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Evolution of the haem synthetic pathway in kinetoplastid flagellates: An essential pathway that is not essential after all?

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ABSTRACT

For a vast majority of living organisms, haem is an essential compound that is synthesised through a conserved biosynthetic pathway. However, certain organisms are haem auxotrophs and need to obtain this molecule from exogenous sources. Kinetoplastid flagellates represent an interesting group of species, as some of them lost the complete pathway while others possess only the last three biosynthetic steps. We decided to supplement a current view on the phylogeny of these important pathogens with the expected state of haem synthesis in representative species. We propose a scenario in which the ancestor of all trypanosomatids was completely deficient of the synthesis of haem. In trypanosomatids other than members of the genus *Trypanosoma*, the pathway was partially rescued by genes encoding enzymes for the last three steps, supposedly obtained by horizontal transfer from a γ-proteobacterium. This event preceded the diversification of the non-*Trypanosoma* trypanosomatids. Later, some flagellates acquired a β-proteobacterial endosymbiont which supplied them with haem precursors. On the other hand, the medically important trypanosomes have remained fully deficient of haem synthesis and obtain this compound from the host.

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1. Introduction

Haem belongs to compounds that are essential for the majority of extant cells, as it is indispensable for the biogenesis of cytochromes and other enzymes which play important biological roles (Rodgers, 1999; Chan, 2000; Panek and O'Brian, 2002; Heinemann et al., 2008). The complete haem synthetic pathway is present in most organisms and is conserved through all three domains of life. However, organisms differ in the synthesis of delta-aminolevulinic acid (ALA), the first commited precursor of haem synthesis. With the exception of α-proteobacteria, all prokaryotes and photosynthetic eukaryotes synthesise ALA via three consecutive enzymatic steps starting with glutamate (Avissar et al., 1989; Beale, 1999), while α-proteobacteria and most non-photosynthetic eukaryotes synthesise ALA by condensation of glycine with succinyl-CoA using the single ALA-synthase enzyme (ALAS) (Jordan and Shemin, 1972; Ferreira and Gong, 1995; Duncan et al., 1999). The remaining seven steps of the haem biosynthesis pathway (from ALA to protoheme) are carried out by the same enzymes in all organisms. Eukaryotes differ not only in the very first precursors they use for the synthesis of haem but also in the intercellular localisation of individual enzymatic steps. For instance, photosynthetic eukaryotes synthesise haem exclusively in the chloroplasts, while in most heterotrophic eukaryotes the pathway is split between the mitochondrion and cytosol (Camadro et al., 1986; Beale, 1999; Dailey et al., 2005). Apicomplexan parasites are an interesting exception, since their haem synthesis starts in the mitochondrion, with ALA being transported to the apicoplast. Several subsequent steps take place in this highly modified plastid, but the last steps appear to be translocated back to the mitochondrion (Sato et al., 2004; van Dooren et al., 2006; Nagaraj et al., 2009).

Organisms that are deficient in haem biosynthesis are rare, especially within eukaryotes. These include anaerobic protists such as *Giardia*, *Trichomonas*, *Entamoeba*, *Cryptosporidium*, *Blastocystis* and *Encephalitozoon*. They possess reduced mitochondria that are called mitochondria-like organelles, hydrogenosomes or mitosomes (Embley, 2006). These protists generate energy by means other than oxidative phosphorylation, thus dispensing with the need for haem co-factor that is incorporated into the respiratory cytochromes. Furthermore, hemoproteins that function in oxidative metabolism such as oxidases, peroxidases, catalases and hydroxylases are not needed in anaerobic conditions (Hörtner et al., 1982; Pugh and Knowles, 1983; Ponka, 1997; Emerling and Chandel, 2005). No gene of the haem biosynthesis has been found in the genomes or expressed sequence tags (ESTs) of the above mentioned protists (Katinka et al., 2001; Abrahamsen et al., 2004; Loftus et al., 2005; Stechmann et al., 2008; Aurrecoechea et al., 2009). However, even organisms that depend on oxidative phosphorylation but are defective in the synthesis of haem are

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known, including slime mould (Daniel et al., 1962), a tick (Braz et al., 1999), a filarial nematode (Ghedin et al., 2007) and even a free-living nematode (Rao et al., 2005). Most parasitic kinetoplastids also belong to this category. These organisms afforded the loss of haem synthesis due to an easy access to this compound from their environment. For example, the tick *Boophilus microplus* has even found a way how to dispose of host haem, which is toxic when present in excess (Lara et al., 2003, 2005). Filarial nematode parasite *Brugia malayi* may uptake host-supplied haem or it can obtain this molecule from the endosymbiotic bacteria (Ghedin et al., 2007), which was also suggested by Foster et al. (2005). The free-living nematode *Caenorhabditis elegans* feeds on bacteria and thus has easy access to haem as well (Rao et al., 2005).

The biosynthesis of haem presents a risk to the organism since the accumulation of intermediates between ALA and protoporphyrin IX in the presence of light and oxygen is damaging to the cell as a result of the generation of free radicals via their oxidation (Sandberg and Brun, 1982). Thus, the levels of these intermediates need to be tightly regulated. Alternatively, some organisms have found a way to obtain haem from other sources, losing their own biosynthetic pathway.

Kinetoplastida (Euglenozoa, Excavata) is a monophyletic group of mostly parasitic protists, some of which are important pathogens of humans and animals, as well as free-living species. These flagellates are unified by the presence of unusual features, such as extensive mRNA trans-splicing, a large catenated mitochondrial genome called kinetoplast DNA and the processing of mtRNA via post-transcriptional uridine insertion/deletion editing (Campbell et al., 2003; Lukeš et al., 2005). In vitro cultivation of these flagellates requires the addition to the medium of haem compounds in the form of haemoglobin, haematin or haemin (Chang and Trager, 1974; Chang et al., 1975). These cells thus appear to be deficient of haem synthesis, possibly lacking many or even all enzymes of the synthetic pathway.

2. Haem synthesis is absent in trypanosomes

We have searched the genomes of *Trypanosoma cruzi* and *Trypanosoma brucei* for enzymes involved in haem synthesis. Neither of them seems to contain any gene encoding enzymes of the pathway (Berriman et al., 2005; El-Sayed et al., 2005a,b). This finding provides quite convincing evidence that trypanosomes are completely deficient of haem synthesis and must therefore scavenge this molecule from their hosts. However, another valid explanation would be that enzymes for haem biosynthesis are divergent enough to be undetectable by homology searches. This scenario is very unlikely due to the conservation of the pathway through all groups of organisms, including those with highly diverse genomes such as *Plasmodium*. Absence of the complete biosynthetic pathway in *T. cruzi* has been pointed out by biochemical studies, in which neither porphobilinogen, porphyrins and free haem, nor the activities of ALA-dehydratase (ALAD), porphobilinogen deaminase (PBGD) and uroporphyrinogen synthase (UROS) were detected (Salzman et al., 1982; Lombardo et al., 2003) (all steps of the pathway are shown in Fig. 1). However, the cell extracts of *T. cruzi* displayed activities of ALAS and ferrochelatase (FeCH) (Salzman et al., 1982). The source of these activities remains questionable, due to possible contamination by enzymes from exogenous sources in the medium. Enzymatic activity of FCS, which is one of the components of trypanosome growth media, was recently described (Lubel et al., 2009). Furthermore, Lombardo et al. (2003) did not detect any ALAS activity in *T. cruzi* but were able to detect small quantities of intracellular ALA. However, 98% of the ALA was found in the extracellular medium. The absence of functional FeCH in trypanosomes is further strengthened by

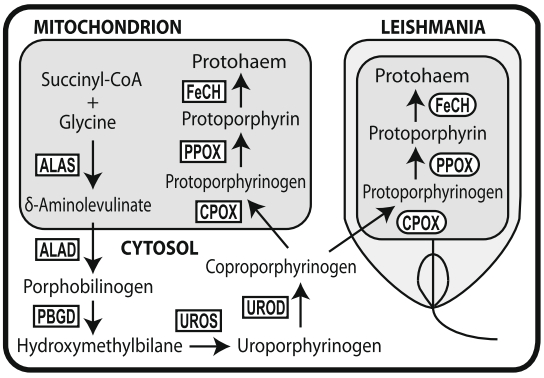

Fig. 1. Intracellular localisation of the haem biosynthesis pathway in the animal cell (enzymes in the sharp-edged rectangles) and the suggested uptake of coproporphyrinogen by *Leishmania*, which uses its own enzymes for the last three steps of the synthesis (rounded rectangles).

the fact that hemin cannot be replaced with protoporphyrin IX in their culture media, which is in contrast to *Leishmania* spp. that encode a clear homologue to the FeCH gene in their genomes (see below). Taking all available evidence into account, we suggest that trypanosomes are fully deficient of haem synthesis.

3. *Leishmania* spp. encode genes for the last three steps of the synthesis

Several trypanosomatids including *Leishmania* spp. and *Crithidia fasciculata* can grow in media in which hemin is replaced by protoporphyrin IX (Chang et al., 1975; Sah et al., 2002; Akilov et al., 2007). This observation provides indirect evidence that at least the last enzyme of the pathway (FeCH) remains functional. As in *T. brucei*, the enzymatic activities of both ALAS and FeCH have also been detected in the promastigotes of *Leishmania donovani* (Srivastava et al., 1997). However, *Leishmania amazonensis* cells that have been transfected with the mammalian genes for ALAD and PBGD, the second and the third enzymes of the pathway, respectively, still required exogenous ALA in order to produce porphyrins. This result indicates that its ALAS protein is not functional (Sah et al., 2002). Since dual xenotransfection with ALAD and PBGD was necessary to produce porphyrins, it is evident that *Leishmania* is deficient in both enzymes. The accumulation of uroporphyrinogen I in these transfectants further suggests a deficiency of UROS, an enzyme downstream in the pathway (Fig. 1). If this enzyme was present, it would have catalysed the formation of uroporphyrinogen III (Sah et al., 2002). Uroporphyrinogen decarboxylase (UROD), the enzyme catalysing the step after UROS, normally converts uroporphyrinogen III into coproporphyrinogen III. However, it is also capable of converting uroporphyrinogen I into coproporphyrinogen I (Chemmanur et al., 2004). The absence of detectable coproporphyrin in the transfectants thus indicates the absence of UROD as well. This finding was further supported by the succeeding study of Dutta et al. (2008) who additionally transfected the double-transfectans of *Leishmania major* with human cDNA of UROD, which resulted in the production of coproporphyrin.

Our extensive search in the genomes of *L. major*, *Leishmania infantum* and *Leishmania braziliensis* identified homologues for the last three enzymes of the haem pathway in all three species: coproporphyrinogen oxidase (CPOX), protoporphyrinogen oxidase (PPOX) and FeCH. No homologues were found for any other genes of the pathway, supporting earlier conclusions (Sah et al., 2002; Dutta et al., 2008). The genes encoding CPOX and FeCH, enzymes of the sixth and eighth steps of the haem pathway, respectively, have been found in the genome shotgun sequences of *Crithidia*

sp. ATCC 30255 (http://www.sanger.ac.uk). The very likely presence of PPOX, an intermediate enzyme representing the seventh step, remains speculative until the genomic sequence of this organism is completed. The available data therefore indicate that, as for *Leishmania*, the monoxenous parasites of insects possess the last three enzymes of haem synthesis. Our phylogenetic analyses grouped all of the genes of haem synthesis from *Leishmania* spp. and *Crithidia* sp. with their homologues from γ-proteobacteria (Fig. 2). This observation strongly suggests that the genes were transferred to all of these protists by horizontal gene transfer (HGT) from a γ-proteobacterium. Moreover, the leishmanial gene that encodes PPOX – *hemG* is predominantly known from γ-proteobacteria (Panek and O'Brian, 2002), but was never found in a eukaryote. In this domain of life, the gene encoding PPOX – *hemY* completely differs in its sequence from *hemG* and is frequently found in bacteria (Nishimura et al., 1995). The sole presence of genes encoding the last three steps of the pathway in all three *Leishmania* genomes raises a question about the raison d'etre of their enzymatic products, as these flagellates are unable to synthesise an earlier precursor of the pathway. It is thus plausible that they are able to uptake the "missing" precursor from their hosts. Taking into account the subcellular localisation within the host cell, it is likely that coproporphyrinogen is accessible to the parasite more easily than haem (Fig. 1).

A complete set of eukaryotic genes for haem synthesis must have been present in the genome of some eukaryote ancestors of kinetoplastids. We propose a scenario in which the pathway was completely lost during the course of evolution of Kinetoplastida, with its parts being either subsequently rescued by horizontally acquired bacterial genes in the non-*Trypanosoma* trypanosomatids, or not rescued at all, resulting in a complete deficiency of the haem synthesis in *Trypanosoma* (Fig. 3). Less plausible is the possibility that the original eukaryotic genes for the haem pathway could have been replaced by bacterial homologues and only later partially (*Leishmania* spp.) or completely lost (*Trypanosoma* spp.).

4. Bacterial endosymbionts in trypanosomatids rescue the synthetic pathway

It has been inferred that a group of insect trypanosomatids consisting of *Herpetomonas roitmani*, *Crithidia deanei*, *Crithidia desouzai*, *Crithidia oncopelti* and *Blastocrithidia culicis* obtain haem or haem precursors from their bacterial endosymbionts, since these cells grow well in a chemically defined medium lacking hemin (Newton, 1957; Chang and Trager, 1974; Mundim and Roitman, 1977; Dutra de Demenezes and Roitman, 1991; Frossard et al., 2006). *Blastocrithidia culicis* and *C. oncopelti* were shown to lose their bacterial symbionts after treatment with antibiotics (Chang and Trager, 1974; Mundim and Roitman, 1977). A medium for cultivation of these aposymbiotic trypanosomatids has to be supplemented with hemin or protoporphyrin IX, and the addition of ALA or porphobilinogen is insufficient (Chang et al., 1975). This observation suggests that, like naturally-occurring symbiont-free trypanosomatids, aposymbiotic flagellates are unable to synthesise haem using earlier precursors. However, they possess the final enzyme of the pathway, FeCH, and perhaps also the two other enzymes preceding FeCH found in the genomes of *Leishmania* spp. and *Crithidia* sp.

In the strains containing symbionts, fluorometric assays showed that both haem and porphyrins were present when the flagellates were grown in a haem-free medium (Chang et al., 1975). Moreover, UROS was found to be active in both *B. culicis* and *C. oncopelti*, while its activity was negligible in the respective symbiont-free species, as well as in *Leishmania tarentolae* and *Trypanosoma conorrhini*. Since the activity of UROS is substantially higher in the isolated endosymbionts than in the cell homogenate, the bacterium is proposed to supply its host at least with this enzyme. Bacterial symbionts may supply their hosts with the final product or the flagellates are able to use precursors of the haem biosynthesis that are supplied by the symbiont and synthesise haem using their own enzymes.

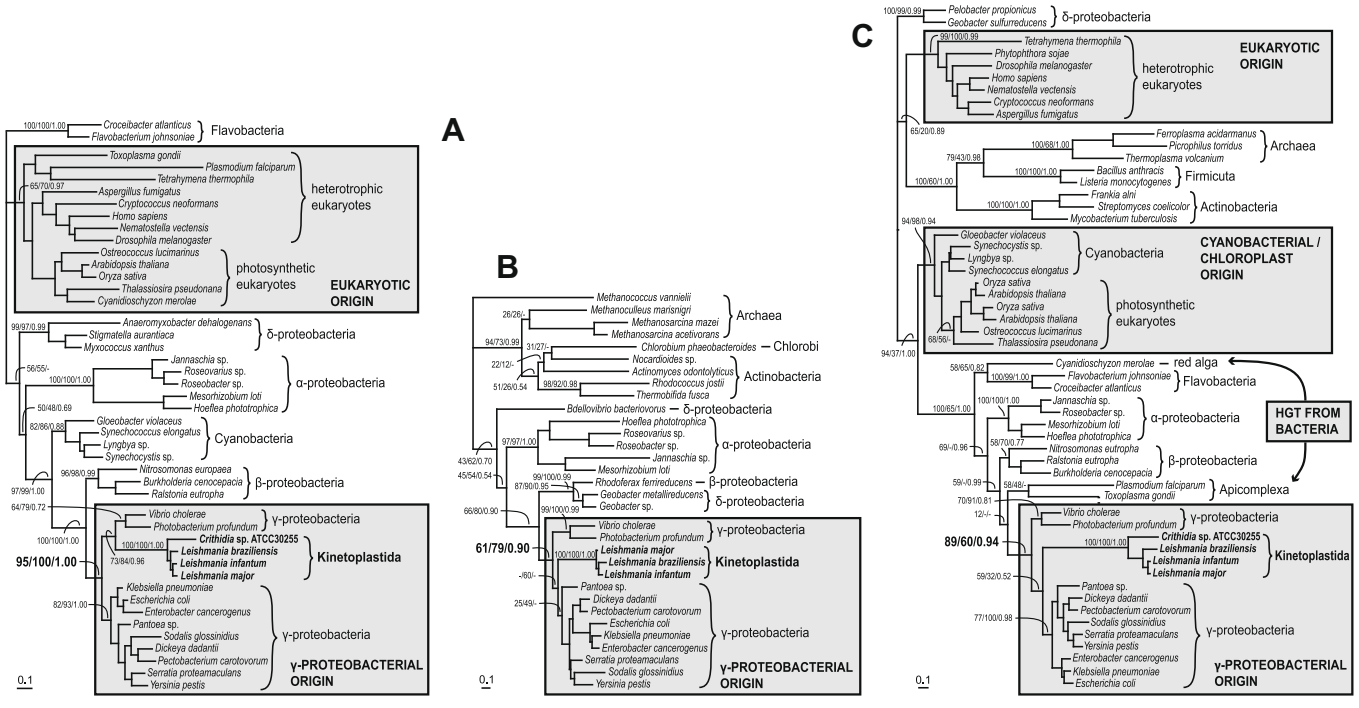

Fig. 2. Maximum likelihood phylogenetic trees of *hemF* (A), *hemG* (B) and *hemH* (C) genes encoding coproporphyrinogen oxidase (CPOX), protoporphyrinogen oxidase (PPOX) and ferrochelatase (FeCH), respectively. Numbers above the branches represent statistical support values: non-parametric bootstrap calculated in RAxML 7.0.3/non-parametric bootstrap calculated with PhyML 3.0/posterior probabilities from PhyloBayes 2.3c. The values supporting monophyly of γ-proteobacteria including Kinetoplastida are highlighted. Different origins of the genes in eukaryotes are exposed in grey boxes.

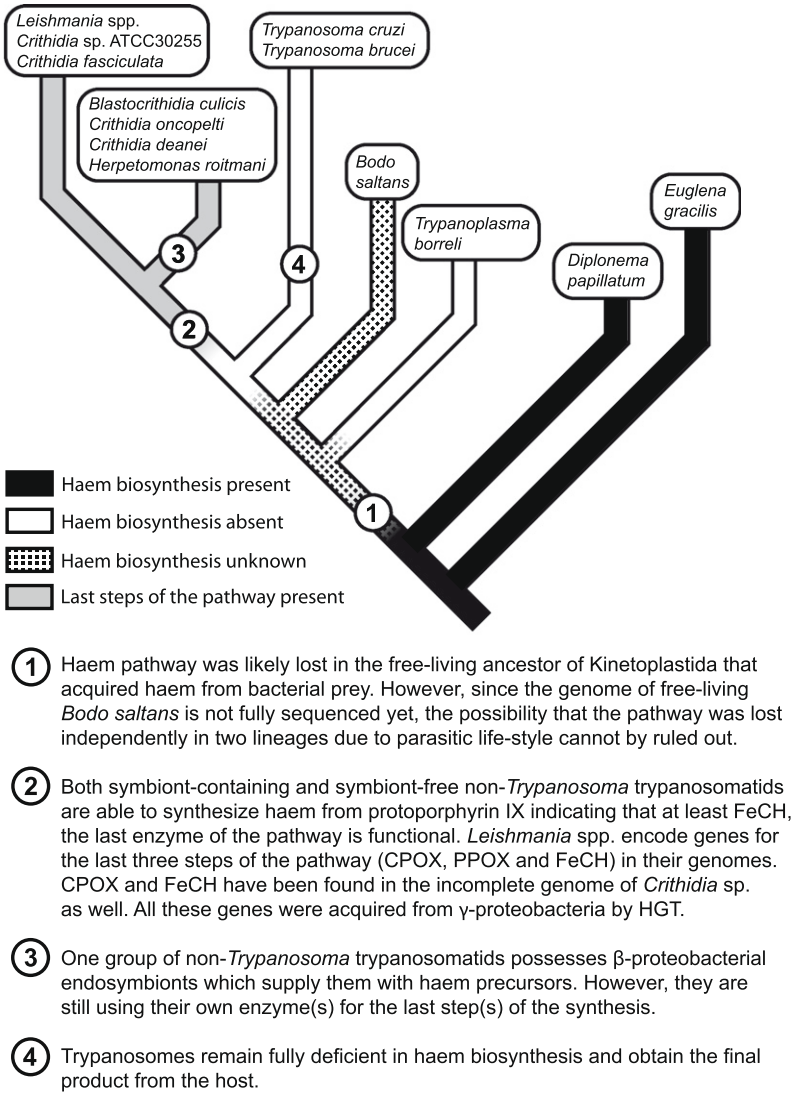

Fig. 3. Proposed scenario for the evolution of haem biosynthesis in Kinetoplastida.

In either scenario, the endosymbiotic bacterium plays an important role in the biosynthesis of the host haem and thus acts as an organelle. Similarly, haem biosynthesis of photosynthetic eukaryotes takes place in chloroplasts and is maintained by the enzymes that originated in the organellar genome, but were subsequently transferred into the host nucleus (Oborník and Green, 2005). The mitochondrion of heterotrophic eukaryotes possesses, in addition to ALA synthesis, enzymes for the last three steps of the pathway using precursors imported from the cytosol (Dailey et al., 2005).

Currently, there is a general consensus that mitochondria and chloroplasts originate from an endosymbiotic relationship between an engulfed bacterium and as yet unspecified host cell (Sagan, 1967; Schwartz and Dayhoff, 1978; Martin et al., 1992; Morden et al., 1992). Being cell-wall deficient, surrounded by two membranes and unable to live outside the host cell, the endosymbionts of trypanosomatids that divide synchronously with the host do nicely resemble a proto-organelle (Chang and Trager, 1974; de Souza and Motta, 1999).

5. Phylogenetic position of bacterial endosymbionts of kinetoplastids

The bacterial endosymbionts of *B. culicis*, *H. roitmani* and some *Crithidia* spp. are closely related to each other (Du et al., 1994). A phylogenic tree of trypanosomatids, as inferred from 18S rRNA gene sequences, shows that the endosymbiont-containing members form a monophyletic lineage, suggesting just a single acquisition of the endosymbiont during trypanosomatid evolution (Maslov et al., 2001; Yurchenko et al., 2009). Based on the 16S rRNA sequences, the bacteria in question were affiliated with the β-division of proteobacteria, branching off the *Bordetella bronchiseptica* lineage (Du et al., 1994), while more recent protein-based phylogenies place them among γ-proteobacteria closely related to the genus *Pseudomonas* (Motta et al., 2004). Finally, a very recently sequenced genome of the endosymbiotic bacterium from *C. deanei* again robustly supports its ranking within β-proteobacteria (Umaki et al., 2009). This finding suggests that γ-proteobacterial genes found in *Leishmania* spp. and *Crithidia* sp. were transferred to the kinetoplastid nucleus from a bacterium that was not related to β-proteobacterial symbionts of extant trypanosomatids.

6. Evolution of kinetoplastids from the haem perspective

The monophyly of the taxon Trypanosomatidae that comprises all kinetoplastids except bodonids is well supported (Simpson et al., 2002). However, the phylogenetic positions of the internal groups of trypanosomatids have been widely discussed. The major debate concerns the monophyly of trypanosomes. 18S rRNA-based

trees proposed two conflicting phylogenies. The first topology implied that the genus *Trypanosoma* is paraphyletic and forms more than one basal lineage (Landweber and Gilbert, 1993; Maslov et al., 1996; Hughes and Piontkivska, 2003; Moreira et al., 2004). The alternative topology supports the monophyly of *Trypanosoma* spp. as the early branch of the trypanosomatid lineage (Haag et al., 1998; Hamilton et al., 2004; Simpson et al., 2006; Yurchenko et al., 2006a). Several evaluations of the influence of different outgroups, methodologies and alignments confirmed the limited information content of the 18S rRNA datasets (Hughes and Piontkivska, 2003; Hamilton et al., 2004; Simpson et al., 2006). Moreover, the phylogenetic trees in the above mentioned studies were rooted using very distant outgroups such as *Euglena* or *Trypanoplasma* that form long branches, which can cause topological artefacts. The controversy was pretty much resolved in favour of the monophyly of the genus *Trypanosoma* by using glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) (Hamilton et al., 2004), which was further strengthened by a concatenated analysis of both 18s rRNA and gGAPDH genes (McInnes et al., 2009). Since 18s rRNA and gGAPDH sequences are now available for a large set of trypanosomatids, we have constructed updated phylogenetic trees of these two markers as well as the concatenated analysis of both of them. *Bodo saltans* served as the most suitable outgroup, since it constitutes a sister lineage to all trypanosomatids (Moreira et al., 2004; Simpson et al., 2006). The resulting trees support the monophyly of both trypanosomes and non-*Trypanosoma* trypanosomatids (data not shown).

Finding a well-supported topology is crucial to our understanding of the causes that led to the loss of the ability to synthesise haem and to the rescue of this function by a bacterial endosymbiont. The hypothesis presented herein suggests that a common ancestor of all extant trypanosomatids was completely deficient in haem biosynthesis. At present we can only speculate whether this pathway was lost in a free-living or already parasitic ancestral kinetoplastid.

Support for the loss of the pathway during the evolution of Kinetoplastida comes from *Euglena gracilis*, a photosynthetic relative, as some genes of its haem pathway are the original eukaryotic ones (L. Kořený and M. Oborník, unpublished data). Several species of diplonemids, which form a sister lineage to Kinetoplastida (Simpson and Roger, 2004), have been cultured in axenic cultures and did not require addition of hemin in the media (Schuster et al., 1968; Porter, 1973; Triemer and Ott, 1990; Sturm et al., 2001; Marande et al., 2005; Roy et al., 2007). Moreover, two genes of the pathway (the second and the sixth step) have been found in the EST database of *Diplonema papillatum* (http://gmod.mbl.edu).

While the loss of the haem pathway in the kinetoplastids could have been a consequence of a parasitic life-style, one can also imagine that it was already lost in the free-living ancestor, which fed on bacteria in the same way as all extant free-living bodonids and thus had easy access to haem. It would not be the first case of eukaryotes that employ this method. A similar strategy is utilised by *C. elegans*, which acquires haem from bacteria it feeds on and lacks haem biosynthesis genes in its genome (Rao et al., 2005; Perally et al., 2008). Indeed, in the available ESTs and partially sequenced genome of free-living *B. saltans* no haem biosynthetic gene has yet been found (http://gmod.mbl.edu; http://www.sanger.ac.uk).

Parasitism evolved several times independently within Kinetoplastida (Simpson et al., 2006). The bodonids contain not only free-living but also some parasitic species. These include well known ectoparasites of fishes such as *Ichtyobodo necator* and several species of the genus *Cryptobia*. Some other *Cryptobia* spp. occupy reproductive systems of molluscs and leeches or guts of planarians, fishes and lizards (Vickerman, 2000). Members of the genus *Trypanoplasma* are dixenous and are similar to trypanosomes, yet belong to the family Bodonidae. They live in the blood of fishes or amphibians and are transmitted by leeches, which are the same host taxa of the most basal clade of trypanosomes (Hamilton et al., 2009), and thus serve as a nice example of convergent evolution. *Trypanoplasma borreli* is the only kinetoplastid outside of Trypanosomatida that is grown in axenic culture. It requires hemin in its culture medium (Maslov et al., 1993; Maslov and Simpson, 2007), indicating that it is a haem auxotroph, similar to trypanosomatids. This finding supports the idea that haem biosynthesis was lost at an early stage of kinetoplastid evolution (Fig. 3). It is also possible that the pathway was lost in both of these lineages independently as a consequence of the parasitic life-style.

Trypanosomes, the early branching group of trypanosomatids, have easy access to haem from the host and are fully deficient of haem synthesis. Furthermore, the bloodstream stage of *T. brucei* does not need haem for the respiratory cytochromes in mitochondria, since it generates energy exclusively by glycolysis (Hannaert et al., 2003). However, other hemoproteins such as peroxidases and cytochromes P450 and b5 have been reported in trypanosomes. Their functions are connected to oxidative stress response, detoxification, synthesis of polyunsaturated fatty acids and sterol biosynthesis (Wilkinson et al., 2002; Tripodi et al., 2006; Bridges et al., 2008; Lepesheva et al., 2008; Chen et al., 2009). Haem proteins in the bloodstream stage of *T. brucei* seem to play an important role in resistance to the oxidative burst induced by macrophages. A haptoglobin–haemoglobin receptor, which enables haem uptake from host blood, has been recently identified (Vanhollebeke et al., 2008). The insect stage has to revive its mitochondrion, generating most of its ATP by oxidative phosphorylation, for which haem-containing proteins are indispensable (Lukeš et al., 2005). It has been proposed that trypanosomes salvage haem from the large amount of vertebrate blood meal that the vector intakes during feeding (Lara et al., 2007). The dependence on haem and perhaps other growth factors from vertebrate blood may be the reason why no monoxenous insect trypanosomes have been found to date. Using an alternative strategy, some trypanosomes evolved the ability to bypass the insect vector (*Trypanosoma equiperdum*), or reduce the transmission time to a very short period (*Trypanosoma evansi*) (Lai et al., 2008).

On the other hand, the non-*Trypanosoma* trypanosomatids are mostly represented by monoxenous parasites of insects. *Leishmania*, which famously infect humans, became dixenous parasites secondarily (Yurchenko et al., 2006b). We postulate that non-*Trypanosoma* trypanosomatids obtained genes coding for last three enzymes of the pathway (CPOX, PPOX, FeCH) through HGT from a γ-proteobacterium. Since these genes have been found in both *Leishmania* spp. and *Crithidia* sp. and the function of FeCH has been confirmed in representatives from different lineages (both symbiont-free and symbiont-containing), we propose that these genes were transferred to their nucleus before the radiation of non-*Trypanosoma* trypanosomatids (Fig. 3). These genes could have been transferred from an endosymbiont that was subsequently lost. Alternatively, they may have been transferred from a bacterial prey of some free-living ancestor having a similar life-style as the recent free-living bodonids, which would be in accordance with the "you are what you eat" theory of horizontal gene transfer (Doolittle, 1998).

The insect trypanosomatids as well as *Leishmania* have fully functional mitochondria (Hannaert et al., 2003; Horváth et al., 2005) and require haem for oxidative phosphorylation and other cellular processes (Berger and Fairlamb, 1993; Tripodi et al., 2006; Castro and Tomás, 2008; Lepesheva et al., 2008). They presumably do not have such easy access to this compound as trypanosomes that live in the blood. Many species parasitise insects that do not feed on blood at all (McGhee and Cosgrove, 1980). They thus need to obtain haem or its precursors directly from the host. They

may take advantage of taking coproporphyrin from the cytosol of the host cell instead of haem from the mitochondrion in the same way that we propose for *Leishmania* (Fig. 1). Indeed, it has been described that insect trypanosomatids closely interact with the epithelial cells of the insect digestive tract and were even observed to be engulfed by these cells (McCulloch, 1919; Fampa et al., 2003). It is possible that the presence of the last three genes of haem biosynthesis in the nucleus of these parasites (both *Leishmania* and insect trypanosomatids) enables them to survive in the host environment where haem is a limiting growth factor.

The ancestor of one lineage of the insect trypanosomatids engulfed a β-proteobacterium that became an endosymbiont. This endosymbiont rescued the haem biosynthesis of these flagellates, which are now totally independent of the haem biosynthesis of their animal hosts. The last three genes of the pathway are probably still encoded in the nucleus of the symbiont-bearing flagellates, indicating that these species are able to use precursors of the haem biosynthesis that are supplied by the symbiont and synthesise haem using their own enzymes. A possible explanation for such a strategy is that it enables them to have control of haem synthesis that has to be tightly regulated to prevent accumulation of intermediates and/or haem, which are toxic for the cell.

7. Conclusions

We propose a new hypothesis on the evolution of haem synthesis in Kinetoplastida, postulating a loss of this pathway in the ancestor of all trypanosomatids. The ancestor of the non-*Trypanosoma* trypanosomatids acquired genes encoding enzymes catalysing the last three steps of the pathway through HGT from a γ-proteobacterium. Alternatively, the initially complete pathway may have been partially lost during the course of evolution. Some trypanosomatids acquired a β-proteobacterium and allowed its transformation into an endosymbiont, which in turn supplies its host with precursors of haem. Those precursors are further processed for haem synthesis using nuclear-encoded enzymes. Aposymbiotic trypanosomatids use the same enzymes for the last three steps of the synthesis, for which precursors are salvaged from the host. Trypanosomes constitute a separate branch that is fully deficient of haem synthesis.

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