Genetic control of oxidative phosphorylation and experimental models of defects

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Energy in the form of ATP is continually produced by all cells for normal growth and function. Anaerobic glycolysis can provide enough ATP for some cells, but energetic cells such as cardiomyocytes and neurons require a more efficient ATP supply, which can only be provided by mitochondrial oxidative phosphorylation. Invented by bacteria that became symbiotically associated with other bacteria to form eukaryotic cells billions of years ago, oxidative phosphorylation carries with it a genetic legacy that is unique. The mitochondrial oxidative phosphorylation complexes are assembled from protein subunits encoded by both the mitochondrial genome (mtDNA) and the nuclear genome (nDNA, located in the chromosomes). The mtDNA is a remnant genome of the bacterial progenitor of mitochondria, and (unlike the biparental diploidy that characterizes the nuclear genome) is present in thousands of copies per cell, is replicated through life, and is inherited (cytoplasmically) only from the female parent. Oxidative phosphorylation comprises five multimeric enzyme complexes that act as a redox pathway, passing electrons from oxidizable intermediates produced by the metabolism of food to molecular oxygen in the mitochondrial matrix, while producing an electrochemical gradient by pumping protons into the intermembranal space. The proton (hydrogen ion) gradient across the inner mitochondrial membrane is used by the \( \text{H}^+ \) transporting ATP synthase to produce ATP from ADP and inorganic phosphate, with the protons released into the mitochondrial matrix then combining with electronated oxygen to form water. Many of the details regarding the control of the synthesis of oxidative phosphorylation enzyme complexes remain to be elucidated. Transmitochondrial cell culture systems have been developed so that defective oxidative phosphorylation can be studied in a controlled nuclear background. Such systems may soon enable the development of mtDNA ‘knockout’ mice in order to better model mtDNA transmission and mitochondrial disease.

Key words: electron transport chain/mitochondrial DNA/oxidative phosphorylation/respiratory chain/transmitochondrial cells

Introduction

A glance at any biochemistry textbook will reveal that mitochondria house a large number
Oxidative phosphorylation and defect models

Figure 1. Genetic map of human mtDNA (upper) and scheme showing how the gene products contribute to the complexes of oxidative phosphorylation (lower). The circular mtDNA genome is represented in the upper figure as a linear map, with tRNA genes indicated by vertical lines, often separating reading frames. Heavy (H-) strand genes are above the line and are transcribed from right to left; light (L-) strand genes are below the line and are read from left to right. As electrons pass from complex I down the chain, oxygen is reduced to water and protons are pumped into the intermembranal space (lower right). *cyt b* encodes apocytochrome *b* of complex III; *ND* genes encode NADH dehydrogenase subunits 1 to 6 of complex I; *CO* genes encode cytochrome oxidase subunits I–III of complex IV; ATP6/8 encode ATP synthase subunits of complex V.

...of metabolic pathways. Some of these, e.g. part of the urea cycle in liver mitochondria and thermogenesis in brown fat, are unique to particular organs; others are common to mitochondria in all cells, e.g. the citric acid cycle and other catabolic pathways that generate reduced (i.e. readily oxidizable) forms of nicotinamide- and flavin-adenine dinucleotide intermediates (NADH and FADH$_2$).

The respiratory chain (or electron transport chain) together with the H$^+$-transporting ATP synthase comprise the final common fuel oxidation pathway, known as oxidative phosphorylation (or OXPHOS). Overall, oxidative phosphorylation involves oxidizable intermediate metabolic substrates, which include NADH and FADH$_2$, generating ATP from ADP and inorganic phosphate, and in the process reducing molecular oxygen to water. The details are explained below. Energetic cells require mitochondrially-produced ATP, whereas quiescent cells can derive sufficient ATP from anaerobic, cytosolic glycolysis, so mitochondria are most abundant in cells with the highest demand for energy. The oxidative phosphorylation pathway comprises five multisubunit enzyme complexes, in turn composed of >80 different polypeptide units, 13 of which are encoded by a separate genome to those contained in the (nuclear) chromosomes, the mitochondrial genome, composed of mitochondrial DNA (mtDNA). The genetic control of oxidative phosphorylation is therefore unusual, requiring the co-ordinated expression of both nuclear and mitochondrial genes. However, mitochondrial biogenesis and self-replication, including mtDNA replication, appear to be regulated entirely by nuclear genes.

The mitochondrial genome

It is now accepted that mitochondria and their genomes are vestiges of aerobic bacteria (prokaryotes) that long ago became symbiotically incorporated into anaerobic prokaryotes to give rise to euukaryotic cells (Margulis, 1981; Gray et al., 1999; Jansen, 2000). Most
of the endosymbiont’s genome was then lost or transferred to the nucleus, leaving a small, circular, histone-free ‘chromosome’ of mtDNA to remain within the cytoplasmic organelle. In humans, mtDNA forms a 16.5 kb circle, which (as in all multicellular animals known) encodes 13 subunits of the OXPHOS enzyme complexes, a complete set of 22 transfer RNAs (for 20 amino acids, two of which have two distinct tRNAs), and two rRNA components of the mitochondrial ribosomes (Figure 1). Replication and transcription of mtDNA are controlled by nuclear-encoded gene products and are discussed elsewhere in this volume (Clayton, 2000).

The proteins encoded by mtDNA include seven subunits of the 42-subunit NADH-ubiquinone oxidoreductase complex (complex I), the apocytochrome b of the 11-subunit ubiquinol-cytochrome c oxidoreductase complex (complex III), three core proteins of the 13 subunits that comprise cytochrome c oxidase (complex IV), and two of the 16 subunits of the H+ATP synthase (complex V) (Anderson et al., 1981; Saraste, 1999; Figure 1).

Cytoplasmic inheritance, suspected since the observations of Ephrussi (1950) with yeast, was confirmed when mtDNA in the fungus Neurospora was shown to be inherited from the female parent (Reich and Luck, 1966). Maternal inheritance of mtDNA was found to be the rule for most of the (multicelled) animal kingdom, including humans (Giles et al., 1980). Plants also show uniparental inheritance of mtDNA (and plastid, or chloroplast, DNA), although the contributing parent can often be the male (Levings, 1983; Hoekstra, 2000). While tiny in comparison to the nuclear genome, animal mtDNA exists in thousands of copies per cell; in typical somatic cells mtDNA comprises ~1% of DNA mass, and in the mature mammalian oocyte it can account for as much as a half or more of total DNA mass (Dawid, 1972). Mitochondria are dependent on the nuclear genome for most of the 80 or so OXPHOS proteins, and all of the regulatory factors for mtDNA replication and transcription. Enzymes of the many other important metabolic functions housed in the mitochondria of different cells are entirely nuclear-encoded.

Plants have relatively large and complex mitochondrial genomes compared with animals (Levings, 1983; Gray et al., 1999). An important class of mtDNA mutations in plants result in cytoplasmic sterility, whereby affected plants suffer an inability to make viable pollen (Levings, 1983, 1996). A wide variety of human diseases (individually rare, but collectively important) are now also attributed to mtDNA mutations (Lightowlers et al., 1997; DiMauro and Schon, 1998; Wallace, 1999; Christodoulou, 2000; Naviaux and McGowan, 2000) and are gradually becoming better understood. Controversy, however, still engulfs the idea that it is an accumulation of wider mtDNA mutations that contributes to the manifestations of age and degenerative disease (Beal, 1996; Lightowlers et al., 1999; Wallace, 1999); discovery of mitochondrial involvement in the signalling for cell death (Newmayer et al., 1994; Kluck et al., 1997; Yang et al., 1997), has heightened interest in this area (Wallace, 1999), and the possibility that altered mitochondrial function consequent to mtDNA mutations could interfere with or potentiate apoptotic signals warrants investigation. ATP is normally exported from the mitochondrion by the adenine nucleotide translocator, which forms part of the mitochondrial permeability transition pore, activated during apoptosis. Generation of reactive oxygen species (see below) and release of cytochrome c through this pore are important events in apoptotic cell death (Matsuyama et al., 1998; Raff, 1998, Martinou, 1999). Oxidative phosphorylation

Organisms need to produce ATP continuously, to do cellular work. Glycolysis, converting
Oxidative phosphorylation and defect models

glucose to pyruvate, produces two ATP molecules per glucose molecule, releasing around 238 kJ/mole. The citric acid cycle and oxidative phosphorylation, housed in the mitochondrion, catalyses the stepwise oxidation of pyruvate to carbon dioxide and water, yielding around 2870 kJ/mole and 38 ATP molecules for each initial glucose molecule (Lehninger, 1964). Long before eukaryotes appeared, bacteria evolved many ways of producing ATP. Oxidative phosphorylation was one of these bacterial inventions and takes advantage of the oxidizing power of molecular oxygen, respiration being the controlled combustion of carbohydrates, producing ATP instead of excess heat.

Oxidative phosphorylation is achieved by five multimeric enzyme complexes present within the inner mitochondrial membrane. Complexes I–IV (the respiratory, or electron transport chain) pass electrons from NADH and FADH₂ (reduced intermediates derived from the oxidation of carbohydrates and fatty acids, ultimately to carbon dioxide) to molecular oxygen, so that electronated oxygen products produced by the respiratory chain enter the mitochondrial matrix. The energy released is partly conserved by the pumping of protons (hydrogen ions) into the intermembranal space (three protons for each electron pair traversing the chain), which produces a proton gradient with respect to both the cytosol and the mitochondrial matrix. The concentration of protons is high enough to reverse an inner mitochondrial membrane-located enzyme H⁺-dependent ATPase (complex V; the reversed ATPase is named H⁺-transporting ATP synthase) and thus to produce ATP from inorganic phosphate and ADP (when, by its presence, the need for ATP-production is signalled). Meanwhile the protons discharged into the mitochondrial matrix reduce the respiratory chain’s electronated oxygen products (which, potentially, constitute ‘reactive oxygen species’) further to form water (Figure 1).

A wealth of exciting new structural information on the respiratory chain enzymes (for review see Saraste, 1999) has paralleled continuing advances in mitochondrial genetics. Complex I is the largest and least understood OXPHOS complex, containing multiple iron–sulphur centres that carry electrons from NADH to ubiquinone. Complex II (succinate dehydrogenase) contains only four subunits, none encoded by mtDNA; this complex also contains several iron–sulphur centres, as well as FAD and a b-type cytochrome, to carry electrons from succinate to ubiquinone. Reduced ubiquinone (ubiquinol) is oxidized in a two-step manner by complex III, as electrons are passed via iron–sulphur centres in the Rieske protein, and via cytochromes b and c₁, finally to reduce cytochrome c. The last step in the respiratory chain sees complex IV, or cytochrome c oxidase, pass electrons through two copper atoms and two a-type cytochromes, finally delivering four electrons to molecular oxygen in the mitochondrial matrix and, by employing four hydrogen ions (protons) there for each molecule of oxygen, producing two water molecules. Complexes I, III and IV also pump a proton across the mitochondrial membrane to the intermembranal space as they pass a pair of electrons, producing the proton gradient and flow utilized by complex V to condense ATP from ADP and inorganic phosphate.

While it is tempting to view the electron transport chain (and hence oxidative phosphorylation) as the linear passage of electrons, the molar ratios of complexes I, II, III, IV and V in the inner membrane is approximately 1:2:3:6:6 (Capaldi et al., 1988). From inhibitor studies it is clear that complex I and II, feeding electrons from NADH and FADH₂ to complex III, exert the greatest control over respiratory chain flux; complexes III, IV and V, however, exhibit specific activities more than an order of magnitude greater.

Figure 2 shows how oxidative phosphoryla-
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mito (0.5 mg)

ADP 125 nmols

100 ng atom O

ADP 125 nmols

DNP

5 mins

Figure 2. Polarographic (oxygen electrode) estimation of oxidative phosphorylation in intact mouse cell mitochondria showing coupling of active respiration (indicated by oxygen consumption) with ADP stimulation, and the effects of uncoupling oxidative phosphorylation with dinitrophenol (DNP) (see text). Oxygen saturation (y axis) is measured as a function of time (x axis), allowing comparison of specific rates of respiration (O$_2$ consumed/min/mg mitochondrial protein). Mito = addition of mitochondria, 0.5 mg.

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Mitochondria from cultured cells can also be studied in vitro using freshly isolated mitochondria in a polarograph chamber. Isolated from cultured mouse cells with their membranes intact, the organelles maintain a proton gradient so that phosphorylation as well as maximal respiratory flux can be directly measured. This (real) polarograph trace illustrates the basic principle of coupling of respiration to ADP availability (Chance and Williams, 1955); substrates are added to the stirred chamber containing mitochondria in an isosmotic buffer with phosphate present, and a slow (state II) rate of respiration follows; additions of ADP stimulate state III respiration (the ‘active’ state in which phosphorylation occurs); then, when added ADP has all been phosphorylated to ATP, the slower state IV respiration ensues. Figure 2 shows how the extraordinary kinetics of oxygen utilization by the respiratory chain ensures that respiration can proceed maximally even at the lowest oxygen levels measurable with the polarograph, ~1% saturated. Respiration can be uncoupled, by adding dinitrophenol, which causes the leakage of protons from the intermembranal compartment, dissipating the proton gradient. The even faster respiration observed with dinitrophenol-uncoupled mitochondria in Figure 2 is typical, and implies that complex V shares some control of respiratory chain electron flux in the phosphorylating state (state III).

Mitochondria from cultured cells can also be used to assay the individual respiratory chain enzyme complexes to gain a full picture of nuclear or mtDNA mutation effects on oxidative phosphorylation (Trounce et al., 1996).

During normal respiratory chain activity some incompletely reduced reactive oxygen species (ROS) are produced, with complexes I and III being the sites of most ROS generation (Boveris and Chance, 1973). An estimated 1–4% of oxygen consumed is directly converted to such free radicals under normal conditions. Inhibition of the respiratory chain greatly increases ROS production (Kwong and Sohal, 1998). When the electron transport chain is inhibited, electrons accumulate in the proximal portion of the electron transport chain (complex I and ubiquinone) (Kwong and Sohal, 1998), and can be added to molecular oxygen, forming superoxide anion (O$_2^-$). Superoxide is converted by mitochondrial manganese superoxide dismutase (MnSOD) to give hydrogen peroxide. Hydrogen peroxide, which in the presence of reduced transition metals can itself be reduced to the highly reactive hydroxyl radical (OH$^-$) by the Fenton reaction, is then converted to water by glutathione peroxidase. Using a MnSOD knockout mouse as a model of acute oxidative stress, Melov et al. (1999) found the iron-sulphur centres of complexes I, II, and III, as well as of aconitase in the citric acid cycle, were inactivated, severely disrupting mitochondrial energy production. Chronic exposure to free radicals
Oxidative phosphorylation and defect models

It can be useful to consider oxidatively-derived ATP as an energy supply that is supplementary (to anaerobic glycolysis) for many cells, but essential for cells of highly energetic tissues, such as myocardium, skeletal muscle, pancreatic islets, the central nervous system, retinal cells, the kidneys and the liver. That human cells can grow in vitro without OXPHOS has been made clear by studies over the past decade using chemically-derived rho-zero (ρ⁰) cells, i.e. cells that have been completely depleted of mtDNA. First described for avian cells (Morais et al., 1988), King and Attardi (1989) succeeded in producing a human ρ⁰ cell line by the same approach, namely treating cells with the γ-polymerase inhibitor, ethidium bromide; as the cells divide in the presence of the ethidium bromide the mtDNA copy number is reduced until clones can be isolated that have no mtDNA.

These ρ⁰ cells, whether primary or transformed, can survive and grow with mitochondria that are without a functional respiratory chain. The mitochondria show grossly abnormal morphology, being greatly enlarged and lacking cristae (Figure 3). The cells produce high levels of lactate and, unlike their ρ⁺ parents, require ‘redox therapy’ (additional pyruvate and uridine in the culture medium) for survival. Such uridine-dependence, or auxotrophy, among ρ⁰ cells was found (Morais et al., 1988) to be because dihydroorotate dehydrogenase (an enzyme of the pyrimidine biosynthesis pathway — thus essential for nuclear DNA synthesis and mitosis — located on the outer surface of the mitochondrion’s inner membrane), normally needs to pass electrons released from the oxidation of dihy-
by knockout of the heart-muscle adenine nucleotide translocator isoform (Graham et al., 1997) and knockout of mitochondrial transcription factor A (mtTFA, or Tfam) (Wang et al., 1999; Rantanen and Larsson, 2000). Knockout mice with different mtDNA defects will no doubt also be valuable tools. By enabling direct study of segregation of deleterious heteroplasmic mutations through development they will greatly aid the study of the pathogenesis of, and possible treatment modalities for, mitochondrial diseases, as well as being useful to researchers investigating nuclear genes that affect OXPHOS (including mediators of apoptosis).

The unique features of mitochondrial genetics present some technical barriers to producing mtDNA knockout mice. Recombination has not been clearly demonstrated in mtDNA (but the subject continues to be controversial; Arctander, 1999), meaning that the targeted knockout approach used for nuclear genes, utilizing homologous recombination of a mutated construct, cannot be used for mtDNA. Transfection approaches using artificial mtDNA constructs have not yet succeeded in transferring the construct into mitochondria within cells.

Transmitochondrial somatic cell techniques provide another approach to producing mtDNA knockout mice, but still require mtDNA mutant cells as the starting point. The inbred mouse strains exhibit little variation in mtDNA sequence, although polymorphic variants between two strains were used to advantage in a pioneering study by Shoubridge’s group (Jenuth et al., 1996). These authors constructed heteroplasmic mouse embryos by fusion of a cytoplast from one strain with a fertilized oocyte from another strain. In other experiments, Meirelles and Smith (1998) have used a similar approach of either cytoplast or karyoplast fusion to further show that oocyte mitochondria of perinuclear (karyoplast) origin tend to show less stringent segregation than peripheral (cytoplast origin) mitochondria during preimplantation development.

A handful of drug-resistant mouse cell mtDNA mutants have been identified in cell culture, the best characterized being chloramphenicol resistance (CAP) due to a 16S rRNA point mutation. CAP cells are not growth-inhibited by chloramphenicol, providing a convenient selectable marker, and the cells have defects in OXPHOS, probably due to disrupted mitochondrial protein synthesis (Howell and Nalty, 1988). Wallace et al. have recently reported an attempt to make a transmitochondrial mouse with the CAP mutation (Levy et al., 1999). While succeeding in transferring the mutant mtDNA into embryonic stem (ES) cells, they failed to get germline transmission of the clone. However, a recent update from the same group claimed to have achieved germline transmission using a different XX ES cell line (Wallace et al., 1999).

Creation of homoplasmic transmitochondrial cells without drug-resistant markers or ρ⁰ cells is possible with the use of the toxic dye rhodamine 6-G (R6G) (Christodoulou, 2000). Pretreatment of cells including ES cells (Levy et al., 1999) with low levels of R6G for several days was shown to kill cells, but the cells could be rescued by fusion with enucleated untreated mitochondrial donor cells. The R6G appears to irreversibly collapse the mitochondrial membrane potential, and the utility of the drug in creating transmitochondrial mouse cells has been demonstrated (Trounce and Wallace, 1996).

Another approach is suggested by the findings of Moraes’s group that mtDNA from closely related primate species can be replicated in human ρ⁰ cells, but that the ‘xenomitochondrial’ cybrids show OXPHOS defects (Kenyon and Moraes, 1997). We have similarly produced a range a xenomitochondrial cybrids by introducing mitochondria from several murinae species into a mouse ρ⁰ cell
Oxidative phosphorylation and defect models

Mouse cell with mtDNA mutation

XX ES cell

OR

enucleate & fuse with rho-zero cells

plasmid
“mtDNA”
construct

transfect mito. or rho-zero cells

enucleate & fuse with R-6G-treated ES cells

R-6G treatment

(HAT select)

rho-zero cell
with homoplasmic
mtDNA mutation

blastoecyst injection

mice

in cultured somatic cells is now well established, as are techniques for karyoplast or cytoplast transfer into fertilized oocytes or early embryos. To mimic human mtDNA mutations in mouse models we now need techniques for creating custom mtDNA knockouts and for isolating mtDNA mutants in cell culture.

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References


I. Trounce


