). Class 3–5 mitochondria are often referred as the mitochondrion-related organelle MROs. Moreover, the term "mitochondrion-like organelle (MLO)" is/was also used when classification remains unassigned, e.g. MROs of *Blastocystis*, *Cryptosporidium*, and *Mastigamoeba* [10,41,49,50].

## 4. Distribution of MROs

MROs are currently demonstratable in a wide range of eukaryotic lineages. Among medically and veterinarily important parasitic protists, which often reside in anaerobic niche in the host, there are a variety of organisms possessing MROs. MROs have been so far characterized in *E. histolytica* (Amebozoa) [7]), *G. intestinalis* (Fornicata) [8], *T. vaginalis* (Parabasalia) [51], *Cryptosporidium parvum*, *Cryptosporidium hominis* (Alveolata) [10], *E. cuniculi*, *Trachipleistophora hominis* (microsporidium, Fungi) [11]), and *Blastocystis hominis* (Stramenopiles) [12]. MROs were also demonstrated in free-living anaerobic unicellular eukaryotes such as an ameba from anaerobic sediments, *Sawyeria marylandensis* (Heterolobosea) [18]), an anaerobic rumen fungus of mammalian herbivores, *Neocallimastix patriciarum* [20], and a free-living ameba, *Mastigamoeba balamuthi* (Amebozoa) [19]).

Hydrogenosomes (class 4) were discovered as an organelle that generates ATP and hydrogen gas via fermentation as a consequence of reduction of proton [42] (Fig. 2). Like other MROs, hydrogenosomes lack cytochromes, a membrane-associated ETC, and oxidative phosphorylation. They are present in the trichomonads, the ciliates, the chytridiomycete fungi, and the heteroloboseid amoeba [18,41]. Mitosomes (class 5) produce no hydrogen gas, no ATP, and are found in *Giardia*, microsporidia, *Cryptosporidium*, and *Entamoeba*. In *Blastocystis*, which is a common inhabitant of the human gastrointestinal tract and a strict anaerobe, its MRO is referred as MLO [12,52–54], but should be refined as the $H_2$-producing mitochondrion, as it retains respiratory complex I and an organelle genome, and its components and functions are more similar to hydrogenosomes than to mitosomes (class 3).

## 5. Morphological features of MROs

As the mitochondria were derived via endosymbiosis, the mitochondria and MROs are always surrounded by double membranes of lipid bilayers, as demonstrated by electron microscopy. While aerobic mitochondria possess a genome [55], MROs, with a few exceptions [12,52,56], contain no genome [42,57].

All MROs from parasitic protists, except *B. hominis* (class 3) [42,58], do not appear to retain cristae, which develop well in the mitochondria from aerobic organisms and organs, e.g., liver, to expand the surface area of the inner mitochondrial membrane, enhancing its ability to produce ATP [10,36–40]. Mitochondria from *Cryptosporidium muris* contain the TCA cycle and AOX using oxygen as a terminal electron acceptor, and thus, are probably classified into class 1 or 2, and *C. muris* mitochondria have tubular cristae (Fig. 1).

The mitosomes of *G. intestinalis* are electron translucent while that of *E. histolytica* and the hydrogenosomes of *T. vaginalis* are consisted of electron dense substances by electron micrography [8,38,59,60]. In *T. vaginalis*, vesicle-like structures were often observed surrounding the periphery of hydrogenosomes [59]. In addition, *Sawyeria* hydrogenosomes were shown to be equipped with a "cup-shaped structure" with an unknown function [18].

**Table 1**
The classification of mitochondrion-derived organelles based on Müller *et al* (2012) [42].

| Class | | ATP production | $H_2$ generation | Electron transport chain | $O_2$ utilization as the terminal electron acceptor |
|---|---|---|---|---|---|
| 1 | Aerobic mitochondria | O | X | O | O |
| 2 | Anaerobic mitochondria | O | X | O | X |
| 3 | $H_2$-producing mitochondria | O | O | O | X |
| 4 | Hydrogenosomes | O | O | X | X |
| 5 | Mitosomes | X | X | X | – |

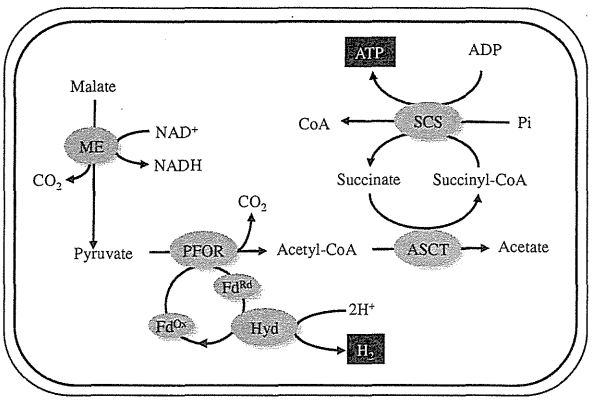


**Fig. 2.** General scheme of ATP and $H_2$ production in hydrogenosomes. Gray circles indicate enzymes. Abbreviations are: ME, malic enzyme; PFOR, pyruvate:ferredoxin oxidoreductase; $Fd^{Rd}$, reduced ferredoxin; $Fd^{Ox}$, oxidized ferredoxin; Hyd, hydrogenase; ASCT, acetate:succinate CoA-transferase; SCS, succinyl-CoA synthetase. ATP and $H_2$ are highlighted by black boxes.

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Together with possible involvement of *S. marylandensis* hydrogenosomes in lipid metabolism (see below), *Sawyeria* hydrogenosomes structurally and functionally differ from those of other organisms that belong to excavate as the same group (Fig. 1).

Electron microscopy also demonstrated that hydrogenosomes lacking organellar genome from *Neocallimastix* contain cristae-like and concentric membranous structures, and an empty vesicle [20,61]. However, the role of these structures has not been demonstrated.

## 6. Proteome of MROs

Aerobic mitochondria, e.g., from human, contain about 1500 proteins [62], which are involved in a variety of functions, mentioned above, including the production of ATP by oxidative phosphorylation, biosynthesis of heme, phospholipid, and Fe–S clusters synthesis, calcium homeostasis, and programmed cell death. In anaerobic parasitic protists, proteins detected by proteomic analyses of their MROs have been largely less than those of aerobic mitochondria (class 1). The recent proteome studies of the representative mitosomes (class 5) from *E. histolytica* and *G. intestinalis* revealed 95 and 638 potential mitosomal proteins, respectively [63,64]. Similarly, 569 hydrogenosomal proteins were identified from *T. vaginalis* (class 4) [65]. It should be noted that significant proportion of candidates for mitosomal and hydrogenosomal proteins apparently possess no N-terminal transit peptide, which is known to be required for the mitochondrial targeting of nuclear-encoded mitochondrial proteins in aerobic eukaryotes [63–66]. Thus, genome- or EST-based prediction of the MRO proteome is potentially erroneous, particularly for the most diversified mitosomes as seen in *G. intestinalis* and *E. histolytica*, in the latter of which a majority of identified proteins isolated by physical purification of mitosomes do not possess the canonical targeting signal [64].

## 7. Genome, pathways, and functions of MROs

In anaerobic parasitic protists, the proteins and functions that are conserved in aerobic mitochondria have been highly modified, lost, or replaced, and consequently the proteins and functions of the extant MROs are largely unique to each lineage. However, MROs from these anaerobes have common features shared by authentic mitochondria, such as a double membrane structure [67] as described above, mitochondrial-type chaperons [7,29,40,68,69], and Fe–S cluster biosynthesis (namely the ISC system) [8,9,11,37,70].

### 7.1. Organelle genome

In contrast to the aerobic mitochondria, which possess a circular or linear form of a separate genome ranging from 6 kbp (in *Plasmodium*) to >2000 kbp (in cucurbit plants) [55], all known MROs, except in *B. hominis* (Fig. 1) and the anaerobic ciliate *Nyctotherus ovalis*, contain no genome [12,52,56]. The lack of organelle genome was demonstrated in most cases by lack of SYTO-61 and DAPI stainings of extranuclear DNA, e.g., [19] or *in situ* nick-translation coupled to immunofluorescence microscopy, e.g., [71].

The organellar genome of *B. hominis* MROs (MLO [49], only MRO is used hereinafter) is a 29,270-bp circular DNA [12], and contains 45 genes: 16 transfer RNAs (tRNAs), 2 ribosomal RNA (rRNA) genes, 26 proteins that have homologs in other organisms, and one protein that has no detectable counterpart. Twenty six known proteins consist of 10 subunits of the NADH:ubiquinone oxidoreductase (Complex I) and 16 ribosomal proteins. The partial 42-kbp sequence of the *N. ovalis* MRO genome, which is expected to be around 48-kb as a whole, contains >30 genes: 2 rRNA genes, 3 tRNA genes, 10 genes encoding subunits of Complex I, 7 genes for ribosomal proteins, and 7 open reading frames with no homolog in 15 unique open reading frames.

### 7.2. Energy production, tricarboxylic acid cycle, and electron transport chain

MROs have lost majority of proteins involved in energy generation via the tricarboxylic acid cycle and oxidative phosphorylation, and β-oxidation of fatty acids. However, some anaerobic MROs in parasitic protists (classes 3 and 4) can still produce ATP via a part of components for respiratory chain (complex I) or substrate-level phosphorylation (e.g., class 4, *Trichomonas*) [42,53,54,72].

#### 7.2.1. Hydrogenosomes in T. vaginalis

Hydrogenosomes are found in multiple lineages in both the Unikonta and Bikonta, e.g., *Trichomonas*, *Sawyeria*, and *Neocallimastix*. The best knowledge of energy production in hydrogenosomes (class 4) is based on *Trichomonas*. *T. vaginalis* hydrogenosomes contain malic enzyme (ME), [Fe]-hydrogenase, ferredoxin, pyruvate:ferredoxin oxidoreductase (PFOR), adenylate kinase, acetate:succinate CoA transferase (ASCT), succinate thokinase α and β subunits, NADH dehydrogenase 24- and 51-kDa subunits, and oxygen scavenging system including thioredoxin (Trx), Trx peroxidase, rubrerythrin, iron superoxide dismutase [65]. These enzymes carry out the substrate level phosphorylation and fermentation to generate equimolar of ATP, acetate, $CO_2$, and $H_2$ from pyruvate or malate. Pyruvate is decarboxylated by PFOR, generating $CO_2$, acetyl-CoA, and reduced ferredoxin, the latter of which in turn carries electrons to ferredoxin-dependent [Fe]-hydrogenase. [Fe]-hydrogenase further donates electrons to protons to generate molecular hydrogen. The CoA moiety of acetyl-CoA is transferred to succinate by ASCT, to form succinyl-CoA and acetate as an end product. Succinyl-CoA synthetase finally yields ATP (or GTP) through substrate-level phosphorylation. The 51- and 24-kDa subunits of complex I of the mitochondrial respiratory chain are also present in *T. vaginalis* hydrogenosomes, and involved in the maintenance of redox balance by reoxidizing NADH consumed in a decarboxylating oxidation of malate to pyruvate by ME [42].

#### 7.2.2. Hydrogenosomes of Neocallimastix and Sawyeria

*Neocallimastix*, the representative anaerobic chytridiomycete together with *Piromyces*, possesses hydrogenosomes [73,74] and [Fe]-hydrogenase [75]. One of the major notable differences from other hydrogenosomes is the formate production, which is catalyzed by pyruvate:formate lyase (PFL) [74,76]. In hydrogenosomes, PFL is responsible for the generation of acetyl-CoA, which is further passed, as shown for in the pathway in *T. vaginalis* hydrogenosomes, for the generation of acetate and ATP (or GTP) via ASCT and succinyl-CoA synthetase and the regeneration of CoA. PFL is the main route of pyruvate breakdown in the hydrogenosomal metabolism of these anaerobic chytrids. The presence of PFOR in *Neocallimastix* appears to be species dependent; PFOR activity, but not PFL activity was detected in two *Neocallimastix* species [20,77,78], while in *Neocallimastix frontalis*, no PFOR activity was found [79]. No complex for the respiratory chain has been demonstrated

*S. marylandensis* possesses, based on EST data, typical anaerobic energy metabolic enzymes, such as PFOR, [Fe]-hydrogenase, and associated hydrogenase maturases with apparent organelle-targeting peptides [18], indicating that its MRO likely functions as a hydrogenosome (class 4). As a genome of *Neocallimastix* and *Sawyeria* is currently not available, there is a possibility that their hydrogenosomes may be classified into $H_2$-producing mitochondria (class 3) once the components of ETC are demonstrated.

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*7.2.3. Mitochondria and MROs of Cryptosporidium*

Components and pathways in mitochondria or MROs to generate ATP seem to be different between *Cryptosporidium* species [80]. Mitosomes of *C. parvum* and *C. hominis*, both of which infect human intestine, lack pyruvate dehydrogenase, most TCA cycle enzymes except malate-quinone oxidoreductase, and most of subunits of ATP synthase except α and β subunits. Thus, ATP appears to be generated only by substrate-level phosphorylation in these two *Cryptosporidium* species. In contrast, in *C. muris*, which parasitizes the rodent stomach, a genome-wide survey showed that all TCA cycle enzymes and ATP synthase are conserved [78]. Therefore, *C. muris* is likely capable, although direct biochemical evidence is lacking, of oxidative phosphorylation with pyruvate-$NADP^+$ oxidoreductase [81–84, in *C. parvum*], and a simple and unique respiratory chain consisting of pyridine nucleotide transhydrogenase [80,85], alternative NADH dehydrogenase 2 [86, in *Plasmodium falciparum*] and cyanide-insensitive alternative oxidase (AOX), the latter of which passes an electron from ubiquinol to oxygen to yield $H_2O$ [87].

AOX is widely distributed in eukaryotes: aerobic organisms including plants, invertebrates, trypanosomes, free-living amoeba *Acanthamoeba castellanii*, as well as *B. hominis*, *Cryptosporidium*, some ciliates, and microsporidia [52,87–94]. AOX is also present in prokaryotes, including α-proteobacteria [95]. It is assumed that AOX was acquired by the ancestral eukaryote in an early step of evolution by the endosymbiosis of α-proteobacterium [88,96]. AOX branches off the main respiratory chain, and receives an excessive electron from ubiquinone, reduces oxygen, and yields water [97–99].

*7.2.4. MLOs (MROs) of Blastocystis*

*B. hominis* possesses cristate mitochondria that can accumulate dyes such as Rhodamine 123 [49], MitoLight [49], and MitoTracker [52], suggesting that it actively maintains an electrochemical proton gradient across its mitochondrial membrane. The *B. hominis* MRO genome encodes proteins involved in respiratory complexes I and II, but lacks components of complexes III, IV, and ATP synthase. Although all components of complex II have been identified in the *B. hominis* genome, fumarate reductase activity has not been demonstrated and thus its function remains uncharacterized [52,54]. Therefore, no ATP production via the ETC seems to be operated, which was also supported by biochemical analyses [58]. It was predicted that a proton translocated to the intermembrane space by complex I is converted to $H_2O$ by AOX, as in *Cryptosporidium*. *Blastocystis* MROs also contain [Fe]-hydrogenase [52]. All these data are consistent with the notion that *Blastocystis* MRO is functionally closer to hydrogenosomes than to mitosomes. Once the fumarate reductase or comparable activity is demonstrated in the functional-undefined complex II, *Blastocystis* MROs may be categorized into anaerobic mitochondria containing organellar DNA (class 2). However, it should be noted that there are notable discrepancies of the presence of particular genes in the TCA cycle among the studies [52–54], and this can be due to at least in part the complex population structure of the species composed of at least nine subtypes [100].

*7.2.5. MROs of Entamoeba and Mastigamoeba*

While the *Entamoeba* mitosomes lack the capacity of energy generation (class 5, mitosomes), the MRO from *M. balamuthi* is equipped with the machinery of energy production, based on the EST survey and targeting sequence screening [19]. *M. balamuthi* MROs are presumed to be able to generate hydrogen as there are cDNAs encoding [Fe]-hydrogenase, ferredoxin, PFOR, malate dehydrogenase, aconitase, isocitrate dehydrogenase, succinate dehydrogenase (SDH) b and c subunits (partial complex II), pyruvate carboxylase, ME, and acetyl-CoA synthetase. At least several of them, including malate dehydrogenase, SDH c, and ferredoxin contain the putative N-terminal targeting sequence [19]. Thus, the *M. balamuthi* MRO is categorized to between class 3 and 4 MROs. *M. balamuthi* MROs also contain partial complex II and more complete components for glycine cleavage system (GCS, see below), compared to *Trichomonas* hydrogenosomes. If other essential components of ETC are discovered by genome analysis and $H_2$ production is biochemically demonstrated, *M. balamuthi* MROs is classified into class 3.

*7.2.6. MROs of Giardia*

*G. intestinalis* mitosomes are not directly involved in energy metabolism (class 5). In *G. intestinalis*, as in *E. histolytica*, PFOR and [Fe]-hydrogenase are in the cytosol. Although *G. intestinalis* produces molecular hydrogen under strictly anoxic conditions [101], ATP is not generated in the mitosomes [42]. However, it was previously demonstrated by proteome analysis that a novel diflavoprotein is isolated from the mitosomes. The diflavo-protein, named GiOR-1, has the mitosomal ferredoxin-independent and NADPH-dependent reductase activity, suggesting that the electron transport by pyridine nucleotides exists in *Giardia* mitosomes [63].

*7.3. Amino acid metabolism*

Human mitochondria harbor a large number of amino acid metabolic pathways. Eleven amino acids can be synthesized in human mitochondria and 17 can be metabolized [102]. When metabolic pathways for amino acids were compared among *H. sapiens*, *Saccharomyces cerevisiae*, *Trypanosoma brucei*, *Tetrahymena thermophila*, and *T. vaginalis*, metabolic pathways for 16 amino acids in *H. sapiens*, 12 in *S. cerevisiae*, eight in *T. brucei*, nine in *T. thermophila*, and six in *T. vaginalis* were identified using KOBAS [65,103,104].

In *T. vaginalis* hydrogenosomes, H and L proteins of the glycine cleavage system (GCS) and serine hydroxymethyltransferase (SHMT) were identified [105]. However, other proteins involved in these two pathways are apparently missing, thus the roles of these proteins are undefined. Three enzymes of the ten additional proteins identified from the hydrogenosome proteome were shown to be involved in the conversion of phosphohydroxypyruvate and homocysteine to cysteine. These enzymes are phosphoserine aminotransferase (PSAT) [106], methionine γ-lyases (MGL) [107,108], and cysteine synthase (CS) [109,110]. While CS was previously demonstrated in the mitochondria, as well as in the plastids and the cytosol of plants (e.g., *Arabidopsis thaliana*), MGL was never demonstrated in the mitochondria and there is only one exception of mitochondrial localization of PSAT in spinach nodules [111]. These proteins lack the putative hydrogenosomal targeting signal, and were predicted to be cytosolic. However, PSAT was demonstrated in hydrogenosomes by immunofluorescence assay [65]. Four alanine aminotransferases, which convert alanine and 2-oxoglutarate to glutamate and pyruvate, were also demonstrated in the proteome. Two additional enzymes, aspartate aminotransferase and glutamate dehydrogenase, which catalyze the reversible metabolism of aspartate, glutamate, oxoglutarate and oxaloacetate, were also present in the hydrogenosomal proteome. Although arginine biosynthesis does not occurs in hydrogenosomes in *T. vaginalis*, one of the mitochondrial enzymes, ornithine transcarbamoylase, used in the arginine biosynthetic pathway in aerobic eukaryotes, is used for a cytosolic arginine dihydrolase pathway for ATP generation [74,112,113].

*N. frontalis* hydrogenosomes contain, based on EST and proteome data, two mitochondrial-type enzymes of arginine biosynthesis, carbamoylphosphate synthase and ornithine transcarbamoylase,

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while activities of the arginine dihydrolase pathway enzymes were not detectable [112]. *Sawyeria* possesses, based on EST data, all of the putative subunits of the glycine cleavage system complex including dihydrolipoamide dehydrogenase (L protein), H, P proteins, SHMT, and all subunits of the branched-chain α-keto acid dehydrogenase including E1 α, E1 β, and E2 subunits [18]. *B. hominis* hydrogenosomes contain, based on genomic data, alanine aminotransferase, branched-chain amino acid aminotransferase, enoyl-CoA hydratase, and 3-hydroxyisobutyryl-CoA hydrolase [54]. In *M. balamuthi,* α-amino-β-ketobutyrate CoA ligase, SHMT, H-, L-, T-, and P-protein of glycine cleavage system, and isovaleryl-CoA dehydrogenase were identified by an EST survey [19]. Among them, at least α-amino-β-ketobutyrate CoA ligase, H-protein, and isovaleryl-CoA dehydrogenase have the N-terminal organellar targeting sequence.

### 7.4. Lipid metabolism

In *B. hominis,* enzymes involved in β oxidation of fatty acids, including long-chain acyl-CoA synthetase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase, as well as enzymes involved in fatty acid biosynthesis including acetyl-CoA carboxylase, acyl carrier protein (ACP), 3-oxoacyl-ACP synthase, 3-oxoacyl-ACP reductase, and 2-enoyl-ACP reductase are compartmentalized to its MROs [54].

In *Giardia* mitosomes, three long chain fatty acid CoA ligases and CDP-diacylglycerolglycerol-3-phosphate 3-phosphatidyltransferase were identified. The localization of the three long chain fatty acid CoA ligases was verified by physical separation on ultracentrifugation [63]. None of these proteins has an apparent N-terminal transit peptide. It should be noted that a VAMP (vesicle-associated membrane protein)-associated protein, GiVAP, which was initially thought to be engaged with membrane trafficking, is involved in lipid metabolism by providing membrane anchors for various lipid binding proteins on the surfaces of the endoplasmic reticulum and the Golgi complex [114] and physically interacting with SNARE proteins, with FFAT-motif containing lipid transport proteins and microtubules. VAPs in general localize on the surfaces of the ER and the Golgi apparatus, and are involved mainly in membrane trafficking and lipid metabolism [114], and there are no report on mitochondrial and MRO-localized VAP. Thus, the function of GiVAP remains largely unknown. Like other VAPs, the *Giardia* VAP protein contains an N-terminal domain that includes the VAP consensus sequence [115], a central coiled-coil domain and a C-terminal transmembrane domain with the putative dimerization motif GxxxG. The presence of a VAP protein has not been reported in mitochondria or other mitosomes so far. Its mitosomal localization was experimentally confirmed [116].

Cardiolipin,1,3-bis(sn-3′-phosphatidyl)-sn-glycerol, is an important phospholipid located in the inner mitochondrial membrane, where it constitutes about 20% of the total lipid composition [117]. Cardiolipin can be also found is in the membranes of most bacteria and chloroplasts. In eukaryotes, cardiolipin is the only lipid that is synthesized in the mitochondria, and essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism. The existence of cardiolipin was previously suggested in *T. vaginalis* hydrogenosomes [118], but the premise was later denied [119]. *E. histolytica* mitosome proteome revealed that a protein with weak similarity to cardiolipin synthase is localized to mitosomes. However, the residues that were shown to be involved in the catalysis of the substrate are not conserved in the *E. histolytica* homolog. Cardiolipin was also undetected in *G. intestinalis,* using biochemical procedures (TLC, HPLC) and fluorescent tools [120].

Although *Sawyeria* hydrogenosomes have not yet been subjected to a proteomic analysis, an EST survey [18] suggested that *Sawyeria* hydrogenosomes contain, unlike hydrogenosomes of parabasalids, branched-chain α-keto acid dehydrogenase, which is a putative mitochondrial protein previously demonstrated in the *Sawyeria*-related heteroloboseid *Naegleria gruberi* [18].

### 7.5. Biogenesis of the Fe–S clusters

The Fe–S clusters are the important cofactors for various Fe–S proteins in bacteria, archeae, and eukaryotes. The assembly of the Fe–S clusters is enzymatically catalyzed by one of four systems [6,121]. The ISC (iron-sulfur cluster assembly) system is most widely distributed, almost ubiquitous in bacteria and the mitochondria of eukaryotes, and in general plays a housekeeping role in the Fe–S cluster biogenesis. The SUF (sulfur utilization factors) system is found in bacteria and plastids of plants and algae, and mainly functions under stress conditions. The NIF system (nitrogen fixation) is mainly present in bacteria including nitrogen-fixing bacteria, and three protists, *E. histolytica, M. balamuthi,* and *T. vaginalis*. The CIA (cytosolic iron-sulfur cluster assembly) system is present in the cytosol of eukaryotes.

It is generally presumed that the minimal common function of MROs is the Fe–S cluster biosynthesis by the ISC system. Consistent with this notion, the ISC system appears to be well conserved in most of MRO-containing eukaryotes except for *E. histolytica* [122,123]. In hydrogenosomes (class 4) from *T. vaginalis* [65], and mitosomes (class 5) from *G. intestinalis* [8], *C. parvum* [124], *E. cuniculi* [11], *T. hominis* [11], and the class 3 MRO from *M. balamuthi* [70], both IscS (the catalytic component, cysteine desulfurase) and IscU (the scaffold component) are conserved. There are additional components required for Fe–S cluster biosynthesis in the mitochondria along with IscS and IscU. These components include alternative scaffolds, molecular chaperons, redox proteins, such as Nfu, IscA, Isd11, Ferredoxin, Frataxin, HscA (Hsp70), HscB (Jac1), and Glultaredoxin (Grx5), and GrpE. They are partially conserved in the MRO-containing protists in a species-dependent manner. For instance, Nfu, IscA, Glultaredoxin, mitochondrial Hsp70, GrpE, Jac1, and [2Fe–2S] Ferredoxin were identified in *G. intestinalis* mitosomes [63]. In *T. vaginalis* hydrogenosomes, IscA, ferredoxins, HscB, GrpE (Mge1), HydF, and HydG, the latter two are necessary for the maturation of hydrogenase, were identified besides IscS and IscU [65]. The genome analysis predicted that Frataxin, HydE, and Isd11, all of which possess a putative N-terminal targeting sequence, are also present in *T. vaginalis*. Moreover, these *T. vaginalis* proteins were also detected in the proteome analysis of its hydrogenosomes [125]. In microsporidia (*E. cuniculi, T. hominis*), Isu1, Isd11, Nfs1, Yfh1 (Frataxin), Ssq1 (HscA), and Grx5 are conserved [11]. Detailed analysis was described in Ref. [126].

One notable exception of the reliance of Fe–S cluster biogenesis on the mitochondrial (and MRO) ISC system is two lineages of the Amoebozoa: *Entamoeba* and *Mastigamoeba*. In these amoebozoan protists, the ISC system is replaced with the NIF system. The NIF system was originally characterized in nitrogen-fixing bacteria such as *Azotobacter vinelandii* [127], and consists of only two, catalytic and scaffold, components (NifS and NifU). It was shown that *NifS* and *NifU* genes were acquired from ε-proteobacteria via lateral gene transfer [122]. While the majority of NifS and NifU proteins are present in the cytoplasm of *E. histolytica*, they were demonstrated to be slightly more (approximately 10 fold) concentrated in mitosomes compared to the cytosol by two independent methods: 1) the quantitation of gold particles per area on immunoelectron micrographs, showing approximately 10-fold concentration of NifS and NifU within mitosomes, compared with that in the cytosol; 2) the quantitation of NifS and NifU in the soluble and pellet fractions of lysates in immunoblot analysis [37]. However, conflicting data

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was presented that neither NifS nor NifU was detected by the proteome analysis of *Entamoeba* mitosomes [64]. Therefore, the compartmentalization of the NIF system to mitosomes is still under debate. So is the minimal common function of mitosomes (see below). It is also of note that *E. histolytica* NIF system is necessary and sufficient for the Fe–S cluster formation of various Fe–S clusters in *Escherichia coli*, but functional only under anaerobic conditions, as demonstrated by heterologous complementation of an *isc/suf*-lacking mutant of *E. coli* [122].

In *M. balamuthi*, possessing class 3 or 4 MRO, NifS and NifU are present as two paralogous isoforms each: one mitosomal and one cytosolic [128]. The former isotype possesses a putative mitochondrial targeting signal, while the latter lacks it. Their localization to the MRO or the cytosol, respectively, was verified by immunofluorescence and immunoblotting assays. A single gene encoding [2Fe2S] ferredoxin that possesses a putative N-terminal targeting sequence was identified. All components of the CIA machinery, except electron transport proteins Tah18 and Dre2 were also identified [128].

*B. hominis* has the mitochondrial-type ISC and cytosolic-type SUF systems [70]. It was shown that *B. hominis* IscS, IscU, and Frataxin were co-localized with the mitochondrial marker MitoTracker by immunofluorescence analysis [70]. *B. hominis* Frataxin rescued the growth defect of the Frataxin-repressed *T. brucei* line, which contains aerobic mitochondria. However, *B. hominis* IscU failed to rescue the growth defect of the corresponding *T. brucei* line, suggesting specific protein–protein interaction unique to individual species [70]. An EST survey revealed genes encoding IscS, Isa2, Frataxin, Ferredoxin, mitochondrial Hsp70, and Grx5 [52]. The recent genome analysis also demonstrated that the scaffold components (IscU and IscA2), Ssq1 (HscA), Jac1 (HscB), Mge1, and Frataxin, the export machinery (Grx5, Atm1, and Erv1) are present [54]. It was also shown that *B. hominis* Isa2 partially rescued the growth defect of and loss of enzymatic activities in the Isa1/2-repressed *T. brucei* line [129]. *B. hominis* also possesses the SUF system, which is usually localized in the plastid in other eukaryotes, but in the cytosol in this organism [70]. SufCB protein has similar biochemical properties to its prokaryotic homologs, and is up-regulated under oxygen stress. Thus, *Blastocystis* appears to have acquired a SUF system from the Archaea to synthesize Fe–S clusters under oxygen stress.

### 7.6. Sulfate activation

Inorganic sulfate is an inert substance [91] and needs to be converted to its biologically active form in order to be transferred onto amino acids, peptides, proteins, carbohydrates, lipids, and small organic molecules [130–133]. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) is one of activated sulfates and is produced from inorganic sulfate and 2 molecules of ATP by the sulfate activation pathway, which consists of a two-step enzymatic process; 1) inorganic sulfate is converted to adenosine-5′-phosphosulfate (APS), in a reaction catalyzed by ATP sulfurylase (AS) and 2) further converted to PAPS in a reaction catalyzed by APS kinase (APSK). PAPS serves as a sulfuryl donor to transfer the sulfuryl moiety to various acceptors by sulfotransferases, resulting in the formation of sulfurylated macromolecules. Alternatively, activated sulfate is reduced to sulfide and either assimilated into cysteine in bacteria and plants or used as a terminal electron acceptor in the anaerobic respiration in sulfate-reducing bacteria, both of which are missing in all parasitic protists including *E. histolytica*, in which AS and APSK are compartmentalized to mitosomes [38,64]. *E. histolytica* is the only organism known to possess a MRO-compartmentalized sulfate activation pathway. In addition to these two enzymes, *Entamoeba* mitosomes also compartmentalize soluble inorganic pyrophosphatase (IPP), which catalyzes the conversion of pyrophosphate (PPi) to phosphates. PPi is a by-product in the reaction of AS and inhibits the forward reaction of AS (and also other PPi-related metabolism) by product inhibition at high concentrations [134]. In *Entamoeba*, PAPS is further used to synthesize at least several species of sulfated lipids (sulfolipids). The detailed structures of these sulfolipids remain unknown. The *E. histolytica* genome contains 10 genes encoding for sulfotransferases [64]. Sulfolipids are a class of lipids containing sulfur. Among them, sulfoquinovosyldiacylglycerol and sulfolipid-I were well characterized in plastids and *Mycobacterium tuberculoris*, respectively [135,136]. Sulfoquinovosyldiacylglycerol was shown to be involved in photosynthesis. Sulfolipid-I was identified as a virulence factor in *M. tuberculoris*.

## 8. The protein import machineries

Most of proteins present in the MRO, except for those encoded by its own organelle genome in *B. hominis* and *N. ovalis*, are nuclear encoded and thus need to be transported to the organelle. No matter how minimal the MRO proteome has become as a consequence of reductive evolution, the specificity of the MRO-targeted proteins needs to be strictly observed. In fungi, it is well established that the protein import into the mitochondrion, and its precise suborganellar compartments, i.e., the matrix, outer, inner membranes, and the intermembrane space, are regulated by at least 52 proteins [137]. These proteins constitute six major complexes: TOM (translocase of the outer membrane of mitochondria) [64,137–140], SAM (sorting and assembly machinery), TIM (translocase of the inner membrane of mitochondria; TIM22 and TIM23 complexes), small TIM complexes [50,137,141], and PAM (presequence translocase-associated motor) complexes, as well as mitochondrial processing peptidase (MPP) involved in the cleavage of the N-terminal targeting peptide. Since many of the central subunits of these complexes, such as Tom40, Tom5, Tom6, and Tom7 for the TOM complex; Sam50 for the SAM complex; Tim22 for the TIM22 complex; Tim23, Tim16, Tim17, Tim18, Tim21, Tim44, and Tim50 for the TIM23 complex; Tim8, Tim9, Tim10, and Tim13 for the small TIMs; hsp70, Cpn60, and Cpn10 as matrix chaperons; and MPP, are conserved in both the Unikonta (fungi) and the Bikonta (plant) [142], these proteins and complexes must have been generated in the early stage of establishment of mitochondria.

As metabolic functions of the MROs are streamlined (some or all components of the important metabolic pathway(s) may have become dispensable in the MROs), a significant proportion of components, either only subunits or as a whole, of the complexes of the canonical aerobic mitochondria appear to have been secondarily lost from the MROs. The presence or absence of all known components of the TOM, SAM, TIM22, TIM23, and small TIM complexes from *S. cerevisiae* mitochondria was examined in representative anaerobic protists (Table 2).

### 8.1. TOM complex

The TOM complex is involved in the first step of the import of nuclear-encoded mitochondrial preproteins into the mitochondria and MROs. The architecture of TOM complex remarkably varies among aerobic eukaryotes, e.g., between mammals and plants and between fungi and trypanosomes. In yeast and mammals, the TOM complex consists of the core translocation channel (Tom40), the membrane-anchored receptors for the recognition of a targeting signal in preproteins (Tom22, Tom20, and Tom70), and the accessory subunits (Tom5, Tom6, and Tom7) [143,144]. In plants, an 8-kDa truncated form of Tom22 serves as translocase [144], and Toc64, which is involved in the import to the chloroplast as a receptor, also functions as a TOM component [145]. Tom20, a presequence binding receptor, appears to have independently evolved

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**Table 2**
Distribution of subunits of the machineries for protein import into mitochondria and MROs from the genomic and EST data.

| Complexes | Translocase of the Outer Mitochondrial Membrane (TOM) | | | | | | | | Sorting and Assembly Machinery of the Outer Mitochondrial Membrane (SAM) | | | | | Intermembrane Space Chaperones | | Carrier Translocase of the Inner Mitochondrial Membrane (TIM22) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunits | ***Tom40*** | Tom22 | Tom20 | Tom70/ Tom71 | Tom5 | Tom6 | Tom7 | Tom60 | ***Sam50*** | Sam37 | ***Sam35*** | Mdm10 | Mim1 | ***Tim9-Tim10*** | Tim8-Tim13 | ***Tim22*** a | Tim54 | Tim18 | ***Tim12*** |
| *Saccharomyces* | 172 | 172 | 172 | 172 | 172 | 172 | 172 | | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 |
| *Encephalitozoon* | 137,140 | | | 137,140 | | | | | 137,140 | | | | | | | 137,140 b | | | |
| *Dictyostelium* | 140,141 | 140,141 | | | | 140,141 | 140,141 | | 140,141 | | | | | 140,141,193 | | 140,141 | | | |
| *Entamoeba* | 140,141,149 | | | | | | | 149 | 140,141 | | | | | | | | | | |
| *Mastigamoeba* | d | | | | | | | | | | | | | | | | | | |
| *Plasmodium* | 168 | 168 | | | | | | | 168 | | | | | 168 | 168 | 168 | | | |
| *Cryptosporidium* | 168 | | | | | | | | 168 | | | | | | 168 f | 168 g | | | |
| *Blastocystis* | | | | 54 | | | h | | 54 | | | | | 54 | 54 | 54 | | | |
| *Giardia* | 63,138 | | | 69,138 i | | | | | | | | | | | | | | | |
| *Trichomonas* | 147 i | | | | | | | | 147 | | | | | 147 | | 147 k | | | |
| *Sawyeria* | | | | | | | | | | | | | | | | | | | |

| Complexes | Presequence Translocase of the Inner Mitochondrial Membrane (TIM23) | | | | Presequence Translocase-Associated Motor (PAM) | | | | | | | Mitochondrial Processing Peptidases | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunits | ***Tim23*** a | ***Tim17*** a | ***Tim50*** | Tim21 | ***mtHsp70*** | ***Mge1*** | ***Tim44*** | ***Pam18*** | Mdj2 | ***Pam16*** | Pam17 | MPPα | MPPβ |
| *Saccharomyces* | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 | 172 |
| *Encephalitozoon* | 137,140 b | 137,140 b | 137,140 | | 137,140 | | | 137,140 | | | | | |
| *Dictyostelium* | 140,141 | 140,141 | | 140,141 | 140,141 | | 140,141 | 140,141 | | 140,141 | | 194 | 195 |
| *Entamoeba* | | | | | 140,141 | 137 c | | | | | | | |
| *Mastigamoeba* | | | | | | 19 | | | | | | 19 e | 19 e |
| *Plasmodium* | 168 | 168 | 168 | | 168 | | 168 | 168 | | | | 168,195 | 168,196 |
| *Cryptosporidium* | 168 g | 168 g | 168 | | 168 | 137 | 168 | 168 | | | | 168,197 | 168,197 |
| *Blastocystis* | 54 | 54 | 54 | 54 | 54 | 54 | | 54 | 54 | 54 | | 54 | 54 |
| *Giardia* | | | | | 63,138 | 63,138 | | 63,138 | | 63,138 | | | 192 l |
| *Trichomonas* | 147 k | 147 k | | | 147 | | 147 | 147 | | 147 | | 191,192 | 191,192 |
| *Sawyeria* | 18 | | | | 18 | | 18 | | | | | | |

Notes:
Black boxes indicate homolog(s) that were predicted in previous studies, while gray boxes dipict possible homologs that are deduced in the present analysis, respectively.
Essential subunits in *Saccharomyces serevisiae* are shown in bold and italics [172].
Numbers indicate references, while alphabets indicate specific notes.
Genome data of the organisms were used except for *Mastigamoeba balamuthi* and *Sawyeria marylandensis*, for which EST data were used for data mining.

[a] Tim17/22/23 are unable to be distinguished on the sequence level.
[b] *Encephalitozoon cuniculi* has a single Tim17/22/23 family protein.
[c] The length of this homolog is larger than that of canonical Mge1.
[d] Based on EST data by personal communication with Courtney Spears and Andrew J. Roger, Dalhousie University.
[e] MPP α and β homologues are absent, and instead a metalloprotease 1 homologue that carries out a MPP-like function in human and yeast is present in the EST data [19].
[f] *Cryptosporidium parvum* has a single gene for small Tim protein family. It was shown that this protein forms a functiona homohexamer [168].
[g] *Cryptosporidium parvum* has only a single Tim17/22/23 family protein.
[h] This homolog was not previously described, and has been discovered in this study (CBK20669), with the e-value of 7.1e-10 to the Tom7 consensus in Pfam database using a hidden Markov model.
[i] *Giardia lamblia* lacks the MPPα homolog. However, *Giardia lamblia* MPPβ homolog recognizes preproteins and cleave its presequence without MPP α.
[j] *Giardia lamblia* posesses a Tom70 homolog, but this protein was not apparently associated with Tom40 homolog [138].
[k] Four paralogs of the Tim17/22/23 family and one Tim17-like protein were detected from *Trichomonas vaginalis* hydrogenosomes [147].
[l] Five paralogs of Tom40 were detected from *Trichomonas vaginalis* hydrogenosomes [147].

from two unrelated ancestral genes in the animal and plant lineages [146]. In trypanosomes, Tom40 was lost and replaced with ATOM, a homolog of Omp85 of archaic origin Ref. [184]. In *T. vaginalis*, six paralogs of Tom40 and four Tim17/22/23 paralogs and one Tim17-like protein exist. However, the functional differences among the paralogs are not understood [147].

The central indispensable component of the protein transport across the outer membrane of the MRO appears to be Tom40. Tom40 homologs are found from all genome-sequenced MRO-possessing organisms, except *B. hominis*. It remains to be determined whether Tom40 has been replaced with an unknown core pore component, as in trypanosomes [148]. Essentiality of *Entamoeba* Tom40 has been demonstrated by gene silencing of Tom40, which caused growth retardation of *Entamoeba* trophozoites [149]. Together, these data indicate that Tom40 is an indispensable component for MROs.

Other TOM components, besides Tom40, including preprotein receptors, Tom20 and Tom70, are not well conserved, although there are a few exceptions: Tom70 and Tom7 in *B. hominis* and Tom70 in *E. cuniculi*. Recently, a novel receptor of premitosomal proteins, Tom60, was identified from the *Entamoeba* TOM complex [149]. Tom60 contains the tetratricopeptide repeats (TPR), which is involved in protein–protein interaction in Tom20 and Tom70 [150], but is detected only in the genus *Entamoeba*. Tom60 is unique in that this receptor component is associated with Tom40 in the TOM complex on the outer membrane and also serves as a carrier of nascently synthesized mitosomal proteins in the cytosol. In addition, Tom60 is capable of binding in vitro to mitosomal matrix proteins directly and to membrane proteins with the assistance of cytosolic Hsp70 and Hsp90. Tom60, similar to Tom40, is essential for cell proliferation [149].

It remains totally unknown when and how *Entamoeba* lost the canonical receptor such as Tom20/Tom70 (or other receptor) and gained Tom60. However, it is conceivable that in the ancestor of *Entamoeba*, the mitosomal proteins, e.g., AS and APSK, acquired from proteobacteria by lateral gene transfer, were poorly imported into mitosomes by canonical receptors. Thus, it was needed for a new receptor to be generated so that exogenous proteins, i.e., mitosome

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proteins with bacterial origin, are efficiently imported into mitosomes. In *E. histolytica*, the mitosome import does not rely on the receptor-mediated recognition of the canonical N-terminal transit peptide [64], as the majority of the putative mitosomal proteins from the mitosome proteome [64] are devoid of the targeting peptide.

*B. hominis* is the only parasitic protist among those examined in this review, that does not apparently have Tom40. Instead, *B. hominis* has Tom70 and is the first organism from the Bikonta that was shown to have Tom70 [151]. Tom70 was also found from other stramenopiles and a haptophyte [151]. Tom70 homologs from different lineages that belong to the Bikonta form a monophyletic group distinct from the Unikonta group, including the animal and the fungal homologs. Although the origin of this protein is still unclear, it was speculated that Tom70 may have been a subunit of the core mitochondrial protein import apparatus of the last common ancestral eukaryote. A possible Tom7 homolog (CBK20669), which was not previously detected, has been discovered in *B. hominis* by our survey of the Pfam database based on a hidden Markov model using the consensus sequence of Tom7 as a query (e-value, 7.1e-10). It is known that in *S. cerevisiae*, Tom7 is involved in the control of the Tom40 and Tom22 assembly via the SAM complex [152].

Unlike *B. hominis*, *E. cuniculi*, which belongs to the Unikonta, together with animals and fungi, has both Tom40 and Tom70. Interestingly, *E. cuniculi* Tom70 is 23% shorter compared to *S. cerevisiae* Tom70 [137], which may reflect the reductive evolution. None of potential receptor subunits, i.e., Tom20, Tom70, Tom60, and Tom22, has been identified from *C. parvum*, *G. intestinalis*, *T. vaginalis*, *M. balamuthi*, and *Sawyeria*, suggesting lineage-specific acquisition or evolution of a premitosomal/hydrogenosomal protein receptor.

### 8.2. SAM complex

The precursors of outer membrane proteins, which typically form a β-barrel structure, are entrapped by the TOM complex, and translocated through the Tom40 channel into the intermembrane space. Subsequently, the precursors bind to the small TIM complexes, Tim9-Tim10 and Tim8-Tim13 chaperon complexes [153] which guide the outer membrane proteins to the SAM complex, which sort and assemble them [154,155]. In yeast, the SAM complex consists of Sam50, Sam35, and Sam37. Sam50, which is a β-barrel protein itself, is the core component of the SAM complex, while Sam35 functions in the binding of the β-signal, which is located at the C-terminus of β-barrel membrane proteins and required for the incorporation into the SAM complex [156]. Sam37 is involved in the release of precursor proteins from the SAM complex [157].

Sam50 homologs are found from all MRO-possessing organisms, except *G. intestinalis*. However, Sam37 and Sam35 homologs are undetected in the MRO-possessing organisms and also aerobic protists such as *Plasmodium* and *Dictyostelium*. These data suggest that Sam37 and Sam35 were probably lost in a majority of lineages early in the eukaryotic evolution. It is puzzling that at least several β-barrel membrane proteins containing the consensus sequence of the β-signal are present in *G. intestinalis*, *T. vaginalis*, and *E. histolytica* [138,141,147], all of which lacks both Sam35 and Sam37. Thus, it remains unknown whether the β-signal still functions as outer membrane targeting signal in MRO-containing eukaryotes. Possible recognition mechanisms for the outer membrane proteins should be present in MRO-containing organisms lacking the canonical components of the SAM complex.

### 8.3. Small TIM

In the intermembrane space, the Tim9-Tim10 and Tim8-Tim13 chaperon complexes (small TIM complexes), bind to the precursors. Tim9 and Tim10 assemble with an additional small Tim protein, Tim12, to form a membrane-bound Tim9-Tim10-Tim12 complex at the outer surface of the TIM22 complex located on the inner membrane [158,159]. On the other hand, the hexameric complex (trimer of Tim9-Tim10 dimer) forms a chaperon complex that guides the hydrophobic precursor proteins to the inner membrane [160–163]. The Tim9–Tim10 complex is important for the translocation of β-barrel membrane proteins and metabolite carriers [160,162–164]. A second hexameric chaperon complex (trimer of Tim8-Tim13 dimer) is homologous to the Tim9-Tim10 complex and functions in a related manner to guide hydrophobic precursors through the intermembrane space [165,166]. The Tim8–Tim13 complex is mainly involved in the import of Tim23 under low membrane potential conditions [153,165–167]. The pairing of small Tim proteins (Tim9-Tim10 and Tim8-Tim13) appears to be important for functional differences, although its underlying mechanisms remain unknown [168]. In yeast, Tim9-10 are essential whereas Tim8-13 are not [169–171].

These intermembrane space chaperon complexes are relatively well conserved among the import machineries, i.e., TOM, SAM, TIM22, TIM23, small TIMs, and PAM. In *C. parvum*, *B. hominis*, and *T. vaginalis*, either one or both of the two complexes seem to be present. These MRO-containing protists also possess Tim23 (see below). On the contrary, the organisms that lack Tim23, e.g., *E. histolytica* and *G. intestinalis* do not possess genes for small Tim proteins, with an exception where *E. cuniculi* possess Tim23. These data also suggest that chaperons and their targets that the chaperons pass the substrates to apparently co-evolved.

### 8.4. TIM23 complex

In model eukaryotes, two distinct TIM complexes are known to be built from distinct members of the Tim17/Tim22/Tim23 family proteins. The inner membrane proteins such as ADP/ATP carrier (or mitochondrial carrier family protein) are assembled by the TIM22 complex (see below), whereas proteins transferred into the matrix are assembled by the TIM23 complex [172]. Tim23 is a channel-forming subunit of TIM23 complex, while Tim17, which is tight associated with Tim23, is involved in the lateral sorting of preproteins. Tim50 is the intermembrane space receptor, gating of the Tim23 channel, while Tim21 interacts with the TOM complex and also the respiratory chain in the matrix, and thus plays a role as a modulator of the TIM23 complex. Conservation of components of the TIM23 complex is similar to that of the small TIM complex, supporting close interaction between the two complexes. That is, at least one of the TIM23 complex is conserved in *C. parvum*, *B. hominis*, and *T. vaginalis*, while none of them is retained in *E. cuniculi*, *E. histolytica*, *M. balamuthi*, and *G. intestinalis*. Only a single gene each that potentially encodes the Tim17/22/23 family and small Tim proteins was found in the *C. parvum* genome [168]. However, it is not possible to determine in silico whether Tim17/22/23 homolog functions as Tim17, Tim22, and/or Tim23.

### 8.5. PAM complex

In model eukaryotes (e.g., yeast), after the proteins are transferred across the inner membrane into the matrix and assembled by the TIM23 complex, they are further recognized and processed by the PAM complex [169]. mtHsp70 is a chaperon and ATPase that binds preproteins for protein folding. Mge1 is a mitochondrial nucleotide-exchange factor for mtHsp70 [173]. Tim44 is a membrane anchor for mtHsp70 [174]. Pam18 [175] and its homolog, Mdj2 [176], are J proteins, and stimulate ATPase activity of mtHsp70. Pam16 J-like protein, forms a module with Pam18 and controls its activity [177]. Pam17 is a component to bind Tim23 of the TIM complex and also involved in the integrity of the Pam18-

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Pam16 module [178–181]. The extent of conservation of the components of the PAM complex varies among the anaerobic protists. mtHsp70 is apparently only component ubiquitously present, with an exception of *M. balamuthi*, for which only EST data are available. While more than or equal to four components of the PAM complex are conserved in *C. parvum* [137,168], *B. hominis* [54], *G. intestinalis* [63,137], and *T. vaginalis* [147], while only one or two components are conserved in *E. cuniculi* and *E. histolytica* (also in *M. balamuthi* and *S. marylandensis*) [18,19,137,140,141].

In aerobic mitochondria, it is well established that the electrophoretic force created on the positive charge of the N-terminal transit peptide of mitochondrial proteins by the membrane potential plays an important role in the transport across the inner membrane [172]. However, the membrane potential is not detected in the MROs of most of anaerobic protozoa, and the N-terminal targeting peptide is often missing in MRO-targeted proteins [37,40,64,182–184]. Therefore, alternative mechanisms to drag proteins into MROs likely exist.

### 8.6. TIM22 complex

The TIM22 complex is involved in the transport of the inner membrane proteins, and consists of four components. The core channel-forming (twin-pore) Tim54 binds the Tim9-Tim10-Tim12 complex via a domain facing the intermembrane space [185,186]. Tim12 is a membrane-bound chaperon, tethering of carrier precursors to the TIM22 complex [187], while Tim18 is also involved in the assembly of the TIM22 complex [188]. Tim22 is conserved in *E. cuniculi* [137,140], *C. parvum* [168], *B. hominis* [54], and *T. vaginalis* [147], but not in *E. histolytica*, *M. balamuthi*, and *G. intestinalis*. None of the other components of the TIM22 complex is present in all organisms examined here.

### 8.7. Mitochondrial processing peptidase and chaperons

In aerobic eukaryotes, once the preproteins enter the mitochondrial matrix, they are removed by a dimeric MPP, which comprises a catalytic MPPβ subunit that binds a zinc cation using amino acid residues of the conserved motif $HXXEHX_{76}E$ [189], and a regulatory MPPα subunit with a flexible glycine-rich loop that is important for substrate recognition [190]. MPP appears to be conserved among all MRO-containing parasitic protists except *E. cuniculi* and *E. histolytica*, both of which lack both subunits. The hydrogenosomal and mitosomal localization of MPP subunits in the *T. vaginalis* and *G. intestinalis*, respectively, were verified [191,192]. *G. intestinalis* lacks a gene encoding for MPPα and *G. intestinalis* MPPβ solely can recognize and cleave the presequence of precursors [191,192].

In *E. histolytica*, a candidate of the MPPβ homolog (XP_656023) was detected. However, this protein tagged with the HA epitope at the C-terminus was localized in the cytosol (Makiuchi *et al.* unpublished data). Together with the lack of the N-terminal transit peptide in *E. histolytica* mitosomal proteins [64], these data indicate that the *E. histolytica* MPPβ homolog may not function as the presequence peptidase in the mitosome. In contrast to *E. histolytica*, *E. cuniculi* seems to retain general targeting signals in its mitosomal proteins [184]. Therefore, it is possible that a unique, not-yet-identified, signal cleavage system has evolved in microsporidia.

In *G. intestinalis* and *T. vaginalis*, the dependence of protein import to MROs on the N-terminal transit peptide is still promiscuous. In *G. intestinalis*, several proteins including Ferredoxin, IscU, and IscA, which were demonstrated in mitosomes, apparently possess the detectable N-terminal targeting signal which contains the conserved arginine residue at the proximal P2 position of the "(ARV)R(F/L)(L/I)T" motif, while such N-terminal presequence is lacking in IscS, mitosomal processing peptidase, Hsp70, Pam18, Cpn60, and Glutaredoxin Refs. [69,182,192]. In *T. vaginalis*, it was recently demonstrated that targeting of PFOR, IscA, and Ferredoxin to hydrogenosomes does not require the N-terminal transit peptide (Gould *et al.*, unpublished data). Taken together, a role of the N-terminal targeting signal in the transport of preproteins to MROs is not well understood.

## 9. Conclusions and perspectives

The number of organisms that possess MRO is expected to increase as more genomes of anaerobic/microaerophilic unicellular eukaryotes, particularly free-living environmental organisms, are sequenced. The diversity of the content and functions will increases accordingly. Since the MRO-targeting signals are promiscuous in anaerobic protozoa including *G. intestinalis* and *T. vaginalis*, and apparently lacking in *E. histolytica*, prediction of organelle-localized proteins is unreliable. Thus, a highly sensitive and unbiased proteomic analysis of highly purified MROs by conventional physical separation, immunoprecipitation, and FACS-based separation of organelles is needed to demonstrate all the components involved in the biogenesis of MROs. Detailed analyses of the constituents and functions of the MROs in such anaerobic eukaryotes, which reside oxygen-deprived or oxygen-poor environments, should illuminate the current evolutionary status of the MROs in these organisms, and give insight to evolutionary constraints that drive organelle diversification.

There are a number of important questions that remain to be answered. First, is there a single minimal functional unit (i.e., components and functions) that is ubiquitously conserved in mitochondria and MROs in life? Despite the previous premise, Fe–S cluster biogenesis does not seem to be the ubiquitous shared function of mitochondria and MROs. The ISC system is replaced with the NIF system in *E. histolytica* and *M. balamuthi*, and at least in *E. histolytica*, the NIF system does not seem to be specifically compartmentalized to mitosomes. Second, are there any metabolic pathways and functions that were additionally acquired by the MRO, other than sulfate activation in *E. histolytica*? Third, if not, why was the sulfate activation gained and retained so uniquely only in this lineage? In other words, how did the *Entamoeba* mitosomes gain the entire sulfate activation pathway? Two major enzymes involved in sulfate activation, AS and APSK, (at least AS) in *Entamoeba* mitosomes are likely acquired from δ-proteobacteria by lateral gene transfer [64]. Is it also possible that these enzymes were simultaneously gained by fusion of two endosymbionts (α- and δ-proteobacteria)? Fourth, what is the selective force for the retention of the sulfate activation pathway in *Entamoeba* and *Mastigamoeba*? Fifth, why are some of amino acid and fatty acid/lipid metabolisms still remained in some lineages that possess MROs? Protein transport across the double membrane of the MROs remains largely unknown except the core components Tom40 and Sam50 on the outer membrane, Tim22 and the components of the TIM23 complex. Sixth, what are the other components in the TOM, SAM, TIM, and PAM complexes? and where are they from? The machineries for the transport across the inner membrane remain, in particular, totally unknown for several anaerobic parasitic protists, including *E. histolytica* and *G. intestinalis*. Seventh, what is the inner membrane translocase in the anaerobic protists lacking TIM complexes? and where did it come from?

The unique metabolism compartmentalized to the MROs is a rational target for the development of chemotherapeutics against infections caused by MRO-possessing pathogens. For instance, although it is still debatable whether the NIF system is exclusively localized to the cytoplasm or partitioned to both the cytosol and mitosomes, the essential non-redundant NIF system in *E. histolytica*, which is absent in humans and animals, represents an

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ideal target for new anti-amoebic drugs. In addition, the sulfate activation pathway in mitosomes of *E. histolytica* can also be exploited as a drug target if a formula for delivering to mitosomes becomes established.

## Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (23390099), a Grant-in-Aid for Scientific Research on Innovative Areas from MEXT of Japan (3308, "Matryoshka-type evolution", 23117001, 23117005), a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare (MHLW) of Japan (H23-Shinkosaiko-ippan-014), a Grant-in-Aid on Bilateral Programs of Joint Research Projects and Seminars from Japan Society for the Promotion of Science, a Grant-in-Aid on Strategic International Research Cooperative Program (SICP) from Japan Science and Technology Agency, a grant for research to promote the development of anti-AIDS pharmaceuticals from the Japan Health Sciences Foundation (KHA1101), and by Global Center of Excellence Program (Global COE for Human Metabolomic Systems Biology) from MEXT, Japan, to T.N.

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Please cite this article in press as: T. Makiuchi, T. Nozaki, Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa, Biochimie (2013), http://dx.doi.org/10.1016/j.biochi.2013.11.018

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Please cite this article in press as: T. Makiuchi, T. Nozaki, Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa, Biochimie (2013), http://dx.doi.org/10.1016/j.biochi.2013.11.018

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Please cite this article in press as: T. Makiuchi, T. Nozaki, Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa, Biochimie (2013), http://dx.doi.org/10.1016/j.biochi.2013.11.018