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Evolutionary significance of anaerobic energy metabolism in eukaryotes

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Endosymbiotic theory is traditionally founded in comparative physiology and biochemistry. That is also where it works best. Mereschkowsky's first formulation of the endosymbiotic origin of plastids was based on the comparison of physiological attributes of plastids and cyanobacteria (Mereschkowsky 1905). The revival of endosymbiotic theory for the origin of mitochondria and chloroplasts by the late Lynn Margulis (Sagan 1967; Margulis 1970) was also very much based on comparative physiology, although her suggestion for an additional endosymbiont for the origin of flagella from spirochaetes was based on morphological similarity. When hydrogenosomes were discovered by Müller in 1973 (Lindmark and Müller 1973), they looked much more like clostridia than mitochondria from the standpoint of comparative biochemistry, but that is mainly because in 1973 the concept of mitochondria was built around investigations on rat liver mitochondria, a strictly aerobically functioning organelle, and in addition very little was known about the mitochondria of anaerobic eukaryotes. As advances have accrued in the understanding of mitochondria of anaerobic eukaryotes, the smaller have become the biochemical differences between mitochondria and hydrogenosomes (Müller et al. 2012). Today it is clear that they share a common ancestry and that they represent related biochemical manifestations of one and the same endosymbiotically derived organelle.

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Introduction

The role of the *Euglena gracilis* mitochondrion as a biochemical missing link between typical mitochondria and hydrogenosomes, has been studied in recent years using the example of wax ester fermentation. While wax ester fermentation in the facultatively anaerobic mitochondria of *Euglena gracilis* harbours undiminished general biochemical interest, most notably because of the biofuels industry and the circumstance that it is easier to transport hydrocarbons than it is to transport H_2, the evolutionary significance of these organelles in the context of endosymbiosis and the transition from prokaryotes to eukaryotic organelles still remains largely defined in their comparison to hydrogenosomes. During the last few years, quite a bit has happened in the field of anaerobic mitochondria (Müller et al. 2012). In particular, the mitochondria of *Chlamydomonas reinhardtii* have, surprisingly, emerged as the most complete biochemical missing links between typical mitochondria and hydrogenosomes currently known, because *Chlamydomonas* harbours genes for virtually all enzymes of core energy metabolism in hydrogenosomes, and expresses them in response to anaerobiosis (Atteia et al. 2006, 2012). The genome sequence of a hydrogenosome-possessing organism, *Trichomonas vaginalis*, has been determined in the meantime (Carlton et al. 2007), opening up new opportunities for the study of eukaryotic anaerobes. The availability of transformation techniques for *Trichomonas* also augments the study of protein import into its hydrogenosomes. That process appears to require both N-terminal and internal — within the imported polypeptide — topogenic signals that specify their import into the organelle (Mentel et al. 2008), the specific roles of which have yet to be fully uncovered.

Known forms of anaerobic energy metabolism in eukaryotes involves a handful of about 50 proteins that are distributed across eukaryotic lineages in sic a way as to indicate that they were present in the common ancestor (Müller et al. 2012). Work on pyruvate:formate lyase (PFL), acetate kinase, and phosphotransacetylase from *Chlamydomonas* showed that all of the enzymes germane to core ATP synthesis from pyruvate in anaerobic eukaryotes are present in *Chlamydomonas*: PFL, pyruvate:ferredoxin oxidoreductase (PFO), iron-only hydrogenase, (Fe-HYD), bifunctional alcohol/aldehyde dehydrogenase (ADHE), the only exception being acetyl-CoA synthase (ADP-forming). Moreover, Mus et al. (2007) studied the expression of enzymes involved in H_2 production in *Chlamydomonas* and found that this whole spectrum of enzymes, that only a few years ago were thought to be specific to anaerobic eukaryotes, are induced within about 30 minutes after the onset of anaerobiosis in *Chlamydomonas*, such that this O_2-producing and O_2-respiring alga basically assumes the physiological identity of *Trichomonas* in response to environmental conditions. Furthermore, the *Chlamydomonas* enzymes share a common ancestry with those of eukaryotic anaerobes, such that, in our view, there can be little doubt that the common ancestor of eukaryotes possessed these enzymes, too. Moreover, Lantsman et al. (2008) even found that the same unusual PFO-fusion protein (pyruvate:$NADP^+$ oxidoreductase) that occurs in *Euglena* is also present in the mitochondria of the anaerobic human parasite *Blastocystis*. What people are finding among anaerobic eukaryotes and anaerobic mitochondria are the same enzymes over and over again, also in agreement with the

working hypothesis that these enzymes were present in the eukaryote common ancestor.

Anaerobic energy metabolism of *Euglena*

Under aerobic conditions, *Euglena* expresses PDH in mitochondria (Hoffmeister et al. 2004) and respires O_2, but using a slightly modified Krebs cycle that involves replacement of α-ketoglutarate dehydrogenase by α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase (Buetow 1989), as is also found among some α-proteobacteria (Green et al. 2000) and cyanobacteria (Zhang and Bryant 2011). Under anaerobic conditions, *Euglena* uses acetyl-CoA as the terminal electron acceptor and produces wax esters as end products of metabolism (Inui et al. 1982; Buetow 1989; Tucci et al. 2010), sometimes at levels up to 57% of dry weight (Tucci et al. 2010).

In general principle, *Euglena*'s wax-ester fermentation is similar to the synthesis of branched short-chain fatty acids in *Ascaris* or butyrate in *Dasytricha* (Müller et al. 2012), where fatty acids are synthesized from acetyl-CoA condensation with an acyl-CoA (starting with acetyl-CoA or propionyl-CoA), reduction of the resulting 3-oxoacid to the 3-hydroxy acid, dehydration thereof and reduction of the resulting *trans*-enoyl-CoA to the elongated acyl-CoA (Müller et al. 2012). The acetyl-CoA dependent *Euglena* route allows net fermentative ATP synthesis from glucose, because acetyl-CoA is condensed without prior ATP-dependent carboxylation to malonyl-CoA (Inui et al. 1982; Schneider and Betz 1985). The step catalyzed by trans-2-enoyl-CoA reductase (NADPH-dependent) circumvents the reversal of an O_2-dependent step in β-oxidation (Hoffmeister et al. 2005). A portion of the fatty acids is reduced to alcohols, esterified with another fatty acid and deposited in the cytosol as wax (wax ester fermentation). The fatty acyl-CoA reductase and the wax synthase involved in the synthesis of *Euglena*'s medium-chain wax aliphatic side chains were recently characterized (Teerawanichpan and Qui 2010). When oxygen again becomes available, the stored waxes are degraded via aerobic dissimilation in the mitochondrion (Inui et al. 1982).

Euglena's wax ester fermentation involves mitochondrial fumarate reduction, and thus utilizes RQ (Hoffmeister et al. 2004) for the synthesis of propionyl-CoA, similar to anaerobic mitochondria of metazoa (Müller et al. 2012), although reduced RQ can also donate electrons to other components of the *Euglena* mitochondrial respiratory chain (Castro-Guerrero et al. 2005), including the cytochrome bc_1 complex and the alternative oxidase (Castro-Guerrero et al. 2004). Propionyl-CoA is the starter for the synthesis of fatty acids of odd-number chain length, which comprise about 50% by weight of hydrocarbon chains in accumulated wax esters under various conditions (Kawabata and Kaneyama 1989) and in different *Euglena* strains (Tucci et al. 2010). Propionyl-CoA is produced via the same short methylmalonyl-CoA route as is found in animal mitochondria (Schneider and Betz 1985) and homologues for the underlying enzymes, methylmalonyl-CoA mutase and propionyl-CoA carboxylase (that provides ATP via substrate-level phosphorylation) are abundantly expressed in euglenid EST data (Ahmadinejad et al. 2007). Methylmalonyl-CoA mutase from *Euglena* was recently characterized (Miyamoto et al. 2010); the enzyme is also present in humans where it is one of our only two vitamin B_{12}- (cobalamin-) dependent enzymes (Roth et al. 1996), whereby *Euglena* was once the standard assay for serum B_{12} levels, because of its B_{12}-dependent ribonucleotide reductase (Torrents et al. 2006).

Under anaerobic conditions *Euglena* expresses pyruvate:$NADP^+$ oxidoreductase (PNO) (Inui et al. 1985, 1987, 1991), which performs oxidative decarboxylation of pyruvate and is a fusion protein, with an N-terminal PFO domain fused to a C-terminal flavoprotein domain with NAD-, FMN, and FAD-binding modules (Rotte et al. 2001; Nakazawa et al. 2003). The flavoprotein domain is found in many other proteins, sometimes alone as in the case of NADPH:cytochrome P450 reductase, and is probably a transducer of one-electron transport (from the FeS clusters of the PFO domain) to two electron transport (NADPH). PNO supplies acetyl-CoA and NADPH for the production of wax esters. PNO was first described in *Euglena* and was long thought to be unique to the Euglena lineage, but the same PNO (PFO fusion) is found in the apicomplexan *Cryptosporidium* (Rotte et al. 2001) as well as in *Blastocystis* (Lantsman et al. 2008). More recently, PNO homologues are turning up abundantly in EST sequencing projects from many disparate eukaryotic lineages (Hug et al. 2010; Atteia et al. 2012). The accumulation of wax esters with even-numbered chains in flufenacet-treated *Euglena* cultures and the inability of the bleached strains to grow anaerobically suggests that there is a considerable intracellular redirection of responsibility for redox balance to the plastid under anaerobic conditions in some strains (Tucci et al. 2010).

Trichomonas hydrogenosomes

Euglena is a thankful system from the standpoint of protein biochemistry, because one can isolate proteins from kilograms of cell material (Hoffmeister et al. 2005). But *Euglena* is so far not transformable, nor is a genome sequence available for *Euglena*. If one wants to study anaerobic mitochondria with genetically based approach, *Euglena* is not the system of choice, nor is *Chlamydomonas*, because only a comparatively small portion of its anaerobic energy metabolism is localized in the mitochondrion (Müller et al. 2012; Atteia et al. 2012). For *Trichomonas*, both a genome sequence and genetic manipulation techniques are available, and this presents some advantages in the investigation of cell biological aspects, for example protein import into hydrogenosomes.

Trichomonas hydrogenosomes possess several components of the mitochondrial protein targeting machinery and import across the two hydrogenosomal membranes is thought to entail components homologous to TOMs and TIMs (translocons of the outer and inner mitochondrial membrane, respectively) of mitochondrial membranes (Carlton et al. 2007; Dolezal et al. 2006; Shiflett and Johnson 2010; Rada et al. 2011). But there are clear differences and clues that the import machinery in *T. vaginalis* is reduced relative to yeast, because Tom70, Tom20 and Tom22 as well as Tim54, Tim50 and Tim21 appear to be lacking (Shiflett and Johnson 2010; Rada et al. 2011). *Trichomonas* hydrogenosomes lack cytochromes (Müller 1993) but seem to generate an electrochemical gradient across the inner membrane (Vilela et al. 2010), which is instrumental in protein import into yeast mitochondria (Dolezal et al. 2005; Neupert and Herrmann 2007). Protein import into hydrogenosomes is ATP-dependent (Bradley et al. 1997) and it is possible, but has not been demonstrated, that the generation ot the membrane potential is as well. *Tv*Tom40 exists as six copies in the *Trichomonas* genome, for *Tv*Tim17 five and for *Tv*Tim9/10 at least two copies exist (Rada et al. 2011) and they all appear to be expressed

(Aurrecoechea et al. 2009). Hydrogenosome import on the order of 250 (Henze 2008) to 500 proteins, about half of which possess a short N-terminal extension (Carlton et al. 2007; Schneider et al. 2011; Burstein et al. 2012; Dyall and Johnson 2000). This extension is cleaved by the hydrogenosomal processing peptidase, a reduced homolog of the yeast mitochondrial processing peptidase (Brown et al. 2007; Smid et al. 2008).

Mentel et al. (2008) found that protein import into *Trichomonas* hydrogenosomes is not strictly dependent upon cleavable N-terminal extensions, but that internal signals contained within the imported protein are, for some imported substrates, sufficient to direct the protein to the organelle. Two hydrogenosomal proteins with known cleavage sites for their N-terminal presequences, the α-subunit of succinyl-CoA synthase (SCSα) and pyruvate:ferredoxin oxidoreductase (PFO), were investigated with respect to the ability of their presequences to target thioredoxin reductase to hydrogenosomes in transformed *Trichomonas*. Neither presequence directed TrxRh1 to hydrogenosomes, indicating that neither extension is, by itself, sufficient for hydrogenosomal targeting. Moreover, SCSα lacking its N-terminal extension was efficiently imported into hydrogenosomes, indicating that this extension is not required for import of this major hydrogenosomal protein. The finding that some hydrogenosomal enzymes require N-terminal signals for import, but that in others the N-terminal extension is not necessary for targeting, indicates the presence of (additional) targeting signals within the mature subunits of several hydrogenosome-localized proteins (Mentel et al. 2008).

The homologous succinyl-CoA synthase promoter works well in *Trichomonas* as does the dihemagglutinin tag (HA tag), which can be added in frame to constructs prepared for electroporation of *T. vaginalis* T1 cells, transformants of which can be selected on standard media containing 100 μg/ml of G418 (Mentel et al. 2008). This technique has permitted study of localization for several hydrogenosomal proteins to date. Three examples include the protein HydG and HydF, involved in [Fe]-hydrogenase maturation (Pütz et al. 2006), rubrerythrin (Pütz et al. 2005), and thioredoxin reductase (Mentel et al. 2008). Good positive controls are also available using antibodies against native acetate:succinate CoA transferase (ASCT), which was recently characterized as the last remaining major protein of core carbon and energy metabolism in trichomonad hydrogenosomes (van Grinsven et al. 2008).

Strain differences

One important aspect that has recently emerged from investigations of eukaryotic anaerobes is that there is significant variability among strains of the same species with regard to the type and amounts of the metabolic end products accumulated. This has been shown for *Euglena* (Tucci et al. 2010) and for *Trichomonas vaginalis* (Rasoloson et al. 2002). The variability among different *Euglena* strains with respect to anaerobic growth and wax ester fermentation is substantial. Eight new strains of *Euglena gracilis* from the Göttingen collection in addition to two bleached mutants generated with standard ofloxacin and streptomycin treatment were examined for their capacity to grow anaerobically, their quantitative capacity to produce wax esters, the effect of flufenacet (Trenkamp et al. 2004) on wax ester accumulation, and the partitioning of carbon species (length and saturation) within the wax ester fraction using GC-MS (Tucci et al. 2010). Substantial differences even with respect to the ability to grow anaerobically were observed, as three of the Göttingen strains (1224-5/10, 1224-/15, and 1224-/27) did not undergo either increases in cell number or increases in biomass over 11 days of culturing, while other strains, such as 1224/-13, produced threefold the amounts of wax esters in comparison to "Z" (1224-5/25), amounting to a wax ester content of >30% dry weight in some cases (note: the dry cell mass of *Euglena gracilis* 1224-5/13 burns like a candle upon ignition). Inclusion of 100 μM of the elongase inhibitor flufenacet in anaerobic cultures generally doubled the wax ester dry weight content, up to 57% of dry weight for 1224-5/13, which burns very nicely indeed, but emitting the scent of burned protein (mainly pellicula), of course. The specific composition of the fatty alcohol and fatty acid (methyl esters) constituents was determined for all of the wax ester fractions, flufenacet inhibited the accumulation of odd numbered chains (methylmalonyl-CoA derived propionyl-CoA starter for mitochondrial synthesis).

Strain-specific differences with regard to the spectrum of end products detected have also been reported for *Chlamydomonas reinhardtii* (Meuser et al. 2009) underscore. Grown anaerobically, *Chlamydomonas* produces acetate, formate, ethanol, and hydrogen as major end products (Mus et al. 2007). Different *C. reinhardtii* strains differ substantially with respect to the relative amounts of different end products accumulated, strain specific variation in formate and glycerol production being particularly pronounced (Meuser et al. 2009). Furthermore, many eukaryotic anaerobes have a life cycle in which pronounced stage-specific differences in energy metabolism exist, for example juvenile *vs.* adult forms of parasitic metazoans (Tielens 1994), or bloodstream *vs.* insect stages of *Trypanosoma* (Bringaud et al. 2006; van Hellemond et al. 2005).

The broader significance

Some anaerobic eukaryotes inhabit environments that are also sulfidic. Mitochondrial sulfide oxidation in animals that inhabit anaerobic and sulfidic environments has also been studied (Theissen and Martin 2008a, 2008b). The evolutionary analysis of the *Chlamydomonas* mitochondrial proteome suggested that the ancestor of mitochondria had a collection of genes that was more similar to that possessed by generalist α-proteobacteria such as *Rhodobacter* than to specialists such as the rickettsias (Atteia et al. 2008). In the larger scheme of things, the widespread occurrence of anaerobic mitochondria and the enzymes of anaerobic energy metabolism in eukaryotes makes sense in the context of the new model of Proterozoic ocean chemistry that has emerged over the last 10 years in the field of geochemistry (Mentel and Martin 2008; Müller et al. 2012). This is an interesting development, because it is rare that major and independent conceptual revolutions in two separate scientific fields converge at the same answers. But in recent years have witnessed major upheavals in views about early eukaryotic evolution and the role of anaerobes therein, developments that have been paralleled by a similar upheaval in the Earth sciences regarding views about the prevalence of oxygen in the oceans during the Proterozoic eon (the time from about 2.5 to 0.57 billion years ago). New views of Proterozoic ocean chemistry indicate that the oceans were anoxic and sulfidic during most of the Proterozoic eon ("Canfield oceans") and only became oxic about 580 million years ago, at the time when the first macroscopic animal forms appear in the fossil record. New views of eukaryote evolution have it that anaerobes are not re-

stricted to primitive or early-branching lineages, but are distributed throughout the eukaryote tree of life. Because of the widespread occurrence of hydrogenosomes and mitosomes among eukaryotic lineages and their evolutionary identity with mitochondria, the possession of mitochondria is no longer synonymous with an oxygen-dependent lifestyle. Eukaryotic anaerobes have mitochondria and many use them for ATP synthesis in the absence of oxygen. Once a puzzle, this is now readily understood in the new geochemical light of our comparatively recent anaerobic past (Mentel and Martin 2008; Müller et al. 2012). Taken together, these independent but mutually compatible developments enrich views of eukaryote ecology and evolution in the context of Earth history.

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