Moonlighting enzymes in parasitic protozoa

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SUMMARY

Enzymes moonlight in a non-enzymatic capacity in a diverse variety of cellular processes. The discovery of these non-enzymatic functions is generally unexpected, and moonlighting enzymes are known in both prokaryotes and eukaryotes. Importantly, this unexpected multi-functionality indicates that caution might be needed on some occasions in interpreting phenotypes that result from the deletion or gene-silencing of some enzymes, including some of the best known enzymes from classic intermediary metabolism. Here, we provide an overview of enzyme moonlighting in parasitic protists. Unequivocal and putative examples of moonlighting are discussed, together with the possibility that the unusual biological characteristics of some parasites either limit opportunities for moonlighting to arise or perhaps contribute to the evolution of novel proteins with clear metabolic ancestry.

Key words: Adhesins, Apicomplexa, glycolysis, motility, *Trichomonas vaginalis*, *Trypanosoma brucei*.

INTRODUCTION

There are numerous examples in the literature of proteins that no longer function as enzymes, but for which metabolic ancestry is evident from either the amino acid sequence or structural data. This is perhaps not so surprising given the likely antiquity of ubiquitous enzymatic domains within the protein evolution world or the innovation that resulted from fission and fusion of protein domains during key transitions in cellular evolution (*e.g.* the appearance of organellar biology during eukaryogenesis) (Ma *et al.* 2008; Caetano-Anollés *et al.* 2009*a*, *b*; Pascual-García *et al.* 2009). In many instances, loss of catalytic activity is often a critical feature in gene speciation and the evolution of enzyme-like proteins with novel functions. However, it has become increasingly apparent over the last twenty five years or so that in addition to their metabolic functions, some enzymes, which are better known for their role(s) in central metabolism, also participate in a non-enzymatic capacity in a diverse variety of cellular processes. This sort of dual-functionality is commonly known as protein moonlighting, and examples have been described in both eukaryotes and prokaryotes (Jeffery, 1999; Gancedo and Flores, 2008; Commichau *et al.* 2009). Since serendipity has generally led to the identification of moonlighting enzymes, it is difficult to predict how many enzymes with key metabolic functions also participate in other cellular processes, but the list is still growing (Jeffery, 2009) and the possibility that moonlighting may represent the norm rather than the exception has been discussed (Jeffery, 2005).

With respect to parasites, the concept of moonlighting is intriguing since biological streamlining (*i.e.* the loss or moderation of well conserved characters in eukaryotic cell biology and biochemistry) is one of the major traits generally associated with adaptation to parasitism, and is evident to a greater or lesser extent from the whole genome analyses of parasitic taxa that have already been subjected to whole genome sequencing. One can therefore anticipate that protein (or more specifically enzyme) multi-functionality will be apparent in many parasites, and could even contribute at some degree to streamlining. Moreover, with the ability to apply metabolomics or other high-throughput technologies towards the analysis of transgenic mutant parasites, it is worth remembering that in some instances mutant phenotypes arising from enzyme inactivation will conceivably be a direct consequence of perturbing cellular process other than metabolism. Our purpose with this review is to provide an overview of the current knowledge regarding enzyme moonlighting in unicellular parasites, and to discuss whether the unusual biology of some parasites either limits opportunities for enzyme moonlighting or even contributes to the evolution of novel enzyme-like proteins.

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MOONLIGHTING IN APICOMPLEXANS

The phylum Apicomplexa comprises a diverse group of over 5000 obligate parasites whose near

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evolutionary neighbours include ciliates and dinoflagellates. Malarial parasites (*e.g. Plasmodium falciparum*, which is the major species responsible for human malaria mortality) and the opportunistic pathogen *Toxoplasma gondii* are among the most well known of the Apicomplexa and, among parasites generally, it is in these organisms that the most clear-cut examples of moonlighting are to be found.

In mammalian hosts, *Toxoplasma* and *Plasmodium* replicate intracellularly, and egress from host cells is followed by active invasion of new host cells (Soldati *et al*. 2004; Baum *et al*. 2008). Glycolysis provides the major source of energy production in *T. gondii* tachyzoites and asexual bloodstage malarial parasites, with mitochondrial oxidative phosphorylation providing little, if any, contribution to cellular processes in cultured parasites (Painter *et al*. 2007; Pomel *et al*. 2008). In both parasites, the localisation of enolase, a glycolytic enzyme, to the nucleus, as well as the cytosol, or in the case of *P. falciparum* the additional localisation of enolase to the parasite's food vacuole, plasma membrane, and cytoskeleton, too is suggestive of functions that extend beyond this protein's classic enzymatic role in glycolysis (Ferguson *et al*. 2002; Pal-Bhowmick *et al*. 2007; Bhowmick *et al*. 2009). The nuclear localisation of enolase in the apicomplexans examined thus far is particularly intriguing since an alternatively-translated, truncated version of enolase has previously been implicated as a transcriptional repressor of the oncogene c-*myc* in a human cell line (Feo *et al*. 2000). Another glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) moonlights within the nuclei of mammalian cells in roles as diverse as transcription, DNA repair, RNA binding and transport, and telomere binding (Sirover, 2005; Demarse *et al*. 2009). These examples represent only a few of the alternative functions proposed for different glycolytic enzymes in a wide variety of organisms. However, the localisations of enolase in *Plasmodium* and *T. gondii* are only suggestive of moonlighting since one cannot rule out a possibility that differential localisation of some glycolytic enzymes helps provide energy-generating capacity at cellular sites where the demand for ATP is particularly acute. Indeed, epitope-tagging and immunofluorescence indicate that several, maybe even all, of the glycolytic enzymes are at least partially re-located from the cytosol to the cell periphery in extracellular *T. gondii* tachyzoites (Pomel *et al*. 2008). Intriguingly, the cell periphery in *T. gondii* and *P. falciparum* provide the sites of the most clear-cut and best delineated examples of enzyme moonlighting in parasites.

A striking example of apicomplexan-specific biology is the way in which parasites move across surfaces and penetrate tissues (Soldati *et al*. 2004; Baum *et al*. 2008). This motility, known as 'gliding' is dependent upon the co-ordinated interaction between adhesins exposed on the outer-face of the parasite plasma membrane and an actin-based cytoskeleton beneath the inner face of the parasite plasma membrane (Fig. 1). Related proteins that span across the plasma membrane of *T. gondii* and *P. falciparum*, respectively and provide the extracellular adhesin activities that engage host cells or other surfaces are MIC2 (standing for 'micronemal protein 2') and TRAP (standing for 'thrombospondin-related anonymous protein'). Both proteins are cross-linked to relatively unstable F-actin filaments by the glycolytic enzyme aldolase (Buscaglia *et al*. 2003; Jewett and Sibley, 2003). The ability of aldolase to bind actin is seen in other organisms, too. An X-ray structure of *P. falciparum* aldolase bound to the C-terminal tail of the TRAP homologue from *P. berghei* (Bosch *et al*. 2007) and homology modelling and mutational analysis of *T. gondii* aldolase (Starnes *et al*. 2009) indicate that actin-binding and TRAP/MIC2-interacting regions overlap, suggesting that the tetrameric structure of catalytic aldolase is likely to be necessary for aldolase to moonlight as a structural bridge between either TRAP or MIC2 and the cytoskeleton that mediates gliding motility (Fig. 1). The substrate-binding active site of aldolase

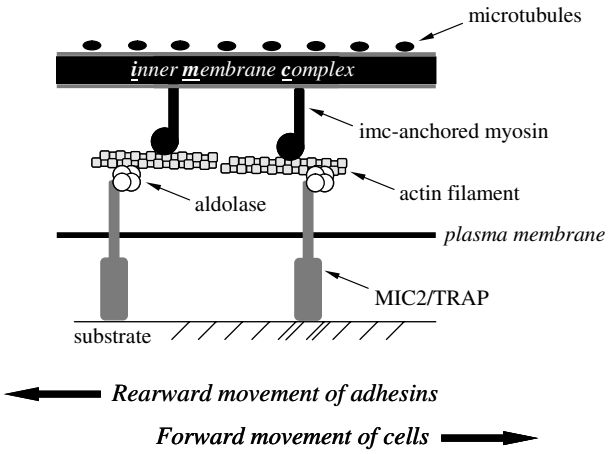

Fig. 1. Organisation of the 'glidosome' in apicomplexan parasites. The transmembrane adhesins TRAP (in malarial sporozoites) and MIC2 (in *T. gondii*) possess extracellular substrate-binding domains, and are cross-linked to F-actin via an aldolase bridge. During extracellular motility and cell invasion, the adhesin is released from apacial micronemes and translocated across relatively unstable actin filaments to the posterior-end of cells by a non-processive myosin motor that is attached to the microtubule-bound 'inner membrane complex'. Posterior-directed movement of the adhesin results in the forward-directed motility or 'gliding' of parasites across the substrate (*e.g.* a host cell surface).

N.B. Based on the studies published to date, aldolase is shown as a tetrameric bridge between adhesin and actin, but there is no indication of how many sub-units are likely to interface with actin and either TRAP or MIC2, respectively.

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also lies in the vicinity of the actin/TRAP/MIC2-interacting region, yet site-directed mutagenesis of the *Toxoplasma* enzyme by the Sibley group nonetheless resulted in the identification of residues required specifically for catalysis, but not for coupling the transmembrane-spanning adhesion to actin filaments and *vice versa* (Starnes *et al*. 2009). The expression of various site-directed aldolase mutants (D33A, K41A, R42A, K41E:R42G, and R148A) in *T. gondii* tachyzoites subsequently not only underscored the importance of glycolysis as an energy-generating pathway during this life cycle stage, but moreover revealed the essentiality of the aldolase-dependent linkage between MIC2 and F-actin for efficient cell invasion and potentially in other situations where motility is challenged by resistance (*e.g.* during penetration between host cells), too (Starnes *et al*. 2009). Comparative genomics indicates that the protein components required for gliding by *Toxoplasma* tachyzoites or malarial sporozoites are conserved across all Apicomplexa examined to date (Baum *et al*. 2006), suggesting that this particular example of aldolase moonlighting appeared early during apicomplexan evolution and was subsequently retained.

THE PROVOCATIVE IMPLICATION OF MOONLIGHTING IN *TRICHOMONAS*

The aldolases from *T. gondii* and *P. falciparum* provide well characterized examples of enzyme moonlighting in parasites. By contrast, a series of data pertaining to the possible expression of classic cytosolic and hydrogenosomal enzymes on the surface of *Trichomonas vaginalis* suggest intriguing, yet far from unequivocal examples of moonlighting in parasites.

T. vaginalis is a sexually transmitted pathogenic protozoan responsible for upwards of 250 million new cases of vaginitis in women annually, with the wider health concerns stemming from infection including increased pre-disposition to cervical cancer and increased transmissibility of HIV virus. The adaptation of *Trichomonas* to the microaerophilic environment of the urogenital mucosa is evident from the secondary loss of the capacity for cytochrome-dependent respiration (Carlton *et al*. 2007). In these parasites, the oxidation of pyruvate occurs within degenerate forms of mitochondria known as hydrogenosomes (for further discussion see Hjort *et al*. 2010) resulting in the formation of hydrogen gas and, following the metabolism of pyruvate-derived acetyl-CoA to acetate, the production of ATP (from substrate level phosphorylation). Carbohydrate and to a lesser extent amino acids provide the carbon sources for energy metabolism in trichomonads.

Biochemical analysis of fractionated cells and localisation experiments using either polyclonal antisera or monoclonal antibodies raised against recombinant proteins indicate the hydrogenosomal localisation of the following key enzymes required for organellar pyruvate metabolism: pyruvate:ferredoxin oxidoreductase (or PFO), malic enzyme, α– and β–subunits of succinyl-CoA synthetase (Williams *et al*. 1987; Brugerolle *et al*. 2000; Hrdy *et al*. 2004). However, several reports document that upon contact of parasites with vaginal epithelial cells or upon exposure to high iron concentrations (250 μM, as opposed to a normal concentration in culture media of 20 μM) these hydrogenosomal enzymes, as well as the glycolytic enzymes enolase and GAPDH, can be found on the outer face of the parasite plasma membrane where they are proposed to moonlight as non-enzymatic adhesins during the adherence of parasites to epithelial cells lining the vaginal tract (Alderete *et al*. 1995; Engbring and Alderete, 1998; Garcia *et al*. 2003; Moreno-Brito *et al*. 2005; Mundodi *et al*. 2008; Lama *et al*. 2009).

Notwithstanding the fact that enzymes have been found to act as adhesins in other pathogens (*e.g.* reviewed in Alderete *et al*. 2001), the suggestion that organellar enzymes and cytosolic glycolytic enzymes in *Trichomonas* could act as adhesins has been met with some uncertainty (*e.g.* Hirt *et al*. 2007). Immunofluoresence analyses using non-transgenic *Trichomonas* have been used to provide evidence of surface localisation of PFO, enolase, malic enzyme and succinyl-CoA synthetase (Engbring and Alderete, 1998; Garcia *et al*. 2003; Moreno-Brito *et al*. 2005; Mundodi *et al*. 2008), but some concern has been voiced that the original discovery of these enzymes as putative adhesins came as a result of indirect studies, including *in vitro* binding assays of parasite whole cell extracts to human cells and the screening of parasite cDNA libraries with sera pooled from patients with trichomoniasis. Although the 'stickiness', or non-specific interactions, mediated by some glycolytic and hydrogenosomal enzymes towards surface exposed receptors on vaginal epithelial cells is conceivably sufficient to select for a moonlighting role that mediates parasite-host cell adherence, the non-specificity of these interactions coupled to an initial difficulty in conceptualising how organellar, or indeed cytosolic, enzymes lacking obvious targeting motifs arrive at, and then remain tethered to the outer-face of the parasite plasma membrane provide obvious counter-arguments to the suggestion that metabolic enzymes play a role in mediating cell adhesion *in vivo*. PFO is a membrane-associated enzyme (Williams *et al*. 1987), but again a major difficulty lies in identifying how this hydrogenosomal protein is also able to putatively locate to the cell surface Moreover, some researchers have only reported the expected hydrogenosomal location for malic enzyme, and succinyl-CoA synthetase in immunofluoresence experiments (*e.g.* Brugerolle *et al*. 2000), and the reported prediction of possible

trans-membrane helices in surface-exposed malic enzyme is supported by neither structural data for recombinant malic enzyme nor a report that malic enzyme excreted from *Trichomonas* retains a soluble, active conformation (Addis *et al*. 1997; Hirt *et al*. 2007). The postulation of a host cell receptor for one of the proposed *Trichomonas* adhesins AP120 (or PFO) could also be treated with some caution since the 'host' cells used in the experiments to identify AP120 (Moreno-Brito *et al*. 2005) were HeLa cells, rather than the vaginal and ureter epithelial cells used in other studies of *Trichomonas* adhesins.

Yet, despite the uncertainties, the case for *Trichomonas* enzymes moonlighting as adhesins remains curious and somewhat compelling. For instance, it is interesting that not merely a single abundant metabolic enzyme is apparently re-located to the cell surface, but several major hydrogenosomal enzymes and cytosolic glycolytic enzymes have been described on the outer-face of the plasma membrane. This suggests large-scale re-modelling of intracellular architecture could underpin the trafficking of enzymes to the cell surface. In that regard it is relevant to note that within minutes of cytoadherence parasite morphology changes significantly from a motile multi-flagellate form to a pseudo-amoeboid form (Arroyo *et al*. 1993). Rapid intracellular remodelling of organelle and cytoskeletal architecture is not uncommon among unicellular eukaryotes, and in some instances selective autophagy pathways are used to initiate rapid organelle turnover within minutes of cells experiencing appropriate environmental cues (*e.g*. Herman *et al*. 2008). In their review on surface proteins in *Trichomonas*, Hirt and co-authors remark that an up-regulation in autophagy could result in the targeting of hydrogenosomes to the lysosome for degradation, and thus, the routing of hydrogenosomal proteins to cell surface via a lysosome-linked trafficking pathway (Hirt *et al*. 2007). The signature footprint for autophagy that is evident in the *Trichomonas* genome (Rigden *et al*. 2009) and suggestive experimental evidence for autophagy in *Trichomonas* (Benchimol, 1999) lend support to this possibility.

An experiment that should unequivocally address the intriguing possibility that several key soluble metabolic enzymes in *Trichomonas* also moonlight as cell surface-exposed adhesins would be to use transgenic parasites expressing GFP-tagged enolase, PFO, malic enzyme or succinyl-CoA synthetase and follow the (re-)localisation of these tagged enzymes, preferably in real-time, in response to high [Fe] exposure and contact with vaginal epithelial cells. In the absence of data from this or other relevant experiments, however, it is perhaps interesting to note that *Giardia lamblia*, an intestinal parasite widely accepted to be distantly related to the trichomonads, was recently observed to secrete the enzymes enolase, arginine deaminase and orthinine carbamoyl transferase in significant amounts following interaction with intestinal epithelial cells (Ringqvist *et al*. 2008). The physiological significance of this enzyme secretion is uncertain, but the characterization of enzyme secretion by cultured parasites is corroborated by data from *in vivo* experiments, too (Davids *et al*. 2006; Ringqvist *et al*. 2008).

TRYPANOSOMATIDS: LIMITED OPPORTUNITIES FOR GLYCOLYTIC ENZYMES TO MOONLIGHT?

The parasitic trypanosomatid family include numerous pathogens of medical, veterinary or agricultural significance. Many of these pathogens are digenetic parasites, transmitted between hosts by blood- or sap-feeding arthropod vectors. The best known are the tropical disease-causing parasites of the genera *Trypanosoma* and *Leishmania*, which are collectively responsible for African sleeping sickness (*T. brucei*), Chagas' disease in South and Central America (*T. cruzi*), and leishmaniasis. Among the many unusual, sometimes unique, biochemical and cell biological characteristics that have been described in trypanosomatids are the complex and elaborate architecture of the mitochondrial genome (or kinetoplast) (Lukeš *et al*. 2002), extensive mitochondrial RNA editing (Stuart *et al*. 2005), and the compartmentalisation of several glycolytic enzymes, as well as other enzymes of carbohydrate metabolism, within peroxisomes (Michels *et al*. 2006). Regarding examples of moonlighting, the suggestion that glutamate dehydrogenase contributes to RNA editing (Bringaud *et al*. 1997) has now been dismissed (Simpson *et al*. 2003), and claims that the Rieske iron-sulphur protein, a sub-unit of cytochrome *c* reductase, is a component of the mitochondrial tRNA import machinery in *L. tropica* have not been replicated in studies with *T. brucei* (Paris *et al*. 2009). However, a recurrent theme in this short review is the ability of glycolytic enzymes to moonlight in different capacities within diverse taxa. Similar to the enolase from *T. vaginalis*, the enolase of *Leishmania mexicana* was found to be present in the cytosol and on the external face of the plasma membrane, acting in the latter location as a plasminogen-binding protein (Quiñones *et al*. 2007; Vanegas *et al*. 2007). Again, the question of how a cytosolic enolase can also be targeted to the external face of the plasma membrane is unanswered, but in the case of *Leishmania* or other trypanosomatids it is also reasonable to ask whether the unique compartmentalisation of glycolysis influences the opportunities for several glycolytic enzymes to moonlight.

The targeting of between six and eight (if one counts pyruvate phosphate dikinase) glycolytic enzymes into *Trypanosoma* and *Leishmania* peroxisomes (Fig. 2) means these metabolically specialised micro-bodies are better known as glycosomes (reviewed by Michels *et al*. 2006). Although it is only

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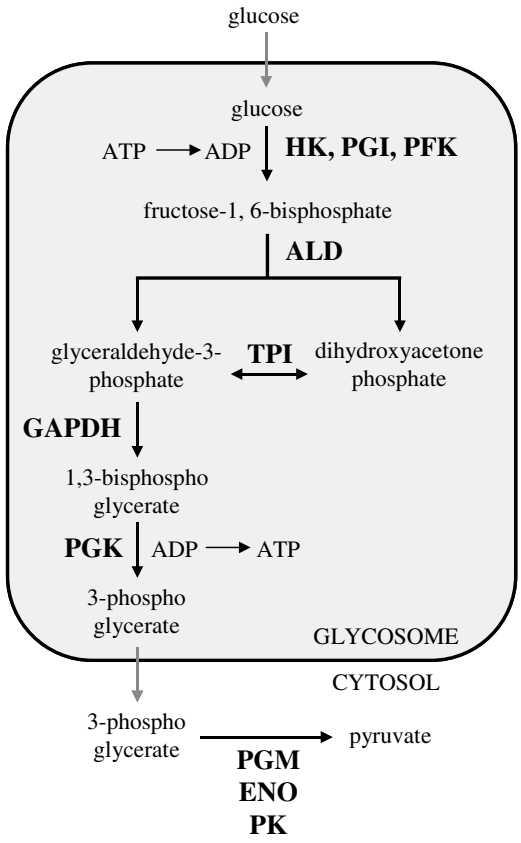

Fig. 2. Peroxisomal compartmentalisation of carbohydrate metabolism in trypansomatids. The scheme illustrates the compartmentalisation of the first seven glycolytic enzymes in the glycosomes of bloodstream *T. brucei*. In other life cycle stages, or indeed in other trypanosomatid species, PGK is not necessarily a glycosomal enzyme, but PPDK (catalysing conversion of phospho-enolpyruvate to pyruvate) is present (Michels *et al*. 2006).

Key: HK, hexokinase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase; ALD, aldolase; TPI, triose-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase.

possible to speculate on the selective pressure(s) and mechanism(s) through which glycolytic enzymes became peroxisomal components in an ancestor of the trypanosomatids and their nearest free-living relations (Ginger *et al*. 2010), mathematical modelling and subsequent experimental studies revealed that re-compartmentalisation was accompanied by significant changes in the regulation of trypanosome glycolysis (Bakker *et al*. 1999*a*, *b*, 2000; Furuya *et al*. 2002; Albert *et al*. 2005; Haanstra *et al*. 2008). In particular, the allosteric feedback regulation of hexokinase and phosphofructokinase seen in other organisms does not occur in trypanosomes – instead the peroxisomal membrane provides a regulatory barrier that prevents uncontrolled hexokinase and phosphofructokinase activities rapidly depleting the cytosolic ATP concentration (Bakker *et al*. 2000; Furuya *et al*. 2002). ATP/ADP balance within glycosomes requires either organellar phosphoglycerate kinase (PGK) activity or, in trypanosomatids other than bloodstream form *T. brucei*, potentially a combination of several glycosomal enzymes, including adenylate kinase, pyruvate phosphate dikinase, and phosphoenolpyruvate carboxykinase. Some, maybe most, glycolytic intermediates, however, are able to move between glycosomes and the cytosol and the consequence of ectopic cytosolic expression normally of glycolytic enzymes can be dire for trypanosome cells (Blattner *et al*. 1998; Helfert *et al*. 2001). Since glycolytic enzymes, which are generally abundant in many organisms or cell-types and can be subjected to dynamic intracellular re-localisation are, to date, the most commonly observed moonlighters, one could reasonably ask whether the unique compartmentalisation of glycolysis and re-programming of glycolysis regulation in trypanosomatids limits the possibilities for glycosomal glycolytic enzymes to moonlight.

There have been reports of glycosomal glycolytic enzymes being found bound to microtubules in biochemically fractionated cytoskeletons and flagella, but these observations are almost certainly an experimental artefact of abundant enzymes with a high pI being able to bind the trypanosome microtubule cytoskeleton once peroxisomal matrix components are solubilised following detergent-extraction of live cells (Robinson *et al*. 1991). Yet, as we discuss below, partial localisation outside of glycosomes of one isoform of hexokinase is suggestive of an additional cellular function beyond glycolysis. This scenario is even more intriguing given how the properties of the hexokinase isoform in question suggest it is catalytically inactive when expressed without an appropriate binding partner. One can therefore pose the question of whether this absence of catalytic activity guards against leaving cells vulnerable to ATP depletion due to unregulated hexokinase activity when this particular trypanosome hexokinase is on moonlighting duties.

Thus, *T. brucei* possess two, tandemly duplicated hexokinase genes; both genes are expressed in bloodstream and procyclic trypanosomes. The protein product from the second of these genes, Tb*HK2*, has independently been found by two groups to be present both inside and outside of glycosomes. Most recently, HK2 protein was shown to partially localise to the trypanosome flagellum (Lyda *et al*. 2009). Several explanations for the dual targeting of HK2 protein to glycosomes and flagella can be put forward, but perhaps more important is that the differences between the C-termini of the otherwise near identical paralogous HK1, which is exclusively glycosomal, and HK2 are sufficient to render the latter protein incapable of phosphorylating glucose when expressed in recombinant form (Morris *et al*. 2006). Gene knockout studies reveal that *HK2* is necessary

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for optimal growth of procyclic *T. brucei* (Morris *et al.* 2006) although it is presently not known whether changes in growth rate and morphology reflect the loss of an important moonlighting function or the effect on HK2 depletion on glycolysis: interestingly catalytically inactive recombinant HK2, when mixed with recombinant *Tb*HK1 produces a hexameric enzyme that exhibits kinetic parameters which are more similar to those reported for native *T. brucei* hexokinase activity purified from cells than to the kinetic parameters of recombinant HK1 hexamers (Chambers *et al.* 2008). The observation that hexokinase activity is reconstituted when *Tb*HK2 is mixed with a recombinant S160A HK1 mutant that is unable to catalyse phospho-group transfer further supports the likelihood that HK2 contributes to glycolytic flux (or its regulation) within glycosomes (Chambers *et al.* 2008).

In *Leishmania donovani*, but not *T. brucei* (Vanhollebeke *et al.* 2008), hexokinase has been suggested to moonlight as a haemoglobin receptor within the flagellar pocket, and thus potentially contribute to haem and/or iron acquisition (Krishnamurthy *et al.* 2005). As cytosolic hexokinase activity is toxic to *Leishmania* promastigotes (Kumar *et al.* 2009), the catalytic activity of hexokinase might need to be masked within the flagellar pocket. If, however, *T. brucei HK2* provides an example of a gene where mutation is likely to have been necessary to facilitate a moonlighting function, then one can reasonably ask whether the evolution of GAPDH-like proteins that are encoded in all trypanosomatid species for which genome sequences are available (*e.g.* the protein encoded by Tb09.211.1370) and genes in *Trypanosoma* species, but not *Leishmania*, that encode large PGK-like proteins (*e.g.* Tb11.22.0003) are at least in part a consequence of constraints that the glycosomal compartmentalisation of glycolysis places upon the ability of some glycolytic enzymes to moonlight. Identifying functions for these proteins will answer this question, but the lack of amino acid conservation at many positions known to contribute to catalysis in *bona fide* GAPDH and PGK enzymes, respectively strongly suggests neither GAPDH-like nor PGK-like proteins in trypanosomatids are likely to be catalytically active. Interestingly, epitope-tagged variants of *T. brucei* GAPDH-like and PGK-like proteins localise or co-purify with cytoskeletal structures, and the PGK-like protein is expressed in both bloodstream and tsetse form trypanosomes (our unpublished data). The function(s) of these novel proteins is under investigation in our laboratory.

CLOSING PERSPECTIVES

Completely sequenced genomes and stable transformation are available for the parasites discussed in this review. Thus, our survey of *bona fide* and putative examples of enzyme moonlighting in parasitic protists provide a timely reminder that in the post-genomic analysis of gene function it is important to remember that for some, maybe many, metabolic enzymes their cellular functions extend beyond classic roles in intermediary metabolism. The widespread use of sensitive mass spectrometry approaches in molecular cell biology means that enzymes will continue to be found in unexpected intracellular locales or structures, although in many instances rather than being due to moonlighting, this will be an artefact of contamination during biochemical fractionation (as in the example of abundant glycosomal enzymes binding to microtubules in trypanosomatid cell-free extracts (Robinson *et al.* 1991)). Work with the apicomplexan aldolases (Bosch *et al.* 2007; Starnes *et al.* 2009), however, illustrates how the individual functions of enzymes that do moonlight can be teased apart experimentally.

In addition to moonlighting enzymes, there are also many examples in diverse taxa of proteins that resemble enzymes, but which perform regulatory or structural tasks rather than catalytic functions. These examples are somewhat tangential and too numerous to cover in detail in a review such as this, but in the case of many regulatory proteins, such as pseudo-protein kinases (Boudeau *et al.* 2006) or the S-adenosylmethionine decarboxylase (AdoMetDC)-like protein that regulates AdoMetDC activity in trypanosomes (Willert *et al.* 2007), it is hard to rationalise how some regulatory functions could have evolved from moonlighting functions. In other instances, however, gene duplication of a multifunctional protein has been followed by paralogue speciation. A classic example is the regulation of galactose metabolism in yeast: in some species a single protein is both a galactokinase and transcriptional activator of galactose metabolism, whereas in other yeast species closely-related paralogues perform either the enzymatic task of galactose phosphorylation or transcriptional activation (Hittinger and Carroll, 2007; Campbell *et al.* 2008). Time will tell whether the examples of GAPDH-like and PGK-like proteins in trypanosomes, or indeed other enzyme-like proteins, such as a GAPDH-like protein from *Giardia lamblia* (Yang *et al.* 2002), are more likely to have evolved as a consequence of ancestral genes that encoded multifunctional proteins (or moonlighters) being subject to duplication or whether acquisition of novel function(s) and loss of catalytic activity are merely the consequences of sequence drift in a duplicated paralogue.

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