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## Protein moonlighting in parasitic protists

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**Abbreviations:** GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PFO, pyruvate:ferredoxin oxidoreductase; ROS, reactive oxygen species; TAC, tripartite attachment complex

### Abstract

Reductive evolution during the adaptation to obligate parasitism and expansions of gene families encoding virulence factors are characteristics evident to greater or lesser degrees in all parasitic protists studied to date. Large evolutionary distances separate many parasitic protists from the yeast and animal models upon which classic views of eukaryotic biochemistry are often based. Thus, a combination of evolutionary divergence, niche adaptation, and reductive evolution means the biochemistry of parasitic protists is often very different to their hosts and to other eukaryotes generally, making parasites intriguing subjects for those interested in the phenomenon of moonlighting proteins. In common with other organisms, the contribution of protein moonlighting to parasite biology is only just emerging, and it is not without controversy. Here, an overview of recently identified moonlighting proteins in parasitic protists is provided, together with discussion of some of the controversies.

### Introduction

A classic association of reductive evolution with the adaptation to obligate parasitism is evident to a greater or lesser degree in all parasitic protists studied to date. For instance, the loss of common biosynthetic pathways, moderation of mitochondrial energy metabolism, and even the degeneration of photosynthetic capability are seen in diverse parasites (*e.g.* [1,2]). Many parasites (*e.g.* the parasitic trypanosomatid family and malarial parasites) undergo complex lifecycles that require differentiation through multiple cellular forms and most also exhibit highly specialised, lineage-specific biological traits, which are often central to virulence – *e.g.* antigenic variation. Due to limitations in the efficacy, toxicity or resistance associated with existing therapies, new medicines are needed for many of the human, veterinary, and plant diseases caused by parasitic protists. Streamlined metabolism and novel biology provide attractive opportunities for chemotherapeutic attack, but life cycle complexity, cellular novelties, and biological streamlining also suggest parasitic protists pose intriguing subjects for those interested in the phenomenon of protein moonlighting. In common with other organisms, the contribution of protein moonlighting to parasite biology is only just emerging, and it is not without controversy. Here, an overview of recently studied moonlighters and some consideration of opportunities to uncover further moonlighting candidates are provided. Further discussion regarding protein moonlighting in eukaryotic parasites can found in recent reviews [3-5].

## Moonlighting at the host-parasite interface: unexpected quirks

Looking broadly at key challenges facing parasites, then life cycle progression often requires host cell invasion or the penetration of host tissue in order to reach niche environments conducive to parasite replication and/or evasion of host immunity. For parasites that cycle through vectors as part of their transmission cycle, it is an invariably small number of parasites that moves from host to invertebrate vector. The small inoculum must adapt rapidly to often surprisingly large changes in temperature, as well as to a new environment in order for productive infection to take place.

The '2-Cys' class of peroxiredoxins are enzymes classically known for a peroxidase activity that is employed in the detoxification of reactive oxygen species (ROS), but in the last ten years or so the role of peroxiredoxins as redox-regulated protein chaperones and activators of signal transduction cascades has been increasingly recognised [6]. *Leishmania* parasites (members of the trypanosomatid family) are unusual in that they are one of the few microbial pathogens that choose macrophage as a safe intracellular haven in which to replicate. In particular, mature *Leishmania* amastigotes do not prevent fusion of the phagosome they occupy with lysosomes, and thus replicate within acidic phagolysosomes, the intracellular compartments in which macrophage-engulfed microbes are normally killed. Life inside macrophage phagolysosomes poses numerous challenges to *Leishmania* parasites including nutrient limitation (to which the solution appears to be an efficient mitochondrial energy metabolism that brings with it ROS production as a consequence [7]) and a vulnerability to ROS generated as part of the macrophage killing system [8]. Quite unexpectedly, therefore, in *L. infantum*, it is the 'moonlighting' chaperone function, rather than the better known peroxidase activity of mitochondrial peroxiredoxin, that is essential for the virulence of this *Leishmania* species in a mouse model [9]. Here, the restoration of virulence to mitochondrial peroxiredoxin-null *L. infantum* through expression of a mitochondrial peroxiredoxin lacking the active site cysteine, and therefore deficient in peroxidase activity, contributes to the evidence that indicates the essentiality of the moonlighting function, but not the protein's better known peroxidase activity, at least in the *Leishmania* life cycle stage responsible for pathology.

Cell surface co-option of the abundant plasma protease plasmin (or co-option and subsequent activation of its zymogen plasminogen) is an apparently ubiquitous trait observed in numerous pathogens of mammals, including diverse bacterial pathogens, unicellular protist parasites and multicellular helminthic parasites [4]. The receptors mediating plasmin binding are often moonlighting proteins. Regarding eukaryotic parasites, available evidence suggests host co-opted plasmin acts in concert with parasite-derived, surface-exposed proteases during cell invasion and traversal through the host's extracellular matrix. Malarial parasites (*e.g. Plasmodium falciparum*, the species responsible for most of the mortality caused by human malaria) appear to have taken things a stage further utilising host-derived plasmin caught by a plasma membrane-localised, moonlighting enolase for penetration of the midgut epithelium within the mosquito vector [10,11]. The initial stages of the *Plasmodium* transmission cycle through the mosquito sees a rapid maturation of gametocytes ingested with the infected blood meal and fusion of the mature gametes; this two-step process takes place within an hour or so following ingestion of the blood meal [12]. Subsequently, ookinetes (the motile form into which zygotes differentiate over the course of 24 h post-fertilization) traverse out of the peritrophic matrix within which the blood meal is digested, and then penetrate the midgut epithelium to reach the basal lamina side of the epithelium whereon they mature to form the oocysts from which mammal-infectious sporozoites ultimately emerge. Collectively, a variety of experiments reveal exposure of enolase on the ookinete cell surface (but not on the surface of either gametes or the zygote) and that interaction between bloodmeal-derived plasminogen and the parasite's surface-exposed enolase is essential for the ookinetes' penetration of the mosquito midgut [10,11]. From a numbers perspective, midgut penetration and traversal has a high attrition rate: an estimate of  $10^5$  gametocytes ingested will yield approximately 1000 ookinetes, of which around two dozen, or less, will go on to form oocysts [10,12]. Recruitment of the abundant, host-derived plasmin protease for midgut invasion therefore points to a requirement for malarial parasites to make best possible use of all available resources in order to overcome a severe bottleneck associated with mosquito colonization and parasite transmission [13].

Intriguingly, the versatility of *Plasmodium* enolase extends still further beyond its best known function as a glycolytic/gluconeogenic enzyme and the more recently characterised role in plasminogen/plasmin binding: in ookinetes surface exposed enolase also provides a ligand for the mosquito midgut epithelial surface protein EBP, thereby contributing to the initial interaction between ookinetes and the midgut glycocalyx. However, in contrast to the essentiality of plasmin recruitment, recently reported studies document that the enolase-EBP interaction is not essential for productive midgut invasion, at least for some *Plasmodium* species or strains [14]. There is also an indication that surface-exposed enolase may act as a parasite adhesin during the invasion of host red blood cells by merozoites (which are responsible for pathology). During the intracellular growth cycle within red blood cells, parasite enolase is found at multiple intracellular sites, including nucleus and the food vacuole, and it is also subject to post-translational modifications. These observations all raise the possibility that enolase moonlights in a variety of other, currently ill-characterised capacities during the malaria life cycle [15-17]. However, caution should be exercised here, since enzyme re-localisation may equally relate to spatially and temporally restricted requirements for ATP production, something which has been noted in another apicomplexan parasite *Toxoplasma gondii* [18].

### Healthy uncertainty

Deployment of normally cytosolic enzymes as cell surface-exposed adhesins is reported for numerous prokaryotic and eukaryotic parasites. In *Giardia intestinalis* and *Trichomonas vaginalis*, two microaerophilic parasites, re-localisation of abundant metabolic enzymes is proposed following contact of the parasite with host epithelial cells [19,20]. In the example of *Trichomonas vaginalis*, the moonlighting candidature of several metabolic enzymes is not without controversy.

*Trichomonas vaginalis*, the most common non-viral sexually transmitted infectious agent, resides extracellularly within the urogenital tract. Its survival depends upon adherence to the host epithelium; unsurprisingly, adherence utilises multiple parasite molecules, including a lipoglycan [21,22] and multiple surface proteins. Proteomic comparisons between surface protein complements of *Trichomonas* strains that differ in their host cell adherence capability have been used to identify candidate protein adhesins, including novelties (e.g. TVAG\_244130 and TVAG\_166850) which have been subjected to experimental validation [23]. More controversially, roles in cytoadherence are suggested for the  $\alpha$ - and  $\beta$ -sub-units of succinyl-CoA synthetase, malic enzyme sub-units, and pyruvate:ferredoxin oxidoreductase (PFO) [24-27]. In *Trichomonas*, these candidate adhesins are found in hydrogenosomes, the name given to the mitochondrial-related organelles present in this parasite [28]. Hydrogenosomes have lost the capacities for carbon flux through pyruvate dehydrogenase and the Krebs (or citric acid) cycle, cytochrome-dependent respiration, and oxidative phosphorylation. In place of these classic mitochondrial pathways, pyruvate is catabolised within hydrogenosomes to acetyl-CoA by the oxygen-sensitive enzyme PFO, organellar ATP is generated via an acetate:succinate CoA transferase cycle that utilises succinyl-CoA synthetase (which is better known for its involvement in a conventional Krebs cycle), and H<sub>2</sub> production provides the sink for electrons transferred during pyruvate decarboxylation.

In keeping with many other moonlighters, malic enzyme, PFO, and succinyl-CoA synthetase are all abundant proteins. However, concerns have been aired regarding the failures of several groups to demonstrate dual localisation of these enzymes within both hydrogenosomes and at the cell surface, as well as some of the indirect approaches employed in the implication of adhesin functions [29,30]. Failure to identify pathways via which hydrogenosomal proteins reach the cell surface has also helped fuel scepticism regarding the candidature of some *Trichomonas* enzymes as putative adhesins. Of course, for any organism defining the route(s) by which cytosolic proteins, such as the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or enolase, reach cell surfaces and, thus, arrive in position to assume reported moonlighting roles provides a puzzle that needs resolving. A variety of secretion mechanisms are now known for eukaryotic cells, of which exosome secretion is perhaps the most widely documented, and glycolytic enzymes, including the well-known GAPDH and enolase moonlighters, are common cargo within these secreted membrane vesicles. For the proposed

hydrogenosomal trichomonad moonlighters, however, there is an added complication of how proteins contained within double membrane-bound organelles, the hydrogenosomes, could be routed onto the cell surface; of the controversial adhesion candidates only the succinyl-CoA synthetase  $\alpha$ -sub-unit is represented within the published *Trichomonas* exosome proteome [31].

Whilst the debate continues as to whether several abundant hydrogenosomal enzymes moonlight at the interface between parasite and host cell or not, it is worth noting that following contact with epithelial cells *Trichomonas* parasites significantly change their appearance and behaviour, switching from spindle-like flagellates to pseudo-amoeboid cells [32]. In other parasites, or indeed in eukaryotic cells generally, dramatic changes in cell morphology or cell differentiation involves activation of the autophagy pathway [33]. Although the change in *Trichomonas* morphology occurs quickly following contact with mammalian epithelial cells, it nonetheless occurs along the same timeline during which autophagy begins to contribute to intracellular re-modelling in other organisms [32]. Cellular targets for turnover via autophagy-dependent pathways include organelles, and hypothetical encapsulation of hydrogenosomes within autophagosomes provides a means whereby hydrogenosomal proteins could be placed in the context of the dynamic eukaryotic vesicular trafficking system with potential opportunity for these proteins to be routed onto the cell surface. Genetic manipulation has been used to prevent the secretion of GAPDH from the bacterium *Streptococcus pyogenes*, thereby confirming a moonlighting role essential for virulence [34], and also discussed in [35]. The genetic tractability of *Trichomonas* is sufficiently robust to attempt the expression of tagged malic enzyme, or other candidate adhesins, and to follow the fate of the tagged proteins in real time following the contact of parasite with epithelial cells. Such experiments have the potential to resolve debate regarding the candidature of somewhat controversial potential moonlighters in *Trichomonas*.

### **Moonlighting and motility**

Cytoskeletal association of various enzymes in proposed moonlighting contexts is widely documented, and sometimes dismissed as an artefact of non-physiological cytoskeletal association of abundant, sticky proteins following their solubilisation during biochemical fractionation. Among parasites, the use of aldolase to connect surface-localised transmembrane adhesins with dynamic actin filaments lying beneath the parasite cell surface in *Plasmodium* merozoites and in *Toxoplasma gondii* perhaps provided the most clear cut evidence for protein moonlighting in parasites [36]. The actin-aldolase-adhesin interaction has been studied at biochemical, cellular, and atomic levels, and the actin filaments form part of an actin-myosin motor complex that is important for the gliding motility of apicomplexan parasites across cell surfaces and host cell invasion [37-39]. Indeed, expression of site-directed aldolase mutants in *Toxoplasma* that was conditionally null for the endogenous aldolase gene had suggested that the catalytic and moonlighting linker functions of the aldolase protein could be distinguished, and that the moonlighting function contributed to the efficiency of host cell invasion [36]. It transpires that this conclusion was premature: whilst a moonlighting role for aldolase in apicomplexan parasites is not called into question, new data indicates the moonlighting function is non-essential as parasites studied in the absence of glucose glide and invade host cells in a normal fashion *in vitro*. Moreover, intracellular accumulation of fructose-1, 6-bisphosphate (a glycolytic intermediate that can be toxic in other organisms) now provides the preferred explanation for motility and invasion defects in *Toxoplasma* cells lacking aldolase [40].

### **A mosaic of unusual metabolism and moonlighting candidature**

Unusual facets of trypanosomatid biochemistry have been studied for several decades not just as a consequence of their importance for virulence, but because of the relative ease with which this family of parasites can be grown in liquid culture and subject to genetic manipulation. Collectively, the family cause three of thirteen neglected tropical diseases and includes parasite species responsible for veterinary and plant diseases, too. Metabolic peculiarities feature heavily in the unusual biochemistry

of trypanosomatids: for instance, the obligate compartmentalisation of most glycolytic enzymes within peroxisomes and organisation of the mitochondrial genome into an elaborate disk-like array of several thousand circular DNA molecules linked by catenation and known as the kinetoplast [41,42]. In the African trypanosome *Trypanosoma brucei*, the trypanosomatid in which metabolic peculiarities have been the most intensively studied, an extreme stage-specific regulation of mitochondrial metabolism sees the pathogenic bloodstream forms dispense with Krebs cycle activity and oxidative phosphorylation as a means for ATP production. Such stage-specific down-regulation of mitochondrial metabolism has provided a background from which one *bona fide* moonlighting protein and another candidate have emerged.

Thus, despite a lack of detectable activity of  $\alpha$ -ketoglutarate dehydrogenase activity in bloodstream *T. brucei*, sub-unit 2 ( $\alpha$ -KDE2) from the  $\alpha$ -ketoglutarate dehydrogenase complex is nonetheless expressed and present in the mitochondrion [43]. It is part of a filamentous multi-protein tri-partite attachment complex (TAC) spanning across outer- and inner-mitochondrial membranes, which attaches the kinetoplast to the flagellar basal body. This physical association of kinetoplast and flagellar basal body ensures that following kinetoplast replication, basal body duplication/new flagellum growth, and finally mitochondrial division each sibling cell inherits a mitochondrial genome [42]. Taking additional advantage of the lack of a Krebs cycle in bloodstream form *T. brucei*, the application of gene-specific RNAi allowed Sykes and Hajduk [43] to reveal moonlighting  $\alpha$ -KDE2 ensures even kinetoplast inheritance between sibling cells, although how  $\alpha$ -KDE2 contributes to kinetoplast inheritance is not yet known.  $\alpha$ -KDE2 also functions in mitochondrial genome maintenance in yeast suggesting, at first glance, conservation of a moonlighting function in eukaryotes that realistically last shared a common ancestor at the dawn of eukaryogenesis. However, here, ‘conservation’ of a moonlighting function is more likely due to convergence since in extant free-living kinetoplastids – the cosmopolitan group from which a monophyletic trypanosomatid family evolved – the several thousand circular DNA molecules that again form the mitochondrial genome are neither catenated nor physically attached to flagellar basal bodies. This indicates the TAC is likely to be a trypanosomatid-specific innovation.

Among the five known TAC components are one evolutionarily conserved protein, TBCCD1, utilised in diverse cytoskeletal structures of filamentous architecture [44], two novel proteins, TAC40 and AEP-1, homologous to conserved protein families involved in other essential aspects of mitochondrial function [45,46], and one moonlighter [43]. The characterisations of all these proteins and their recognisable domain architectures perhaps point to the importance of pre-existing proteins during the evolution of new cellular features. Yet, more significantly, within the context of this overview, the example of  $\alpha$ -KDE2 also illustrates how significant biochemical differences between parasite life stages can help in the identification of moonlighting proteins. It is therefore intriguing that high-throughput analysis of transcript abundances in different trypanosome lifecycle stages shows an unexpectedly high level of mRNA in bloodstream parasites for the  $\beta$ -sub-unit, but not the  $\alpha$ -sub-unit, of another Krebs cycle enzyme, succinyl-CoA synthetase (SCoAS) [47]. This result, together with the essentiality of the SCoAS  $\beta$ -sub-unit in bloodstream *T. brucei* [47] points to another moonlighting function within trypanosomatid mitochondria.

Finally, the *T. brucei* hexokinase isoform HXK2 and a protein homologous to GAPDH (GAPDHL) are worthy of mention. An unusual compartmentalization of glycolytic enzymes within peroxisomes in trypanosomes and their ancestors and the absence of feedback regulation acting hexokinase and phosphofructokinase, as seen in other organisms, are probably not coincidental [41]. Ectopic expression of glycolytic enzymes outside of their normal peroxisomal compartmentalization is also invariably deleterious for trypanosomes grown in the presence of glucose. It is therefore curious that HXK2 is found in the flagellum, as well as peroxisomes, in bloodstream *T. brucei* [48], and that this hexokinase isoform is catalytically inert unless mixed with its paralogue HXK1, whereon recombinant enzyme with kinetic properties similar to native enzyme is reconstituted [49]. A plausible explanation of these data is HXK2 moonlights in the flagellum – there is certainly no indication that the early reactions of glycolysis can occur in trypanosome flagella – and that loss of catalytic activity was a necessary event during the development of that moonlighting function. Regarding GAPDHL, the possibility that its presence within the paraflagellar rod, a filamentous flagellar structure in trypanosomatids required for

motility, provides evidence of a degenerate moonlighting enzyme has also recently been discussed [50]. Looking forward, these studies suggest careful mining of genome sequences that sample comprehensively across the breadth of parasite families and their near free-living relatives will allow perhaps complex histories of protein moonlighting to be revealed.

## Concluding comments

It is not yet clear just how widespread within nature the phenomenon of protein moonlighting might be. What is currently understood is that evolutionarily conserved moonlighting functions exist for assorted ancient, ubiquitous enzymes. Some of these moonlighting commonalities may reflect convergence, but among parasitic protists it seems that moonlighting traits also seen in other organisms are used in unexpected ways (*e.g.* the exploitation of host-derived plasminogen during traversal of the mosquito midgut by malarial parasites). By making good use of tractable genetic tools, exploiting differences in the life stage-specific biology of some parasites (as illustrated by the identification of  $\alpha$ -KDH2 moonlighting in African trypanosomes), and through cross-species comparisons it should be possible to produce a comprehensive compendium of moonlighting proteins in parasitic protists, to resolve some of the current controversies, and possibly even consider opportunities for chemotherapeutic intervention.

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