Biochemical and evolutionary aspects of anaerobically functioning mitochondria

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Mitochondria are usually considered to be the powerhouses of the cell and to be responsible for the aerobic production of ATP. However, many eukaryotic organisms are known to possess anaerobically functioning mitochondria, which differ significantly from classical aerobically functioning mitochondria. Recently, functional and phylogenetic studies on some enzymes involved clearly indicated an unexpected evolutionary relationship between these anaerobically functioning mitochondria and the classical aerobic type. Mitochondria evolved by an endosymbiotic event between an anaerobically functioning archaeobacterial host and an aerobic α -proteobacterium. However, true anaerobically functioning mitochondria, such as found in parasitic helminths and some lower marine organisms, most likely did not originate directly from the pluripotent ancestral mitochondrion, but arose later in evolution from the aerobic type of mitochondria after these were already adapted to an aerobic way of life by losing their anaerobic capacities. This review will focus on some biochemical and evolutionary aspects of these fermentative mitochondria, with special attention to fumarate reductase, the synthesis of the rhodoquinone involved, and the enzymes involved in acetate production (acetate : succinate CoA-transferase and succinyl CoA-synthetase).

Keywords: evolution; succinate dehydrogenase; fumarate reductase; rhodoquinone; anoxia

1. INTRODUCTION

Mitochondria are generally described in textbooks as the aerobic powerhouses of the cell, because most mitochondria are oxygen-consuming, ATP-producing organelles. These mitochondria utilize Krebs-cycle activity for the complete oxidation of acetyl-CoA to CO₂. Energy is mainly produced via oxidative phosphorylation, where electrons from NADH are transported to oxygen via a proton-translocating electron-transport chain, and the resulting gradient of protons across the mitochondrial inner membrane enables the mitochondrial ATP-synthase to convert ADP and P_i into ATP (figure 1). Oxygen is the final electron acceptor in these classical mitochondria and, in the absence of oxygen, Krebs-cycle activity and mitochondrial ATP synthesis will stop.

However, living with hypoxia, or even anoxia, is an everyday experience for many groups of eukaryotes. Some anaerobically functioning eukaryotes, such as parasitic helminths, are highly adapted for prolonged survival or even continuous functioning in the absence of oxygen, whereas others, like some marine invertebrates, are adapted to alternating periods in the presence and absence of oxygen. All these eukaryotes contain anaerobically functioning mitochondria, which produce ATP with the help of proton-pumping electron transport, but they use terminal

electron acceptors other than O₂. Some other anaerobically functioning eukaryotes, such as yeast and certain fish, can survive without mitochondrial energy metabolism via cytosolic fermentations in which the NADH produced during glycolysis is consumed during the reaction of pyruvate to lactate or ethanol, which are subsequently excreted as end-products. Several other eukaryotes, like several anaerobic ciliates, amoeboid flagellates, chytridiomycete fungi and parabasalids (Müller 1993; Embley *et al.* 1995; Ahkmanova *et al.* 1998; Hackstein *et al.* 1999; Rotte *et al.* 2000), contain other types of anaerobic ATP-producing organelles, the hydrogenosomes, which are H₂-producing, membrane-bounded organelles, that are evolutionarily related to mitochondria (Voncken *et al.* 2002; Van der Giezen *et al.* 2002). This review will not discuss these hydrogenosomal pathways nor the cytosolic fermentation pathways of energy generation, but will focus on anaerobic mitochondria.

Anaerobically functioning mitochondria have to maintain redox balance without aerobic respiration, as they cannot use oxygen as terminal electron acceptor. Therefore, the reduced cofactors produced by the catabolic pathways have to be oxidized by an alternative process.

Organisms with anaerobic mitochondria can be broadly divided into two different types: those that use an electron acceptor present in the environment, such as NO₂⁻, and those that use an endogenously produced, organic electron acceptor, such as fumarate (Martin *et al.* 2001; Tielens *et al.* 2002). An example of the first type is the nitrate respiration that occurs in several ciliates (Finlay *et al.* 1983) and the fungi (Kobayashi *et al.* 1996; Takaya *et al.*

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Figure 1. Schematic comparison of mitochondrial energy metabolism in aerobically and anaerobically functioning mitochondria. As an example, the main pathways of aerobic (open arrows) and anaerobic (filled arrows) metabolism in *Fasciola hepatica* are shown. Transport of electrons is shown by dashed arrows and the end-products are shown in boxes. Enzyme names are indicated in *italics*. AcCoA, acetyl-CoA; C, cytochrome *c*; CI, CIII, CIV, complex I, III, IV of the respiratory chain; CITR, citrate; FUM, fumarate; MAL, malate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUCC, succinate; Succ-CoA, succinyl-CoA.

1999) which use NO_3^- and/or NO_2^- as terminal electron acceptor of a membrane-associated electron-transport chain, producing nitrous oxide as the reduced end-product. The metabolism of these anaerobically functioning mitochondria resembles that of their aerobic counterparts, as reduced cofactors are produced that are re-oxidized by the electron-transport chain, which contains special terminal oxidoreductases that donate electrons to an acceptor present in the environment, in this case nitrite or nitrate instead of oxygen.

The other class of anaerobic mitochondria is present in parasitic helminths, freshwater snails and some lower marine organisms (Tielens & van Hellemond 1998). The energy metabolism of these anaerobic mitochondria differs principally and significantly from that in aerobic mitochondria, as no external final electron acceptors are used. Therefore, this mitochondrial metabolism has to be truly fermentative; in other words, the number of NADH-producing reactions has to equal the number of NADH-consuming reactions without the use of oxygen or other external electron acceptors. This mitochondrial fermentation pathway is called malate dismutation, and involves the endogenous formation and subsequent use of fumarate as electron sink of the electron-transport chain.

2. FERMENTATIVE MITOCHONDRIAL METABOLISM: MALATE DISMUTATION

Anaerobically functioning mitochondria that do not use external electron acceptors are present in several invertebrates (Tielens 1994). Most of these anaerobically functioning eukaryotes, like adult parasitic helminths, are

constantly dependent on this process. On the other hand, several lower marine animals, like mussels, oysters and lugworms, are intermittently dependent on this process when the tides of the sea force them to function anaerobically (De Zwaan 1991; Collicutt & Hochachka 1977; Grieshaber *et al.* 1994). All these organisms are known to be able to survive in an anaerobic environment via the fermentation pathway malate dismutation, which involves the use of an especially adapted electron-transport chain and the reduction of endogenously produced fumarate as electron sink (Tielens & van Hellemond 1998). In those organisms that are adapted to anoxic functioning via malate dismutation, carbohydrates are degraded by the usual glycolytic pathway to phosphoenolpyruvate, which is then converted to malate. This malate, produced in the cytosol, is transported into the mitochondria for further degradation (figure 1). In a split pathway, one-third of this malate is oxidized via pyruvate to acetate and two-thirds is reduced to succinate, which is often further metabolized to propionate (Tielens 1994). The reduction of malate to succinate occurs in two reactions that reverse part of the Krebs cycle, and the reduction of fumarate is the essential NADH-consuming reaction to maintain redox balance. Fumarate reduction is linked to electron transport via electron-transferring enzyme complexes in an anaerobically functioning electron-transport chain (figures 1 and 2). Striking differences of these anaerobically functioning mitochondria compared with the classical aerobic mitochondria are the presence of: (i) ASCT, the enzyme that converts acetyl-CoA into acetate; (ii) FRD, the enzyme catalysing the reduction of fumarate to succinate; and (iii) RQ, the quinone that provides in its reduced form

Figure 2. Schematic representation of mitochondrial respiratory chains. Boxes indicate electron-transport chain complexes, whereas ovals represent the electron transporters UQ, RQ and cytochrome *c*. The black boxes represent complexes involved in malate dismutation, whereas the open boxes represent complexes involved in the classical aerobic respiratory chain. The vertical bar represents a scale for the standard redox potentials in mV. Translocation of protons by the complexes is indicated by $H^+ \rightarrow$. CI, CIII and CIV, complex I, III and IV of the respiratory chain; bc_1 , complex III of the respiratory chain; cyt *c*, cytochrome *c*; FUM, fumarate; SUCC, succinate.

the electrons for the reduction of fumarate (figures 1 and 2).

3. ACETATE PRODUCTION AND ASCT

In the oxidative branch of malate dismutation, malic enzyme oxidizes malate to pyruvate, which is then further oxidized to acetyl-CoA. Subsequently, the CoA moiety of acetyl-CoA is transferred to succinate by ASCT, after which the resulting acetate is excreted as an end-product (Saz *et al.* 1996; van Hellemond *et al.* 1998). The produced succinyl-CoA is subsequently recycled to succinate by SCS, a process concomitantly producing ATP from ADP (or GTP from GDP). ASCT is present not only in fermentative mitochondria but also in the strictly aerobically functioning mitochondria of the trypanosomatids *T. brucei* and *Leishmania* (van Hellemond *et al.* 1998), as well as in the hydrogenosomes of trichomonads (Steinbüchel & Müller 1986). The localization of ASCT in these mitochondria identified the first metabolic pathway common to mitochondria and hydrogenosomes: an ASCT/SCS cycle, which is further evidence for their common evolutionary origin (van Hellemond *et al.* 1998). Interestingly, organisms lacking compartmentalization of metabolism, such as *Giardia* and *Entamoeba*, also lack this cycle and instead produce acetate from acetyl-CoA by a single-step reaction catalysed by acetyl-CoA synthetase. This suggests that compartmentalization and the presence of the acetyl/succinyl-CoA cycle are linked.

ASCT is not present in the mitochondria of all organisms, and has so far only been detected in anaerobically functioning mitochondria of parasitic worms and the aerobically functioning mitochondria of trypanosomatids. These latter mitochondria are interesting as these protozoa are considered to be the earliest-branching eukaryotes that contain mitochondria. Hence, these mitochondria could contain enzymes present in the ancestral mitochondrial organelle. The presence of ASCT in hydrogenosomes on the one hand, and, on the other hand, in aerobically as well as anaerobically functioning mitochondria, is very intriguing. ASCT has not been purified yet, nor have genes encoding ASCTs been cloned, and therefore, phylogenetic analysis of ASCT genes of various mitochondria and hydrogenosomes can unfortunately not be performed yet.

So far, three families of CoA-transferases have been described. Type I CoA-transferases form the best characterized group. Most type I CoA-transferases are known to operate with acetyl-CoA or succinyl-CoA as possible CoA donor. These CoA-transferases are a heterogeneous family of enzymes, present in both prokaryotes and eukaryotes, and they appear in a variety of oligomerization states, ranging from homodimers, in the case of pig heart 3-oxoacid CoA-transferase, to hetero-octamers, in the case of glutaconate CoA-transferase from the strictly anaerobic bacterium *Acidaminococcus fermentans* (Jacob *et al.* 1997). The α -subunits of prokaryotic CoA-transferases are homologous to the N-terminal half of eukaryotic

CoA-transferases, whereas the β -subunits are homologous to the C-terminal half. This strongly indicates the occurrence of a gene fusion at a certain time during evolution of CoA-transferases (Parales & Harwood 1992). Type II CoA-transferases catalyse the transfer of an acyl carrier protein, which contains a covalently bound CoA derivative, but also accept free CoA-thioesters *in vitro*. The prokaryotic CoA-transferases of this type are referred to as non-heterotetrameric and lack both an $\alpha_2\beta_2$ -subunit arrangement and significant amino-acid sequence similarity to eukaryotic CoA-transferases (Heider 2001). The few type III CoA-transferases studied so far are known to be involved in anaerobic metabolic pathways. Recently characterized members are from bacteria, but genes for similar enzymes are also found in Archaea and eukaryotes. Their amino-acid sequences do not resemble those of the CoA-transferases of any of the other two types. Oxalate CoA-transferase from *Oxalobacter formigenes*, a monomer of 45 kDa, is specific for oxalate and formyl-CoA, exhibiting partial activity only with succinyl-CoA and none with acetyl-CoA as alternative CoA donor (Baetz & Allison 1990). Based on the small and hydrophilic substrates involved, ASCT might belong to either family 1 or 3, but so far no homologues of the type III CoA-transferase family can be found in the various trypanosomatid genome databases, which suggests that ASCT is a type I CoA-transferase.

SCS, which catalyses succinyl-CoA hydrolysis coupled to substrate level phosphorylation, is found in acetate-producing organisms as well as in most aerobically functioning organisms, since SCS is also a Krebs-cycle enzyme. SCS is a multimeric enzyme composed of α - and β -subunits, and homologues of both α - and β -subunits have been cloned from many aerobically functioning species and from several acetate-producing organisms. Phylogenetic analysis of both α - and β -subunits of SCS (figure 3) shows that the SCS subunits of acetate-producing organisms do not cluster as a distinct group, but do resemble the consensus 'tree of life' irrespective of their involvement in acetate production or Krebs cycle. Since the catalytic function of SCS in Krebs cycle and acetate production is identical, no specific additional selection is expected for SCS functioning in acetate production, which is in agreement with the absence of phylogenetic clustering of SCS genes of acetate-producing organisms.

4. FRD VERSUS SDH

The interconversion of succinate and fumarate may have been important in several critical ways during the early phases of biochemical evolution. At the outset of one possible evolutionary sequence, the reduction of pyruvate to succinate, a process in which NADH is reoxidized, might have initially functioned as a simple electron sink, helping to maintain redox balance in the cell (Gest 1987; Bryant 1994). Such early fumarate-reducing systems would most likely have been soluble and relatively simple, and might have functioned primarily to ensure redox balance, similar to a fermentation (Gest 1987) (figure 4a). In the further course of evolution it may have been energetically advantageous to couple the electron transport to fumarate with proton pumping and chemiosmotic phos-

phorylation, which would require association of the enzyme with the membrane (figure 4b). A prerequisite for this, however, would have been the pre-existence of intermediate electron carriers of suitable redox potential: iron-sulphur clusters, menaquinone and cytochrome *b* (Gest 1987; Blaut *et al.* 1989), and such electron carriers were suggested to have been present in the earliest cells (Martin & Russell 2003).

Such early developments were paralleled by the evolution of several related biosynthetic pathways, such as the conversion of succinate to succinyl-CoA for the synthesis of tetrapyrroles, and the production of 2-oxoglutarate from acetyl-CoA and oxaloacetate via citrate for the synthesis of glutamate. These biosynthetic processes, together with the production of succinate from pyruvate via fumarate reduction and the development of 2-oxoglutarate dehydrogenase and succinate thiokinase, might have been combined and resulted in the origin of the citric acid cycle (Gest 1987). The FRD present at that point in time would have been used in the direction of succinate oxidation. During further evolution, this enzyme system might have specialized towards this succinate oxidation through the covalent binding of the flavin, through increased standard redox potentials of the iron-sulphur clusters of the enzyme, and through the preferred use of UQ instead of menaquinone as a cofactor in order to raise the redox potential of the electron acceptor (figure 2, figure 4c). Such a hypothetical scenario would explain the existence of an SDH system functioning with UQ and an FRD system functioning with menaquinone (figure 4c and 4b, respectively). Both systems are still present in many prokaryotes and the parallel existence of these two systems most likely evolved via a very early gene duplication.

5. PHYLOGENETIC RELATIONSHIP BETWEEN SDHS AND FRDS

FRD and SDH complexes are structurally very similar and each comprises usually four non-identical subunits: a flavin-containing A subunit (F_p subunit), a B subunit that contains three iron-sulphur clusters (I_p subunit), and mostly two hydrophobic (cytochrome *b*-containing) subunits C and D that are essential for the attachment of the catalytic subunits A and B to the membrane and for the interaction of the catalytic subunits with the quinones (Ackrell *et al.* 1992; van Hellemond & Tielens 1994). The F_p and I_p subunits of SDH are highly conserved in different species and are also closely related to the F_p and I_p subunits of FRD. Nevertheless, these two enzymes are clearly distinct and are differentially expressed in *Escherichia coli*: depending on external conditions, either SDH or FRD is expressed. It has been shown for the parasitic helminth *Haemonchus contortus* that it possesses two different genes for the I_p subunit that are differentially expressed during the development of this parasite (Roos & Tielens 1994). This differential expression during development has also been shown to occur in another parasitic helminth, *Ascaris suum*, in which the existence of two different stage-specific forms of complex II was also demonstrated (Saruta *et al.* 1995). Studies on complex II of anaerobically functioning eukaryotes have mostly been restricted to the parasitic helminth *Ascaris* (for reviews on

Figure 3. Unrooted neighbour-joining phylogenetic trees of SCS subunits A (a) and B (b). Full-length amino-acid sequences were aligned using CLUSTALX and phylogenetic trees were prepared by neighbour joining using the PHYLIP package. Bootstrap values for 100 independent random order analyses are shown when greater than 50. Similar trees were obtained by maximal-parsimony methods. Eukaryotic species that are known to produce acetate are boxed.

Figure 4. Schematic representation of the proposed evolution of eukaryotic FRDs. (a) Shows a soluble FRD, which became membrane bound and coupled to electron transport (b), after which SDH evolved from FRD (c). (d) Represents eukaryotic FRDs, which are structurally more similar to SDHs (rounded structures) and they interact with a benzoquinone (oval) instead of a naphthoquinone (diamond), although they function as a FRD (grey structures) and not as an SDH (open structures). FUM, fumarate; SUCC, succinate.

these extensive studies see Kita 1992 and Kita *et al.* 1997). The enzyme complexes responsible for fumarate reduction in eukaryotes other than parasitic helminths have not yet been studied. However, the presence of a specialized quinone, RQ, in lower marine organisms, such as oysters, indicates that these facultative anaerobic organisms possess FRD complexes comparable with those in parasitic helminths (van Hellemond *et al.* 1995).

Analyses of enzyme kinetics, as well as the known differences in primary structures of prokaryotic and eukaryotic complexes that reduce fumarate, prompted us earlier to

the suggestion that fumarate-reducing eukaryotes possess an enzyme complex for the reduction of fumarate that is structurally related to SDH-type complex II, but has the functional characteristics of the FRD complexes of prokaryotes (van Hellemond *et al.* 1995). Although the amount of data is still very limited, phylogenetic analysis of SDHs and FRDs demonstrated that the sequences of parasitic enzymes that reduce fumarate are more closely related to the eukaryotic SDH sequences than to the bacterial FRDs (figure 4d; Tielens & van Hellemond 1998; Tielens *et al.* 2002).

6. ELECTRON TRANSPORT IN AEROBIC AND FERMENTATIVE MITOCHONDRIA

For the oxidation of succinate to fumarate ($E^{\circ'} = +30$ mV) SDH uses preferentially an electron acceptor with a relatively high standard electron potential, such as UQ ($E^{\circ'} = +70$ mV). FRD, on the other hand, catalyses the opposite reaction, in which an electron donor is oxidized and two electrons are subsequently transferred to fumarate, reducing this to succinate. Therefore, FRD complexes interact in general with quinones having a lower standard electron potential ($E^{\circ'} = -70$ mV) compared with SDH complexes (figure 2; Ackrell *et al.* 1992; van Hellemond & Tielens 1994).

Most prokaryotes are known to use menaquinone for electron transport to FRD. We showed, on the other hand, that in eukaryotes, RQ fulfils this function. RQ is an indispensable component for efficient electron transport in all eukaryotic organisms investigated so far that perform malate dismutation in anaerobic mitochondria (van Hellemond *et al.* 1995). These organisms included not only parasitic helminths but also freshwater snails, some protists and the lower marine animals, mussels, oysters and lugworms (Powls & Hemming 1966; van Hellemond *et al.* 1995). RQ is also present in a few prokaryotic organisms, as *Rhodospirillum rubrum* and some other members of the family Rhodospirillaceae (facultative phototrophic purple non-sulphur bacteria) contain, apart from UQ, also RQ (Parson & Rudney 1965). Those members of Rhodospirillaceae that contain RQ were found to possess FRD activity, whereas the species containing exclusively UQ showed no significant FRD activity (Hiraishi 1988), which confirms the essential function of RQ in fumarate reduction when menaquinone is absent.

Although RQ and menaquinone are functional equivalents (having a low standard electron potential), their structures differ significantly. In contrast to both RQ and UQ, menaquinone (present in prokaryotes) is not a benzoquinone but a naphthoquinone. Therefore, the structure of the quinone involved in electron transport during malate dismutation in eukaryotes (RQ) is more similar to the quinone involved in aerobic mitochondrial electron transport (UQ) than to its functional equivalent in prokaryotes, menaquinone. This suggests, similarly to the primary sequence analysis of eukaryotic FRDs, that the electron transporters of anaerobically functioning mitochondria are also more closely related to those of aerobically functioning mitochondria than to their functional homologues in prokaryotes.

7. UQ AND RQ BIOSYNTHESIS

The biosynthesis of UQ is known (figure 5) and the genes involved were recently studied. However, many details of the mechanism of all the reactions involved are not yet resolved (Meganathan 2001). Menaquinone biosynthesis has also been studied in detail (Bentley & Meganathan 1993; Meganathan 2001), but the pathway of RQ biosynthesis has not yet been thoroughly investigated. The structure of RQ is almost identical to that of UQ, apart from an amino group instead of a methoxy group on position 5 of the benzoquinone ring structure, respectively (figure 5). Hence, in our opinion, it is most

likely that the early reactions in the biosynthesis of the benzoquinone structure are identical for both RQ and UQ. The benzoquinone structure for UQ synthesis starts with acetate for the synthesis of the isoprenoid side chain. This so-called mevalonate pathway results in the formation of farnesyl pyrophosphate which can then be used for the synthesis of cholesterol, dolichol and UQ as well as for the isoprenylation of proteins. *p*-Hydroxybenzoate is used as starting material for the benzoic ring structure of UQ. In eukaryotic cells long-chain all-*trans* polyprenyl diphosphates are produced by the enzyme *trans*-prenyltransferase. These all-*trans* polyprenyl compounds, containing six to ten isoprene units, are then used to form the side-chain of the benzoquinone. The number of isoprene units attached to the quinones varies among organisms. For instance, *Saccharomyces cerevisiae* mainly produces quinones with six isoprene units, *E. coli* mainly produces quinones with eight isoprene units, whereas humans produce UQ with ten isoprene units. These differences are believed to be determined by the availability of polyprenyl diphosphate in the cell (Okada *et al.* 1996). The all-*trans* polyprenyl diphosphate unit is condensed with *p*-hydroxybenzoate by a polyprenyltransferase, and a number of subsequent modifications of the ring are required for the completion of the benzoquinone structure and subsequently UQ (figure 5). These modifications of the ring start with *C*-hydroxylation, followed by *O*-methylation and decarboxylation. Two additional *C*-hydroxylations, one *C*-methylation and one *O*-methylation are necessary for the completion of UQ. This sequence of reactions is characterized in bacterial as well as eukaryotic systems. In bacteria the sequence of the reactions is slightly different, the first step after the condensation being the decarboxylation rather than the *C*-hydroxylation.

The pathway of RQ synthesis has not yet been elucidated, but pulse-chase experiments suggested that UQ might be used for the synthesis of RQ in *Rhodospirillum rubrum* and *Euglena gracilis* (Parson & Rudney 1965; Powls & Hemming 1996). Our earlier investigations on the biosynthesis of RQ in parasitic helminths indicated that *Fasciola hepatica* and *Schistosoma mansoni* do not use exogenous UQ as a precursor for RQ synthesis (J. J. van Hellemond and A. G. M. Tielens, unpublished data). Furthermore, differences in the number of isoprene units present in the quinones of the parasite compared with that of the host, demonstrated that also *in vivo* quinones are not scavenged from the host, and studies using radioactive mevalonate showed that these parasites synthesize both UQ and RQ *de novo* (van Hellemond *et al.* 1996, 1997).

RQ differs from UQ only in the amino group on position 5 of the benzoquinone structure instead of a methoxy group (figure 5). Interestingly, this methoxy group is formed during the last step of UQ biosynthesis and, therefore, we proposed earlier that RQ synthesis diverges only in one reaction from UQ biosynthesis, via introduction of an amino group in either 5-hydroxy-6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone or 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone (figure 5; van Hellemond *et al.* 1996). Database searches for analogous enzymes catalysing the last three reactions in UQ biosynthesis, in organisms known to synthesize RQ, did not reveal genes for the possible enzymes for this last reaction in RQ biosynthesis. This genetic database approach is hampered

Figure 5. Biosynthesis of UQ and possible pathways of RQ biosynthesis in eukaryotes.

by the fact that no genome projects exist for RQ-synthesizing organisms. Furthermore, the putative enzyme involved in RQ biosynthesis that introduces the amino group does not necessarily have to contain recognizable stretches of sequences, as quinone-binding sites are not universal and are inadequate for prediction of quinone-binding sites in unknown structures (Fisher & Rich 2000). Hence, even if genes of enzymes involved in RQ synthesis have already been sequenced, these genes would not be recognized as such. Classical biochemical approaches are, therefore, probably needed to elucidate the biosynthetic pathway of RQ and to characterize the enzyme(s) involved.

8. EVOLUTIONARY ASPECTS OF ANAEROBIC MITOCHONDRIA

It is now widely accepted that the classical aerobically functioning mitochondrion evolved by an endosymbiotic event between an anaerobically functioning archaeobacterium and an α -proteobacterium. Such an endosymbiotic theory was already postulated in the 19th century and the investigations of Margulis in the second half of the 20th century raised evidence in support of this theory (Margulis 1970). More recently, several refinements of the endosymbiont theory were suggested. One of these, the hydrogen hypothesis (Martin & Müller 1998), starts from the concept that the hydrogen, produced as waste product by the α -proteobacterium, functions as substrate for the hydrogen-dependent autotrophic Archaeal host, and was the driving force for the development of mitochondria, instead of the ATP produced by the α -proteobacterial symbiont and consumed by the anaerobic host, as suggested originally. It is proposed that in aerobic environments the anaerobic capacities of the original symbiont were then lost, and this resulted in the evolution of the classical mitochondria (Martin & Müller 1998).

In anaerobic environments, on the other hand, the aerobic capacities of the endosymbiont were lost. This resulted in the formation of two types of amitochondriate organ-

isms, those where the symbiont evolved into anaerobically functioning organelles, hydrogenosomes (as are still present in, for instance, trichomonads), and those where (part of) the contents of the organelle was retained, but the ATP-producing organelle as such disappeared, such as in *Giardia* (Martin & Müller 1998). The evolutionary origin of the anaerobically functioning mitochondria, such as those in parasitic helminths, has been less debated until now. We have recently postulated a possible evolutionary origin of these unconventional mitochondria (Tielens *et al.* 2002).

It could be envisaged that these true anaerobically functioning mitochondria originated from the pluripotent ancestral organism by adaptation to an anaerobic environment, like the hydrogenosomes of trichomonads. In our opinion, however, these anaerobically functioning mitochondria evolved later in evolution from the more conventional aerobic type of mitochondria after these were adapted to an aerobic way of life by losing their anaerobic capacities (Tielens *et al.* 2002).

Several observations indicate that these anaerobically functioning mitochondria evolved from the classical mitochondria, and did not originate (by adaptation to an anaerobic environment) directly from the facultative anaerobic pluripotent ancestral cell that was the result of the endosymbiosis of an α -proteobacterial symbiont and the Archaeal host. First, as discussed above, all sequence data available up to now on the FRDs of these organisms demonstrate that these enzymes are closely related to the SDHs of classical aerobic mitochondria. These classical mitochondria are supposed to have evolved by losing the anaerobic capacities of the pluripotent ancestral cell, which probably already also possessed a comparable enzyme for anaerobic fumarate reduction (FRD). Therefore, it is more likely that this adaptation towards an anaerobic metabolism did not occur immediately after the symbiotic event, but after the earlier prokaryotic fumarate-reducing enzyme for anaerobically functioning was lost. Second, the same argument holds true for the quinone used for this anaerobic

fumarate reduction. All anaerobically functioning fumarate-reducing eukaryotes investigated so far use RQ for the transport of electrons from complex I to the fumarate-reducing enzyme, while the more ancient prokaryotic systems use menaquinone. Again, the anaerobically functioning mitochondria use a molecule that is structurally more related (a benzoquinone) to the molecule of the classical mitochondria, UQ (also a benzoquinone), than to the molecule used by the anaerobic prokaryotes, menaquinone (a naphthoquinone). As the synthesis of RQ probably differs only in the last step from the synthesis of UQ, this indicates that also the electron transporter was adapted to anaerobically functioning after the synthesis of the originally present transporter (menaquinone) was lost by adaptation to an aerobic environment. Third, in our opinion, the fact that both classical and anaerobically functioning mitochondria are known to occur within the same organism, for instance parasitic helminths, points more in the direction of a later evolution of classical aerobic mitochondria to an anaerobic environment than in the direction of an immediate adaptation to an anaerobic environment, as parasitic helminths are supposed to have evolved from free-living worms, which were probably aerobically functioning, like free-living worms nowadays.

For these reasons we suggest that anaerobically functioning mitochondria evolved from the classical type by later adaptation to an anaerobic environment. This adaptation could have occurred by small structural modifications of the SDH enzyme, which, for instance, changed the redox potentials of the iron-sulphur clusters, making the enzyme more suitable for reduction of fumarate, resulting in an enzyme that is structurally more related to the original SDH, but functionally more related to the older FRDs of prokaryotes. Next to this, an adaptation could have occurred in the synthesis of the quinone involved in fumarate reduction. The biosynthetic pathway of RQ is still unknown, as is the origin of the enzyme(s) of the last steps in this biosynthesis. It is conceivable that it is acquired by lateral gene transfer from, for instance, a *Rhodospirillum*-like prokaryotic organism, which is also known to synthesize RQ.

Anaerobic metabolism is usually considered to be an old and rather primitive way of life, but the above-described scenario would implicate that these anaerobically functioning mitochondria are an adaptation of the traditional type of mitochondria to anaerobic environments, and are in fact a further evolution instead of a more primitive form.

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Discussion

D. S. Horner (*Dipartimento di Fisiologia e Biochimica Generali, University of Milan, Milan, Italy*). As regards your scheme of the different pathways of conversion, between the forms, I was wondering with particular respect to the fungal hydrogenosomes whether you could envision a model where they were actually derived through an anaerobic mitochondrial sort of pathway.

A. G. M. Tielens. I agree, I had too many arrows.

D. S. Horner. I mean, as a generalization, you had an early loss of oxidative phosphorylation in the pathway to hydrogenosomes and I appreciate it is a network, but I just wondered whether the fungal hydrogenosomes could have come via anaerobic mitochondria as a sort of intermediate stage?

A. G. M. Tielens. I am not sure I quite understand what you mean. I have drawn on the hydrogenosomes from the anaerobic....

D. S. Horner. No, you have got to the type II hydrogenosomal phenotype as a fundamentally early split from the pathway from the classical mitochondria, and I am just wondering whether you could even have an arrow between anaerobic, fumarate-using mitochondria, in the case of the fungal ones, in particular.

A. G. M. Tielens. They have absolutely nothing in common.

D. S. Horner. Well, that is the question.

A. G. M. Tielens. No, no, definitely not allowed. This of course did not happen the split second after the α -proteobacterium was engulfed, so certainly something has to be happening. Of course a lot of things have happened here and in the beginning before they went apart, but these definitely, if I understand, went into an anaerobic thing by losing all the aerobic capacities.

D. S. Horner. The reason I am thinking this is that the hydrogenase in the anaerobic, well the ciliates, and particularly for the hydrogenosomes, you have got these pyridine dinucleotides acting like components fused on to the hydrogenase, in the case of the pyruvate NADH oxidoreductase-type fusions you have pyridine dinucleotides interacting with the components of fused into pyruvate : ferredoxin oxidoreductases (PFOs), whereas in the basal-type hydrogenosomes it is generally assumed that ferredoxin is the electron carrier.

A. G. M. Tielens. As I said, there would have to be at least several different blue boxes containing the type II hydrogenosomes, so definitely they also split at different moments.

W. Martin (*Institute of Botany III, Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany*). What kinds of prokaryotes synthesize rhodoquinones?

A. G. M. Tielens. What kinds of prokaryotes? Not too many, *Rhodospirillum*... and that is about it.... Euglenas.

W. Martin. Euglenas? I said prokaryotes.

A. G. M. Tielens. OK, sorry, no, not too many, so it is possible that this enzyme has to come from somewhere. Maybe it would even be a nice idea to obtain that from, say, *Rhodospirillum*-like bacteria, but I have absolutely no evidence for that because the enzyme is unknown; it is not an annotated enzyme. We are in the business of trying to purify it, but it is difficult to do because we do not even know what reaction it catalyses. One of my students is isolating an enzyme of which she does not know what it does, and she does not know what it looks like.

T. Cavalier-Smith (*Department of Zoology, University of Oxford, Oxford, UK*). You mentioned that trypanosomes are known to be an early branching. That is not really the case.

A. G. M. Tielens. No, I was afraid that that was wrong.

T. Cavalier-Smith. They are related to the other colidoplastids, which are related to *Euglena*, and they in turn are related to another group, which is also anaerobic, but the common ancestor of the sister group—the Euglenazoa. Andrew Roger's laboratory has recent evidence that they probably had a photosynthetic ancestor, and I would put them in part of this Cabazoa group. They are probably quite derived among the eukaryotes, and in fact very little is likely to be early-branching. We have a paper coming out in *Science* next month, indicating that the root is where I showed it on the tree.

A. G. M. Tielens. The only point I was trying to make was that they are aerobic, but I do not know why they do not use it because that is the main thing. I do not know if you knew it but you can have mitochondria without using them, at least without using them properly, so they keep fermenting without using their full capacity. Well, in most papers the trypanosomes are at least kind of separated from the rest. I do not know of any example where you can find these mitochondria; as far as we know you can only find them in the trypanosomes.

J. F. Allen (*Plant Biochemistry, Lund University, Lund, Sweden*). On that scheme, which we do not see, we have a nice tidy...

A. G. M. Tielens. This one or that one?

J. F. Allen. [With reference to a display corresponding to fig. 4 of Tielens *et al.* (2002).] Well, on the top row, on the extreme left, there are beef heart mitochondria; good, classical aerobic mitochondria, and therefore coloured red—because they are on the left. And on the extreme right you have a blue box, depicting anaerobic mitochondria, and there is this slight drift towards the right I have noticed, which I believe is in keeping with your thesis. But just before you get to the far right you have hydrogenosomes. I think you would agree with me that the obvious and logical position of the hydrogenosomes is as an anaerobic mitochondrion whose job is to make ATP by anaerobic oxidative phosphorylation; you almost said that.

A. G. M. Tielens. No, no, I hope I did not say that, because hydrogenosomes do not have oxidative phosphorylation; in my opinion they are no longer entitled to be called mitochondria.

J. F. Allen. I am a bit vague about this. I think Martin Embley actually flashed over the key to what hydrogenosomes do.

A. G. M. Tielens. No, no. We all agree hydrogenosomes once were mitochondria (that is probably the correct way of putting it), but you can also call them hydrogen-producing mitochondria. However, it is easier to call them hydrogenosomes. While they were once mitochondria they are no longer entitled to that title because they have lost oxidative phosphorylation. You have to be strict about that.

J. F. Allen. They cannot use hydrogen as an electron donor to reduce fumarate?

A. G. M. Tielens. No, I do not think so.

J. F. Allen. Is it impossible?

A. G. M. Tielens. There is no way they can use it.

S. Ferguson (*Department of Biochemistry, University of Oxford, Oxford, UK*). On the diagram you have got there, where it says terminal oxidases leading to the box with external electron acceptors, is that referring to the nitrate–nitrite reductases? Should it say terminal reductases?

A. G. M. Tielens. Yes, I already tried to explain that it is very unclear. This is a kind of respiratory-chain assembly of all these things and so you have the fumarate reductases that I showed you. You go from the quinone and give it up to reduced fumarate; these are the other types, and this is the aerobic, the usual complex I, III, IV, and oxygen, but that these are the nitrate reductases and the nitrite reductases, using also the ubiquinone pool or the cytochrome *c* pool depending on the organism, and it is very unclear and has hardly been investigated where these things come from. Some of them probably originated in what we like to call the ancestral host and the ancestral organism, and others might have been obtained later, but I have absolutely no idea.

S. Ferguson. Are these the *Fusarium* kind of fungi? Is that what you are referring to with this nitrate–nitrite?

A. G. M. Tielens. Yes, they were just included to make the spectrum complete.

S. Ferguson. So just to take the evolutionary argument a step further forward, in the bacterial respiration field these days most people of course think nitrite–nitrate respiration came before oxygen respiration, but you have considered that point and cannot imagine that the mitochondria did that first and used oxygen later?

A. G. M. Tielens. I have no idea what will be the last answer on this one.

S. Ferguson. There is actually just an interesting point about that. As you said, the fact that the final product in these *Fusarium* organisms seems to be nitrous oxide, N_2O , rather than NO , and of course it is the NO reductases in these *Fusaria* that are not actually linked to the respiratory chain, but a cytochrome P450 protein. This is rather strange because the whole argument about the evolution of these respiratory pathways was based on the surprising finding in bacteria that the nitric oxide reductase appears to be the precursor of the cytochrome oxidase, so it would fit in with your scheme that these nitrate–nitrite reductase enzymes have been recruited later from the bacterium,

and that the P450 type of NO reductases is some other kind of adaptation, whereas it could still be the case that oxygen respiration evolved from denitrification, which is what some people believe the geological record also now shows.

A. G. M. Tielens. Yes, but both types are present so it is not really certain to what extent each is important.

J. Tovar (*School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK*). Just to comment in relation to the evolution of anaerobic mitochondria, and the evolution of organelles from facultative anaerobic organisms like trichomonads and *Entamoeba*, it seems to me that it makes sense that an anaerobic mitochondrion would be a neat pathway to the evolution of the mitosome, for example, yet you say that there is no way that could be the case. Am I correct? Did I understand you?

A. G. M. Tielens. Well, that was when I was referring to the hydrogenosomes. As we do not know exactly what is in the mitosome, I do not want to be blamed for saying where they came from. I say that there is no resemblance; actually the only resemblance they have is the acetate-succinate CoA transferase, and the accompanying succinate thio-kinase, so that is one of the only systems that is present both in mitochondria and in hydrogenosomes, which is why I am saying that their divergence is early. I thought that I had convinced you that the anaerobic mitochondria evolved out of the aerobic mitochondria, after the hydrogenosomal aspects were lost; that is the whole idea.

J. Tovar. But you are not ruling out the possibility that organelles like the hydrogenosomes could also be derivative of an anaerobic mitochondrion, so anaerobic mitochondria could be an intermediate.

A. G. M. Tielens. No, I do not mean to imply that; of course nothing is impossible, but that would mean that you go several steps again, that you are again gaining things you already had that had been lost and regained again. I mean that is all possible, but that that would not fit in this scheme. Am I saying something wrong?

D. S. Horner. It is the phylogenies on which we agree, but the hydrogenase phylogenies and the distribution among eukaryotes suggest that it is quite plausible that those enzymes were retained very early, and thus they may still be there at the point of divergence to the anaerobic mitochondria.

A. G. M. Tielens. No, but I think we agreed that they came from the same kind of ancestors. That is not a disagreement, is it?

D. S. Horner. No.

A. G. M. Tielens. I am only trying to say that I have tried to show that these things regained anaerobic capacities after they lost comparable ones from their earlier beginnings, while the hydrogenosomes already had

these anaerobic but completely different types. They had the hydrogenase and so on, and these anaerobic mitochondria do not have that; they found another way of remodelling their aerobic enzymes.

D. S. Horner. Yes, I understand, but notwithstanding the ciliate case, it looks like the fungal hydrogenase shares a common ancestry with all of the other eukaryote hydrogenases, and, thus, was there in the common ancestor of fungi, for example. It was not acquired independently by *Neocalamastix* necessarily, but was there, so it was potentially there in the common ancestors of *Physarum* and *Fusarium* and *Neocalamastix*. Therefore it is not necessarily a case of having to regain the hydrogenase.

W. Martin. I think Jorge [Tovar] and David [Horner] perhaps had the same problem as I have had, and that is they are thinking about protists and protist diversity and possibly ancient lineages. In intense work with Louis [A. G. M. Tielens] over the past months, I have learned that when Louis says anaerobic mitochondria he means primarily the anaerobic mitochondria of parasitic helminths, the ones he has worked on for decades.

The parasitic helminths are metazoans; they are relatively advanced animals if you will, and if one examines a phylogeny of early eukaryotes, the anaerobic forms that have fumarate reductase and the rhodoquinone arise from within forms that have mitochondria that are very similar to ours. So if the question, 'do you see a hydrogenosome arising from an anaerobic mitochondrion?' is asked, Louis is thinking about a parasitic helminth with a mitochondrion turning into a *Trichomonas* hydrogenosome, and he says 'no', and he is absolutely right. What he did not show in the figure is the phylogenetic distribution of these different physiological mitochondrion types, the diversity of organisms in which these different kinds of mitochondria are found. That might help clarify part of that point.

A. G. M. Tielens. That, at least, is how I try to start, to show these are all metazoans, real organisms.

Additional reference

Tielens, A. G. M., Rotte, C., Van Hellemond, J. J. & Martin, W. 2002 Mitochondria as we don't know them. *Trends Biochem. Sci.* **27**, 564–572.

GLOSSARY

ASCT: acetate : succinate CoA-transferase

CoA: coenzyme A

FRD: fumarate reductase

NADH: nicotinamide adenine dinucleotide, reduced form

P_i: inorganic phosphate

RQ: rhodoquinone

SCS: succinyl-CoA synthetase

SDH: succinate dehydrogenase

UQ: ubiquinone