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Physiological uncoupling of mitochondrial oxidative phosphorylation. Studies in different yeast species

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Abstract Under non-phosphorylating conditions a high proton transmembrane gradient inhibits the rate of oxygen consumption mediated by the mitochondrial respiratory chain (state IV). Slow electron transit leads to production of reactive oxygen species (ROS) capable of participating in deleterious side reactions. In order to avoid overproducing ROS, mitochondria maintain a high rate of O_2 consumption by activating different exquisitely controlled uncoupling pathways. Different yeast species possess one or more uncoupling systems that work through one of two possible mechanisms: i) Proton sinks and ii) Non-pumping redox enzymes. Proton sinks are exemplified by mitochondrial unspecific channels (MUC) and by uncoupling proteins (UCP). *Saccharomyces. cerevisiae* and *Debaryomyces hansenii* express highly regulated MUCs. Also, a UCP was described in *Yarrowia lipolytica* which promotes uncoupled O_2 consumption. Non-pumping alternative oxido-reductases may substitute for a pump, as in *S. cerevisiae* or may coexist with a complete set of pumps as in the branched respiratory chains from *Y. lipolytica* or *D. hansenii*. In addition, pumps may suffer intrinsic uncoupling (slipping). Promising models for study are unicellular parasites which can turn off their aerobic metabolism completely. The variety of energy dissipating systems in eukaryote species is probably designed to control ROS production in the different environments where each species lives.

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Introduction

In mitochondria, oxidative phosphorylation results from the coupling between the redox-primary proton pumps in the respiratory chain and the F_1F_0-ATP synthase. The redox H^+ pumps create a pH gradient (ΔpH) used by the F_1F_0-ATP synthase to phosphorylate ADP. The efficiency of this system varies when electrons enter or exit the respiratory chain at different enzymes or when the H^+ gradient is used by secondary pumps for the active transport of proteins, ions and metabolites (Nicholls and Ferguson 2002) (Fig. 1).

Three of the four respiratory complexes in an orthodox respiratory chain are proton pumps. These enzymes oxidize substrates, transferring electron(s) to the next acceptor in the chain and expelling H^+(s) to the intermembrane space. Recycling of the electron within a given pump often results in H^+/e^- stoichiometries higher than 1 (Brandt 2006; Hosler et al. 2006; Trumpower 1990). This high efficiency comes at a price, as redox reactions involve several steps where incomplete reductions transiently convert coenzymes into reactive free radicals (Drose and Brandt 2008; Kushnareva et al. 2002). Therefore, when the mitochondrial ADP concentration drops, the rate of electron flux through the respiratory chain decreases (State IV respiration) and mitochondria become an important source of superoxide and other reactive oxygen species (ROS) (Chen et al. 2003). ROS production has diverse functions, such as signaling and apoptosis (Forman et al. 2010; Perrone et al. 2008).

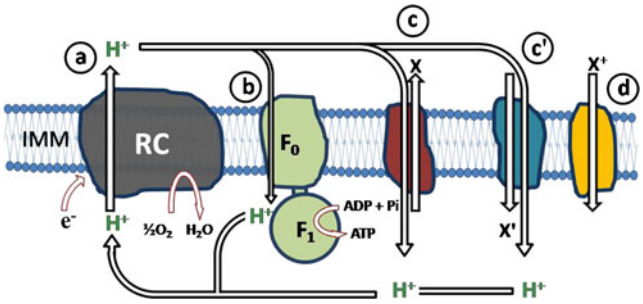

Fig. 1 Oxidative phosphorylation efficiency variations due to different systems that use protons. The proton gradient generated by the respiratory chain may be used by (**a**) the F_1F_0-ATP synthase (F_0F_1) for ADP phosphorylation (**b**) the transport of ions or metabolites across the inner mitochondrial membrane (IMM) either as (**c**) antiporter or (c') symporter. (**d**) ion uniport. RC, respiratory chain

However, overproduction of ROS may lead to ageing and disease (Drakulic et al. 2005; Wilhelm et al. 2006).

The labile nature of the superoxide radical has made difficult the identification of all its mitochondrial sources. Still, it is known that the ubiquinone and flavin oxido-reduction centers produce ROS (Chen et al. 2003; Starkov et al. 2004; Zundorf et al. 2009). During the redox ubiquinone/ubiquinol reaction, oxidized ubiquinone is partially reduced by one electron in the Qo site of the bc_1 complex becoming a potential superoxide source (Drose and Brandt 2008). At a high mitochondrial transmembrane potential, semiquinone accumulates participating in a side reactions that produce ROS (Koshkin et al. 2003; Rottenberg et al. 2009).

In cells and mitochondria there are different enzymes that eliminate ROS, such as Mn^{2+} SOD-dismutases, catalase and glutathione peroxidases. However, ROS overproduction may overwhelm these systems and thus different energy-dissipating uncoupling mechanisms may be activated to prevent such overproduction. These "physiological uncoupling" mechanisms would prevent ROS over-accumulation by inducing increased electron flux (Czarna and Jarmuszkiewicz 2005; Maxwell et al. 1999).

Among plants, yeast and fungi, there are different strategies aimed at preventing ROS overproduction (Kowaltowski et al. 1998; Magnani et al. 2008). In different yeast species it has been observed that oxidative phosphorylation can be uncoupled by different mechanisms (Fig. 2). Oxidative phosphorylation may be uncoupled through dissipation of the H^+ gradient through proton sinks, also termed extrinsic uncouplers (Kadenbach 2003): these may be channels or transporters and are represented by two well studied systems. These are the yeast mitochondrial unspecific channel (MUC) (Manon et al. 1998), which in mammals is known as the permeability transition pore (PTP) (Haworth and Hunter 1979), and the uncoupling proteins (UCP) (Nicholls and Rial 1999) that specifically dissipate H^+ gradients (Fig. 3). The second respiratory chain uncoupling mechanism, also termed intrinsic uncoupling (Kadenbach 2003) is the catalysis of redox reactions without pumping protons. Non-pumping redox enzymes are widely represented in the branched mitochondrial respiratory chains observed in plants and unicellular organisms (Rasmusson et al. 2004; Umbach and Siedow 2000; Wagner and Moore 1997). Among these enzymes, there are type-II NADH dehydrogenases (NDH2) and alternative oxidases (AOX). In addition, the variations in H^+/e^- stoichiometry (slipping) are another source of uncoupling.

Proton dissipating pathways

The mitochondrial unselective channel

Mitochondrial unspecific channels (MUCs) have been detected in yeast such as *Saccharomyces cerevisiae* ($_{Sc}$MUC) (Guerin et al. 1994; Prieto et al. 1992) and *Debaryomyces hansenii* ($_{Dh}$MUC) (Cabrera-Orefice et al. 2010). MUC opening results in a mitochondrial permeability transition (PT) similar to that described in mammals, i.e. a large

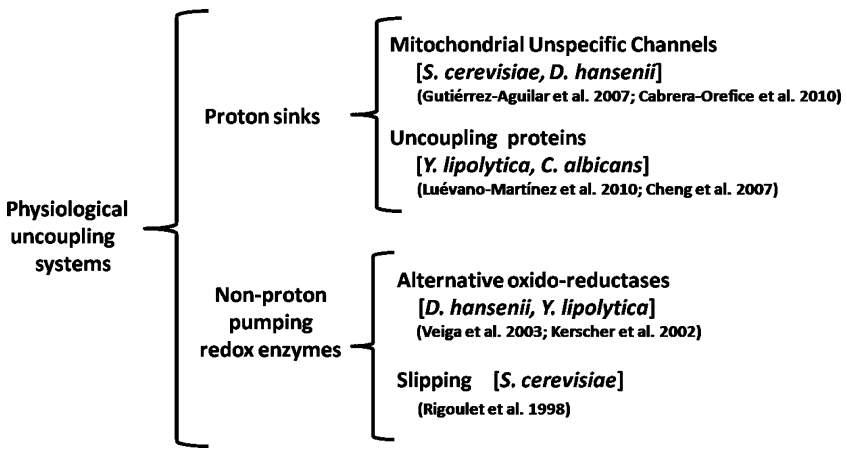

Fig. 2 Physiological uncoupling systems in yeast mitochondria. The rate of oxygen consumption may be accelerated independently of the synthesis of ATP by either depleting the transmembrane pH gradient or by reducing oxygen without contributing to the proton gradient. These mechanisms are present in different yeast species

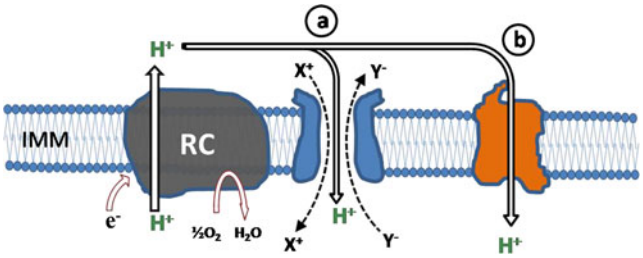

Fig. 3 Proton Sinks. Two proton sink systems are exemplified. Once the (**a**) Respiratory chain establishes a proton gradient, protons may be returned to the matrix through (**b**) unspecific channels or through (**c**) uncoupling proteins that are specific protonophores

increase in conductivity that depletes electrochemical gradients (Azzolin et al. 2010).

The $_{Sc}$MUC has been thoroughly characterized. $_{Sc}$MUC opens in response to ATP, while it is closed by Pi or ADP (Prieto et al. 1995). This suggests that $_{Sc}$MUC is controlled by the phosphorylation potential (Wallace et al. 1994). In addition, both the $_{Sc}$MUC (Perez-Vazquez et al. 2003) and the $_{Dh}$MUC (Cabrera-Orefice et al. 2010) are closed by Mg^{2+} and by Ca^{2+}. Furthermore, the $_{Sc}$MUC seems to be controlled cooperatively by Ca^{2+}, Mg^{2+} and Pi [to be published]. In *S. cerevisiae*, a rise in cytoplasmic [Ca^{2+}] precedes processes such as division, mating (Nakajima-Shimada et al. 2000; Ohya et al. 1991); or even a death program resembling apoptosis (Nakajima-Shimada et al. 2000; Ohya et al. 1991; Pozniakovsky et al. 2005). That is, a rise in $[Ca^{2+}]_{cyt}$ indicates that the cell is about to spend a large amount of energy (Anraku et al. 1991; Manon and Guerin 1998). Both the $_{Sc}$MUC and the $_{Dh}$MUC close in response to low [ATP] or high [Pi] while in contrast, when there is a surplus of ATP and no signals this indicates an oncoming need for energy, yeast MUCs open, dissipating the transmembrane potential and thus allowing the rate of oxygen consumption to increase (Prieto et al. 1992) and the production of ROS to decrease (Korshunov et al. 1997).

In *S. cerevisiae*, Ca^{2+} closes MUC, probably through its interaction with the voltage-dependent anionic channel (VDAC) (Gutierrez-Aguilar et al. 2007). The Ca^{2+}-VDAC interaction has also been proposed for vertebrates (Gincel et al. 2001). In both cases, the possibility that VDAC is a regulatory pore component has been suggested (Baines et al. 2007; Gutierrez-Aguilar et al. 2007). In regard to the possible component of MUC in the IMM, in *S. cerevisiae* it has long been evident that Pi is a strong MUC regulator (Azzolin et al. 2010; Cortes et al. 2000; Jung et al. 1997; Manon and Guerin 1997; Prieto et al. 1992; Velours et al. 1977). From this, it should not be surprising that recent evidence suggests that the mitochondrial phosphate carrier (PiC) is a constituent of the $_{Sc}$MUC: in the absence of PiC, $_{Sc}$MUC changes its solute size exclusion size and Pi sensitivity (Gutierrez-Aguilar et al. 2010). In mammals, PiC has also been proposed to be part of this channel (Leung et al. 2008).

Different modulators of MUCs have been reported depending on the species, strain or even tissue under study (Berman et al. 2000; Fortes et al. 2001; Friberg et al. 1999; Manon et al. 1998), suggesting that MUCs have evolved in response to selective pressure, e.g. in *D. hansenii*, the MUC is closed by monovalent cations (Cabrera-Orefice et al. 2010). This closure probably results in higher production of ATP, as it correlates with increased growth rate and mass yield (Gonzalez-Hernandez et al. 2004) and probably constitutes an adaptation to the high Na^+ contents of sea water (Gustafsson and Norkrans 1976).

Adding to the ongoing debate on the physiological role of MUCs, it is suggested that their role as physiological uncouplers should be considered; i.e. MUCs probably are highly regulated energy dissipative systems that decrease mitochondrial gradients when the demand for energy is low.

PT does not seem to be universal. *Yarrowia lypolytica* and *Endomyces magnusii* undergo PT only upon forced conditions which include incubation with the Ca^{2+} ionophore ETH129 (Kovaleva et al. 2009; Yamada et al. 2009). If MUC- mediated uncoupling is important to inhibit ROS production, and *Y. lipolytica* and *E. magnusii* seem to lack such a structure, then these yeast species should possess alternative uncoupling systems. Indeed, in *Y. lipolytica* mitochondria there are two such systems that might function as uncouplers: an uncoupling protein (Luevano-Martinez et al. 2010) and a branched respiratory chain (Guerrero-Castillo et al. 2009; Kerscher et al. 2002).

Uncoupling proteins

Uncoupling protein (UCP)-like activities have been detected in mitochondria from unicellular organisms, higher eukaryotes and plants (Jarmuszkiewicz et al. 2010). The physiological role of UCPs in unicellular organisms is still debated: the small size of unicellular eukaryotes makes a thermogenic role unlikely, as it is impossible to form a temperature gradient between the cell and the environment although, in *Acanthamoeba castellanii* UCP expression does increase in cells growing at 4 °C (Jarmuszkiewicz et al. 2004). Here, it is proposed that unicellular UCPs are capable of decreasing the mitochondrial $\Delta\Psi$ with the aim of decreasing production of ROS. Also, in unicellular organisms resistance to exogenous ROS is enhanced by UCP activity (Kowaltowski et al. 1998; Ricquier 2005), probably because UCP decreases endogenous ROS production (Krauss et al. 2005) and thus detoxifying enzymes are free to deal with the exogenous species: e.g. strains of *Candida albicans* devoid of UCP are less invasive that the wild type (Cavalheiro et al. 2004; Cheng et al. 2007).

In addition to the available functional evidence, recently a protein exhibiting UCP-like activity was identified in *Y. lipolytica* (Luevano-Martinez et al. 2010). The UCP activity was regulated similarly to the UCP1 from brown adipose tissue. After an extensive phylogenetic search for a UCP ortholog in this yeast, it was demonstrated that the mitochondrial oxaloacetate carrier (OAC) from *Y. lipolytica* is a *bona fide* UCP. The *Y. lipolytica* OAC displayed both, a sulfate/oxaloacetate transport and a UCP behavior. It is noteworthy that in the unicellular organisms where UCP activity has been reported, the green algae *Chlamydomonas reinhardtii*, the amoeba *Dictyostelium discoideum* (DictyBase) and the yeast *Candida albicans* (Cavalheiro et al. 2004; Jarmuszkiewicz et al. 2002) the only UCP-like proteins seem to be the mitochondrial oxaloacetate carriers (results not published). In regard to whether a UCP might prevent ROS overproduction, in *Y. lipolytica,* it has been demonstrated that this protein is over-expressed in the stationary phase, where a degree of uncoupling would be needed to maintain a high rate of oxygen consumption in the absence of ATP synthesis (Luevano-Martinez et al. 2010).

Redox enzymes that do not pump protons

Branched mitochondrial respiratory chains

Redox enzymes lacking pumping activity are constituted by a single protein subunit. These enzymes probably appeared early in the reducing world, before the appearance of oxidative phosphorylation, fulfilling the need to detoxify oxygen from the vicinity of enzymes and membranes. Some prokaryotes still use oxidoreductase-mediated detoxification of oxygen to protect their fragile nitrogen reducing enzymes (Flores-Encarnacion et al. 1999).

Alternative redox enzymes do not contribute to the proton gradient. Branched mitochondrial respiratory chains may contain a number of different enzymes that donate electrons to the quinone pool including complex I (the only proton pump), succinate dehydrogenase, glycerol phosphate dehydrogenase, dihydroorotate dehydrogenase and internal or external type II NADH dehydrogenases. Then the electrons in reduced ubiquinol follow two possible pathways reaching either the cytochrome pathway (complexes III and IV), or the alternative oxidase (AOX). In these respiratory chains, different electron pathways may be envisioned that bypass energy-conserving respiratory complexes I, III and/or IV, i.e. branched chains seem to be able to reduce oxygen while using 0, 1, 2 or 3 proton pumps (Fig. 4).

In mitochondria, the most widely distributed monosubunit redox enzymes are type II NADH dehydrogenases (NDH2) and alternative oxidases (AOX). NDH2s may be located on either surface of the IMM. External NDH2s (NDH2e) oxidize cytosolic NADH, while internal NDH2s (NDH2i) oxidize NADH from the matrix in a rotenone-insensitive reaction. The structure (Fisher et al. 2007; Fisher et al. 2009; Gonzalez-Meler et al. 1999; Kerscher 2000; Melo et al. 2004; Schmid and Gerloff 2004) and kinetics (Fisher et al. 2009; Velazquez and Pardo 2001) of NDH2s from different organisms have been reported. AOX is a single subunit enzyme (Albury et al. 2002; Andersson and Nordlund 1999; Berthold et al. 2000; Moore and Siedow 1991). AOX activity is regulated by nucleotides, by dimerization and/or by α-ketoacids (Hoefnagel et al. 1995; Millar et al. 1993; Millenaar et al. 1998). Some yeast species contain two AOX isoforms, one being constitutively expressed and a second one induced by stress (Siedow and Umbach 2000). It is noteworthy that AOX is present only in fungi that express complex I, possibly because in a respiratory chain without Complex I, any electron reaching AOX would be totally unproductive (Joseph-Horne et al. 2001).

In mitochondria with alternative components, the pathway that electrons follow has to be strictly controlled. A direct reaction between NDH2, ubiquinone and AOX would result in a non-productive, uncoupled pathway, i.e. no protons would be pumped. Furthermore, at the external face of the inner membrane, NDH2 receives the hydride from NADH and takes one H^+, transferring both hydrogen atoms to ubiquinone. Then ubiquinone is regenerated by AOX which in turn transfers its hydrogen atoms to oxygen producing water. This sequence of reactions results in the dissipation of a H^+, i.e. it has a H^+/e^- pumping stoichiometry of −0.5. Therefore, when energy is required, alternative redox enzymes need to be isolated from each other, probably by binding to the proton-pumping complexes. In contrast, when phosphorylation is not active, as in the stationary phase, the non-producing electron transfer between NDH2 and AOX would be useful to maintain a high rate of oxygen consumption at a high transmembrane potential, preventing semiquinone accumulation and decreasing ROS formation (Joseph-Horne et al. 2001).

Proton/electron stoichiometry variations. Slipping

Non-branched respiratory chains seem to use other mechanisms to regulate the efficiency of oxidative phosphorylation (van Dam et al. 1990). Uncoupling may result from increased proton conductance at the lipid bilayer (Luvisetto and Azzone 1989; Luvisetto et al. 1991). A second mechanism would be the decrease in the efficiency of a respiratory pump (slipping) (Pietrobon et al. 1981; Pietrobon et al. 1983). Intrinsic uncoupling or slipping is defined as a decrease in the efficiency of a

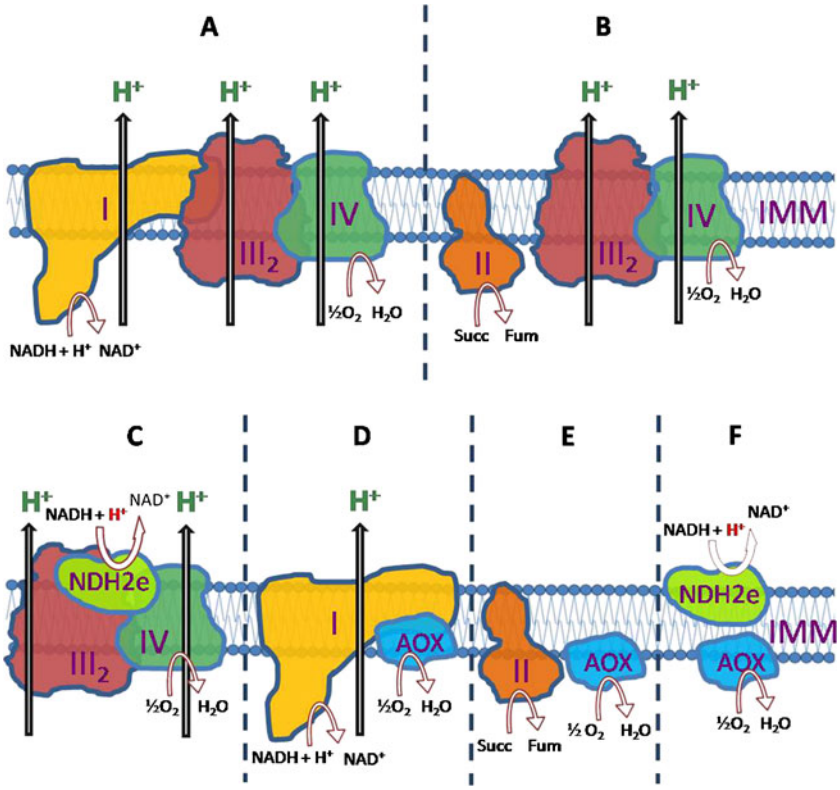

Fig. 4 In a branched respiratory chain the number of proton pumps participating in electron transfer may vary from three to zero. In branched respiratory chains electrons may follow different routes to reach oxygen. Thus the number of proton pumps involved may change: **a** complexes I, III and IV: three proton pumps are involved. **b** from succinate dehydrogenase through the cytochrome pathway, two proton pumps. **c** from NDH2e through complexes III-IV; two proton pumps, although H^+/e^- is 2.5 instead of 3 as in (**b**). **d** from complex I though AOX; one pump. **e** from succinate dehydrogenase through AOX; No proton pumps participate. **f** NDH2e through AOX; zero proton pumps participate and in addition, the combined activity of NDH2e with AOX would consume a H^+ from the intermembrane space, yielding a negative stoichiometry of −0.5 H^+/e^-. Numbers I, II, III_2 and IV represent each of the four respiratory complexes; NDH2e, external NADH dehydrogenase; AOX, alternative oxidase; IMM, inner mitochondrial membrane. Protons in red are used for ubiquinone reduction in the intermembrane side of the IMM, i.e. they do not contribute (**c**) or contribute negatively to the H^+/e^- stoichiometry (**f**)

proton pump (decrease of the H^+/e or H^+/ATP stoichiometry) resulting in a diminished P/O ratio (Kadenbach 2003).

Slipping has been reported in cytochrome c oxidase (Azzone et al. 1985; Frank and Kadenbach 1996). The F_1F_0-synthase can also undergo slipping, hydrolyzing ATP without pumping protons (Feniouk et al. 2005). In addition, protons can reenter the matrix through the pumps without moving electrons backwards or making ATP (Pietrobon et al. 1983).

Slipping accelerates the rate of oxygen consumption as more electrons are needed to maintain a high ΔpH. Normally in the proton pump the chemical reaction and the transport of protons are tightly coupled, while during slipping both processes become independent (Mourier et al. 2010). Upon slipping, the rate of electron flux increases while the proton motive force remains constant and energy is dissipated as heat (Kadenbach 2003).

In *S. cerevisiae* mitochondria, a remarkable change in the stoichiometry of proton pumping has been described. Feeding the respiratory chain with substrates for different quinone reductases leads to an increase in the rate of oxygen consumption without increasing the rate of ATP phosphorylation (Mourier et al. 2010). This phenomenon has been termed active leak and is probably due to slipping of an oxidative phosphorylation pump, although an increase in the proton conductance of the bilayer has not been ruled out.

Interactions between the cytoplasm and mitochondria regulate the efficiency of oxidative phosphorylation

At any given moment the cell´s energy needs determine which metabolic pathways are activated or inhibited (Devin and Rigoulet 2007). The catabolism/anabolism activity ratio is determined by metabolic fluxes (Cascante et al. 1994; Moreno-Sanchez et al. 2010; Ovadi and Saks 2004; Srere 1987). Upon oxygenation, the rate of glycolysis decreases. This may be explained by the allosteric regulation of glycolytic enzymes by ATP and fructose 2,6-bisphosphate and by the competition for ADP and for reducing equivalents observed between glycolysis and

oxidative phosphorylation (Beauvoit et al. 1993; Gosalvez et al. 1974).

In *Saccharomyces cerevisiae*, glycolysis is the main source of ATP; however, in the presence of non-fermentable substrates oxidative phosphorylation becomes the main energy source. During fermentation the genes that encode for oxidative metabolism enzymes stop their expression (Takeda 1981), e.g. glucose addition inhibits the expression of cytochrome c (Thevelein 1994; Zitomer and Nichols 1978), while glycolytic intermediates are accumulated to induce the expression of glycolytic enzymes (Boles et al. 1993).

In *S. cerevisiae* the addition of glucose induces the transition to fermentative metabolism, where glycolysis is increased and oxidative phosphorylation is decreased (den Hollander et al. 1986). This is the Crabtree effect. There are both Crabtree-positive and negative yeast species. Recent sudies indicate that fructose1,6-bisphosphate inhibits oxygen consumption through an interaction with complexes III and IV. In contrast, physiological concentrations of glucose 6-phosphate and fructose 6-phosphate stimulate the respiratory flux, possibly inducing slipping (Diaz-Ruiz et al. 2008).

Unicellular organisms other than yeast

Protists make up the bulk of the eukaryotes, while vertebrates and fungi represent only a small fraction. Protists present a wide variety of physiological properties. There are very few bioenergetics studies on these organisms. *Giardia lamblia* (Hashimoto et al. 1994) and *Entamoeba histolytica* (Tovar et al. 1999) have lost their mitochondria. Other protists, such as some Trichomonadidae and ciliates, have organelles called hydrogenosomes, which are related to mitochondria (de Souza et al. 2009; Mather and Vaidya 2008).

Unicellular parasites have evolved to adapt their metabolism for survival within the host. Depending on the environment and stage in their life cycle, *Plasmodium, Trypanosoma* and *Leishmania* can make a complete switch from a glycolytic to an aerobic metabolism and back, such that in *Plasmodium falciparum* the activities of complex III, IV and dihydroorotate dehydrogenase, are 10 times higher in the sexual than in the asexual stage (Monzote and Gille 2010). Likewise, mitochondria have adapted to the metabolic conditions found within the host, e.g. in the mosquito, *Plasmodium* gametocytes are aerobic and mitochondria are typical. In contrast, in the vertebrate host, sporozoites and merozoites are adapted to microaerophilia and contain few, underdeveloped mitochondria (Segura and Blair 2003).

Throughout the trypanosomatid life cycle, mitochondrial activity varies widely (Schneider 2001). In the bloodstream, these protozoans are anaerobic while in the gut of the insect they perform oxidative phosphorylation. In *Toxoplasma* most energy is obtained from glycolysis, although the mitochondrial DNA sequence of these parasites shows significant differences from the mammalian host, suggesting possible drug targets (Monzote and Gille 2010). Remarkably, the mitochondrial DNAs from trypanosomatids and Apicomplexa lack genes for transfer RNA (Mather and Vaidya 2008).

Concluding remarks

Aerobic metabolism is at the same time highly efficient and very dangerous. The reactive oxygen species produced by the respiratory chain can react with, and damage different components of the cell. Diverse mechanisms have evolved to prevent the deleterious effect of ROS. There are many detoxifying enzymes such as glutathione reductase, superoxide dismutase or catalase. In addition, upriver from these reactions, there are diverse mitochondrial systems designed to prevent ROS overproduction. These systems promote physiological uncoupling to ensure that the redox enzymes in the respiratory chain work at a fast rate, thus preventing reactive intermediates from participating in collateral reactions.

There are two mitochondrial uncoupling mechanisms: a) Those that dissipate the pH gradient and b) Non-productive redox reactions. Both mechanisms are widely spread in nature. Physiological proton sinks are the uncoupling proteins and the mitochondrial unspecific channels, while non productive redox reactions are catalyzed by redox/non-pumping alternative dehydrogenases and by orthodox complexes that undergo slipping.

The relationship between the cytoplasmic and the mitochondrial metabolic pathways needs to be better understood. The ability of some products from glycolysis to regulate oxidative phosphorylation is illustrative. The comparison between Crabtree positive and Crabtree negative yeast species may help understand the mechanisms and consequences of these interactions.

Understanding the mechanisms underlying the control and production of ROS may help to select more resistant organisms for biotechnological applications. Also, various ROS-related diseases have to be understood in order to design better treatments. In this light, it seems useful to know that uncoupling prevents ROS production.

During evolution, each eukaryote species preserved one or more ROS overproduction-prevention mechanism(s). Yeast species are ideal to study each mechanism. Other unicellular organisms may be helpful to understand their ability to shut down aerobic metabolism without being overwhelmed by ROS production.

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