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REVIEW

Anaerobically functioning mitochondria: evolutionary perspective on modulation of energy metabolism in *Mytilus edulis*

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Abstract

The mitochondrion represents a compelling biological model of complex organelle development driven by evolutionary modification of permanently enslaved primordial purple non-sulphur bacteria. As an evolutionary modification, the dynamic nature of the mitochondrion has been observed to exhibit biochemical and functional variation, including the capacity for energy production driven by anaerobic respiratory mechanisms. In invertebrates, mitochondrial anaerobic respiration allows the organism to survive at a lower energy state while yielding more ATP than can be achieved by glycolysis alone. Furthermore, a preferred physiological state of lower energy production operationally yields diminished free radical generation, thereby offering a protective existential advantage. It has been established that energy production by the blue mussel, *Mytilus edulis*, is functionally dependent on anaerobic respiratory mechanisms within the mitochondrion. Importantly, under hypoxic conditions metabolic pathways in *M. edulis* have been demonstrated to synthesize and utilize amino acid adducts termed opines as chemically defined energy reserves. In addition to the utilization of opines as anaerobic metabolic intermediates by invertebrate organisms, opines were also discovered and characterized as metabolic intermediates in plant parasites, specifically crown gall tumors. A careful review of the biomedical literature indicates mechanistic similarities between anaerobically functioning mitochondria in *M. edulis* and crown gall tissues and metabolic processes in human tumors. The anaerobically functioning mitochondrion in *M. edulis* tissues is a potentially valuable high resolution model system for development of novel anticancer therapeutic agents.

Key Words: anaerobic respiration; anaerobic mitochondria; opines; *Mytilus edulis*; mollusc

Introduction

Mitochondria represent an endosymbiont model of complex organelle development driven by evolutionary modification of permanently enslaved primordial purple non-sulphur bacteria (Gray *et al.*, 1999). From a teleological perspective, endosymbiotic enhancement of eukaryotic cellular energy requirements indicates a convergence of metabolic processes within the mitochondrial matrix for optimal synthesis of ATP from ADP and inorganic phosphate. Bacterial and mitochondrial ATP synthases (F-ATPases) require a defined membrane potential to achieve transductive transmembrane proton-motive force across the inner membrane linked to high efficiency of ATP production (Stefano *et al.*, 2012). This necessitates an evolutionarily driven retrofit of the bacterial plasma membrane into the inner mitochondrial membrane. The proton-motive force is functionally coupled via mechanical transductive events within discrete protein subunits localized to the transmembrane domains of F-ATPases and involves sequential protonation and deprotonation of glutamate side-chains of cytochrome c-subunits within functional pores. Evolutionary pressure is predicted to provide an existential advantage to the host eukaryotic cell at this primal level of energy production (Stefano *et al.*, 2012). Recent elegant work has confirmed this key contention by demonstrating an enhanced efficiency of 2.7 *vs.* 3.3 - 5 protons per synthesized ATP molecule by eukaryotic *vs.* prokaryotic F-ATPases,

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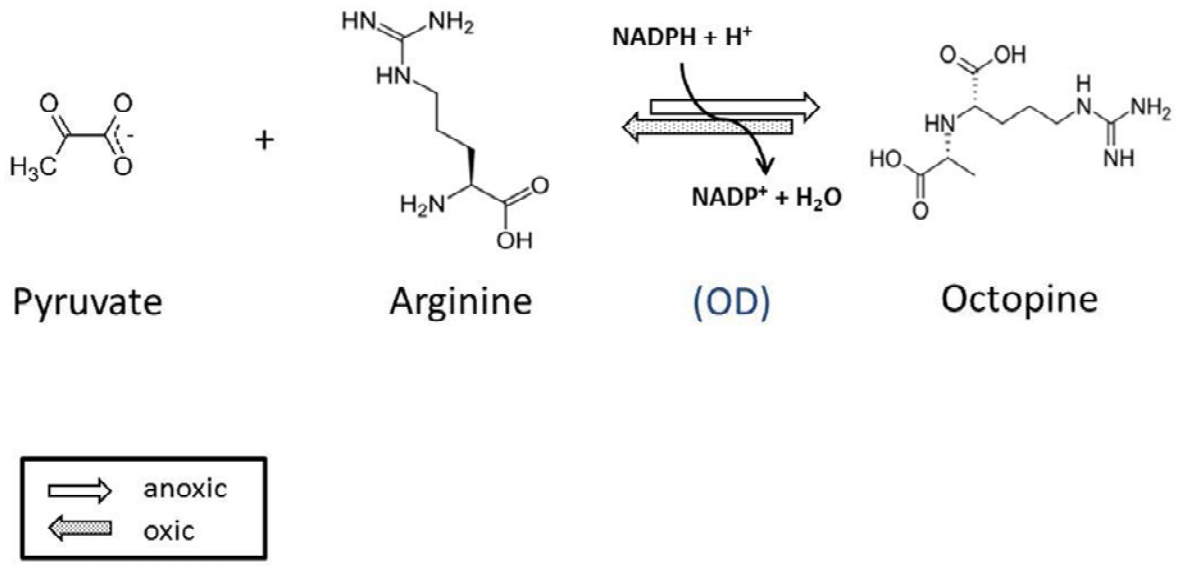

Fig. 1 Anaerobic production and re-oxidation of the opine, octopine, in *Mitylus edulis*. Pyruvate is condensed with the amino acid, arginine, to produce octopine. The reaction is catalyzed by octopine dehydrogenase (OD). Opines are stored until oxygen becomes available to reverse this reaction and produce pyruvate for the Krebs cycle.

respectively (Watt *et al.*, 2010).

Mechanistically, endosymbiosis has apparently resulted in seamless coupling of cytochrome c oxidase (COX) to F-ATPase for maximal ATP production in respiring mitochondria, thereby effecting essential partitioning of glycolytic and TCA cycle metabolic processes within discrete cellular domains. COX is an inner mitochondrial multi-subunit enzyme complex expressed and assembled as a mosaic from nuclear and mitochondrial genomes. A recent review presents the case for COX as a key regulator of mitochondrial ATP production (Pierron *et al.*, 2012). The authors propose that the evolutionarily driven addition of nuclear-encoded COX subunits provides the host eukaryotic cell with high order control over the ancestral activity of COX subunits encoded by mtDNA genes in the face of fluctuating mitochondrial oxygen tensions and potentially dangerous reactive oxygen species.

Anaerobic respiration in invertebrates

The intertidal habitat of the marine mussel, *M. edulis*, poses unique metabolic challenges to the survival of the species. During low tide, the mussel must close its valves to avoid water loss and therefore experiences hypoxic conditions. *M. edulis* has evolved to cope with hypoxia by switching to anaerobic respiration (de Zwaan *et al.*, 1976; Connor *et al.,* 2012). This strategy is not only employed by mussels; it has also been observed in other marine invertebrates (Hochachka *et al.*, 1977), numerous other eukaryotes [see review (Muller *et al.*, 2012)], plants (Igamberdiev *et al.*, 2009; Shingaki-Wells *et al.*, 2014), and of course in prokaryotes. To effectively mediate anaerobic metabolic demands, *M. edulis* synthesizes and utilizes amino acid adducts termed opines as chemically defined energy reserves (de Zwaan *et al.*, 1976).

Opines were first discovered in the mollusc, *Octopus* (Morizawa, 1927), notably the prototypic compound octopine, the enzymatically derived condensation product of arginine and pyruvate. In addition to the utilization of opines as anaerobic metabolic intermediates by invertebrate organisms, opines were also discovered and characterized as metabolic intermediates in plant parasites, specifically crown gall tumors (Holsters *et al.*, 1978; Guyon *et al.*, 1980; Toothman, 1982; Dessaux *et al.*, 1993). Synthesized opines are effectively stored until oxygen levels are sufficient to resume aerobic respiration followed by enzymatic oxidation to release pyruvate as an essential TCA cycle substrate (Grieshaber *et al.*, 1994) (Fig. 1). The amino acids used in the biosynthesis of opines are alanine, arginine or glycine (Fields *et al.*, 1980, Siegmund *et al.*, 1983; Grieshaber *et al.*, 1994). The enzyme required for production of octopine from arginine and pyruvate has recently been isolated and purified (Vazquez-Dorado *et al.*, 2011). Presumably, this strategy evolved to maintain osmolality and to produce a by-product less acidic than lactate (Ballantyne, 2004). This metabolic pathway, like glycolysis, only produces 2 ATP per mole of glucose.

Anaerobically functioning mitochondria in invertebrates

In recent times the dynamic nature of the mitochondrion has been observed to exhibit biochemical and functional variation, including the capacity for anaerobic respiration (Muller *et al.*, 2012). In this regard, *M. edulis* has been well studied (Doeller *et al.,* 2001; Connor *et al.*, 2012). When a prolonged period of hypoxia leads to anoxia

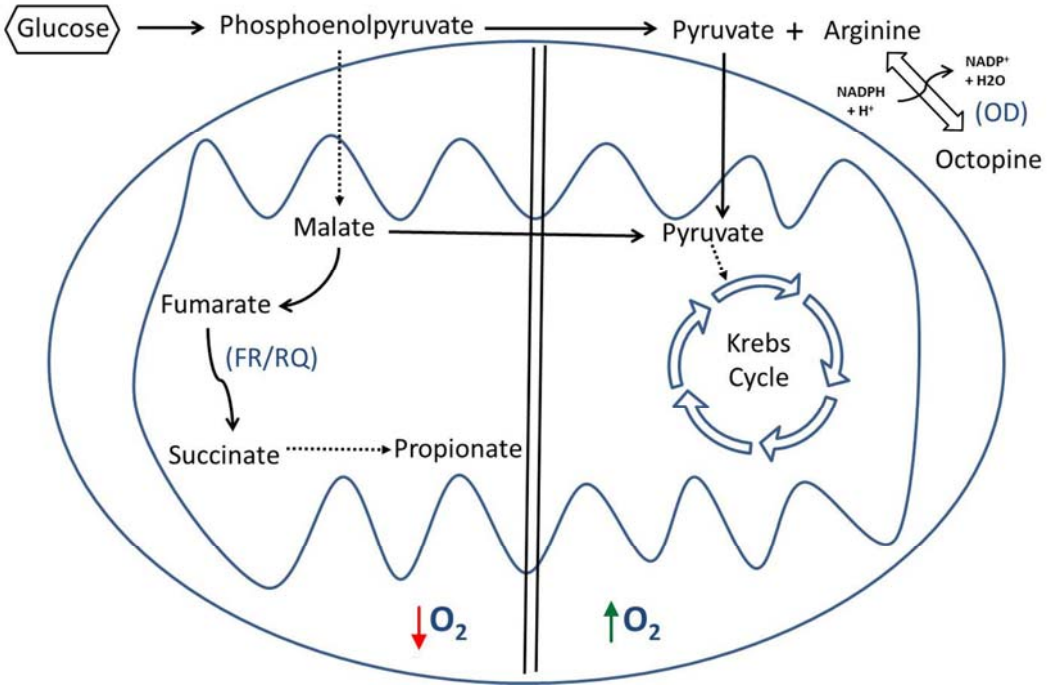

Fig. 2 (adapted from (Muller *et al.,* 2012) Anaerobic and aerobic metabolic pathways within the cytoplasm and mitochondria of the mussel, *Mytilus edulis*. Glucose can be converted to phosphoenolpyruvate and to pyruvate. Pyruvate can be used in the mitochondrial Krebs cycle or condensed with amino acids to produce opines. Phosphoenolpyruvate can be converted to malate before being simultaneously reduced to fumarate and oxidized to pyruvate (malate dismutation). Pyruvate can be used in the Krebs cycle. The fumarate is further reduced by fumarate reductase (FR) and rhodoquinone (RQ) to succinate. Succinate is then transformed into propionate as an end product.

in the mussel, an additional metabolic pathway is employed instead of opine production (Woo *et al.*, 2011). Malate dismutation contains the favored reactions and malate's reduction to fumarate, via a reaction that is essentially part of the Krebs cycle running in reverse, leads to the production of succinate (Muller *et al.*, 2012). *M. edulis* utilizes fumarate reductase and rhodoquinone to reduce fumarate to succinate (Tielens *et al.*, 2002). Succinate is further metabolized to propionate resulting in approximately 5 ATP (Tielens *et al.*, 2002) (Fig. 2). This process allows the organism to survive at a lower energy state while yielding more ATP than can be achieved by glycolysis alone. Furthermore, in this state of lower energy production there are less free radicals generated, offering a degree of protection while in this physiological state (Rivera-Ingraham *et al.*, 2013).

Interestingly, each tissue type in the mussel responds differently to hypoxia as a result of mitochondrial functional differences in gene expression. The gills, digestive glands, mantle, and adductor muscle have been shown to respond to hypoxia by switching to anaerobic respiration, (Ibarguren *et al.*,1989; Lushchak *et al.*, 1997; Bacchiocchi *et al.*, 2000, Doeller *et al.*, 2001; Diaz-Enrich *et al.*, 2002). In the case of gill ciliated epithelium, which is most important for the survival of the individual, the metabolic process is kept on. This can be surmised by the fact that the gill cilia are densely packed with mitochondria (Paparo, 1972). The ciliated gill epithelium of *M. edulis* has been studied not only for its ciliary activity but for its innervation as well (Paparo, 1972; Stefano *et al.*, 1975, 1976). This epithelium is innervated via serotoninergic and dopaminergic neurons, providing for cilio-excitation and cilio-inhibition, respectively. Clearly, this necessitates greater energy requirements, which may be difficult at intertidal intervals. We surmise this difficulty is overcome by way of nervous system integration of the tissue, exerting specific and rapid responses to respiratory and waste needs carried out by the ciliated epithelium (Stefano, 1990; Stefano *et al.*, 1991).

Anaerobically functioning mitochondria and cancer biology

A careful review of the biomedical literature indicates functional similarities between anaerobic mitochondrial subtypes in *M. edulis* and crown gall tissue and metabolic processes in human tumors. Cancer cells utilize anaerobic energy metabolism under hypoxic, anoxic and even during normoxic conditions (Gonzalez *et al.*, 2012; Amoedo *et al.*, 2013; Witkiewicz *et al.*, 2013; Chen *et al.*, 2014). It has been suggested that carcinogenic processes might target normal mitochondrial functioning and cause a disruption of the Krebs cycle and electron

transport enzymes (Gonzalez *et al.*, 2012). It has been recently proposed that normative mitochondrial function in non-proliferating cells affects relatively high cytosolic ATP/ADP ratios resulting in functional inhibition of aerobic glycolysis (Maldonado *et al.*, 2014). In contrast, the bioenergetics of the "Warburg" effect that has been extensively linked to the metabolic phenotype of numerous cancer cell types is characterized by enhanced aerobic glycolysis and suppression of aerobic mitochondrial metabolism (Gonzalez *et al.*, 2012; Amoedo *et al.*, 2013; Witkiewicz *et al.*, 2013; Chen *et al.*, 2014). Furthermore, aerobic respiration in proliferating cells leads to deleterious production of free radicals that can damage DNA and proteins. Accordingly, free radical damage is proposed to exacerbate compromised mitochondrial functioning thereby diminishing the existential viability of cancer cells. Along these lines, Davila and Zamarano (2013) posit that cancer can be viewed as a cell that has phenotypically reverted to the last common eukaryotic ancestor of the host cell. They surmise that a cancer cell is functioning as a facultative anaerobic microbe with unlimited replication potential (Davila and Zamarano, 2013). Interestingly, anaerobic mitochondria in gill cilia of *M. edulis* have evolved to utilize the phenotype of a facultative anaerobe (Doeller *et al.*, 1993, 2001).

Mytilus *mitochondrial DNA, tRNA and a link to cancer*

For over a decade, an ostensibly unresolved issue relating to essential genes expressed by mitochondrial DNA (mtDNA) from *M. edulis* and related species of marine mussels is the absence of a traditionally defined gene encoding subunit 8 of the mitochondrial ATP synthase complex (ATP8) (Boore, *et al*., 2004; Breton *et al.,* 2010; Smietanka *et al.,* 2010). The protein expressed by the ATP8 gene has been established as an integral component of the ATP synthase stator stalk in yeast and all metazoan phyla and is essential for coupled ATP production within the mitochondrial matrix. Recently, two laboratories have independently defined an open reading frame (ORF) corresponding to a never before annotated ATP8 variant in the mtDNA of several *Mytilus* species and have speculated that evolutionary resolution of mtDNA contributions by both male and female underlies its novel representation within the mitochondrial genome (Breton *et al*., 2010; Smietanka *et al*., 2010). A very recent publication reinforces the functional role of ATP8 mtDNA gene expression in the process of carcinogenesis (Grzybowska-Szatkowska *et al.,* 2014). Five identified mutations and polymorphisms of the ATP8 gene were identified in tissues obtained from breast cancer patients, thereby supporting the contention that functional modification/impairment of an essential subunit of the mitochondrial ATP synthase complex represents causative factor in carcinogenesis.

Another interesting characteristic of *Mytilus* mitochondrial genome is the presence of an additional novel methionyl tRNA. Its UAU anticodon makes it unique among taxa (Hoffmann *et al.*,1992; Boore et al., 2004). The presence of this additional tRNA raises questions in regard to potential similarities with tumor cells since these tend to exhibit elevated levels of initiator methionyl tRNA expression (Kanduc, 1997; Kanduc *et al.*, 1997; Marshall *et al.*, 2008; Pavon-Eternod *et al.*, 2009; Zhou *et al.*, 2009). It has been postulated that altering the tRNA expression profile in cells might influence the regulation of translation of growth factors, proto-oncogenes and other proteins involved in cell cycle (Kanduc, 1997; Marshall *et al.*, 2008; Kolitz *et al.*, 2009; Pavon-Eternod *et al.*, 2013). In particular, it has been demonstrated that increasing the levels of initiator $tRNA^{met}$ caused a concomitant elevation of other tRNA molecules, resulting in increased metabolic activity and cell proliferation (Pavon-Eternod *et al.*, 2013). Accordingly, after partial hepatectomy, levels of initiator $tRNA^{met}$ increase in rat hepatocytes compared to those of elongator $tRNA^{met}$ during cell-cycle progression (Kanduc, 1997). Similar $tRNA^{met}$ pattern shift was observed in human colorectal and gastro-intestinal tumors (Kanduc *et al.*,1997). Moreover, in embryonic fibroblasts from mice, overexpression of initiator tRNA resulted in induction of tumorigenesis (Marshall *et al.*, 2008), and in breast cancer and multiple myeloma cell lines initiator tRNA levels were also found to be elevated (Pavon-Eternod *et al.*, 2009; Zhou *et al.*, 2009).

Mytilus *as a model to study cancer*

In humans, the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene encodes a small GTPase involved in key regulatory signaling cascades (Franks *et al.*, 1987) and in tumorigenesis (Chetty *et al.*, 2013). Amplification of KRAS gene expression and/or oncogenic activating gain-of-function KRAS mutations have been functionally linked to enhanced growth, survival, and metastasis of major classes of human tumor types included in small-cell lung cancer (Minuti *et al.*, 2013) colorectal cancer (Brand *et al.*, 2012), pancreatic cancer (di Magliano *et al.*, 2013, Fang *et al.*, 2013), and intrahepatic cholangiocarcinoma (Robertson *et al.*, 2013). Of equivalent importance, dysregulation of the cellular epidermal growth factor receptor (EGFR) signaling pathway has been demonstrated to be critically important in promoting tumor growth, survival, and metastasis in human tumors (Goffin *et al.*, 2013) and development of several frontline anticancer therapeutic agents have attempted to achieve efficacious selective targeting of the oncogenic EGFR signaling pathway (Kohler *et al.*, 2013). Recent studies indicate that KRAS tumorigenicity is functionally linked to the "Warburg" phenotype favoring a high rate of aerobic glycolysis and anaerobic mitochondrial function (Weinberg *et al.*, 2010). This establishes the facultative anaerobic mitochondrion in *M. edulis* tissues as a potentially valuable high resolution model system for the development of novel anticancer therapeutic agents.

Conclusions

This review documents the phenomenon and existence of anaerobically functioning mitochondria in *M. edulis* as a model for invertebrate energy generating systems. In this regard, this mechanism

is used to benefit the organisms when large amounts of energy translocation are not present. It is clear *Mytilus* may use this pathway to survive when an abundant source of oxygen is not present *e.g.*, intertidal periodicity. Accordingly, if mitochondria represents evolutionary defined endosymbiont organelles, they have retained part of the anaerobic process associated with bacteria. This dynamic capacity would have survival value under hypoxic environmental conditions. In part, we surmise, that dysfunctional mitochondria in cancer cells may have their origin in the early evolution of eukaryotic cells by retaining this information and/or processes to implement this phenomenon in times of stress. However, in metastatic processes this pathway may emerge due to poor chemical messenger regulation. Importantly, *Mytilus* may yet be another invertebrate that can be used as a model system because of its broad scope of energy balance and dynamic capacity to adapt.

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