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Evolution of the couple cytochrome *c* and cytochrome *c* oxidase in Primates

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Abstract

Mitochondrial energy metabolism has been affected by a broad set of ancient and recent evolutionary events. The oldest example is the endosymbiosis theory that led to mitochondria and a recently proposed example is adaptation to cold climate by anatomically modern human lineages. Mitochondrial energy metabolism has also been associated with an important area in anthropology and evolutionary biology, brain enlargement in human evolution. Indeed, several studies have pointed to the need for a major metabolic rearrangement to supply a sufficient amount of energy for brain development in primates.

The gene encoding for the coupled cytochrome *c* (cyt *c*) / cytochrome *c* oxidase (COX, complex IV, EC 1.9.3.1) seems to have an exceptional pattern of evolution in the anthropoid lineage. It has been proposed that this evolution was linked to the rearrangement of energy metabolism needed for brain enlargement. This hypothesis is reinforced by the fact that the COX enzyme was proposed to have a large role in control of the respiratory chain and thereby global energy production.

After summarizing major events that occurred during the evolution of COX and cytochrome *c* on the primate lineage, we review the different evolutionary forces that could have influenced primate COX evolution and discuss the probable causes and consequence of this evolution. Finally, we discuss and review the co-occurring primate phenotypic evolution.

Keywords

Evolution; Oxidative phosphorylation; Mitochondria, Regulation

X.1 Introduction

Mitochondrial energy metabolism has been associated with a broad set of both ancient and recent evolutionary events. The oldest example would be the endosymbiosis event in which an ancient bacterium was incorporated into another cell (Mereschkowski, 1905; Sagan, 1967); indeed, the increase by more than 15× of available ATP from a glucose molecule due to mitochondrial oxidative phosphorylation (OxPhos) is proposed as a necessary step for the emergence of pluricellular organisms (Lane and Martin, 2010). A more recent example would be the adaptation to cold climate by human populations. In this latter proposal,

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several OxPhos polymorphisms are suspected to increase heat production by decoupling oxygen consumption from energy production and would have been positively selected in the coldest parts of the world (Mishmar et al., 2003; Ruiz-Pesini et al., 2004; Pierron et al., 2008).

Mitochondrial energy metabolism has also been associated with one of the main topics of anthropology and evolutionary biology, brain enlargement during primate evolution (Grossman et al., 2001; Grossman et al., 2004; Uddin et al., 2008; Williams et al., 2010). Indeed, several authors have pointed out the need for a major rearrangement of metabolism to supply a sufficient amount of energy necessary for brain development in primates (Leonard et al., 2007; Isler and Van Schaik, 2009). In parallel, numerous studies have shown that the genes encoding the constitutive proteins of the OxPhos complexes have been through an adaptive evolutionary process during primate evolution, and specifically on the anthropoid lineages (Old World monkey, New World monkey, ape, and human) (Adkins and Honeycutt, 1994a; Adkins et al., 1996; Schmidt et al., 1997; Wu et al., 1997; Schmidt et al., 1999; Andrews and Easteal, 2000; Wu et al., 2000; Grossman et al., 2001; Schmidt et al., 2002; Goldberg et al., 2003; Doan et al., 2004; Grossman et al., 2004; Schmidt et al., 2005; Uddin et al., 2008).

The fourth OxPhos complex (cytochrome *c* oxidase, COX, EC 1.9.3.1) and cytochrome *c* (Cyt *c*) seem to have evolved exceptionally during the anthropoid descent (Doan et al., 2004; Schmidt et al., 2005). Furthermore, this evolution has been proposed to be linked to the energy metabolism rearrangement needed for brain enlargement (Grossman et al., 2004; Schmidt et al., 2005; Goodman et al., 2009). This hypothesis is reinforced by the fact that the COX enzyme was proposed to have a large role in the control of the respiratory chain and so of global energy production (Pacelli et al., 2011). Indeed, it has been shown that *in vivo* COX has a high control coefficient on OxPhos activity and its activity is tightly regulated by several cellular mechanisms (Follmann et al., 1998; Fontanesi et al., 2006). Furthermore, COX and Cyt *c* are the only components of primate OxPhos with known tissue-specific isoforms (reviewed in (Hüttemann et al., 2008). Arguably the control function of the COX/Cyt *c* couple could have been a specific evolution target affecting global primate metabolism.

In this chapter we will trace the evolution of Cyt *c* and COX genes among the primate lineages and discuss the probable causes and consequence of this evolution. First we will present a general view of the biochemical Cyt *c*/COX mechanisms within a macro-evolutionary perspective. Then we will review the COX/Cyt *c* differences both across primates and compared to non-primate mammals, as well as the timeframe of the appearance of these differences. We will also review the different evolutionary forces that have been suggested to influence primate COX gene evolution. Finally, we will discuss the possible causes and consequences of this evolution and attempt to integrate COX/Cyt *c* evolution into a broader view of primate evolution.

X.2 COX function and macro-evolution in OxPhos

X.2.1 OxPhos system

OxPhos consists of the electron transport chain (ETC) and ATP synthase. The ETC transfers electrons derived from the breakdown of food that are captured in reduced molecules such as NADH. The electrons of the latter enter the ETC via NADH dehydrogenase (complex I). The Krebs (i.e., Citric Acid) cycle feeds additional electrons into the ETC through succinate dehydrogenase (complex II). Both complex I- and II-derived electrons are then transferred to ubiquinone (coenzyme Q or CoQ), which is thereby reduced to ubiquinol. The bc_1-complex (complex III) is an electron relay, converting packages of two electrons delivered from

ubiquinol into single electron packages taken up by Cyt *c*. Finally, Cyt *c* binds to COX (complex IV), which transfers electrons to molecular oxygen.

Electron transfer reactions catalyzed by the ETC are coupled to the pumping of protons across the inner mitochondrial membrane by complexes I, III, and IV, but not complex II. The pumped protons generate the mitochondrial proton motive force (Δp_m), which consists of a chemical component, the *p*H difference across the inner membrane, and an electrical component, the mitochondrial membrane potential ($\Delta\Psi_m$), which constitutes the major part of Δp_m in mitochondria. In the last step of OxPhos, Δp_m is utilized by ATP synthase (complex V) to generate ATP from ADP and phosphate, which is coupled to the backflow of protons from the mitochondrial intermembrane space (IMS) to the matrix.

X.2.2 Electron transfer from cytochrome *c* via cytochrome *c* oxidase to oxygen

The precise mechanism of how electron transfer is coupled to proton pumping is still a matter of controversy. Several models exist, some of which involve the heme propionate side chains as the sites where protons formally leave the matrix space and enter the intermembrane space within COX. The electron pathway is well-established: after Cyt *c* binds to COX subunit II, a single electron leaves the Cyt *c* heme group and enters COX subunit II *via* the binuclear CuA site. From there the electron enters subunit I and reaches the heme *a* group located near the middle of the membrane and horizontally transfers to the heme a_3-Cu_B binuclear site where oxygen binds. The heme groups are perpendicular to the membrane at an angle of 108° to each other, and their closest edge-to-edge distance is only 5Å (Ludwig et al., 2001). Four subsequent electron transfer reactions in addition to four protons, which are taken up from the matrix space, are required to reduce dioxygen to water:

$$4\,\mathrm{Cyt}c^{2+} + \mathrm{O_2} + \left(n_{\mathrm{pumped}} + 4_{\mathrm{chemical}}\right)\mathrm{H^+}_{\mathrm{matrix}} \rightarrow 4\,\mathrm{Cyt}c^{3+} + 2\mathrm{H_2O} \quad n\,\mathrm{H^+}_{\mathrm{cystol}}$$

X.2.3 Regulatory mechanisms of cytochrome *c* oxidase

The last step of the ETC, the reaction catalyzed by COX, is the proposed rate-limiting step of the ETC in intact mammalian cells (Villani and Attardi, 1997; Villani et al., 1998) but not in isolated mitochondria, likely due to loss of regulatory properties of the OxPhos complexes during mitochondrial isolation (Hüttemann et al., 2011). The essentially irreversible terminal reaction catalyzed by COX has to be well regulated and adapted to temporal and tissue-specific energy requirements. This adaptation is mediated by the presence of tissue-specific and developmentally-regulated isoforms only found in COX and Cyt *c* among OxPhos complexes, further supporting the important role of COX in the regulation of overall ETC flux. To date six isoforms have been reported for the nuclear encoded subunits of COX, which are encoded by separate genes (Hüttemann et al., 2011). Three are liver- and heart-type isoform pairs of subunits, VIa, VIIa, and VIII. Liver-type COX is found in organs such as liver, brain, and kidney. In contrast, the heart-type isozyme is expressed in heart and skeletal muscle. In addition, there is a lung-specific isoform of COX subunit IV (*COX4i2*), a testis-specific isoform of subunit VIb (*COX6B2*), and a third isoform of subunit VIII (*COX8C*) (Huttemann et al., 2003b). Of those enzymes their basal activities follow the sequence heart-type studied, < liver-type < lung type, which inversely correlates with the mitochondrial capacity of the corresponding tissues.

Several additional regulatory mechanisms act on COX including allosteric regulation by a built-in energy sensor, an ATP/ADP binding pocket located on subunit IV that senses the energetic state of the cell and adjusts energy production to demand (see chapter KADENBACH). Other modulators that affect COX activity include nitric oxide (NO), which competes with oxygen for binding at the binuclear heme *a*-Cu_B center and inhibits

COX, and thyroid hormone 3,5-diiodothyronine, which binds to subunit Va and releases the allosteric ATP inhibition, thus allowing higher ETC flux even in the presence of high ATP/ADP ratios. This finding was proposed to explain the short-term activating action of thyroid hormones on metabolism and it may also be involved in non-shivering thermogenesis (Ludwig et al., 2001).

COX is also targeted by cell signaling pathways through phosphorylation of specific serine, threonine, and tyrosine residues, which in some instances decisively regulate enzyme activity (see chapter HÜTTEMANN). Therefore, the multitude of regulatory properties found in COX underlines its unique position as the oxygen burning engine of the ETC. Analyzing COX evolution can thus provide insight into important mechanistic and regulatory aspects of aerobic energy metabolism, and it may also help to answer unsolved basic questions, such as the identification of proton exit pathways and oxygen channels within the enzyme that are presumably evolutionarily conserved.

X.2.4 COX macroevolution

It is now broadly accepted that mitochondria and OxPhos complexes are the relic of an endosymbiosis between a pre-eukaryotic cell and an α-proteobacterium (Sagan, 1967; Zimmer, 2009; Richards and Archibald, 2011). Interestingly, mammalian COX activity is mainly regulated by the nuclear subunits, which are absent in bacterial COX. Genomic data has shown that nuclear subunits accumulated on the various eukaryotic lineages after the endosymbiosis (for review see Pierron et al., 2011b). Because the catalytic activity of human COX is not higher than the proteobacterial one, the macro-evolutionary process leading to the doubling of COX's size seems not caused by the selection of a more active enzyme. Instead, the appearance of nuclear subunits could have respond to the need for tight regulation of the COX enzyme by the nuclear genome of eukaryotic pluricellular organisms. We speculate that because endosymbiosis has increased energy production by about 15-fold, selective pressures acting on the new eukaryotic cells were not acting to produce more energy but to produce it when and where necessary, and otherwise to reduce the production of toxic products and by-products of accompanying free radicals.

Recent studies have highlighted that the mammalian respiratory chain has one component less than the eukaryotic ancestral respiratory chain: an alternative oxidase (Mcdonald et al., 2009). This enzyme was a substitute for complexes III and IV (COX), performing the oxidation of CoQ and the reduction of oxygen, but without any proton translocation, and was probably lost at the vertebrate stem (Mcdonald et al., 2009). The function of this enzyme is not clear but it has been shown that in plants this enzyme responded to oxygen concentration stress (Szal et al., 2003). The loss of this COX substitute has probably modified the evolutionary constraint acting on COX. Interestingly, whole genome duplication was another concomitant key event modifying the early vertebrate respiratory chain (Wotton and Shimeld, 2006). Indeed, it has been shown that several COX subunits duplicated at this point have been retained in the subsequent vertebrate genome, allowing the specialization of different isoforms for the same subunit (i.e., the *COX4i1-COX4i2* couple, which are regulated based on the oxygen concentration (Huttemann et al., 2007)). Such gene duplication has been a continuous process throughout mammalian genome evolution, promoting organ specific isoforms for the same subunits (i.e., liver or heart isoforms) to allow tight regulation of COX activity based on organ needs (Pierron et al., 2011b).

X.3 Evolutionary events of primate COX and cytochrome *c*

Despite their relative evolutionary stability, both Cyt *c* and the majority of subunits of COX have accumulated amino acid replacements in the primate lineage at a faster rate than would

be predicted from either their rate during previous mammalian descent or by the slowdown in the mutation rate in primates (Wu and Li, 1985; Bailey et al., 1991; Li et al., 1996). COX appears to have developed the role of its subunits further than the other complexes, adapting them both during development and differentiation. We review here their role in these processes and their evolution.

X.3.1 Expression of subunits

The overall timing of subunit expression reflects the metabolic program of the embryo. In mouse, a Cyt *c* null mutant is able to develop until mid-gestation, signaling the time of demand for oxidative metabolism (Li et al., 2000). After the start of oxidative metabolism, the earliest expression of COX subunits in tissues that are destined after differentiation to produce tissue specific forms is of the ubiquitously expressed isoforms. In an early study, Kadenbach and co-workers compared human fetal tissues from liver, skeletal muscle, heart, and intestine with those of the corresponding adult tissues (Bonne et al., 1993). They found for the contractile tissues heart and skeletal muscle that both COX6A and COX7A transcription between fetal weeks 20-28 was predominantly of the L isoform (*COX6A1* and *COX7A2*), turning on the H isoform (*COX6A2* and *COX7A1*) after birth. Similar timing was seen for subunit 8 (switching from *COX8A* to *COX8B*) (Bonne et al., 1993) and subunit 4 (*COX4I1* to *COX4I2*) (Hüttemann et al., 2001). Depending on the organism and tissue, the developmental isoform is transcribed in addition to, rather than in place of, the initially expressed isoform. For example, there was a complete switch of isoform expression for subunit 6A and a partial one for 7A in human heart and skeletal muscle (Bonne et al., 1993) as well as a partial one for subunit 4 in rodent lung (Hüttemann et al., 2001).

Determining the functional difference between tissue specific isoforms of the same subunit has proven challenging. Little is known about the functions of COX7A and COX8. In the case of subunit 4, the holoenzyme containing COX4I2 was shown to be at least twice as active as that containing COX4I1 (Hüttemann et al., 2007). The transcription of *COX4I2* has been shown to respond to oxygen concentration (Horvat et al., 2006; Fukuda et al., 2007; Hüttemann et al., 2007), supporting the original suggestion that a pair of cysteines in COX4I2, which are absent from COX4I1, could serve as a hypoxia sensor (Hüttemann et al., 2001). The COX6A isoforms have been associated with energy transduction efficiency. The contractile muscle specific subunit COX6A2 contains an adenine nucleotide binding site whose occupancy can result in a change of H^+/e^- efficiency. At low ATP/ADP the H^+/e^- ratio is close to 1, contributing to maximum efficiency of energy transduction, whereas at a high ATP/ADP ratios it is 0.5, which may contribute to thermogenesis (Frank and Kadenbach, 1996; Kadenbach et al., 1998). For COX6A1, binding of palmitate can produce a similar reduction of the H^+/e^- stoichiometry to 0.5, an effect not produced by other fatty acids tested (Lee and Kadenbach, 2001). Thus, in this way, depending on the tissue COX can respond to a readout of either energy supply or metabolic cues and appropriately parse potential energy into heat for non-shivering thermogenesis and chemical energy as ATP. Although these isoforms are also present in invertebrates (with less confidence for COX8) (Little et al., 2010), no information is available about whether comparable isoform specific functions are present.

X.3.2 Subunit duplication

COX is the only one of the electron transport complexes to contain subunit isoforms[1], presumably reflecting its central role in regulation. Each of these appears to have arisen by gene duplication, although in almost all cases the duplicates reside on different

[1]We note that possible paralogs in complex I have been reported.

chromosomes. The subunits currently known to have more than one form are 4, 6A, 6B, 7A, and 8 (Table 1).

Subunit 4 was shown to have a second form in mammals with the discovery of a lung-specific isoform (Hüttemann et al., 2001). As had been previously shown for the muscle-specific isoforms, *COX4I2* is also developmentally induced. Its likely origin during a whole genome duplication (Wotton and Shimeld, 2006) was previously discussed. Its current function may have been molded in part by subsequent increases in the oxygen content of the earth's atmosphere to a level about 50% higher than the present, a level highest in at least the last billion years. The molding of this isoform during a maximum in atmospheric oxygen concentration suggests a role in dealing with high oxygen concentration, a suggestion reinforced by its presence in the lung (and, as subsequently found, in the placenta, the embryonic gas exchange tissue). Its precise function, however, is not yet clear: although functioning in the body's highest oxygen concentration, its transcription is stimulated at 4% oxygen (Hüttemann et al., 2007) and it has been said to adjust respiration in hypoxic cells (Fukuda et al., 2007). As originally suggested (Hüttemann et al., 2001), the presence of cysteines that are absent from COX4I1, and that are appropriately located to serve as a potential disulfide redox sensor, supports a role at oxidizing versus reducing oxygen concentrations and/or high reactive oxygen (ROS) environment.

One difficulty in defining a role is in arriving at an understanding of whether the in vitro determined optimum of 4% oxygen is the normal physiological level in some tissues or whether it represents a state of physiological stress we call hypoxia. For example, in the central nervous system in particular, 4% oxygen has been suggested to represent physiological normoxia (Lamanna, 2007; Chadwick et al., 2011). In lungs oxygen is about 6% in venous blood and lower within tissues (Kinnula and Crapo, 2003). Since no evidence has yet tied COX4I2-containing COX to physiological hypoxia or ischemia, but COX4I2-containing COX has been shown to be a more active enzyme (Hüttemann et al., 2007), it may well be that its main function is to provide a higher turnover to more metabolically active tissues.

Whatever the function carried out by the subunit 4 isoforms, it has arisen more than once: The yeast isoforms COXVa and COXVb have been proposed to be homologous to the mammalian COX4 pair but it is clear from the evolution pattern (Hüttemann et al., 2007) that they arose independently. Furthermore, for the mammalian pair, a recent analysis suggests that *COX4I2* is the ancestral gene and that *COX4I1* arose by duplication (Little et al., 2010).

Little is known about COX6B2 function. COX6B connects the COX monomers in its physiologic (dimeric) form. Its existence as a testis-specific isoform (Hüttemann et al., 2003) is satisfying since Cyc *c*, which has a testis isoform in mammals, appears to bind to COX by first interacting with COX6B (Sampson and Alleyne, 2001; Hüttemann et al., 2003). However, Cyc *c* testis (Cyct) is lost at the stem of primates (Pierron et al., 2011a) whereas *COX6B2* is not. Interestingly, in rodents, which express Cyct, Cox6b2 is the exclusive testes form; humans, which have lost Cyct, express both COX6B isoforms in testes.

The remaining isoforms, discussed in Section 3.1, are contractile muscle-specific. However, they are not the exclusive isoform in contractile muscle. Detailed studies are lacking in cases where both isoforms are expressed in the same tissue as to whether there is positional segregation within the cell. It is also unknown whether any hybrid forms occur of the dimer, which under some conditions is the more stable form of COX (Stanicova et al., 2007).

X.3.3 Subunit silencing in primates

The presence of a testes-specific Cyt *c* is well known (Hake et al., 1990). It was lost on the primate stem about 65 mya via a nonsense mutation that is present in all primates. The loss of the testes isoform produces lower fecundity (Narisawa et al., 2002). A detailed recent analysis of the somatic form of Cyt *c* across primates suggested parallel evolution in both the platyrrhine and catarrhine stems (Pierron et al., 2011a). Examination of the sites of evolutionary changes suggests they are focused on the respiratory chain rather than on other Cyt *c* functions, such as apoptosis. In addition to Cyt *c*, a COX subunit was lost as well. The smallest COX subunit, COX8, is present as an L and an H isoform throughout mammals. Where this has been examined — in cow (Yanamura et al., 1988; Lightowlers et al., 1990) and rat (Kadenbach et al., 1990) — subunit 8 is expressed in heart exclusively as the H form. COX8H (COX8B) is also present in strepsirrhine primates (lorises and lemurs) and platyrrhine primates (New World monkeys). Surprisingly, however, COX8B is absent in catarrhines (Old World monkeys, Apes, including human) (Goldberg et al., 2003), where exon 2 has been replaced by repeat elements. Of the three contractile muscle isoforms, perhaps the least is known about the function of COX8. Based on an evolutionary study, *COX8A* (the ubiquitous or L form) evolved under the force of positive selection, including at apparently functionally important positions, in several anthropoid terminal lineages (Goldberg et al., 2003). These changes presumably subsumed the advantages that the H isoform provided to contractile tissues.

X.3.4 Amino acid replacement and “rapid evolution”

The original discovery that human *COX4I1* evolves more rapidly than expected (Lomax et al., 1992) led to more detailed examination of its evolution (Wu et al., 1997; Wildman et al., 2002) and later that of other subunits of cytochrome oxidase and then of complex III (Grossman et al., 2001; Doan et al., 2005). The COX genes found also to show accelerated evolution in primates are subunit I (Wu et al., 2000), II (Adkins and Honeycutt, 1994), 5A (Uddin et al., 2008), 6B (Doan et al., 2004), 6C (Doan et al., 2004), 7A2 (Schmidt et al., 1999), 7C (Doan et al., 2004), and 8A (Goldberg et al., 2003).

For a given subunit, the designation rapid evolution is based on the ratio of a standard measure at each branch of the phylogenetic tree of its non-synonymous substitution rate (K_A) and its synonymous substitution rate (K_S). The conclusion of positive selection rather than reduction of evolutionary constraints on replacement for these subunits are based on observing a period of increased K_A/K_S followed by a reduction — that is, positive selection followed by purifying selection (Goodman, 1982). A K_A/K_S ratio >1 is in accord with the generally accepted criterion of positive selection and is observed explicitly for several subunits (Wu et al., 1997; Wildman et al., 2002). For most subunits, however, K_A/K_S is <1 in anthropoid primates but significantly higher than seen in other mammals. As discussed in Section 4.4, we have interpreted this as representing positive selection of highly conserved proteins that are undergoing adaptive evolution in the presence of functional constraints.

Although the amino acid replacements can be documented, their functional significance awaits a better understanding of subunit function. The best developed picture stems from the insight that a moderate fraction of the amino acid replacements in mammals are concerned with the binding of Cyc *c* to COX, and that the nature of the interaction between Cyc *c* and COX has changed in anthropoid primates (Schmidt et al., 2005). Both simulated binding (Roberts and Pique, 1999) and enzyme kinetic analysis (Osheroff et al., 1983) established that the binding between Cyc *c* and COX is electrostatic. Schmidt et al. (2005), using structural data for Cyc *c* and COX and an interaction model (Roberts and Pique, 1999; Zhen et al., 1999), identified 57 of the >1500 COX residues that take part in binding Cyc *c*. At least 27 of these 57 residues were replaced in the anthropoid primate lineage; what is more,

22 of these 57 residues are charged and 11 of the 22 have been replaced within anthropoids by uncharged residues. The binding of Cyc *c* to COX thus appears to have gone from primarily electrostatic to more hydrophobic. The effect of this remarkable change on the intrinsic reaction parameters has not been investigated so that it remains unclear how this restructuring of the binding site has benefitted anthropoid primates.

X.3.5 Xenocybrids and coevolution

The introduction of transmitochondrial cybrids (Wallace et al., 1975) allowed dissecting nuclear versus mitochondrial contributions to phenotype (e.g., Hayashi et al., 1991). The ability to replace cytoplasms was soon applied also to co-evolution. One example was for assessing the ability of human nuclear mitochondrial genes to interact productively with mitochondrial genes from increasingly diverse species (Kenyon and Moraes, 1997). Kenyon and Moraes found that they could replace human mtDNA with that from chimpanzee, bonobo, and gorilla with resultant oxygen consumption of the cell lines roughly equal and about 80% the value of the human parental line. However, when human mtDNA was replaced with that from species that diverged as recently as 7-14 mya, no oxygen consumption could be demonstrated. This was surprising compared to the expected model of oxygen consumption falling off gradually with increasing evolutionary divergence.

Examination of the mtDNA changes showed about twice as many amino acid replacements between orangutan and human as was found between the chimpanzees or between gorilla and human. With respect to the electron transport chain complexes, the least were found for complex IV. When considered in light of the accelerated evolution reported for many of the nuclear encoded subunits of complexes III and IV during primate descent, it seems likely that the coevolution taking place between subunits of a complex coded by the mitochondrial and nuclear genomes, which presumably acts to optimize protein-protein, protein-RNA, and protein mtDNA interaction, and therefore function, has become a victim of co-evolution when genomes from different species are mixed. The same effect possibly occurs in a more limited way in somatic cell nuclear transfer (Evans et al., 1999).

To investigate the basis of the abrupt drop-off in function after 7-14 mya of evolutionary separation, transfer of orangutan chromosomes into human cells lacking mtDNA (ρ°) and repopulated with orangutan mtDNA was investigated (Barrientos et al., 2000). Hybrids that produced some respiratory function were only seen containing many orangutan chromosomes. When examined for activity of individual complexes, they showed a complex IV deficiency. Furthermore, more detailed analysis showed normal synthesis but defective assembly of complex IV components. The most consistent hypothesis was that human components were exerting a dominant negative effect on attempted assembly of an orangutan enzyme so that the low level of activity found resulted from homologous association. Furthermore, it can be speculated that the lack of interference with the other complexes resulted from the higher evolutionary divergence seen for them (Kenyon and Moraes, 1997), such that the loose association of interspecies intermediates during the assembly process of the complexes would have been avoided. These studies suggest that as little as seven million years is sufficient to change key amino acids that can disrupt harmonious assembly and function of these interacting proteins.

The greater evolutionary change seen in complex I components was utilized to study this effect and its role on cell physiology in some detail. Given that there appears to be excess complex I activity, and that reducing it has potential effects on ROS production, mitochondrial membrane potential, and apoptosis, the coevolution of the complex's subunits to optimize function is a clear advantage for long lived primate species. This was emphasized in detailed studies of complex I deficiencies in transmitochondrial hybrids. In human-chimpanzee hybrids, which suffered a 20-30% decrease in oxygen consumption,

only complex I was affected (Barrientos et al., 1998). Furthermore, decreased complex I impairment was correlated with free radical (ROS) production, mitochondrial membrane potential, and apoptosis (Barrientos and Moraes, 1999).

The steep effects of evolutionary distance observed for primates were reproduced qualitatively but were quantitatively less significant for rodent xenomitochondrial hybrids. When mtDNA-less cells derived from the common mouse (*Mus musculus domesticus*) were fused to cytoplasts prepared from *Mus musculus*, *Mus spretus*, or rat (*Rattus norvegicus*), a comparable number of respiring clones could be obtained (Dey et al., 2000). However, mouse xenomitochondrial cybrids harboring rat mtDNA had a slower growth rate in medium containing galactose as the carbon source, suggesting a defect in oxidative phosphorylation. Nevertheless, mitochondrial protein synthesis was unaffected. The fact that mild defects could be seen in rodent xenomitochondrial hybrids (Pinkert and Trounce, 2002; Trounce et al., 2004), coupled with the difficulties of direct alteration of the mitochondrial genome, led to attempts to use such hybrids as animal models for mitochondrial dysfunction (Cannon et al., 2011; Dunn et al., 2011). The impact thus far has been limited, however, owing to lack of an overt phenotype.

Although the present focus is on primates, it is interesting to consider that mitochondria–nuclear co-adaptation has been ongoing probably since the time of the origin of mitochondria. A well-studied and useful system has been the co-evolution of nuclear and mitochondrial genes in the copepod *Tigriopus californicus*. When the mitochondria from one population are placed by repeated backcrosses into the nuclear background of a different population, reduced COX activity is observed, suggesting that co-adaptation has occurred (Edmands and Burton, 1999). This result could be confirmed by showing that Cyc *c* from each population functioned optimally when the COX from the same population was also present (Willett and Burton, 2004). In some cases, incompatibilities between populations were present that could be traced to a single amino acid replacement in Cyc *c* (Harrison and Burton, 2006). The incompatibilities observed in hybrids led Burton and his collaborators to suggest that the classical reduced fitness of F2 hybrids may stem from reduction of the co-evolutionary optimization of mitochondrial function in the parental strains (Burton et al., 2006; Ellison and Burton, 2008).

X.4 Evolutionary mechanism of primate COX

It is clear that most of the COX subunits and Cyt *c* proteins have evolved rapidly during primate evolution. The reason for this rapid evolution is less clear, however, and the specific function acted upon by evolution is least clear. Indeed, this evolution is necessarily due to the combination of several evolution mechanisms.

X.4.1 Mutation rate

The accumulation of amino acid changes on the proteins could be due to the imperfect replication of DNA by DNA polymerase. While copying a protein-coding locus, the polymerase can by chance substitute one nucleotide for another and thereby modify a codon. When the codon substitution results in the replacement of the encoded amino acid during protein synthesis the mutation is called “non-synonymous”; however, due to the redundancy of the genetic code, the substitution sometimes does not result in a change to the encoded amino acid sequence and thus is called “synonymous.”

When one mutation event occurs in a germinal stem, the new nucleotide is transmitted to the offspring and so two alleles of this position exist in the same population, the new nucleotide (in the offspring) and the ancestral nucleotide (in the other individuals of the population). Sometimes the new nucleotide spreads over the rest of the population and the ancestral

nucleotide disappears. In this chapter the term substitution event defines this fixation of the new allele in the population. By assuming that the fixation of a synonymous mutation or a mutation on a non-coding position is not subject to selective pressure, it often assumes that the rate of synonymous substitution is proportional to the mutation rate.

Nucleotide mutation is often seen as a random binomial event, with accumulation following Poisson's distribution. However, although the accumulation rate of non-coding and synonymous substitutions is variable, primates appear to have a slower rate of substitution than rodents (Wu and Li, 1985; Li et al., 1996) and hominoids have a slower rate than other primates (Wu and Li, 1985; Bailey et al., 1991; Steiper et al., 2004). By studying intron (non-coding sequence) and synonymous substitutions on isoform 1 of COX4 (*COX4i1*), Wildman et al. have shown that neutral substitution slowdown was occurring in parallel with the acceleration of non-synonymous substitution on COX genes (Wildman et al., 2002). Therefore, the rapid accumulation of amino-acid replacement on COX is not due to an increase of the mutation rate. Surprisingly, in fact, the rapid accumulation of amino acid replacement on COX appears to be opposite to the general slowdown of evolution observed throughout the primate genome compared to the rodent genome.

The primate substitution slowdown was much more drastic for mitochondrial DNA (mtDNA) than for nuclear DNA (nDNA) (Nabholz et al., 2008). Consequently, the mutational ratio has changed between the nuclear and mitochondrial genomes. In Muridae the mitochondrial substitution rate can be 100 times higher than the nuclear one, whereas this ratio is lower than 20 in Hominidae (Nabholz et al., 2008). Nabholz and collaborators have proposed that this could be due to the divergent phenotypic evolution between rodents and primates.

The main hypothesis regarding the nDNA primate slowdown is the increase of generation time (Li et al., 1996; Tsantes and Steiper, 2009). Indeed, mutation accumulation occurs during germinal cell duplication. Because the number of germinal cell duplications by generation is almost constant between mammals, the greater the number of generations in a time period, the higher is the number of mutations accumulated during this period. The number of generations on the spermatozoid lineage is much greater than on the egg lineage; thus, most of the mutations occur on the spermatozoid lineage. This "male-driven model" is supported by the fact that the locus on chromosome Y only transmitted by the spermatozoid lineage has a higher mutation rate than other loci (Li et al., 1996).

Interestingly, a study on strepsirrhines has shown a particularly strong correlation between generation time and nDNA neutral replacement accumulation rate but found a lesser correlation with the mtDNA substitution accumulation rate (Tsantes and Steiper, 2009). It seems logical that mtDNA escapes in the male-driven generation time model because of its maternal inheritance. Instead of an impact of spermatozoid stem cell division, it has been proposed that the high mtDNA mutation rate observed in rodent could be directly due to the amount of ROS produced by the high metabolism rate of these species (Pamplona and Barja, 2007; Min and Hickey, 2008). However, a higher metabolism rate does not necessarily imply higher free radical production and higher free radical production does not directly imply a higher mutation rate (Galtier et al., 2009a). Samuels et al. have instead proposed that mtDNA mutation rate itself could be subject to specific selective pressures due to lifespan increase (Samuels, 2004). The neutral mtDNA substitution rate is quite variable across the species of short-lived mammals such as rodent, but constrained to low values in long-lived mammals such as some primates (Nabholz et al., 2008). The low mutation rate of these animals may be an adaptive phenomenon to avoid the accumulation of deleterious mutations on important mtDNA genes, such as mt-COX subunits, on somatic cells across a long life (Galtier et al., 2009a). This type of selective pressure could act on the replication mechanism

and it would be interesting to compare the accuracy of gamma polymerase between rodent and primate species but to our knowledge data allowing this kind of comparison are not yet available. Thus, the observed primate mutation slowdown for mtDNA encoded subunits is maternally driven, possibly constrained by the long lifespan phenotype of primates, whereas the mutation slowdown for nuclear encoded COX subunits is a paternal-driven phenomenon passively due to the increased generation time. What is clear is that the mutation accumulation rate on COX primate genes is not random but is closely linked to the evolution of primate phenotypic life traits.

X.4.2 Mutational bias

Another major difference between nDNA and mtDNA is that mtDNA presents a strong bias in base composition between the two strands. The transcribed strand (for mt-COX genes) is C-rich and called the light strand based on its buoyant density in a CsCl gradient, whereas the other is G-rich and called the heavy strand. This compositional asymmetry is due to a mutation bias on heavy strand cytosine to thymine (C ->T) and adenine to guanine (A->G) as shown on the rat phylogeny of the mt-COII gene (Brown and Simpson, 1982) and confirmed by later studies on vertebrates (Reyes et al., 1998; Faith and Pollock, 2003). As proposed by Brown and Simpson (1982), the nucleotide bias could be due to mode of mtDNA replication, asymmetric and slow. Indeed, during mtDNA replication the heavy strand remains single-stranded for a long time and is exposed to oxidative damage, increasing the deamination of cytosine and adenine, causing transitions to thymine and guanine. This hypothesis is supported by the fact that the proportion of these transitions is related to the time that the heavy strand remains single-stranded during replication (Reyes et al., 1998; Faith and Pollock, 2003). The mt-COX locus is slightly less sensitive to the phenomenon since it is close to the second replication origin and thus remains single-stranded a shorter time.

Anthropoid primates show a stronger compositional shift, with an enrichment of C on the light strand compared to other mammals and non-anthropoid mammals (Schmitz et al., 2002; Gibson et al., 2005). By studying synonymous mutations on the human mtDNA phylogeny, Kivisild *et al.* have confirmed a strong mutational bias with 250 transitions of T->C and only 162 C->T despite an already biased composition of 596 codons NNT and 1444 codons NNC (Kivisild et al., 2006). Interestingly, the transitions A<->G equilibrate with about 230 transitions in each direction, explaining the finding that the percentage of G is constant across mammals (Gibson et al., 2005).

By comparing mtDNA sequences among different orders of mammals, Min and Hickey have shown that the C enrichment is not primate specific but instead is significantly correlated with longevity and generation time (Min and Hickey, 2008). No mechanisms have been proposed to explain this correlation yet. Nevertheless, it means that if mutations due to the asymmetric replication are proportionally more important in long-lived species, then some other mutational mechanisms such as inherent misincorporation rate of gamma polymerase has proportionally decreased. This provides a first clue to the selective pressure acting on the global decrease of mutation rate observed in long lifespan species. Finally, it appears that the evolution of primate life traits has strongly influenced the mutation rate and the composition bias of mt-COX genes.

The strong mutation bias observed in primates should necessarily affect the amino-acid composition of mtDNA encoded subunits. Indeed, Gibson et al. have shown a strong correlation between the compositional bias of cytosine (measured by the third position composition) and the amino acid composition. On the most cytosine biased genome, they have highlighted the over-representation of amino acids with a C in the codon position (leucine (CTN), proline (CCN), histidine (CAY), threonine (ACN)), and an

underrepresentation of those with T (isoleucine, methionine, serine, tyrosine, phenylalanine) (Gibson et al., 2005). Consequently, in primates Schmitz et al. have shown that non-anthropoid primates such as Tarsius and slow loris exhibit an affinity toward the amino acids isoleucine, lysine, phenylalanine, and tyrosine, which are encoded by T-rich codons, compared to the rest of primates (Schmitz et al., 2002).

It has been shown that mammalian and primate lifespans exhibit a positive correlation with the mtDNA frequency of cysteine and negative correlation with the mtDNA frequency of threonine (Kitazoe et al., 2008; Moosmann and Behl, 2008). The first hypothesis was that it was a functional adaptive response to reduce free radical production. However, because this correlation has been seen only on the mt-COX genes and not on nuclear encoded COX genes, Jobson et al. argue that it is a passive phenomenon due to the shift of cytosine mutation pattern linked to increased lifespan (Jobson et al., 2010). Nevertheless, the two hypotheses are not exclusive and both link limitation of deleterious mutations on mtDNA encoded genes with long lifespan. Primate mt-COX gene evolution appears to be strongly constrained by the imperative of limiting deleterious mutations.

X.4.3 Negative selection, recombination and effective population size

MtDNA encodes the COX catalytic subunits, which are highly conserved across eukaryotic life. The evolution of these subunits is constrained by strong negative selection — pressure against the spread of new deleterious mutations. This negative selection has to be particularly strong because of the high and biased mutation rate of mtDNA.

Paradoxically, population genetic theory predicts that negative selection should be less efficient on a mtDNA encoded subunit than on a nuclear encoded subunit due to the fact that recombination on primate mtDNA can be considered as marginal (Galtier et al., 2009b). Indeed, maternal inheritance of mtDNA encoded subunits reduces the effect of negative selection in two ways: (i) by reducing the effective size of the population, so that the fluctuation of mutation frequency is more influenced by random events and less by selective pressures. This larger random effect allows deleterious mutations to become fortuitously fixed despite the negative selection; (ii) by excepting reversion, recombination is the only way that offspring do not inherit parental deleterious mutations.

As a consequence, mtDNA of primate lineages should be accumulating deleterious mutations irreversibly, and eventually be condemned to a meltdown. This phenomenon, known as Muller’s ratchet, is indeed one of the proposed evolutionary advantages for recombination in the nucleus (Muller, 1964; Felsenstein, 1974).

Despite this theoretical paradox, the evidence shows that deleterious mutations are actually rapidly removed. Fan et al. have shown severe mutation (on ND6, complex I) to be selectively eliminated during oogenesis within four generations (Fan et al., 2008). Similarly, Stewart et al., following the mtDNA for a few generations of mutator mice expressing a proofreading-deficient mitochondrial DNA polymerase, have shown a greater accumulation of synonymous than non-synonymous mutations (Stewart et al., 2008). This is due to a rapid and strong elimination of non-synonymous changes in protein-coding genes, a hallmark of negative selection. This result shows that negative selection against mutations in mtDNA protein-coding genes is strong enough to be monitored. Interestingly, they found that the negative selection occurred more strongly on mt-COX I and mt-COXII than on any other mtDNA encoded gene.

These results suggest that deleterious mutations are rapidly removed under efficient negative selection. It not clear why, but a simple model would be that due the importance of mtDNA-encoded subunits in cell metabolism, mtDNA mutations are directly deleterious for the cell

lineage and this lineage disappears by itself or due to competition with other cells. This scenario is strengthened by the fact that mt-COI of cancerous cell lines accumulates much more synonymous than non-synonymous mutations, suggesting strong purifying selection against mt-COI even for cells less dependent on OxPhos to produce energy (Stafford and Chen-Quin, 2010). This result confirms the importance of mt-COI because there was an absence of selection on mt-COIII, NAD3, and NAD4L. Others have proposed a role of the bottleneck phenomenon (drastic reduction of mtDNA copy number during oogenesis) (Wai et al., 2008; Wai et al., 2010); however, its role as a potential promoting or limiting factor for negative selection is still unresolved (Neiman and Taylor, 2009).

Whatever the cause, deleterious mutations do not ordinarily spread in the population. The frequency of common mtDNA deleterious mutations such as A3243G is due to mutational hot–spots and not to inefficient negative selection (Pierron et al., 2008). However, the fate of mildly deleterious mutations is less clear. For example, deleterious LHON mutations on complex I can spread over several human generations (Carelli et al., 2006). This result was confirmed also on COX genes: the mouse V421A mutation on mt-COI can be transmitted for numerous generations even if the mice are showing mitochondrial pathology symptoms (Fan et al., 2008). Recent results suggest that mildly deleterious mutations can spread across a population to achieve a state of polymorphism before being eventually eliminated. Indeed, based on amino-acid properties, young mtDNA polymorphisms are on average more deleterious than old polymorphisms, suggesting a long term effect of negative selection (Pereira et al., 2011). The long term effect of negative selection on mtDNA is in agreement with the current explanation of the phylogenetic observation that younger lineages seem to accumulate mutations faster than older stems (Soares et al., 2009).

Nevertheless, deleterious mutations can fortuitously reach fixation. Furthermore, such fixation of deleterious mutations on mtDNA are more frequent in large animals than in small animals (Popadin et al., 2007). The reason proposed is that larger animals have a smaller effective population size. And the smaller is the population, the stronger is the random effect. Therefore, the accumulation of deleterious mutations could have accelerated during the primate lineage along with the mass increase and the decrease of population size. This raises the possibility that the observed rapid replacement of several amino acids of COX is due to a relaxation of negative selection constraints.

X.4.4 Positive selection

The Ka/Ks ratio is often used to test whether an accumulation of amino-acid replacements is due to a relaxation of negative selection or due to positive selection, as in the case of COX (see for review Harris, 2010). The Ka/Ks ratio is the ratio of non-synonymous replacement over synonymous replacements. As noted earlier, because synonymous replacements do not impact the protein amino-acid sequence, these replacements are considered as minimally affected by any selective force and their accumulation can be considered as a neutral marker of the mutation rate. By contrast, non-synonymous replacements change the amino-acid sequence and potentially impact phenotype and fitness. A Ka/Ks below 1 indicates that non-synonymous accumulation is slower than synonymous replacement accumulation. This case can be interpreted as non-synonymous replacements being subject to negative selection and therefore reaching fixation less often than synonymous replacements randomly reaching fixation. A ratio of Ka/Ks over 1 indicates that non-synonymous replacements reach fixation more often than neutral synonymous replacements, a signal of positive selection.

Although this principle is broadly accepted, it is important to realize the reverse principle is not true. Indeed, a Ka/Ks ratio under 1 does not necessarily reveal the absence of positive selection and conversely a Ka/Ks ratio over 1 does not necessarily mean an absence of negative selection on particular sites.

In agreement with the importance of COX, Ka/Ks on its genes are very low across the mammals, confirming the strong negative selection (Uddin et al., 2008). However, on primates a Ka/Ks higher than 1 has been shown for several subunits (Wildman et al., 2002; Doan et al., 2004). This result clearly shows positive selection on these subunits. For other subunits, Ka/Ks is higher in primates compared to other mammals, but somewhat lower than 1. For these subunits it could be tempting to conclude that Ka/Ks close to 1 means that these proteins are not affected by selective pressure constraints, either positive or negative, but evolve neutrally. However, “neutral” evolution is quite rare and is a short-term state for a protein because it implies the random replacement of amino acids in the sequence. Such random evolution will eventually change the functionality of the protein and allow appearance of a nonsense mutation. Because deleterious mutations on COX genes induce life-threatening symptoms due to mitochondrial pathology, we speculate that a relaxation of negative constraints on these subunits is not likely. Therefore, we propose that the Ka/Ks near 1 observed on primate phylogeny shows the existence of positive selection on these very conserved proteins, such as cytochrome *c*.

Recently Osada and Akashi (2011) proposed that the positive selection acting on the nuclear subunits results from compensatory evolution due to an accumulation of deleterious mutation on mt-COX genes. Indeed, they argue that only nuclear-encoded components of COX show evidence for positive selection. However, this result is inconsistent with a previous finding (Wu et al., 2000) and may have resulted from the choice of species (5 anthropoid primates and 2 non primates but no non-anthropoid primates), missing the evidence of positive selection on mt-COI on the anthropoid stem. Taking all the evidence together, we propose that rapid evolution and positive selection on both nuclear and mitochondrial subunits reinforces the notion of adaptive selection acting on the whole COX complex.

X.5 COX and primate phenotypic evolution

If we allow that the accumulation of amino acid substitutions on COX during the primate lineage is due to positive selection, the next issue is to identify the selective pressure(s) that has acted on COX. It would be tempting to begin by considering that because COX rapid evolution is specific to primates, the selective pressures should also be specific to anthropoid primates. However, for similar pressures it is not unlikely that different taxons can develop different adaptive strategies. For example, amino acid replacement on COX during anthropoid diversification is co-occurring with a change of oxygen concentration in the atmosphere (Falkowski et al., 2005); because oxygen is a substrate of COX, the two events could be linked. But does the fact that other mammals do not present the same COX evolution invalidate this hypothesis? There is no obvious answer to this question and it may well be difficult to move it beyond the speculation level.

Instead we propose here a broader view, to study how COX evolution can be integrated in two major phenotypic primate evolution processes: (i) the history of life evolution, and (ii) brain enlargement.

X.5.1 History of life

As we have seen, COX evolution was closely linked to the evolution of history trait of life in primates. Due to mutation bias, the mutation rate decrease and the lifespan increase has shaped the COX mtDNA subunits. However, due to OxPhos ROS production, COX has the ability to influence its own mutation rate and mutation bias. Indeed, COX is one of the respiratory chain regulators and thus influences free radical production (Pacelli et al., 2011). It is worth noting that the other OxPhos complexes have also experienced a rapid evolution in primates. Galtier et *al.* have proposed that the decrease of mtDNA mutation rate was

positively selected along with the increase of lifespan (Galtier et al., 2009a). The effect of OxPhos primate evolution on ROS production has to be investigated in order to know if COX evolution could be due to the selective pressure favoring the decrease of mutation rate on somatic cells.

The primate substitution slowdown suggests that this evolution has also led to a decrease of ROS production in the female germ line. In contrast (but not in contradiction), we suggest that evolution of the Cyt *c*-COX couple in primates allowed an increase of ROS production in the male germ line. Indeed, we have shown that a Cyt *c* testis isoform was present in ancestral mammals and it became a pseudogene before primate differentiation (Pierron et al. 2011a). Interestingly, when Cyt *c* testis isoform is silenced in knock-out mice, these mice produce functional but less efficient spermatozoids due to reduced free radical scavenging (Narisawa et al., 2002; Liu et al., 2006). Even if mice are not the best model for ancestral primates, this result suggests that one function of the Cyt *c* testis isoform is to maintain efficient spermatozoids. We propose that the loss of testis Cyt *c* in primates could be due to a decrease of selective pressure for efficient spermatozoids on primates compared to rodents. This idea is supported by the fact that primates indeed have a generally smaller litter size and reduced offspring number compared to rodents (De Magalhaes and Costa, 2009). It remains to be seen, however, how the ROS scavenging ability of the single primate Cyt *c* compares with the mouse somatic and testis enzymes.

Lifespan, reproduction age, reproduction rate, and population size are parameters that have drastically changed during primate evolution. Because numerous authors have linked these life history parameters to metabolism evolution, we propose that these parameters should be seen as both potential cause and consequence of OxPhos evolution and particularly the couple COX-Cyt c (Figure 1).

X.5.2 Brain enlargement

The human brain is approximately six times larger in mass than is expected for a mammal species of its body mass (Jerison, 1973). This increase in relative brain mass has a long phylogenetic history. Indeed, all anthropoid primates (i.e., New and Old World monkeys and apes including humans) are at least twice as encephalized as would be expected for creatures of their size, and besides *Homo*, capuchin monkeys (*Cebus spp.*) are the most encephalized mammalian genus (Boddy et al., in review). Therefore, the process of encephalization must have begun at least 40 million years ago at the time of the last common ancestor of anthropoid primates, and it has continued at variable rates in a wide range of primate taxa such as humans. It should be noted that the encephalization that characterizes humans is due more to expansion of the neocortex than expansion of phylogenetically more ancient brain regions such as the cerebellum (Clark et al., 2001). In general, the human neocortex can be characterized as A) larger than expected given the species body mass; B) possessing a significantly more convoluted prefrontal cortex than is seen in other species; and C) containing a high glia/neuron (i.e., white matter/gray matter) ratio than is found in other anthropoids (Stephan et al., 1988; Rilling and Insel, 1999; Sherwood et al., 2006). This increase in the relative amount of white matter in human evolution may serve to enable more connections among the existing neurons. This finding implies that the human neocortex does not have a greater number of neurons associated with its recent expansion, but rather there are more connections among the existing neurons, and it has been proposed that this increase in connectivity enables the associative prowess connected with higher human cognitive abilities (Sultan, 2002). It is clear that the expanded neocortex present in humans requires more energy in the form of ATP, but little is known regarding the evolution of energy production in glycolysis and oxidative phosphorylation in neurons vs. glial cells. A promising area of research would involve the quantification and in vivo imaging of mitochondrial activity in the brains of various mammalian species. Studies of gene

expression (Caceres et al., 2003; Uddin et al., 2004) have demonstrated that human mRNA expression is upregulated in genes categorized as being involved in aerobic metabolism and neuronal function in humans relative to other primates, but these studies have yet to be confirmed at the protein level and in vivo.

The brain is a metabolically costly organ, and as such, it has been proposed that the expansion of the human brain came with the price of reducing the metabolic needs of other organs such as the gut (Aiello and Wheeler, 1995). Some have argued that the massive encephalization seen on the human lineage after divergence from chimpanzees was facilitated by the emergence of cooking, and that this innovation both freed up metabolic energy for the brain and enabled early humans to spend more time engaged in social activities instead of chewing food for a significant portion of their waking hours (Wrangham, 2009). One recent study compared the weight of visceral organs to brain size in 100 mammal species, but found no negative correlation between brain size and the size of the digestive tract (Navarrete et al., 2011). Instead, in line with Wrangham's proposal, these authors suggested that the evolution of increased brain size in humans was facilitated by the stabilization of energy inputs made possible by the advent of efficient foraging strategies, cooking, and the use of tools. Indeed, the Navarette study indicated that a negative correlation between adiposity and brain size was observed in non-primate mammals, although that study did not sample the highly encephalized and adipose rich cetaceans. Regardless, humans do possess several means for increasing their energy intake, and it is possible that these innovations played some sort of role in providing the raw material for fueling the energetically costly brain.

The human brain requires energy to function, and much work has suggested that humans possess distinctive aspects of the glucose metabolism pathway. PET studies have shown, on a mass-specific basis, that certain neonatal brain regions consume less glucose than the adult brain (Chugani et al., 1987). Cerebral metabolic rate later exceeds that of the adult by roughly 2-3 years of age and, in at least some regions (e.g., cortex), remains 150-200% higher than the adult brain until late childhood or early adolescence. That human brain metabolic requirements change as a function of age suggests that metabolism of humans has undergone dynamic evolution. In addition to adaptive evolution of the components of the electron transport chain (reviewed in Grossman et al., 2004), the promoters of genes involved in glucose metabolism have been shown to have evolved adaptively during human evolution (Haygood et al., 2007).

Another aspect of molecular evolution that bears directly on energy metabolism in the human brain involves glutamate dehydrogenase, a key mitochondrial enzyme in cellular energy metabolism that plays a role in generating ATP through the Krebs cycle. Humans and other apes possess two genes encoding glutamate dehydrogenase whereas other species of mammals contain only one gene (*GLUD1*) (Burki and Kaessmann, 2004). This hominid-specific gene (*GLUD2*) is specifically targeted to the mitochondria due to positive selection in its targeting sequence (Rosso et al., 2008). Conversely, GLUD1 is found in both the mitochondria and cytoplasm, Moreover, it has been posited that the functional role of *GLUD2* is increased in astrocyte metabolism of glutamate (Burki and Kaessmann, 2004), and Rosso et al. (Rosso et al., 2008) further suggest that *GLUD2* has evolved a more positively charged targeting sequence in order to compensate for the relatively low mitochondrial membrane potential in astrocytes compared to tissues where membrane potential is higher (e.g., heart). Taken together, these findings indicate that many aspects of metabolism that are crucial for proper neuronal function have evolved adaptively during primate evolution. It is further interesting, but not thus far connected to brain metabolism, that glutamate dehydrogenase was previously identified as a (bovine) 3′-UTR RNA binding

protein for the mRNAs of *COX6A1*, *COX7A2* and *COX8A* (Preiss et al., 1995; Preiss and Lightowlers, 1993).

X.6 Conclusions

The special role of COX and Cyt *c* in terms of isoform expression, allosteric control, and phosphorylation has been emphasized in chapter X, "Phosphorylation of mammalian cytochrome *c* and cytochrome *c* oxidase in the regulation of cell destiny: respiration, apoptosis, and human disease." In addition to the cited properties, each also shows an evolution pattern that is notable. One of the notable features is the degree to which changes have taken place at the stem of anthropoid primates or within primate lineages. Cyt *c* has undergone several periods of accelerated evolution followed by consolidation (purifying selection) in which replacements were concentrated in regions that participate in OxPhos (Baba et al., 1981; Pierron et al., 2011a) and in addition the testes specific isoform that is present in mammals becomes inactivated at the stem of anthropoid primates (Pierron et al., 2011a). For the 13 subunits of COX, 10 have undergone accelerated evolution in anthropoid primates (Grossman et al., 2004).

The functional nature of the primate evolution changes is not clear. Of the approximately 1500 amino acids in COX, about 20% or 300 were replaced in anthropoid evolution. Of these replaced residues, nearly 10% or 27 are part of the COX binding site for Cyt *c.* Furthermore, of the binding site residues, 12 out of 22 were charged and are replaced by noncharged residues. Thus, a significant product of anthropoid evolution for the COX and Cyt *c* couple has been a reconfiguration of the binding interface from an electrostatic to a more hydrophobic surface. How this has modified their functional properties is an ongoing puzzle.

The temporal correlation between the evolution of the electron transport chain and the expansion of the neocortex, which after heart and kidney is the most energy utilizing tissue per gram, has led us to develop a model in which the events are coupled (e.g., Grossman et al., 2004). This correlation is also supported by the increased glia to neuron ratio with brain size (Sherwood et al., 2006). Glia-produced lactate appears to be able to cross-feed adjacent neurons to supply an oxidative energy source (Ames, 2000). Anthropoid primates such as humans, in addition to high energy requirements to take account of their size (Isler and Van Schaik, 2006; Hasenstaub et al., 2010) and complex social interactions, are also long-lived and thus require long-lived neurons (i.e., ones that operate in a low radical environment). At the end of the day, it may be that what has been most refined is the ability to precisely regulate energy expenditure, the brain's most valuable resource, in time and space.

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X.8 References

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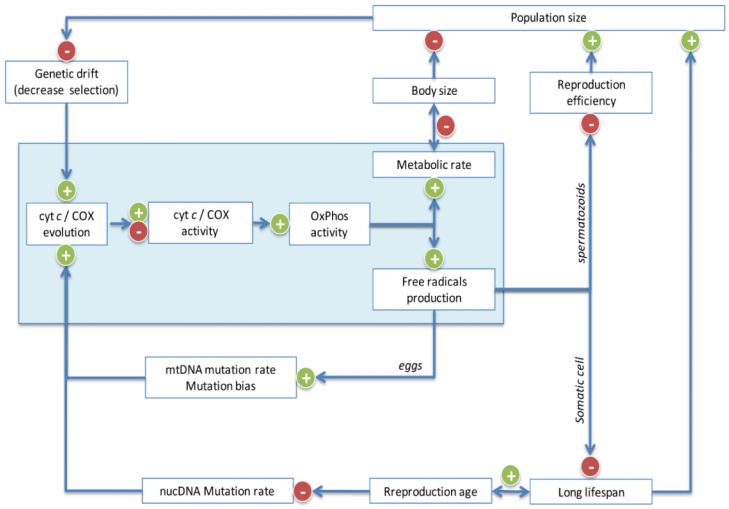

Figure 1.
Putative influences of the Cyt *c* / COX couple on their own evolution through non-selective processes.

Table 1

Isoforms of cytochrome *c* oxidase. Known sites of expression of tissue-specific isoforms are shown in Tissues column. (P) indicates loss of that gene as pseudogene in human and some ancestral lineages.

Subunit	Ubiquitous	Tissue-specific	Chr	Tissues	Ref
4	*COX4I1*	*COX4I2*	16, 20	Lung, placenta	*1, 2*
6A	*COX6A1*	*COX6A2*	12, 16	Heart, skeletal muscle	*3, 4*
6B	*COX6B1*	*COX6B2*	19, 19	Testis	*5*
7A	*COX7A2*	*COX7A1* *COX7A2L* *	6, 19, 2	Heart, skeletal muscle	*6-8*
8	*COX8A*	*COX8B(P)* *COX8C* **	11, 11, 14	Heart, skeletal muscle	*9 – 11*
Cyt *c*	*CYCS*	*CYCT(P)*	7, 2	(Testis)	*12-14*

* Localizes to Golgi.

** It is not yet known where COX8C is expressed.

1 (Hüttemann et al., 2001)

2 (Hüttemann et al., 2007)

3 (Yanamura et al., 1988)

4 (Ewart et al., 1991)

5 (Hüttemann et al., 2003a)

6 (Arnaudo et al., 1992)

7 (Van Kuilenburg et al., 1992)

8 (Van Beeumen et al., 1990)

9 (Lomax et al., 1995)

10 (Rizzuto et al., 1989)

11 (Hüttemann et al., 2003b)

12 (Hake et al., 1994)

13 (Goldberg, 2003)

14 (Pierron et al., 2011a)