EVOLUTION OF CYTOCHROME C OXIDASE SUBUNIT 4 IN RELATION TO HYPOXIA

by

Katrinka Maria Kocha

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Abstract

Cytochrome c oxidase (COX) is complex IV of the electron transport system, and catalyzes the reduction of molecular oxygen to water. It possesses ten nuclear-encoded subunits, the largest of which is COX4. Bayesian analysis suggests the isoform pair for this subunit arose early in vertebrate evolution, and tissue distribution of the COX4 paralogs is similar in mammals and teleosts: COX4-1 is ubiquitously transcribed while COX4-2 is present in large amounts only in brain and respiratory tissue. This subunit is of interest due to its apparent sensitivity to oxygen. During hypoxia, transcription switches from COX4-1 to COX4-2 in some mammalian tissues. However, questions remain about the regulation of this response as well as its pervasiveness across vertebrates. I investigated these uncertainties by measuring the transcriptional response of the COX4 paralogs to hypoxia in a variety of vertebrate models, and assessing the hypoxic induction of putative oxygen-responsive elements (HRE1, HRE2, and ORE) from candidate vertebrate species in a transfection experiment. I also examined the conservation of key elements of the COX4-2 gene and polypeptide in vertebrates. It was found that the hypoxia-responsiveness of COX4-2 may not be vital to the cellular response to hypoxia. COX4-1 transcripts remained in excess during hypoxia in all of the vertebrate models used with the exception of western painted turtle (Chrysemys picta), where COX4-2 transcripts remained in excess during control and hypoxic treatments. Only the HRE2 element from human COX4-2 was activated with hypoxic exposure, yet this along with the other features of the gene and polypeptide were not well conserved across mammals, and nearly absent outside of this lineage. These results provide evidence that COX4-2 may respond to hypoxia in only select few mammalian tissues, or that the function of this gene is not related to the cellular hypoxic response.
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<th>Description</th>
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<tbody>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cycle threshold value</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EF-1α</td>
<td>Elongation factor 1 alpha</td>
</tr>
<tr>
<td>ETS</td>
<td>Electron transport system</td>
</tr>
<tr>
<td>FIH-1</td>
<td>Factor inhibiting HIF-1</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia responsive element</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NRF-1</td>
<td>Nuclear respiratory factor-1</td>
</tr>
<tr>
<td>ORE</td>
<td>Oxygen responsive element</td>
</tr>
<tr>
<td>ox</td>
<td>Oxidation</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>red</td>
<td>Reduction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPL13α</td>
<td>60S ribosomal protein L13α</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>WGD</td>
<td>Whole genome duplication</td>
</tr>
<tr>
<td>X-gal</td>
<td>Bromo-chloro-indolyl-galactopyranoside</td>
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Chapter 1

General Introduction and Literature Review

1.1 Overview

The ability of a cell to survive stress often hinges on its capacity to modulate energy production to match demands. For most organisms, this means matching rates of ATP synthesis to ATP utilization. In non-photosynthetic eukaryotes, ATP may be produced through the anaerobic process of glycolysis, or the aerobic process of mitochondrial oxidative phosphorylation (OXPHOS). While glycolysis does not rely on the presence of oxygen to function, it is far less efficient, producing only 2 molecules of ATP per molecule of glucose. OXPHOS supplies the majority of ATP in eukaryotes, producing between 30 and 36 molecules of ATP per molecule of glucose. Reducing equivalents (NADH, FADH$_2$) produced by the tricarboxylic acid cycle (TCA) and other reactions are oxidized by the electron transport system (ETS). Proton pumping by ETS complexes generates an electrochemical gradient, the proton motive force. This gradient is then used to drive phosphorylation by an $F_1F_0$ ATPase.

While regulation of energy production can occur at numerous points, many of the known regulatory mechanisms occur within the complexes involved in OXPHOS. These regulatory mechanisms act through the production of reducing equivalents, the electrochemical gradient present across the inner mitochondrial membrane, the concentration of ATPase substrates and products (ADP, phosphate, and ATP), and the modulation of various subunits of the OXPHOS complexes. Cells can change the content of the enzymes through transcription and translation of nuclear and mitochondrial genes. This remodeling process may include changes in the expression of isoforms for proteins, which confers regulatory or structural differences. Largely unknown is the influence of evolutionary variation in the structure of isoforms of OXPHOS complexes. Such
a large and variable array of mechanisms able to modify OXPHOS capacity and cellular energy production suggests that fine control of this system has been vital to the survival and radiation of eukaryotes.

My goal in this thesis was to investigate the regulation of a gene that encodes isoform 2 of subunit 4 of cytochrome c oxidase (COX). COX is Complex IV in the ETS and is responsible for the final electron transfer step, which translocates four protons across the inner mitochondrial membrane and reduces molecular oxygen to water. By investigating the presence or absence of a response to hypoxia in the COX4-2 gene both within tissue types of a species and across several vertebrate species, I have shed light on the evolution of a gene purported to increase survival under hypoxic conditions in mammals, as well as provided a cautionary tale for limiting the investigation of gene function and regulation to a few species from a single lineage.

1.2 Oxidative metabolism

Although animal cells may produce limited energy via the anaerobic glycolysis pathway, most energy is produced via OXPHOS. This is a step-wise process where metabolic substrates are oxidized, and the collected electrons transferred to reducing equivalents, which become the substrate for the ETS.

The ETS is a pathway consisting of four complexes (complexes I to IV) and two electron carriers (ubiquinone and cytochrome c) embedded in the inner membrane of the mitochondrion (Figure 1). NADH and FADH₂ transfer electrons to Complex I (NADH: ubiquinone oxidoreductase) and Complex II (succinate dehydrogenase), respectively. Electrons are then passed from ubiquinone to cytochrome c by Complex III (cytochrome c reductase). The final transfer occurs at Complex IV, hereafter called by its more familiar name, COX. This enzyme
complex oxidizes cytochrome c, and transfers the electrons to a molecule of oxygen bound in the COX reaction center in order to form water (Figure 1).

Complexes I, III, and IV translocate protons across the inner mitochondrial membrane as they funnel electrons through the ETS. The electrochemical gradient generated across the membrane is termed the proton motive force ($\Delta p_m$), and is harnessed by $F_1F_0$ ATPase to generate ATP from ADP and a molecule of inorganic phosphate. Through this process, OXPHOS is able to generate approximately 18 times more ATP per molecule of glucose used than can be derived by glycolysis.

Figure 1. Schematic of the electron transport system (ETS).
Blue arrows represent the flow of electrons from Complexes I (NADH: ubiquinone oxidoreductase) or II (succinate dehydrogenase) to the electron carrier ubiquinone (Q), then to complex III (cytochrome c reductase), the electron carrier cytochrome c (cyt c), and finally to complex IV (cytochrome c oxidase). Red arrows represent the translocation of protons from the matrix to the mitochondrial intermembrane space.

1.3 COX structure and function

In vertebrates, a COX monomer possesses 13 subunits. The three largest are highly conserved across eukaryotes, are encoded in the mitochondrial genome and are all that is required
to perform the catalytic functions of COX. These subunits possess primary sequence similarity to the prokaryote COX subunits (Saraste, 1990). The other ten subunits are encoded in the nuclear genome and are much more variable in sequence across vertebrates. The precise functions of the nuclear-encoded subunits are still the subject of research; in general they serve to provide structure and regulate the catalytic functions of COX. Also contained within the COX complex are seven metal ions. In addition to a zinc and a magnesium ion, 3 copper ions and two hemes form four redox-active centers: the low-spin heme \( \alpha \), the high-spin heme \( \alpha_3 \), the single ion \( \text{Cu}_B \) and the mixed valence ion pair \( \text{Cu}_A \) (Malmström and Malmstroem, 1990). Once synthesized, COX forms a dimer within the inner mitochondrial membrane, enabling the enzyme to take up oxygen to make water.

The crystal structure of the COX complex has been determined for three bacteria (Paracoccus denitrificans, Rhodobacter sphaeroides and Thermus thermophilus; Iwata et al., 1995; Svensson-Ek et al., 2002; Soulimane et al., 2000) and one vertebrate (Bos taurus; Tsukihara et al., 1995). While the complexity of COX increases with organismal complexity, the three subunit structure of the catalytic core of the bacterial enzymes display a high similarity to that of the vertebrate structure. The three mitochondrial-encoded subunits, which form the catalytic core, are composed mainly of transmembrane helices except for the cytochrome c binding domain of COX2 which is found in the intermembrane space (Tsukihara et al., 1995). COX1 and 2 subunits coordinate two hemes, two copper centers, and a magnesium ion. The ten nuclear-encoded subunits in metazoan COX are involved in the structure and regulation of the complex, though some questions about their specific functions remain. Of the ten nuclear-encoded subunits, COX5A and 5B are bound to the complex on the matrix side, and COX6B is present in the intermembrane space. Additionally, COX5B houses a zinc atom (Tsukihara et al.,
Binding sites for various ligands are present on the nuclear-encoded subunits, which serve regulatory purposes (see section 1.4.2).

The catalytic cycle directly involves only subunits 1 and 2, and produces the net reaction:

$$4 \text{ cyt c}^{\text{red}} + 8 H^+_\text{(in)} + O_2 \rightarrow 4 \text{ cyt c}^{\text{ox}} + 2 H_2O + 4 H^+_\text{(out)}$$

Thus, a full turn of the COX reaction involves pumping eight protons and transferring four electrons (Antonini and Brunori, 1970). The electron transfer pathway is the better understood process, and involves the consecutive docking and oxidation of four units of cytochrome c followed by four electron transfer steps. After cytochrome c binds to COX2, an electron enters the complex via Trp-106 and is passed to the mixed valence copper center CuA (Witt et al., 1998; Malatesta et al., 1998). Heme a accepts the electron from CuA and transfers it to the binuclear reaction center of the complex, consisting of heme a3 and CuB. When the four metal centers have been reduced, oxygen may bind to the binuclear center. The covalent bond between the atoms of the oxygen molecule is broken within 200 µs of binding to COX as two electrons are donated to each oxygen; one proton is likely added at this stage as well (Proshlyakov et al., 1998). The remaining three protons are subsequently added to the oxygen molecule to complete the formation of two water molecules. The newly formed water is then released, and the catalytic cycle can begin again. It is of note that the reduction of oxygen to water catalyzed by COX is irreversible and never fails to proceed to completion. Because of this one-way gated reaction, oxygen radicals are never produced through this mechanism in a cellular environment.

As mentioned previously, translocation of protons from the mitochondrial matrix to the intermembrane space is coupled to the reduction of oxygen. The number of protons and the pathway they take across the inner mitochondrial membrane during one catalytic cycle are however still subjects of debate. The driving force of the proton pumping action by COX has
been recently determined to be a gating mechanism involving heme $a_3$, transfer of the protons to oxygen and the release of the produced water molecules. When an oxygen molecule binds to the binuclear reaction center, heme $a_3$ is oxidized as it breaks the dioxygen bond. This creates electrostatic repulsion that drives the protons away from the center of COX and the addition of protons to the now electronegative oxygen atoms enhances this effect (Tsukihara et al., 2003; Faxén et al., 2005; Belevich et al., 2006). Unidirectionality is achieved through a molecular gate created by an ionic bond between a conserved arginine and heme $a$. This gate prevents backflow of protons as they migrate away from the positively charged reaction center. When water is released, it exits through this gate, opening it and creating the potential for proton flow reversal (Wikström et al., 2005; Qin et al., 2009).

Bovine COX is currently known to possess three proton pathways, termed the D-, K-, and H-channels due to residues which play important roles in each (Tsukihara et al., 1995; Yoshikawa, 1998). Each pathway is a network of hydrogen bonds which have been proposed to allow protons to translocate through COX. However, concrete experimental evidence for the use of these pathways has proven to be difficult to obtain. Only the H-channel has been observed to function in mammalian cells through the mutation of key residues in the pathway (Shimokata et al., 2007; Tsukihara et al., 2003). The K- and D-channels appear to be of more importance in the bacterial enzyme, as no corresponding H-channel is present in these complexes. The D-channel may play a role as an alternative proton pathway in mammalian COX and mutation of residues in the D-channel have produced evidence for the functionality of this pathway, but the mutations have either prevented the oxygen reduction reaction or failed to fully inhibit the ability of protons to use this pathway (Yoshikawa et al., 2011). The ratio of protons pumped to electrons transferred ($H^+/e^-$) has been generally been defined in vertebrate COX as 1 (Babcock and Wikström, 1992). It has also been established that the coupling of proton pumping to electron transfer occurs at a rate
of one pair during the binding of oxygen to COX, and one during the reduction of the enzyme before the next oxygen molecule binds (Bloch et al., 2004). This native $\text{H}^+/\text{e}^-$ stoichiometry can be altered during several different situations, such as a high proton gradient across the inner mitochondrial membrane, cytoplasmic ATP levels, and differential expression of COX subunit isoforms (Papa et al., 1991; Frank and Kadenbach, 1996; Hüttemann et al., 1999). Such stoichiometric alterations are termed proton slip or OXPHOS uncoupling, and serve to regulate the rate of ATP production by OXPHOS. The regulation of OXPHOS is a striking example of the exquisite control often displayed by cells to allow for survival in constantly changing conditions, and involves a number of mechanisms which are still a topic of intensive research.

### 1.3.1 COX regulation through the nuclear genes

The three mitochondrial subunits in COX are transcribed in a polycistronic fashion that is regulated by well-defined transcription factors (e.g. mitochondrial transcription factor A; Fisher and Clayton, 1988). However, the regulation of the nuclear-encoded subunits is less clear-cut and only recently has progress been made on the topic. It has been found that nuclear respiratory factor (NRF)-1 and NRF-2, transcription factors which are important in the regulation of OXPHOS components, directly regulate the transcription of all ten nuclear-encoded COX subunits in mammals (Dhar et al., 2008; Ongwijitwat and Wong-Riley, 2005).

When the energy status of a cell changes, NRF-2 is able to sense and respond to this change by coordinately upregulating the transcription of the COX nuclear-encoded subunits (Ongwijitwat et al., 2006). To achieve a synchronized increase in ten gene transcripts from different genomic loci, transcription of the subunits has been seen to occur in ‘transcription factories,’ where the genes for each nuclear-encoded subunit co-localize around a group of RNA
polymerase II molecules and other components necessary for transcription to occur (Dhar et al., 2009).

While the mammalian COX genes appear to be predictably regulated by NRF-1 and NRF-2, and respond transcriptionally in in a highly coordinated manner, this exquisite coordination is not seen in fish. Although there is sometimes a correlation between COX activity and NRF levels in certain fish species, this pattern is by no means universal across teleosts (Bremer and Moyes, 2011; LeMoine et al., 2008, 2010; Bremer et al., 2012).

In zebrafish (Danio rerio), the ten COX nuclear-encoded subunits are present in highly variable stoichiometries both within and across several tissues under normal conditions (Little et al., 2010). This absence of pattern holds true in the nuclear-encoded COX transcript levels in the white muscle tissue of two other Cyprinid minnow species (Crassius auratus and Chrosomus eos). Additionally, no coordinated transcriptional response is seen in any of the three minnow species in response to cold acclimation, a common stimulus of mitochondrial biogenesis (Duggan et al., 2011). In contrast to mammals, fish may exercise an additional level of regulation in limiting the transcripts of key nuclear-encoded COX genes, which themselves may be regulated in a fundamentally different manner than in mammals (LeMoine et al., 2008; Little et al., 2010; Bremer and Moyes, 2011; Duggan et al., 2011; Bremer et al., 2012). To gain an understanding of the function and evolution of these regulatory mechanisms across vertebrates, it is useful to compare what is known of mammalian function to what occurs in lower vertebrate taxa.

1.4 Regulation of OXPHOS in mammals

Aside from proton pumping abilities, Complexes I, III, and IV of the ETS share an additional feature in that they are composed of subunits encoded by both the nuclear and
mitochondrial genomes. Compatibility between the subunits originating from each genome is crucial for the efficient function of the complexes. This, combined with the fact that a single unit of the ETS with the accompanying F$_1$F$_0$ ATPase contains more than 80 uniquely-encoded polypeptides, provide a basis for explaining why mitochondrial dysfunction is one of the most common birth defects, occurring in approximately 1 of every 10 000 live human births (Smeitink et al., 2001). Such findings have led some researchers to argue that most human diseases are caused by mitochondrial dysfunction, and involve some combination of inadequate energy production and excessive production of reactive oxygen species (ROS; Hüttemann et al., 2008).

To ensure the proper balance between ATP and ROS in cells, OXPHOS is regulated in a highly complex manner.

The “classical” regulatory mechanisms include the availability of OXPHOS substrates such as reducing equivalents and inorganic phosphate (Lardy and Wellman, 1951), and the magnitude of the $\Delta p_m$. As the F$_1$F$_0$ ATPase generates ATP, the $\Delta p_m$ is diminished. This stimulates ETS complexes to increase activity, restoring the magnitude of the $\Delta p_m$ in the process. These two regulatory mechanisms alone cannot explain modulations in OXPHOS activity, however. More recently proposed mechanisms include the previously discussed transcriptional control of OXPHOS components, physical locations of ETS components, allosteric regulation, phosphorylation of the complexes, and alternative isoform expression. Much of the regulatory mechanisms that have been identified to this point involve COX, and many researchers now believe COX to be the central regulatory point in OXPHOS (Poyton et al., 1988; Erecinska and Wilson, 1982; Villani and Attardi, 2000; Arnold, 2012).
1.4.1 ETS structure

Within mitochondria COX exists as a dimer. However, the dimer also associates with other ETS components to form larger structures termed supercomplexes. These structures can vary with regards to the composition of their ETS complexes and their abundance within a given inner mitochondrial membrane, and appear to allow for an increased efficiency in electron transmission between complexes. Supercomplex structures are therefore thought to form as needed to increase OXPHOS efficiency (Dudkina et al., 2010; Boekema and Braun, 2007).

Mitochondria are now understood to be dynamic in shape and size, and the morphology of the inner mitochondrial membrane is hypothesized to have effects similar to those seen in supercomplexes. By modulating the composition of the cristae in the inner mitochondrial membrane, ETS complexes may be brought together in particular conformations, or allowed access to other factors involved in the regulation of OXPHOS (McBride et al., 2006)

1.4.2 Allosteric regulation

Only cytochrome c and COX are known to be regulated allosterically, and in both cases by the relative availability of ATP. One ATP/ADP binding site is found on cytochrome c, and prevents the binding of cytochrome c to COX when cytosolic ATP concentrations are high (Ferguson-Miller et al., 1976). Another seven such sites have been located on COX (Napiwotzki et al., 1997), although two have been shown to provide what has been termed the “second mechanism of respiratory control,” acting in tandem with regulation via Δp_m. Replacing ADP with ATP at two sites located on subunit 4, one on the matrix domain and one on the cytosolic domain, results in allosteric inhibition of COX (Arnold and Kadenbach, 1997; Napiwotzki et al., 1997; Napiwotzki and Kadenbach, 1998). This allows OXPHOS to sense energy conditions in the
cell independently of the magnitude of $\Delta p_m$. Additionally, it appears that this mechanism is able to maintain a $\Delta p_m$ below 140 mV, which would not be possible if it was only controlled by thermodynamic principles (Ramzan et al., 2010). This may aid in preventing the formation of damaging ROS (Liu, 1997; Lee et al., 2002). Interestingly, the ATP/ADP regulation at subunit 4 is switched off by the allosteric binding of a metabolite of thyroid hormone, 3,5-diiodo-thyronine, to subunit 5A of the COX enzyme (Arnold et al., 1998). One other ATP/ADP site located on COX subunit 6A seems to allow for an alteration in the ratio of protons pumped into the intermembrane space to electrons transferred to oxygen. This has only been found in mammals and has been suggested to contribute to thermogenesis (Frank and Kadenbach, 1996).

In addition to adenylate ligands, COX can be regulated through ligands which bind directly to its catalytic core. These ligands are gas molecules, and include carbon monoxide (CO), nitric oxide (NO), hydrogen sulfide (H$_2$S), and hydrogen cyanide (HCN$^-$. By binding to the catalytic center, COX activity is inhibited in a way that is either competitive or non-competitive with oxygen, depending on the ligand. Of this group of ligands, CO and NO play the most important regulatory roles. CO was the first molecule to be recognized for its ability to bind to COX in place of oxygen (Chance et al., 1970). Like oxygen, CO will only bind to fully reduced COX complexes; thus it competes directly with the COX-oxygen interaction. However, the intracellular concentration of CO is so minimal that until recently it was not thought to play any role of importance in COX regulation. New research suggests that CO produced endogenously via heme oxidases is able to significantly reduce COX activity, particularly under hypoxic conditions (D’Amico et al., 2006). CO is currently believed to be an important regulator of COX under inflammatory and hypoxic conditions.

The other important gaseous molecule which binds to COX is NO. This molecule has a more complex relationship with COX, and will bind to different regions of the catalytic center.
under different redox states. When COX is reduced, NO is able to bind to heme $a_3$ in the binuclear reaction center and compete directly with oxygen. When COX is oxidized, NO may bind to Cu$_b$ and be reduced to nitrite ($\text{NO}_2^-$) without competing with oxygen (Cooper and Brown, 2008). The latter process is an important NO sink and occurs particularly under conditions where cytosolic oxygen concentrations are high and COX remains in a mostly oxidized state. This is an important function as COX has been shown to have a greater affinity for NO at its binuclear reaction center than oxygen (Brown and Cooper, 1994). When oxygen concentrations are low, COX tends towards a reduced state and the conversion of NO to $\text{NO}_2^-$ can no longer occur. Under these conditions, COX capacity is stimulated to increase to continue to meet cellular ATP demands until a critical ratio of $\text{O}_2$:NO is reached, and NO begins to actively inhibit COX activity (Taylor and Moncada, 2010). Like CO, this complex relationship with NO is thought to be important in the cellular response to hypoxia.

### 1.4.3 Phosphorylation

Phosphorylation of a protein is a rapid and efficient way to fine tune the activity of a particular protein or complex. While OXPHOS seems a likely candidate for extensive phosphorylation to finely control ATP production, the dynamic nature of phosphorylation states has made it a difficult research subject. Only in the past decade have studies on the phosphorylation targets in OXPHOS emerged, and the contradictory nature of many findings makes the interpretation of the results difficult.

Complex I has been shown to contain phosphorylation sites on five of its 45 subunits (Palmisano et al., 2007). The purported functions of these subunits range from allowing the assembly of the complex and increasing its activity, preventing excessive formation of ROS, and triggering apoptosis (Technikova-Dobrova et al., 2001; Bellomo et al., 2006; Angell et al., 2000;
Huang et al., 2004). F$_1$F$_0$ ATPase also appears to possess four phosphorylation sites on its β subunit which affect the stability and activity of the complex (Kane et al., 2010).

The most heavily studied OXPHOS component in this field is, by far, COX. To date, 22 phosphorylation sites have been identified in bovine heart and various other mammalian models (Fang et al., 2007; Helling et al., 2008; Lee et al., 2005a; Tsukihara et al., 2003; Olsen et al., 2010; Hüttemann et al., 2012; Zhao et al., 2011; Lee et al., 2002; Acin-Perez et al., 2011; Helling et al., 2012; Lee et al., 2009), while a total of 53 sites have been predicted based on serine/threonine phosphorylation (Pearson and Kemp, 1991). The functions of some of these sites remain to be identified (e.g. Tyr-218 of COX2, Ser-1 of COX5A, and Ser-71/72 of COX4; Helling et al., 2008; Zhao et al., 2011), but many others have been found to play direct roles in the regulation of COX activity. In general, it has been found that phosphorylated COX complexes possess lower turnover rates than those that are unphosphorylated (Hüttemann et al., 2008). Further, the regulation of COX phosphorylation appears to be controlled by cAMP, while dephosphorylation is controlled by intracellular calcium levels, acting through an unknown protein phosphatase (Bender and Kadenbach, 2000; Lee et al., 2005b; Hüttemann et al., 2008).

The picture that emerges is one of COX activity being altered quickly and reversibly through its phosphorylation state. This is an elegant means by which the cell may ratchet the energy production and oxygen consumption up or down, based on its particular needs.

Of the known phosphorylation sites, the majority have been linked to altering the inhibition of COX by ATP, such as Ser-441 of COX1, Ser-126 of COX2, Tyr-11 and Ser-34 of COX4, and Ser-4 of COX5A (Lee et al., 2002; Hüttemann et al., 2012; Tsukihara et al., 2003; Helling et al., 2008). Paradoxically, while the phosphorylation of these sites appears to enable ATP binding to COX, Acin-Perez and colleagues recently identified a residue on COX4 (Ser-58) which prevents the binding of ATP when phosphorylated, allowing COX to function at higher
turnover rates even in the presence of high ATP concentrations (Acin-Perez et al., 2011). The phosphorylation pattern of a COX complex at these sites may fine tune the level of inhibition exhibited during high cellular ATP concentrations based on tissue or cell type.

Another research group has identified four sites on the COX complex which are exclusively phosphorylated during ischemia in rabbit heart tissue: Ser-115 and Ser-116 of COX1, Thr-52 of COX4, and Ser-40 of COX5B (Fang et al., 2007). The group saw a decrease in COX activity when these sites were phosphorylated, and hypothesize that a hypoxia sensitive pathway is responsible for the phosphorylation of these sites, which may serve to inhibit COX activity during periods of low oxygen. Finally, two sites have been identified that do not seem to affect COX activity in any way, but instead aid in maintaining the structure of COX (Ser-2 of COX5B and Thr-11 of COX6A; Helling et al., 2012; Tsukihiara et al., 2003). With such a large number of characterized phosphorylation sites which play myriad roles in COX function, this mechanism could potentially play the largest role in COX, and perhaps also OXPHOS regulation.

One area still lacking in elucidation is the identification of kinases and phosphatases involved in the regulation of OXPHOS phosphorylation. Some common factors such as protein kinase A (PKA), PKC, and Raf kinases have been linked to the mitochondria, but few direct interactions with OXPHOS complexes have been experimentally confirmed (Foster et al., 2009; Acin-Perez et al., 2009). Again, pathways involving the phosphorylation of COX amino acids are the most heavily studied. PKA has been found to successfully phosphorylate several sites on COX that are linked to both increasing and decreasing COX activity (Helling et al., 2008; Acin-Perez et al., 2011), while the tyrosine kinase c-Src has been found to phosphorylate COX and increase its activity (Miyazaki et al., 2003). However, concluding that these are the kinases responsible for COX phosphorylation in vivo has proven to be difficult; for example Miyazaki and colleagues found that even when c-Src was not present in mouse kidney tissues, COX was
still able to function normally, suggesting that other kinases are able to perform the same phosphorylation functions on COX (Miyazaki et al., 2003). Intriguingly, there is some indication that ETS complexes may be able to autophosphorylate in the absence of any accessory proteins (Phillips et al., 2011). However, this too must be further investigated.

1.4.4 Isoforms

Different forms of proteins, or isoforms, may arise from the alternative splicing of transcripts prior to translation, or the transcription of different genes. The latter isoform type is known to play important roles in the regulation of OXPHOS components. There are two cytochrome c genes (paralogs) in many mammalian taxa, such as rodents (Hennig, 1975). One isoform is the somatic isoform found in all mammals, while the other is specific to testes and results in cytochrome c which is able to reduce hydrogen peroxide and induce apoptosis at 3 times the levels of the somatic protein (Liu et al., 2006). Interestingly, this testes-specific isoform is not found in humans, where the gene is present as a pseudogene (Hüttemann, Jaradat, et al., 2003).

There are also isoforms of nuclear-encoded subunits for COX. The three mitochondrial-encoded subunits are derived from mtDNA, and thus beyond heteroplasmy, there is little option for alternative structures. However, the 10 nuclear-encoded subunits display various isoform patterns across vertebrate taxa. For example mammalian COX subunit isoforms are present for COX4, 6A, 6B, 7A, 7B and 8, while teleost fish lack isoforms for 7B but possess additional isoform pairs for COX5A and 5B (Little et al., 2010). Surprisingly, little is known about the specific functions of these isoforms; current knowledge of the COX isoforms will be discussed in more detail below.
The preceding discussion of known regulatory mechanisms of OXPHOS is by no means exhaustive. In addition to the need to determine the phosphorylation patterns of the major OXPHOS components and the pathways which regulate them, the extent of the role that supercomplexes play in the control of energy production also remains to be fully understood. It is also likely that other mechanisms exert control over OXPHOS rates. Other cell factors may act as ligands; for example, a metabolite of thyroid hormone and both carbon monoxide (CO) and nitrous oxide (NO) are known to bind to COX in place of molecular oxygen and alter the activity of the complex (Arnold et al., 1998; Palacios-Callender et al., 2004; Taylor and Moncada, 2010). RNA molecules have also become major candidates for gene regulation, and it is conceivable that they also play a role in OXPHOS regulation. However, no specific interactions between RNA and OXPHOS genes have been reported to date.

1.5 COX Isoform evolution in vertebrates

1.5.1 Mechanisms of isoform origin

The accumulation of mutations was originally believed to be the sole contributor to increased biological diversity. However, in 1970, Ohno proposed a novel mechanism through which such diversity may be achieved. This mechanism is composed of two steps: gene or genome duplication followed by sequence divergence (Ohno, 1970). Currently, this mechanism is considered to be the chief mechanism responsible for vertebrate diversity.

Gene duplication events may occur through two main mechanisms. Tandem or single gene duplication generally results from unequal crossover events during meiosis. This is by far the most common occurrence in eukaryotic genomes (Friedman and Hughes, 2001). A rarer way
in which the gene copy numbers of an organism may increase is through whole genome duplication (WGD) events. Three WGD events are hypothesized to have occurred early in vertebrate evolution. The precise timing of these events is still controversial, but it is generally believed that the first event occurred after the divergence of cephalochordates, the second after the divergence of cyclostomes and before the divergence of Chondrichthyes, and the third only in the Actinopterigian line, before the divergence of teleost fish (Holland, 1999; Taylor et al., 2003). These excess genes experience relaxed selection pressures, and may undergo mutations, often without harming the overall fitness of the organism. Eventually, the genes may develop new functions, and increase biological diversity in the process.

Within eukaryotes, duplicate genes are thought to arise on average at a rate of 0.01 per gene every million years. On average, 40% of the eukaryotic genome is composed of duplicate genes (Zhang, 2003). However, within a few million years, the overwhelming majority of these excess genes accumulates deleterious mutations, and undergoes the process of becoming functionally silent, a process called pseudogenization (Lynch and Conery, 2000). Novel gene function arises through one of two processes. Subfunctionalization occurs when the duplicates of a gene diverge to the point that each may provide a supportive role to accomplish an overall function within the organism (Lynch and Force, 2000). In the process of neofunctionalization, mutations may accumulate in the duplicate gene to the point that it now possesses a function that differs from its progenitor and confers a selective advantage on the organism (Hughes, 1994). These neofunctionalized genes sometimes perform functions unrelated to the ancestral gene, but more often become genetic isoforms which have slightly modified activity and play important roles in cellular regulation. In this way, eukaryotic cells are able to perform novel functions without increasing the number of genes maintained by the cell. Evidence for this can be seen in
the increasing complexity of organisms without a significant increase in the number of genes (Ohno, 1999; Friedman and Hughes, 2001).

1.5.2 Vertebrate COX isoforms

Gene isoforms have been discovered for a number of the nuclear-encoded COX subunits. The number of isoforms varies across vertebrate taxa. For example, mammals possess isoform pairs for six of the ten nuclear-encoded subunits, while teleosts possess isoforms for seven of the ten subunits. Based on recent phylogenetic analysis, it is likely that the three WGD events early in the vertebrate lineage largely governed the distribution of isoforms across taxa. The ancestries of five of the ten nuclear-encoded COX genes appears to be identical across vertebrates, including the COX4 paralogs. There are two subunits which have paralogs only in teleost fish, and three which have unique paralogs in mammals. Of those subunits with similar ancestries, we saw examples of isoform pairs which had likely arisen from a WGD event before the divergence of fish and tetrapods (e.g. COX4), as well as those which had likely arisen from lineage-specific gene duplications (e.g. COX6A). Subunits with extra copies only in the teleost lineage may have arisen from the actinopterygian-specific WGD. No such events are known to have occurred in the tetrapod lineage, so it is more likely that extra copies in these species arose from single or tandem gene duplications (Little et al., 2010).

The functions of nuclear-encoded COX isoforms are only beginning to be understood. To date, the vast majority of research has focused on the mammalian isoform functions, more specifically isoforms for COX 4, 6A, 6B, 7A, and 8. Four of these isoform pairs are expressed in a tissue- and developmental-specific manner in mammals. COX4 isoforms, on the other hand, appear to be regulated by environmental conditions.
In general, the tissue-specific isoform pairs display a major-minor pattern of expression, where one is deemed the ubiquitously-expressed or liver-type isoform and the other is the tissue-specific, or heart-type isoform. Mammalian COX6A and COX7A are transcribed in a tissue- and developmental-specific pattern. The liver-type isoforms (COX6A1 and COX7A2) are expressed in all tissues during embryonic development. Before the end of the gestation period, transcription in heart and skeletal muscle tissues switches to the heart-type isoforms, COX6A2 and COX7A1 (Bonne et al., 1993). It should be noted that while COX7 possesses a third isoform in vertebrates, it has been determined to be involved exclusively in Golgi body function; this is an example of neofunctionalization (Segade et al., 1996; Schmidt et al., 2003). COX8 has two paralogs which display a similar liver-type and heart-type isoform organization, although no evidence of developmentally-regulated switching of isoforms has yet been found (Ewart et al., 1991). Additionally, a third COX8 isoform (COX8C) has been recently discovered in mammals, and has yet to be classified in terms of expression patterns or functions (Hüttemann, Schmidt, et al., 2003). COX6B, conversely, possesses one ubiquitous isoform in mammals and one which is specific to testes tissue (Hüttemann, et al., 2003).

In terms of the unique functions of these mammalian isoforms, few have yet been determined. When the liver-type isoform COX6A1 is incorporated into the COX complex, it maintains an H+/e- ratio of 0.5. Conversely, the heart-type isoform COX6A2 is able to adjust the COX H+/e- ratio from 0.5 to 1.0 when there is a high ATP/ADP ratio in the cell. Allowing a reduced proton-pumping efficiency is hypothesized to aid in thermogenesis via the uncoupling of COX function with ATP production (Hüttemann et al., 1999; Lee and Kadenbach, 2001). Thus, in muscle tissues where energy demand is highly variable, COX may switch between a more thermogenic mode and a highly efficient energy-producing mode. As only Mammalia and Aves are concerned with thermoregulation and the latter class does not possess isoforms for COX6A,
the purported function of COX6A2 is likely specialized to mammals. The testes-specific COX6B2 may increase the binding efficiency of the testes-specific cytochrome c isoform and allow highly energetic sperm cells to maintain energy homeostasis (Hüttemann et al., 2003). The functions and tissue profiles of the nuclear-encoded COX subunit isoforms found in mammals should not necessarily be expected to be conserved across vertebrates. The evolutionary pattern of these isoforms must be taken into consideration when predicting the presence or absence of these features in extant taxa. For example, the ancestry of the COX6A paralogs suggests that although they are present in all major vertebrate lineages, they arose from separate duplication events (Little et al., 2010). This implies that the mammalian COX6A2 isoform evolved separately from the teleost COX6A2, and likely do not share functional mechanisms. Conversely, COX4 isoforms do appear to be orthologous across vertebrates, suggesting that mammalian structures and functions may be conserved in teleost fish. As the COX nuclear-encoded subunit which has aroused perhaps more interest than any other subunit, it is the major concern of my thesis, and is discussed in more detail below.

1.6 COX4 structure and function

COX4 is the largest of the nuclear-encoded subunits. It is composed of one transmembrane domain flanked by an extramembrane domain on either end, and interacts with the catalytic subunits COX1 and 2, as well as the nuclear-encoded subunits COX7B and 8 (Tsukihara et al., 1995). COX4 is the first nuclear-encoded subunit, along with COX5A, to be inserted into the growing COX complex during its biogenesis, giving it the potential to play a regulatory role in the assembly of new COX complexes through the availability of its transcripts or polypeptides (Fornuskova et al., 2010; Fontanesi et al., 2006; Ugalde et al., 2002; Nijtmans et
Indeed, murine cells with COX4-1 levels reduced to 4-14% of normal levels displayed COX activities of 37-51% of normal levels, and were sensitized to apoptosis. Conversely, increased COX4-1 levels resulted in increases in COX activities (Li et al., 2006).

COX4 has been associated with several additional regulatory functions in the COX complex. This includes a tentative link to the regulation of the $\text{H}^+/\text{e}^-$ ratio by mediating the access of protons to the transmembrane proton channel via its N-terminal region in the matrix (Capitanio et al., 1994). While this facet of regulation remains controversial, a far better understood function involves the ability to sense and respond to cellular energy levels. COX4 contains two adenylate binding sites, one found in the matrix domain and one in the cytosolic domain (Napiwotzki et al., 1997; Napiwotzki and Kadenbach, 1998). These sites experience competitive binding by ATP and ADP, such that the amount of ATP or ADP bound to the COX complex is a reflection of the cytosolic ATP/ADP ratio. The binding of ATP to these sites induces allosteric inhibition of COX and has been termed the second mechanism of respiratory control (see section 1.4.2; Napiwotzki and Kadenbach, 1998; Arnold and Kadenbach, 1999; Kadenbach and Arnold, 1999).

Additionally, of all 13 subunits, COX4 possesses the largest number of described phosphorylation sites to date. As discussed in section 1.4.5, the roles of most of these sites remain elusive, although a site on Ser-58 has been shown to abolish ATP-mediated regulation, and phosphorylation Thr-52 during ischemic episodes is thought to inhibit COX activity (Acin-Perez et al., 2011; Fang et al., 2007). Such a range of regulatory mechanisms argues that COX4 plays an important role in the regulation of COX, and indirectly, OXPHOS.

In addition to the known functions of the COX4 subunit in mammals, a pair of isoforms was discovered for the subunit; first in three fish species, and later in mammals as well (Hüttemann, 2000; Hüttemann et al., 2001). In mammals, both genes are structured into 5 exons, with the ATG start site located in the second exon. The paralogs of COX4 share only 44%
identity, while orthologs share a 70% identity across mammals (Hüttemann et al., 2001). In humans, COX4-1 and COX4-2 are composed of 169 amino acids and 171 amino acids, respectively. COX4-1 weighs 19.6 kilodaltons while COX4-2 weighs 20 kilodaltons. Currently, it is accepted that the COX4 paralogs exist in vertebrates from actinopterygians to mammals, with a notable exception in the Aves lineage. It is also believed, as stated previously, that these paralogs arose during one of the two WGD early in vertebrate evolution. Of the two isoforms, COX4-2 appears to possess a higher sequence similarity to the single invertebrate COX4 gene, implying that this is the ancestral isoform while COX4-1 is more diverged (Little et al., 2010). The phylogeny of COX4 isoforms in vertebrates is displayed in Figure 2. Interestingly, in addition to possessing orthologous isoforms for COX4, teleosts and mammals display similar transcription patterns for the paralogs. COX4-1 is transcribed ubiquitously in most tissues except for brain and respiratory tissue, where COX4-1 and COX4-2 are transcribed in comparable amounts (Hüttemann et al., 2001; Little et al., 2010).

The important structural differences between the two isoforms are still uncertain, although COX complexes containing COX4-2 tend to have higher activities and appear to lack sensitivity to the ATP/ADP ratio (Fukuda et al., 2007; Hüttemann et al., 2007). Based on this observation, it has been suggested that a pair of cysteine residues which are found in the matrix ATP binding site of COX4-2 only may form a disulfide bond and prevent the binding of adenylates to this site (Hüttemann et al., 2007). COX4-1 is therefore thought to allow COX to switch between higher and lower turnover rates as energy demands fluctuate in the cell, while COX4-2 allows COX to continuously function at “full speed.” If the hypothesis is correct, the pair of cysteine residues found in some mammalian COX4-2 polypeptides should be well-conserved across vertebrates. In my thesis, I aim to assess the available vertebrate COX4-2 sequences to determine if these residues are in fact conserved, implying that they do serve a functional
significance in the COX4-2 subunit. It remains to be determined if COX4-2 is present in some tissues to allow for high ATP production in energetic tissues such as in brain, or for continuous COX activity during large fluctuations in oxygen concentration such as in respiratory tissue.

Figure 2. Cladogram of deduced amino acid sequences of COX4 isoforms determined by Bayesian analysis.
Values below branches represent Bayesian posterior probabilities and are labeled only when values <0.90 (Adapted from Little et al. 2010).
1.7 Hypoxia responses of COX4 genes

The COX4 isoforms have recently become the subject of intense research as it has been found that they are sensitive to cellular oxygen levels. While COX4-1 is considered to be the ubiquitously-expressed isoform in the majority of mammalian tissues, expression has been shown to switch to COX4-2 when oxygen levels fall below normal atmospheric levels (Hervouet et al., 2006; Fukuda et al., 2007; Hüttemann et al., 2007). This raises two issues that I am concerned with, namely if COX4-2 transcription is induced during hypoxia, and if the increased abundance of COX4-2 subunits is beneficial to the cellular hypoxic response.

1.7.1 The cellular response to hypoxia

When oxygen levels become depleted in aerobic non-photosynthetic eukaryotic cells, several processes are set in motion which negatively affects homeostasis. As OXPHOS becomes inhibited by the lack of available oxygen molecules, COX complexes tend to remain reduced, and can no longer function as NO sinks (see section 1.4.2; Taylor and Moncada, 2010). This allows for the accumulation of NO, which both acts to further inhibit COX activity through competitive binding to the binuclear reaction center, and can be converted to the reactive oxygen species (ROS) peroxynitrite (Taylor and Moncada, 2010; Poyton et al., 2009). Also occurring under hypoxic stress is the increased production of other ROS, particularly by Complex III (Guzy et al., 2005; Dirmeier et al., 2002). ROS may play important roles in cellular signaling, particularly under hypoxic stress, but also act as strong oxidizing agents which can damage proteins, nucleic acids, and lipids (Poyton et al., 2009; Castello et al., 2008). Under hypoxic conditions, cells employ mechanisms to endure oxidative stress which vary with both the cell’s type and its native
environment. For example, a vertebrate tissue will induce angiogenesis to increase the perfusion of oxygen when experiencing hypoxia, whereas isolated cells will decrease their aerobic metabolic pathways (Prabhakar et al., 2009; Snyder and Chandel, 2009).

The coordinated cellular response to hypoxic stress has been found to be controlled in metazoans by the master regulator hypoxia-inducible factor 1 (HIF-1). This transcriptional activator is a heterodimeric complex made up of the ubiquitously-transcribed HIF-1β, and the oxygen-sensitive HIF-1α (Semenza and Wang, 1992). The mechanism of HIF-1 activation and regulation has been well characterized in vertebrates. Both subunits possess the necessary domains for dimerization and DNA binding (Jiang et al., 1996). HIF-1β is present in excess and is able to heterodimerize with other proteins containing the necessary domain (Semenza et al., 1996). Thus, it is HIF-1α which determines the activity of the complex. Under normoxic conditions, HIF-1α is continuously transcribed and degraded through the hydroxylation of a proline residue by the prolyl hydroxylase PHD2 (Epstein et al., 2001). Hydroxylated HIF-1α transcripts are recognized and bound by the von Hippel-Lindau protein. This protein in turn recruits an ubiquitin ligase which targets HIF-1α for proteasomal degradation (Ohh et al., 2000). In addition to this transcript degradation pathway, factor-inhibiting HIF-1 (FIH-1) prevents the activation of HIF-1α polypeptides by hydroxylating an asparagine residue, which inhibits the binding of the coactivator protein p300 (Mahon et al., 2001). Both PHD2 and FIH-1 use oxygen as substrates in their hydroxylation reactions, and become inhibited when oxygen molecules are unavailable. This allows for the stabilization and translation of HIF-1α transcripts, and the association of HIF-1α subunits with HIF-1β and p300 (Epstein et al., 2001; Ohh et al., 2000; Mahon et al., 2001). Once assembled, the HIF-1 complex binds to the promoter sites of genes termed hypoxia responsive elements (HREs, consensus site RCGTG) and recruits transcriptional machinery (Semenza et al., 1996). Through this mechanism, HIF-1 is able to activate a suite of
genes which aid the cell in surviving hypoxic conditions. Although the activation of this master regulator is known to occur under hypoxic conditions, there is increasing evidence which suggests that HIF-1 stabilization is caused not by the decrease in oxygen availability, but by the increase in ROS which occurs during hypoxia (Bellomo et al., 2006; Brunelle et al., 2005; Bell et al., 2007; Mansfield et al., 2005). Regardless, it is clear that HIF-1 is known to be among the most important factors in the cellular response to hypoxia, and has been identified in all animal lineages examined to date (Loenarz et al., 2011).

1.7.2 Hypoxia response mechanism in yeast COXV genes

Before isoforms of COX4 were discovered in vertebrates, isoforms of the orthologous subunit in the budding yeast (Saccharomyces cerevisiae) were identified (Trueblood and Poyton, 1987). Following the general rule of increasing enzyme complexity with evolutionary complexity, yeast COX complexes contain 11 subunits instead of 13 (Poyton et al., 1995). The only subunit which possesses isoforms in yeast is the ortholog of vertebrate COX4, termed COXV. The paralogs (COXVa and COXVb) share 66% sequence identity, and have been studied to the extent that their functions and regulation are well known (Cumsky et al., 1987). The COXV transmembrane domain interacts directly with COX1 and can alter the rate of electron transfer between heme $a$ and heme $a_3$. When COXVb is incorporated into the COX complex, this transfer step occurs three to four times faster than when COXVa is present (Allen et al., 1995). Further, these isoforms are known to be regulated by cellular oxygen concentration. Under normoxic conditions, COXVa transcribed while COXVb is repressed. The COXV isoforms are regulated in a heme dependant manner: COXVa transcription is regulated by the heme-dependant Hap2p/3p/4p/5p complex, while COXVb transcription is repressed by the Hap1-regulated Rox1p
and the oxygen-regulated Ord1p (Trueblood et al., 1988; Trueblood and Poyton, 1988; Lambert et al., 1994). When oxygen levels fall below 0.5 µM, heme levels decrease, COXVa is repressed, and transcription switches to COXVb (Burke et al., 1997). The increased efficiency in COX activity provided by COXVb is believed to increase ATP production and decrease ROS production during episodes of hypoxia, when ROS production tends to be increased (Barrientos et al., 2008).

Interestingly, the slime mold (*Dictyostelium discoideum*) also possesses a pair of isoforms which respond to environmental hypoxia (Bisson et al., 1997). *Dictyostelium* COXVII is the ortholog of COX6C in mammals, and is the smallest of the four nuclear-encoded subunits present in the slime mold. COX isoforms VIIe and VIIIs are transcribed during normoxia and hypoxia, respectively. The genes of these isoforms are arranged in a head-to-tail orientation with an oxygen responsive element between them. Although the differential functions of these isoforms is not known, COXVIIIs possesses more charged amino acids in the region which interacts with the electron entry site on COX2. Similar to yeast COXVb, this could increase the speed of electron transfer within the complex and thus the overall turnover rate (Bisson et al., 1997).

The yeast COX complex is a commonly used model for the study of eukaryotic COX due to the relative ease of its isolation and its resilience under experimental conditions. However, one must be cautious when extrapolating data gathered from the yeast complex to higher eukaryotes such as mammals, as there are several key differences. As mentioned above, yeast COX contains only 11 subunits, and of those, only 9 are required for normal function (Poyton et al., 1995; Taanman and Capaldi, 1992). One of the three hypothesized proton pathways through the enzyme, the H-channel, does not appear to be functional in yeast COX (Maréchal et al., 2012). Further, the vertebrate COX4 possesses two adenylate binding sites not found in yeast COXV, and lacks the ability to modify electron transfer efficiency as yeast COXV does (Tsukihara et al.,
In terms of the oxygen-sensitivity of this isoform pair, the mechanism functions independently of the well-characterized HIF-1 response pathway, as yeast lack this complex (Bunn and Poyton, 1996). Thus, although the well-described functions of COXV in yeast provide knowledge on the response of eukaryotic COX to hypoxia, the detailed function and regulation of these isoforms cannot be applied to vertebrate COX4.

1.7.3 Hypoxia response in mammalian COX4 genes

The oxygen sensitivity of mammalian COX4 paralogs was hypothesized at the time of discovery of COX4-2 (Hüttemann et al., 2001). It was not until 2007 that evidence for this feature was produced by two separate groups. Each demonstrated that the COX4-2 gene responded to hypoxia, though each attributed this response to different mechanisms of transcriptional activation.

The Fukuda group (2007) was able to show that COX4-2 transcription as well as expression is increased during both hypoxia (1% O₂) and chemical hypoxia (cobalt choride, desferrioxamine, and dimethyloxalylglycine) in two human cell lines (HeLa and Hep3B). They also demonstrated that COX4-2 transcription is induced in some mice tissues when they are exposed to 10% O₂ for three weeks. COX4-1 transcription was concordantly decreased in these experiments. Upon examination of the promoter region of COX4-2, the group identified two putative HREs, one present in the proximal promoter region and one in the first intron, and was able to show through transfection experiments that these regions conferred a hypoxia response in COX4-2 through transactivation by HIF-1. The decrease in COX4-1 transcripts was demonstrated to occur during hypoxic episodes, as well as the degradation of COX4-1 proteins through the action of the LON protease which is also activated by HIF-1 (Fukuda et al., 2007).
The functional significance of this switch for the COX complex was hypothesized to be an optimization of electron transfer under particular oxygen conditions. The group showed that under normoxia, complexes containing COX4-1 minimized production of the ROS species H$_2$O$_2$ and maximized ATP production, while complexes containing COX4-2 displayed the same results under hypoxic conditions. Thus, Fukuda and colleagues concluded that the COX4 paralog switch is regulated by HIF-1 and functions to optimize COX activity while minimizing ROS production during differing oxygen concentrations (Fukuda et al., 2007).

The Hüttemann (2007) group analyzed the COX4 switch and came to a different conclusion. They examined the mammalian COX4-2 gene and found that the putative HRE sites were poorly conserved across human, mouse, rat, and cow. Further, the activation of the COX4-2 promoter occurs at 4% oxygen, while a typical HRE is only activated to any great extent at anoxia. This caused the group to search for an alternative activation site in the COX4-2 promoter. The site they identified is conserved across the four mammalian species listed above and is composed of a 13 bp region with the consensus sequence 5’-GGACGTCCCAG-3’. They named this site the oxygen responsive element (ORE), and showed through transfection experiments that this site, located approximately 20 bp downstream of the proximal promoter HRE, confers hypoxia-responsiveness to COX4-2 independently of the HREs (Hüttemann et al., 2007). The transcription factor which binds to the ORE has yet to be identified.

In terms of the function conferred on the COX complex, Hüttemann and colleagues generally agree with the Fukuda group that COX4-2 allows for the more efficient utilization of oxygen and a decreased rate of ROS production (Hüttemann et al., 2007). However, they note that the highest levels of COX4-2 transcripts appear in lung tissue and brain (Hüttemann et al., 2001; Horvat et al., 2006). They suggest that COX4-2 may function to allow tissues with high degrees of fluctuation in oxygen supply to maintain homeostasis and minimize ROS production,
as opposed to participating in a generalized cellular response to hypoxic stress (Hüttemann et al., 2007).

Since the publication of these contradictory studies, most of the attention has been given to the suggestion of the regulation of COX4 through the HIF-1 pathway. Several other studies have examined the response of COX4 isoforms to hypoxia with varying results. One of the main cell types in mammalian brains, cortical astrocytes, has displayed a three- to four-fold increase in COX4-2 transcripts after six hours of exposure to anoxia in cells isolated from neonatal mice. As seen by the Fukuda and Hüttemann groups, COX activity in these cells did lacked sensitivity to ATP concentrations. Conversely, another major cell type in brain tissue, cerebellar granule neurons, were also isolated from the neonatal mice and showed no change in COX4 isoforms with exposure to anoxia. As astrocytes are the more energetic of the two cell types, the authors suggested that maintaining ATP production during hypoxic episodes may be more important in these cells (Horvat et al., 2006). A group examining the causes of exocrine pancreatic insufficiency found that COX4-2 is also relatively highly expressed in the acinar cells of the pancreas in humans during normoxia. They identified a mutation in the COX4-2 gene in four patients with the pancreatic disorder, and found that this prevented a response in COX4-2 transcription during hypoxia. However, they noted that even with the increase in COX4-2 transcription of wild-type COX4-2 during hypoxia, COX4-1 remained in vast excess (a ratio of COX4-1:COX4-2 of 206.5; Shteyer et al., 2009). Other studies have examined the COX4 hypoxic response in the muscles of high-performance thoroughbred horses and high-altitude specialist pikas (Ochotona dauurica, Link) and failed to find any response (Luo et al., 2008; Hill et al., 2010; Eivers et al., 2010). Thus, although COX4 paralogs appear to confer functional differences on the COX complex, and appear to be differentially regulated by oxygen in some species, the extent of their role in the hypoxic response has yet to be elucidated.
My aim in this thesis is to address the distribution of the hypoxia-responsiveness of COX4 paralogs across vertebrates and tissue types.

1.8 Hypotheses

In this thesis, I sought to investigate the evolution of the structure and function of COX4-2 across vertebrate lineages. I examined the DNA and amino acid sequences of COX4-2, and assessed the transcriptional activities of the genes and their promoters when exposed to hypoxia. Using these methods I addressed three hypotheses:

1. Given that the ancestry of the COX4 paralogs appear to be identical across vertebrates, and the tissue distribution of the transcripts is similar in mammals and teleosts, I hypothesized that COX4-2 would be responsive to hypoxic exposure in all other vertebrate systems I examined, including mammals (mice muscle and human prostate cancer cell lines) and lower vertebrate taxa (zebrafish, rainbow trout, and western painted turtle).

2. Given that there are features of the COX4-2 gene (HREs, ORE) and protein (cysteine residues) which are believed to be important for its regulation and function, I hypothesized these would be present in COX4-2 across most vertebrate lineages.

3. If the putative oxygen-sensitive promoter elements (HREs, ORE) are present in the COX4-2 promoter of a species, I further hypothesized that the activity of the promoter elements would increase with hypoxic exposure.
Chapter 2

Materials and Methods

2.1 COX4-2 sequence analysis

COX4-2 genes and their promoters were characterized from candidate vertebrate species. Sequences were obtained from the online databases Ensembl and NCBI, and the presence or absence of the putative elements responsible for the hypoxic response of COX4-2 was determined by alignments with the human element sequences found by the Fukuda and Hüttemann groups using MultAlin (Corpet, 1988; Fukuda et al., 2007; Hüttemann et al., 2007).

The human COX4-2 elements are as follows: HRE1 is present approximately 100 bp upstream of the first exon and has the sequence 5’-CTGTGCGCTCCCACGCC-3’, HRE2 is present in the first intron, approximately 700 bp downstream of the first exon and has the sequence 5’-CTGTGTGCACGTA-3’, the ORE is present approximately 50 bp upstream of the first exon and has the sequence 5’-GGACGTTCCCACGCTGGGG-3’ (Fukuda et al., 2007; Hüttemann et al., 2007).

Polypeptide sequences of COX4-2 were also examined from a wide array of vertebrate species. The presence or absence of three cysteine residues thought to be important in the isoform-specific function of COX4-2 was determined for the polypeptides. The three residues present in the human sequence are Cys-16, Cys-30, Cys-84 (Hüttemann et al., 2001). Polypeptides were obtained from the online database Ensembl for candidate species from several vertebrate classes and aligned using ClustalW2 (Larkin et al. 2007; Goujon et al. 2010).
2.2 Animal experiments

Zebrafish (Danio rerio, Hamilton) were donated from a wild type population at Université de Montréal. They were held in a 140 L aquarium with dechlorinated water maintained at 25°C for at least six weeks to allow for acclimation. The fish were kept under a 12:12 light:dark photoperiod and fed Omega One Freshwater Flakes (OmegaSea, Painesville, OH, USA) daily ad libitum.

Hypoxia experiments were carried out in air-tight 8 L jars. Groups of eight fish were exposed to either 4% oxygen balanced with nitrogen using a pre-mixed gas cylinder or air for 8 h. After an initial period of 2 h during which the appropriate gas mixture was pumped directly into the experimental jars via an air stone to equilibrate the water to the correct oxygen levels, the apparatus was switched to a flow-through system where 25°C water equilibrated to the correct oxygen level was pumped into the sealed jars at a rate of 4 ml min⁻¹. Immediately following the experiment, fish were lethally anaesthetized in a solution of 0.4 g L⁻¹ of tricaine methanesulphonate and 0.8 g L⁻¹ sodium bicarbonate. Gills, liver, brain, and white muscle were dissected, immediately flash frozen in liquid nitrogen and stored at -80°C.

Western painted turtle (Chrysemys picta bellii, Schneider) liver and pectoralis muscle samples were generously donated by Dr. Leslie Buck (University of Toronto). Tissues were dissected from individuals that were from a control group or had been submerged in freshwater for 24 h, and immediately frozen in liquid nitrogen, as described in (Ramaglia and Buck 2004).

2.3 Tissue culture experiments

Two fish cell lines and two mammalian cell lines were employed in this study. Fish cell lines were grown in Leibovitz’s L-15 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented
with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) under regular atmospheric conditions. ZEB2J zebrafish epithelial cells were maintained at 26°C and RTG-2 rainbow trout (Oncorhynchus mykiss, Walbaum) gonadal fibroblasts were maintained at 19°C. Mammalian cell lines were grown in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and held at 5% CO₂ and 95% humidity. Sol8 mouse (Mus musculus, Linnaeus) myoblast cells and LNCaP human (Homo sapiens, Linnaeus) prostate cancer cells were all maintained at 37°C.

Hypoxia and anoxia treatments were done using gas mixtures of 2% oxygen/98% nitrogen or 100% nitrogen, respectively and lasted for 8 or 24 h. Control treatments were done under normal atmospheric conditions for 24 h. Cells were grown in 6-well plates until they were approximately 90% confluent. The media was then switched to L-15 with 10% FBS that had been pre-equilibrated to the appropriate temperature and oxygen levels. Hypoxia treatment plates were kept in an air-tight chamber containing the appropriate gas mixture and control treatment plates were left at normal atmospheric conditions. All treatments were carried out at the same temperatures in which the cells were previously grown. LNCaP cells were not harvested after 24 h and Sol8 cells were not harvested for either time point of the anoxic treatments (100% nitrogen) because they did not survive these conditions.

Cells were harvested immediately following these treatments by removing the media and scraping in RLT buffer from Qiagen’s RNeasy kit (Qiagen, Valencia, CA, USA) containing 1% β-mercaptoethanol. Cell suspensions were lysed by vortexing for approximately 10 s and then stored at -80°C.
2.4 RNA extraction and cDNA synthesis

RNA was extracted from whole animal tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), using 1 ml of TRIzol per 50 mg of tissue. Samples were homogenized in TRIzol with a tissue homogenizer (Fisher, Ottawa, ON, CAN) and then centrifuged at 12 000 × g for 10 min at 4°C. The supernatant of each sample was collected and mixed with 0.2 ml of chloroform per 1 ml of TRIzol. Samples were incubated at room temperature for approximately 3 min and then centrifuged at 12 000 × g for 15 min at 4°C. The upper aqueous layer was collected and mixed with 0.5 ml of isopropanol per 1 ml of TRIzol. After 5 min of incubation at room temperature, the samples were centrifuged at 12 000 × g for 10 min at 4°C. The supernatant was removed and an equal volume of 75% ethanol was used to wash the pellet. Samples were re-suspended in RNase-free water and quantified using a spectrophotometer reading at 260 nm. Quality of the RNA samples was also assessed during the quantification using the 260/280 ratio.

Extraction of RNA from cell lysates was done using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purified RNA samples were quantified by reading absorbance at 260 nm.

RNA was reverse-transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 1 μg of RNA in a 20 μl reaction, as per the manufacturer’s instructions.

2.5 Quantitative PCR

Primers for qPCR were designed to amplify 50-200 bp (Table 1) in a manner that was linear with respect to template concentration and produced only a single dissociation peak. When possible, primers were designed using sequences available online. For those genes measured in this study that lack a published sequence, primers were designed from consensus sequences of
published genes of related species. While consensus primers served for qPCR in most instances, some turtle genes required partial sequencing using consensus primers before qPCR primers could be designed from these resulting partial sequences. Thus, for turtle I amplified fragments of the transcripts of COX4-2 and VEGFA: (COX4-2: Forward 5’-CCCTGAGCAGAAAGCCCTGAAA-3’, reverse 5’-CATTCTTCTTCTTATAATCCCACTTG-3’; VEGFA: Forward 5’-GCAGCTTCTGAGAATTGA-3’, reverse 5’-GCAAGTGCGCTTAACTCGAATTGA-3’). Large fragments were amplified, cloned and sequenced. To confirm that the amplified fragment was of the desired gene, the obtained sequence was aligned with the consensus sequences used to design the primers. qPCR primers were then designed from the resulting sequences.

Cloning and sequencing was carried out for all primer sets used. They were first tested by PCR using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). All reactions were run in 25 μl volumes containing 1× PCR buffer, 1 mM MgCl₂, 0.75 units Taq DNA polymerase (Qiagen, Valencia, CA, USA), 0.4 μM dNTPs (Promega, Madison, WI, USA), 0.3 μM of each primer, 50-100 ng of cDNA template, and the balance of nuclease-free water. PCR runs involved a 3 min denaturation at 94°C followed by 35 cycles of 15 s at 94°C, 30 s at the appropriate annealing temperature, and 30 s at 72°C. There was a final extension period of 10 min at 72°C. PCR products were visualized on 1% agarose gels with ethidium bromide. DNA amplicons of the expected sizes were removed and purified from the gel, ligated into the pDrive cloning vector (Qiagen, Valencia, CA, USA), transformed into cloning-competent DH5α cells (Invitrogen, Carlsbad, CA, USA), and grown on agar plates containing 0.05 mM IPTG, 0.2 mM X-gal, and 50 ng μl⁻¹ ampicillin. Colonies testing positive for inserts were grown overnight in Lysogeny broth with 50 ng μl⁻¹ ampicillin. Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA), quantified by spectrophotometric readings at 260 nm,
and then sequenced (Robarts Research Institute, London, ON, Canada). Samples were sequenced with an Applied Biosystems 3730 Analyzer (Applied Biosystems, Carlsbad, CA, USA) and aligned with published sequences to verify the amplification of the correct gene fragment.

Real-time quantitative PCR was run on an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using GoTaq qPCR Master Mix (Promega, Madison, WI, USA). Reactions were done in 25 μl volumes with 12.5 μl of the GoTaq Master Mix, 25-100 ng of cDNA template, 0.58 μM each of the forward and reverse primer, and the balance of water. qPCR runs consisted of an initial 15 min denaturation at 95°C followed by 40 cycles of 15 s at 95°C, 15 s at the appropriate annealing temperature, and 36 s at 72°C. All samples were run in duplicate and each primer set was run with a no template control.

For every transcript level measured in qPCR, the threshold cycle (Ct) of each cDNA sample was normalized to a housekeeping factor obtained from the geometric mean of the Ct recorded for β-actin and RPL13A (zebrafish only) or EF-1α (all other species). Thus changes in Ct levels for a particular transcript between samples from different treatments could be corrected for any changes due to the efficiency of cDNA synthesis.
Table 1. Primers used for qPCR quantification of target mRNAs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
<th>(°C)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish</td>
<td>COX4-1</td>
<td>CAAGTTTGTGACACAGCTG</td>
<td>CAAAGAAGAGATTCCTGCAAA</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>COX4-2</td>
<td>CGCAGACCTTACAAAGACATCC</td>
<td>GAAGATAGATAGCTCCAGCCTG</td>
<td>63</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>IGFBP-1</td>
<td>ACGCAAAGAAGATTCCTGCAAA</td>
<td>GGCGCTGCTGAGTTCGATA</td>
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<td>3</td>
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<td></td>
<td>VegFA</td>
<td>ATGATCCCGAGAGACTGACAGCA</td>
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<td>β-actin</td>
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<td>RPL13A</td>
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<td>Rainbow Trout</td>
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<tr>
<td></td>
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<td>EF-1a</td>
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<td>COX4-2</td>
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<td>GAGACAGCTGGAGTGCAATGCA</td>
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<tr>
<td></td>
<td>VegFA</td>
<td>ATAGCTGCTGACATGAGTGG</td>
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<td>EF-1a</td>
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<td>COX4-2</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>COX4-2</td>
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<td>TCGTGAGGGAGGGCTGTCATCTC</td>
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<td>1</td>
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</table>

* Primers were designed from consensus sequences from closely related species
† Primer set was used for all other species listed except mouse
‡ Primer set was used for mouse and turtle as well.
Sources: 1 this study; 2 Little et al. (2010); 3 Marques et al. (2008); 4 Duggan et al. (2011); 5 Tang et al. (2007); 6 Fukuda et al. (2007); 7 Dehne et al. (2007); 8 Kocha et al. (2011).

2.6 Transfection experiments

To address the question of the hypoxia-sensitivity of COX4-2 promoter elements across vertebrates, plasmid constructs were developed for human (Homo sapiens, Linnaeus), Carolina anole (Anolis carolinensis, Voigt), and western clawed frog (Xenopus tropicalis, Wagler), and tested for hypoxic activity in a transfection experiment. The constructs consisted of four tandem repeats of each of the three putative elements responsible for the hypoxic response seen in
mammalian COX4-2 (HRE1, HRE2, and ORE). Oligonucleotides of the tandem repeats were ordered, along with a universal reverse primer containing an MluI restriction site (Table 2). As a positive control, a tandem repeat of the human iNOS HRE (5’-GTGACTACGTGCTGCCTAG-3’) was also designed, as outlined in Table 2.

Double-stranded products were obtained through reactions using the Klenow fragment of DNA polymerase I and an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Reactions were run in 25 μl volumes containing 1× PCR NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT), 12.5 units Klenow (New England Biolabs, Ipswich, MA, USA), 0.4 μM dNTPs (iNtRON Biotechnology, Seongnam-Si, Gyeonggi-do, Korea), 20 mM each of an element oligonucleotide and the universal reverse primer, and the balance of nuclease-free water. The oligonucleotides were first allowed to anneal for 10 minutes before the addition of Klenow by decreasing the thermocycler temperature from 99 °C to 4°C at a rate of 0.3°C/s. The synthesis reaction was carried out at 25 °C for 15 minutes, after which Klenow was inactivated by holding at 75 °C for 20 minutes. To allow for the ligation of these elements into the target plasmid vector, the double-stranded products were phosphorylated by 2 units of T4 polynucleotide kinase per μg DNA (New England Biolabs, Ipswich, MA, USA).

The plasmid vector used in this experiment was the pGL4.23[luc2/minP] Vector (Promega, Madison, WI, USA), which contains a multiple cloning site upstream of a minimal promoter and the luciferase gene luc2. To prepare the vector for ligation with the element inserts, it was digested by 40 units of EcoRV per μg DNA (New England Biolabs, Ipswich, MA, USA). To prevent self-ligation of the vector, terminal phosphate groups were removed by 4 units of calf intestinal phosphatase per μg DNA (New England Biolabs, Ipswich, MA, USA). The resulting cut plasmid was run in a 1% agarose gel, excised, and purified.
The ligation of the plasmid and inserts was performed with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and a 3:1 ratio of plasmid vector to insert. Plasmid constructs were then transformed into cloning-competent DH5α cells (Invitrogen, Carlsbad, CA, USA), and grown on agar plates containing 0.13 mg L⁻¹ ampicillin. Multiple colonies for each construct were grown overnight in LB broth with 50 ng µl⁻¹ ampicillin. Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA), quantified by spectrophotometric readings at 260 nm, and tested for the presence of inserts by digestion reactions with MluI and XhoI followed by visualization on a 2% agarose gel with ethidium bromide. Those plasmids showing inserts of the correct size were then sequenced (Robarts Research Institute, London, ON, Canada). Samples were sequenced with an Applied Biosystems 3730 Analyzer (Applied Biosystems, Carlsbad, CA, USA) and aligned with the designed oligonucleotide sequences to verify the correct insertion and orientation of the insert.

Transfection experiments were carried out using the prostate cancer cell line PC3 in 24-well plates. Cells were grown and kept under the conditions outlined for the mammalian cell lines in section 2.4. When cells were confluent, the media was changed and a mixture of the experimental plasmid construct and a control pRL Renilla vector (Promega, Madison WI, USA) was added to each well using the transfection agent FuGENE6.0 (Roche Applied Science, Laval, Quebec, Canada). Transfected cells were then incubated in an incubator under normal conditions for the control group or under 2% O₂, 5% CO₂, and the balance nitrogen for the hypoxia treatment. The anoxia treatment plates were incubated in an air-tight chamber containing 5% CO₂ and the balance nitrogen. All plates were held at 37 °C for the duration of the experiment.

Transfected cells were harvested after 24 h of treatment by incubating with 1× passive lysis buffer (Promega, Madison, WI, USA). The resulting cell suspensions were transferred to 1.5 ml tubes and frozen at -80 °C for approximately 16 h. Luciferase assays were carried out using a
Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as per the manufacturer’s instructions. Assays were measured using an Lmax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), and resulting values were determined relative to the control Renilla luminescence values for each sample.

Table 2. Element sequences used in transfection experiment.
The plasmid construct for each element contained 4 tandem repeats of the element followed by a 12 bp sequence containing a MluI restriction site (5’-ACGCGTATCGAT-3’).

<table>
<thead>
<tr>
<th>Species</th>
<th>HRE1 (5’-3’)</th>
<th>HRE2 (5’-3’)</th>
<th>ORE (5’-3’)</th>
<th>Accession no.</th>
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<tbody>
<tr>
<td>Human (Homo sapiens)</td>
<td>CTGTGCGCTCCACGCC</td>
<td>CAGGCCCTGTGTCACGTA</td>
<td>GGACGTTCCACGC</td>
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</tr>
<tr>
<td>Anole (Anolis carolinensis)</td>
<td>GTTTGCACTCTTTGGCC</td>
<td>CAGGCATGGGCACTTG</td>
<td>AGGCTATCCTCGC</td>
<td>NW_003339315.1</td>
</tr>
<tr>
<td>Xenopus (Xenopus tropicalis)</td>
<td>ATTTTCGATACCACATA</td>
<td>CGGGTCTCTGGGAGTAG</td>
<td>GGAGTTGCTCCTAAT</td>
<td>NW_003171408.1</td>
</tr>
</tbody>
</table>

2.7 Statistical analysis

All data are presented as means + SEM, and are expressed relative to the control group for each treatment. To determine significance between control and treatment groups, Mann-Whitney U tests were used. Differences were considered significant if \( P < 0.05 \).
Chapter 3

Results

3.1 Mammalian tissues

We employed two mammalian cell lines from two different species (mouse and human) and representing two different tissue types (myoblasts and prostate adenocarcinoma cells), yet neither displayed the expected shift from transcription of COX4-1 to COX4-2 during exposure to hypoxia. The results of these experiments are shown in Figure 3. Neither cell type was able to survive exposure to anoxia, so only results from exposure to 2% oxygen are displayed.

With mouse Sol8 cells, there were significant increases of 2 – 2.6-fold in VEGFA transcripts for the hypoxic treatments (Figure 3B). A significant decrease by half in COX4-1 mRNA levels was seen for both hypoxic treatments as compared to the control group. There was a significant 1.4-fold increase in COX4-2 transcripts only after 24 hours of exposure to hypoxia, but for all treatments, COX4-2 transcripts accounted for less than 0.1% of all COX4 transcripts (Figure 3A and C).

As with Sol8 cells, LNCaP cells displayed significant increases of 4.4 – 11-fold in VEGFA mRNA levels when exposed to hypoxia (Figure 3B). COX4-1 mRNA levels remained steady across treatment groups. Interestingly, although we were able to successfully amplify fragments of the COX4-2 gene using LNCaP DNA, we could not detect any COX4-2 transcripts under any condition, suggesting the COX4-2 paralog is not transcribed in this cell line.
Figure 3. Transcriptional response in murine myoblast cells (Sol8) and human prostate cancer cells (LNCaP) exposed to 8 or 24 h of 2% O₂, n = 6 per treatment. (A) Relative mRNA levels for COX4-1 and COX4-2. (B) Relative mRNA levels for a known hypoxia-responsive gene. (C) Proportion of COX4 transcripts present as COX4-1 or COX4-2. Asterisks represent a significant difference from the control treatment as determined by a Mann-Whitney U test. Values are presented ±SEM.

3.2 Fish tissues

Although we examined two species of teleost fish as well as multiple tissues and experimental approaches using zebrafish, we found no indication that COX4 transcription switches from COX4-1 to COX4-2 during exposure to hypoxia. The results for the whole zebrafish experiment can be seen in Figure 4. Significant increases in the mRNA levels of the
known hypoxia-responsive gene insulin-like growth factor binding protein 1 (IGFBP1) were found in every tissue except for liver, where there was an insignificant 12-fold increase (p = 0.054, Figure 4C). Within the COX4 paralogs, brain, liver and white muscle displayed significant decreases in COX4-1 mRNA levels, and there was a significant 1.4-fold increase in COX4-2 mRNA levels in zebrafish brain (Figure 4A). When both paralogs are taken into account, however, COX4-1 remained in excess of COX4-2 in all tissues during hypoxia, including brain where COX4-2 transcripts never account for more than 35% of all COX4 transcripts (Figure 4B).

Similar results were obtained for the experiments involving cultured fish tissues, also summarized in Figure 4. For the ZEB2J cells, COX4-1 was found to decrease significantly with increasing severity of hypoxic treatments to a minimum of a 0.6-fold decrease relative to the control group after 24 hours of anoxia. COX4-2 mRNA levels remained unchanged except for the group exposed to 24 hours of 2% oxygen, where transcript levels increased significantly by 1.7-fold relative to the control group. Despite the significant results found within COX4-1 or COX4-2 paralogs, COX4-1 transcripts account for more than 99% of total COX4 transcripts across all treatment groups (Figure 4D and E). In experiments with RTG-2 cells, COX4-1 mRNA levels decreased significantly by 1.4-fold after 8 and 24 hours of exposure to 2% oxygen, but did not change during either anoxia treatment. No significant changes were seen in COX4-2 mRNA levels for any treatment group. When taking both COX4 paralogs into account, COX4-1 accounted for between 76% and 85% of total COX4 transcripts (Figure 4E).

While the known hypoxia-responsive gene vascular growth factor A (VEGFA) increased significantly in ZEB2J cells with increasing severity of hypoxia treatment (Figure 4F), no significant differences were seen in RTG-2 cells. Given that the same incubation conditions resulted in a hypoxic response in ZEB2J cells, I believe the lack of response in RTG cells reflects
a different VEGF signaling pathway, rather than a failure to impose hypoxia. Regrettably, I did not explore other options for hypoxia positive controls.

3.3 Turtle tissues

It was previously not known if the Testudine lineage possesses two isoforms of COX4. Some species from another reptilian order (Squamates) possess both paralogs, while the closely-related Aves super-order has been found to possess only COX4-1 (Little et al. 2010). We were able to clone fragments of two distinct COX4 isoforms which amplified in a mutually exclusive manner in quantitative PCR. When a neighbour-joining tree was constructed using these and other COX4 sequences from candidate species representing other taxa, each showed greater homology to one of the COX4 paralogs. Thus we conclude that western painted turtles possess both COX4 isoforms, and we were able to measure their respective transcript levels accurately using quantitative PCR.

Results of the experiment were in agreement with our other findings. We found that although VEGFA increased significantly in hypoxic muscle tissue by 2-fold, no switch in COX4 isoforms was detected during exposure to hypoxia in either liver or muscle tissues (Figure 5A and B). However, turtle COX4 transcript levels were unique in that COX4-2 was the dominant isoform under all treatments in both tissues. COX4-2 accounted for between 69% and 80% of all COX4 transcripts in turtle tissues (Figure 5C).
Figure 4. Transcriptional response of COX4 isoforms to hypoxic treatments in whole zebrafish, and ZEB2J and RTG-2 cell lines.

(A – C) Transcriptional response in gills, brain, liver, and white muscle (WM) of zebrafish exposed to 8 h of 4% $O_2$, $n = 8$ per treatment. (D – F) Transcriptional response in zebrafish epithelial cells (ZEB2J) and rainbow trout gonadal fibroblasts (RTG-2) exposed to 8 or 24 h of 2% or 0% $O_2$, $n = 6$ per treatment. Panels (A) and (D) display the relative mRNA levels for COX4-1 and COX4-2 while panels (B) and (E) display the proportion of COX4 transcripts present as COX4-1 or COX4-2. Panels (C) and (F) display the relative mRNA levels for known hypoxia-responsive genes. No response was seen in any hypoxia-responsive gene in RTG-2 cells (data not shown). Asterisks represent a significant difference from the control treatment as determined by a Mann-Whitney U test. Values are presented ±SEM.
Figure 5. Transcriptional response in muscle or liver tissues of western painted turtles exposed to 24 h of anoxia, n = 6 per treatment. (A) Relative mRNA levels for COX4-1 and COX4-2. (B) Relative mRNA levels for a known hypoxia-responsive gene. (C) Proportion of COX4 transcripts present as COX4-1 or COX4-2. Asterisks represent a significant difference from the control treatment as determined by a Mann-Whitney U test. Values are presented ±SEM.

3.4 Structure of the COX4-2 promoter and polypeptide

The promoter sequences of COX4-2 genes from a selection of vertebrate species were examined and the presence or absence of the putative oxygen-responsive elements was determined (Figure 6). The ORE is better conserved across mammals than either of the HREs. Single nucleotide differences are seen in the ORE for pika of the order Lagomorpha and hyrax of
the order Afrotheria. The HREs are conserved only within the primate family, although there is a single nucleotide substitution from C to G in macaques (*Macaca sylvanus*, Linnaeus). Outside of Mammalia, the elements are poorly conserved. Of the three elements, HRE2 is the most conserved in Xenopus and anole: 61% and 67%, respectively for HRE2 compared to 53% – 59% for the other elements. No recognizable elements were found in the *COX4-2* genes of any teleost species.

The polypeptide sequences of *COX4-2* from vertebrate species were also examined to look for the conservation of three cysteine residues. The results are displayed in Figure 7 The first two residues are hypothesized to form a disulfide bond in an ATP binding site of *COX4-2* which prevents the binding of adenylates (in humans, Cys-16, Cys-30, Cys-84; Hüttemann et al., 2001; Hüttemann et al., 2007). The latter two cysteines are conserved across all examined mammalian species, while the first is not present in all species. Outside of Mammalia, only the middle cysteine is found in anole, and a unique cysteine residue is found near the C-terminus in some teleost species.
Figure 6. Comparison of the putative oxygen sensitive elements in vertebrate COX4-2. The human sequences for the two hypoxia responsive elements (HREs) and the oxygen responsive element (ORE) were used to search for orthologous elements in other candidate vertebrate species. (A) The location of the three elements in relation to the 5′ untranslated region (5′UTR) and the ATG start site (ATG) of COX4-2 is shown for major classes of vertebrates. (B) The determined elements are presented in relation to the vertebrate phylogeny. Differences from the consensus (human) sequence are indicated by filled boxes containing the alternative nucleotide or deletion (represented by a dash). Species names and the accession numbers used for each COX4-2 gene can be found in Appendix 1.
Figure 7. Comparison of three cysteine residues in COX4-2 across vertebrates.
The polypeptide sequences of COX4-2 were aligned. The conservation of the three cysteine residues found in human COX4-2 (Cys-16, Cys-30, Cys-84) was determined across vertebrates. All conserved cysteines are red and bolded. There is a single cysteine near the N-terminus of Xenopus which is one residue away from the conserved cysteine site (underlined C). Species names and accession numbers for each polypeptide sequence used can be found in Appendix 1.

3.5 Reporter analysis of the COX4-2 oxygen sensitive elements

The three putative elements in the COX4-2 promoter were developed into reporter constructs in four tandem repeats and analyzed for their response to hypoxia in a reporter experiment. The elements from human, anole, and Xenopus were studied. The well-characterized HRE from the human iNOS gene responded to 24 h of hypoxia and anoxia by increasing 11- and 15-fold, respectively. Of the COX4-2 elements, only the human HRE2 from COX4-2 was seen to respond 8- and 12- fold, respectively to the treatments. The other element constructs remained unchanged in activity from the control treatment. The results can be seen in Figure 8.
Figure 8. Reporter gene activities for luciferase (pGL4.23) under control of the putative COX4-2 hypoxia responsive elements from human (Hu), anole (An), and xenopus (Xe). Activities were determined from constructs containing four tandem repeats of each hypoxia responsive element (HRE1 and 2) and the oxygen responsive element (ORE). Also included are a known hypoxia-responsive element from the iNOS gene and the baseline activities for the empty pGL4.23 vector (EV). Asterisks represent a significant difference from the control treatment as determined by a Mann-Whitney U test. Results are presented relative to normoxic activities, +SEM.
Chapter 4

Discussion

The notion that COX plays a central role in the regulation of OXPHOS has been gaining popularity in recent years. While doubts about this remain, it is well accepted that the nuclear-encoded genes are vital in the regulation of COX function in mammals. One of the more heavily-investigated nuclear-encoded subunits is COX4, which is encoded by two gene isoforms. These paralogs are of particular interest due to the recent discovery that they differ in the oxygen sensitivities of each gene, and encode proteins that are differentially regulated by adenylates.

*COX4-1* is the ubiquitously-transcribed isoform under normoxic conditions, while transcription switches to *COX4-2* during bouts of hypoxia. The presence of COX4-2 in the COX complex appears to benefit mammalian tissues in two ways: it allows the COX complex to function at high turnover rates regardless of the current energy demands of the cell, and it decreases the production of ROS under low oxygen conditions. These unique functions are thought to be conferred by the two cysteine residues that preclude ATP-mediated inhibition of COX activity. The transcriptional regulation of the *COX4-2* isoform is believed to be controlled by some combination of three putative oxygen-sensitive elements present in the gene. Many uncertainties remain about COX4 isoform function and mechanism even in mammals, but my work focused on the evolution of the protein and gene regulation more broadly in vertebrates. By combining physiological, genetic, and molecular approaches I sought to elucidate the evolutionary history of the oxygen-responsiveness of the COX4 isoforms, and assess their transcriptional response to hypoxic stress in a variety of vertebrate taxa.
4.1 Sensitivity of COX4 isoforms to hypoxia

Since its discovery in 2007, the hypoxia-responsive switch in COX4 paralogs has been largely accepted by the scientific community. However, several shortfalls in the collective knowledge of this function remain. To date, the transcriptional response of COX4 paralogs to hypoxia has only been measured in one whole animal model (mouse) and a select few number of human cancer cell lines (Fukuda et al., 2007; Horvat et al., 2006; Shteyer et al., 2009). Within the mouse hypoxia experiments, a transcriptional response in COX4-2 was seen in only lung and liver out of the five tissues tested, and only one of two types of brain cells when mouse primary cell cultures were examined (Fukuda et al., 2007; Horvat et al., 2006). The use of human cancer cell lines, while common in functional studies, allows for the possibility of generating false positive results as these cells are fundamentally different from other somatic cells in that they have been immortalized through some combination of genetic and epigenetic alterations. Therefore, the current data regarding the hypoxic-response of COX4 isoforms raise questions about the pervasiveness of this hypoxia response across mammalian taxa and tissues, as well as other vertebrate lineages.

To address the current uncertainties, I investigated the transcriptional response of the COX4 isoforms to hypoxia. I used both cellular and whole animal approaches to assess the transcript levels of COX4-1 and COX4-2 in candidate species of fish, reptiles, and mammals. Overall, I found little evidence of the hypoxia-induced switch from COX4-1 to COX4-2 in any of the models I used, suggesting that the hypoxia-responsiveness of COX4-2 is not as pervasive as originally thought, and further that COX4-2 may not play a major role in the cellular response to hypoxia.
4.1.1 COX4 transcriptional response in mammalian cells

A large body of work has been completed involving COX4 function in mammals, yet their hypoxia responsiveness of COX4 isoforms has only been experimentally measured in select tissues of human and mouse. I added to this body of knowledge by measuring the transcriptional response of COX4 isoforms to hypoxic treatments for 8 and 24 h in mouse muscle fibroblasts and a human prostate cancer cell line. Interestingly, the expected switch in COX4 transcripts from COX4-1 to COX4-2 was not seen in either cell type, highlighting the tissue specificity of this response in mammals.

Previously, no mammalian skeletal muscle had been examined for the switch in COX4 isoforms. I expected to see a decrease in COX4-1 and an increase in COX4-2 mRNA levels with exposure to hypoxia in this tissue with large energetic demands. While I did see the expected 0.5-fold decrease in COX4-1 transcripts and a 1.4-fold increase in COX4-2 transcripts after 24 h of hypoxia, COX4-2 comprised less than 0.1% of COX4 transcripts in all treatments (Figure 3). These results are surprising as muscle tissue is generally rich in mitochondria and requires large amounts of ATP. In support of my results, thoroughbred horses (*Equus ferus caballus*, Linnaeus) were found to decrease the transcription of COX4-2 in response to muscular oxygen deprivation, achieved through bouts of sprint exercise (Hill et al., 2010). Fukuda and colleagues similarly found no increase in COX4-2 mRNA levels in the hearts of adult mice exposed to hypoxia (Fukuda et al., 2007). While the latter study leads to the assumption that mice prioritize the delivery of oxygen to vital organs such as the heart, my findings raise the question of whether COX4-2 is able to respond to hypoxia in mammalian muscle tissues.

Of the human cell types previously used in studies on COX4 hypoxia responsiveness, all have displayed a pronounced increase on COX4-2 transcription during hypoxia, and all but one, a primary culture of pancreatic tissue, have been derived from tumors (Fukuda et al., 2007; Shteyer
et al., 2009). The use of tumorigenic cells in studies relating to hypoxia presents conflicting issues. These cells possess fundamentally altered genetic material capable of conferring immortality, and thus may also possess other abnormal genetic features. Conversely, tumors are hypoxic environments where cells must develop alternative strategies to survive a constant lack of oxygen, making them interesting models in which to investigate cellular mechanisms employed in a hypoxic response.

I examined the hypoxic response of the COX4 paralogs in the relatively oxidative prostate cancer cell line LNCaP, and was surprised to find undetectable levels of COX4-2 transcripts under normoxic, hypoxic, and anoxic conditions (Figure 3A). I repeated this experiment with the more glycolytic prostate cancer cell line DU145 and achieved the same result (data not shown). In both cases, regions of the COX4-2 gene could be amplified in a predictable manner in regular and qPCR, demonstrating that the gene has not been lost in these cell lines. Connections between increased COX4-2 expression and cancer have been made by some groups; it is thought that COX4-2 is required in the relatively hypoxic tumor environment to prevent cell damage caused by the accumulation of ROS (Gogvadze et al., 2008; Semenza, 2009). My results provide no evidence to support this theory, even though LNCaP are considered more oxidative than some other prostate cancer cell lines (Higgins et al. 2009). The necessary elements for transcription of COX4-2 may have undergone mutations which silenced the gene in both of the cell lines I examined. Thus, COX4-2 does not seem to be required for the survival of cancerous cells in hypoxic environments.
4.1.2 COX4 transcriptional response in fish

To address the question of the pervasiveness of the hypoxic switch in COX4 isoforms across vertebrates, I investigated the presence of oxygen sensitivity in the COX4 isoforms of teleost fish, employing both cellular and whole animal methods. I measured the mRNA levels of COX4-1 and COX4-2 in four hypoxic and normoxic zebrafish tissues, as well as hypoxic, anoxic, and normoxic zebrafish epithelial cells and rainbow trout gonadal fibroblasts. The results suggest that the COX4-1/COX4-2 switch seen during hypoxia in some mammalian tissues is not present in the teleost lineage.

Whole zebrafish were exposed to 4% oxygen levels for 8 h. Gills, liver, white muscle, and brain tissues from these fish were analyzed and compared to the analogous tissues from normoxic zebrafish. Zebrafish epithelial cells and rainbow trout gonadal fibroblasts were exposed to both hypoxia and anoxia for 8 and 24 h and compared to normoxic cultures. I found that COX4-1 transcripts predominated in all tissues under all experimental conditions, suggesting that COX4-1 is the primarily transcribed isoform in fish tissues under both normoxic and hypoxic conditions. COX4-2 mRNA levels increased significantly only in brain, by 1.4-fold, and 24 h of hypoxic treatment in ZEB2J, by 1.7-fold (Figure 4). However, as COX4-2 accounted for 35% of COX4 transcripts in hypoxic brain tissue, and less than 0.1% of COX4 transcripts in ZEB2J, it appears unlikely that COX4-2 is being incorporated into a significantly larger proportion of COX complexes than COX4-1.

Although this is the first study to measure the hypoxic response of COX4 isoforms in teleost brain tissue, two other studies have examined this response in mammalian brain tissue, both involving mice. Adult mice exposed to 10% oxygen for two weeks showed significant increase in COX4-2 mRNA levels (Fukuda et al., 2007). In contrast to these findings, another study exposed primary cultures of two different brain cell types from postnatal mice to anoxia for
A significant increase in COX4-2 mRNA was found in cortical astrocytes, but not in cerebellar granule cells (Horvat et al., 2006). The disagreement between the results of each study is likely due to the different experimental models used. While the former study did not differentiate between brain cell types, the latter study considered only isolated cells from immature mice. When considered with my results, it remains unclear if COX4-2 transcription responds to hypoxia in mammalian or teleost brain tissue. In fish, studies involving hypoxia treatments on primary brain tissue cultures or other species which are more tolerant to fluctuating oxygen levels would help to clarify this issue.

Significant decreases in COX4-1 mRNA levels were detected in brain, liver, and white muscle of whole animals, and both cell lines after exposure to hypoxia (Figure 4A, D). Again, the proportion of COX4 transcripts remained skewed towards COX4-1 in all cases. COX4-1 transcripts were also found to decrease in human cell lines by Fukuda and colleagues to approximately half of their normoxic levels (Fukuda et al., 2007). This, combined with increases in COX4-2 transcripts during hypoxia, led the researchers to conclude a hypoxic switch in COX4 isoforms had occurred during these treatments. However, as no comparison between COX4-1 and COX4-2 transcript abundance was done in the study, it may be that these shifts in transcript levels seen by the Fukuda group have little effect on the overall proportion of COX4 transcripts, as I saw in my experiments. If this is the case, COX4-2 would likely not play a large role in the physiological response of cells to hypoxic stress.
4.1.3 COX4 transcriptional response in turtle tissues

To bridge the gap in knowledge with respect to the hypoxia responsiveness of COX4 paralogs between teleost fish and mammals, I looked at muscle and liver tissues of hypoxic and normoxic western painted turtles. The freshwater turtle group to which this species belongs is remarkably tolerant to anoxia, being obligate air-breathers that are able to survive anoxia for up to five months (Jackson, 2002). For this reason, they are often used to study the cellular mechanisms of hypoxia response in higher vertebrates. While I failed to see any significant changes in the mRNA levels of each isoform when exposed to hypoxia, I found that COX4-2 was the dominantly-transcribed COX4 paralog in both tissues (Figure 5 C). COX4-2 transcripts were 2–4-fold more abundant than COX4-1 under both normoxic and hypoxic conditions. Such a unique isoform distribution may be explained by the general response of freshwater turtles to anoxic exposure, or a chance alteration in the evolutionary history of this lineage.

When submerged in water, western painted turtles conserve energy via a coordinated down-regulation of both ATP producing and consuming processes within their tissues. This results in a decrease in energy metabolism by at least 49% (Buck et al.; Doll et al., 1994). During the hypoxic stress and contrary to what occurs in mammalian tissues, ROS production in the brains of western painted turtles decreases during bouts of anoxia (Pamenter et al., 2007). This may be due to the global decrease in activity of most cellular processes, or the increased level of COX4-2 present in COX complexes. In mammalian tissues, the presence of COX4-2 lowers ROS production during hypoxia, but increases ROS production during normoxia, relative to complexes containing COX4-1 (Fukuda et al., 2007). Anoxia-tolerant turtles may have made a trade-off between increased ROS production under normoxic conditions and decreased production during hypoxic conditions as a result of their frequent exposure to anoxic conditions.
This is the first study to my knowledge that has measured the normoxic levels of COX4 paralogs or the response of COX4 paralogs to hypoxia in turtles, and I have only examined brain and liver tissues. Further, I was only able to sequence a portion of each COX4 gene, so it is yet unknown if they possess similar gene and polypeptide structures to their mammalian counterparts, and what functional consequences each isoform has on the COX complex. Therefore, it cannot be ruled out that the switch in the dominant COX4 paralog to COX4-2 found in turtles may have arisen from an alternative evolutionary subfunctionalization of the COX4 paralogs. That is, the COX4 paralogs in turtles may have evolved to both serve the same function in the COX complex, or they may have evolved to preform complimentary functions which differ from other vertebrates.

4.1.4 Implications

The finding that COX4-2 replaces COX4-1 transcription during hypoxic stress in mammalian tissues has led to the hypothesis that this occurs as a cellular survival mechanism to combat increased ROS production. I was interested in determining if this mechanism is widespread across vertebrates. My results indicate that not only does this appear to be a unique mammalian feature, but that it may be limited to a select number of species or tissue types within the mammalian lineage. COX4-1 transcript levels remained dominant during hypoxia in all fish tissues I examined, from both whole animals and cultured cells. Similar results were obtained for cultured cells from mouse muscle and human prostate cancer. Additionally, no functional COX4-2 gene is found in the Avian class (Little et al., 2010).

Studies have shown that ROS production is decreased by the presence of COX4-2 within the COX complex in mammals, but the importance of this function in the cellular hypoxic...
response is called into question by my results (Fukuda et al., 2007; Hütttemann et al., 2007). Alternatively, the induction of COX4-2 may be coincidental. Transcription of this gene may instead be of importance in highly oxygenated tissues such as brain and respiratory tissue, where COX4-2 transcription is the highest under normoxic conditions. Brain tissue requires a nearly constant supply of oxygen to prevent damage (Floyd, 1990), while respiratory tissue is the site of oxygen uptake from the environment. ROS tend to form at higher rates in these tissues than in others, so incorporating COX-2 into COX complexes in these tissues may play a role in preventing cell damage caused by ROS (Hüttemann et al., 2007). COX-2 transcription may occur in a hypoxia responsive or oxygen sensitive manner in vertebrates, or some combination of both which is dependent on species and tissue type. The transcriptional response seen in turtle tissues was unique in that COX-2 was the dominant isoform under all conditions. While these results argue in favour of the importance of COX-2 in a hypoxic response, the high levels of COX-2 transcripts may be a strategy limited to the extreme anoxia-tolerant life history of freshwater turtles.

I was able to employ both whole animal and cultured cell models in this study from a selection of vertebrate species. In the case of fish, this allowed me to directly observe differences in transcriptional response between the two models. While the results I obtained from whole fish and cultured fish cells were in agreement, it cannot be ruled out that the mammalian and turtle responses were unique to the tissues and cell types I used. Each model possesses its own strengths and weaknesses. While the use of cultured cells allows for a highly-controlled hypoxic treatment and greater survivability of tissues during hypoxia than whole animals, the cells are immortalized and long separated from an intact animal, and thus may function in a way that is not relevant to what is seen in a whole organism. The use of whole animals may produce responses more physiologically relevant than isolated cells, but it can be difficult to isolate the effects of a single
environmental stimulus such as oxygen levels from others, such as the age, diet, and reproductive stage of individual animals. The innate hypoxia tolerance of each species may also have played a role in my results. For example, my studies in fish were limited to the relatively hypoxia-tolerant zebrafish and the hypoxia-intolerant rainbow trout. COX4-2 may play a more important role in fish with higher hypoxia thresholds, such as goldfish (*Carassius auratus*, Linnaeus). Conversely, the use of a less anoxia-tolerant reptile may have produced results more in accordance with the COX4 isoform transcript abundance seen in the other vertebrates I examined.

4.2 Evolution of COX4-2 in vertebrates

The function and regulation of COX4-2 is widely accepted to hinge on cellular oxygen concentrations in mammals, and play a major role in cellular hypoxic responses. This led researchers to study COX4-2 evolution in thoroughbred horses. It was theorized that since thoroughbreds have been artificially selected for their speed and stamina, they must possess unique genetic features that give rise to the extreme exercise-performance phenotypes, among these features an increased ability to deal with local oxygen depletion in muscle (Gu et al., 2010). Thus COX4-2 was tested for single nucleotide polymorphisms associated with high performance race horses. However, only a weakly-significant correlation was found between a SNP in the COX4-2 gene and racing performance, implying that this gene is not an important factor in the extreme exercise-performance of these horses (Gu et al., 2010). Similarly, due to the apparent presence of orthologous COX4 isoforms across most vertebrate lineages, I hypothesized that the hypoxia responsiveness of COX4-2 would pervade vertebrates. However, my transcriptional data provide evidence that this is not the case. To gain a better understanding of the function and
regulation of COX4-2 in mammals and lower vertebrates, I was interested in examining the evolution of the COX4-2 gene and polypeptide across vertebrates.

4.2.1 Regulatory elements in COX4-2

The regulation of the COX4 paralogs has proven difficult to ascertain. One study concluded the regulation of COX4-1 and COX4-2 was due to the well-defined HIF-1 pathway. The authors showed that COX4-2 transcription was activated through the binding of HIF-1 to a pair of HREs while COX4-1 proteins were selectively degraded through the action of the protease LON which is also activated through the HIF-1 pathway (Fukuda et al., 2007). A second study argued that COX4-2 is regulated by an unknown pathway independently of HIF-1. This group was able to show that the COX4-2 promoter was activated not by its putative HREs, but by an element they termed the ORE which is bound by an as yet unknown transcription factor (Hüttemann et al., 2007). Both studies restricted their promoter analysis to human cell lines. Such perplexingly contradictory results leave uncertainties about both the importance of the putative elements in the hypoxia-responsiveness of COX4-2, as well as their pervasiveness across vertebrates.

Although I saw no hypoxia responsiveness in COX4-2 transcription in any of the experimental models I used, it is possible that the collection of cells and tissues have features with prevent the hypoxia sensitivity from being seen. Since the hypoxia responsiveness of COX4-2 is already known to be tissue-specific in mammals, it is highly likely that lower vertebrate taxa would possess similar specificity. I did not examine every tissue type within zebrafish or western painted turtle, and was only able to use single cell lines for rainbow trout, human, and mouse. Other tissues from these species may display the hypoxia responsive switch in COX4 paralogs.
At the cellular level, the necessary transcription factors and cofactors may not have been present, or the COX4-2 gene may have been epigenetically silenced in the tissues used in this study. I sought to clarify these issues by examining the promoter structures and element activities from a variety of vertebrate species.

I first compared the promoters of the COX4-2 gene from a wide range of taxa to determine the conservation of the two HREs and the ORE across vertebrates. The first HRE is present in the proximal promoter, the second is present within the first intron of the gene, and the ORE is found slightly downstream of HRE1 within the proximal promoter (Figure 6A). None of the putative elements were completely conserved across mammals, though the ORE was far more conserved than either HRE (Figure 6B). This suggests that the ORE is of more regulatory importance than the HREs. Outside of the mammalian class, there was little conservation of the three elements. No recognizable sequence homology for any element could be found in any teleost species, and the elements found in amphibian and reptilian species were as low as 41% conserved compared to the human element sequences. Such a low level of homology outside of Mammalia implies that these putative elements may have arisen after the divergence of the mammalian order, and that the hypoxia-responsiveness of COX4-2 may be specific to mammals. However, as many transcription factor-DNA interactions are species-specific, oxygen-sensitive factors may be able to recognize and bind the non-mammalian elements (Dowell, 2010).

To test the ability of the three hypoxia responsive elements to activate COX4-2 during exposure to hypoxia, I carried out a reporter gene experiment. This allowed for the elimination of the cell type- and species-specific differences discussed above, placing the reporter constructs containing the putative elements in a common context. I compared the efficacy of the hypoxia response of the two HREs and the ORE from the human, anole, and Xenopus COX4-2 genes to a well-known HRE from the human iNOS gene. Only the human HRE2 reporter construct
displayed a response to the hypoxic and anoxic treatments comparable to the \textit{iNOS} HRE response (Figure 8). This is surprising because the Fukuda and Hüttemann groups found a hypoxia-dependent activation of reporter plasmids containing HRE1 or HRE2, and ORE, respectively (Fukuda et al., 2007; Hüttemann et al., 2007). Further, the ORE is the most conserved across mammals while the HRE2 tends to be the least conserved, although HRE2 was more conserved relative to the human sequence in anole and Xenopus than the other elements (61% and 67%, respectively for HRE2 compared to 53% – 59% for the other elements). My results lend some support to the Fukuda group’s assertion that the HRE elements are responsible for the hypoxia responsiveness of \textit{COX4-2}, although only the second HRE found in the first intron. It is not known if the regulation of the HRE in an intron would differ if the same HRE was present in the promoter. However, the elements in a common context in a minimal promoter showed pronounced differences in activity.

It is unclear why the other two human putative oxygen sensitive elements did not respond to the hypoxia treatments. The constructs were exposed to both hypoxic (2% O$_2$) and anoxic conditions for 24 h. I have no reason to believe this was insufficient time for the activation of an oxygen-sensitive element, as the \textit{iNOS} reporter was activated 11- and 15-fold more than the control treatment during hypoxia and anoxia treatments, respectively. However, the possibility of false negative results cannot be excluded, for any of the species. While the HRE in \textit{iNOS} is known to bind HIF-1 during hypoxic stress, the factors responsible for the induction of \textit{COX4-2} remain unknown. Though it is reasonable to assume that each HRE would bind HIF-1, it is not known if other factors, such as coactivators, would be differentially recruited. The cell line I employed may have lacked the necessary factors for the induction of the other hypoxia responsive elements. The elements may also require specific oxygen levels for activation which were not tested in the experiment. The human ORE was found to be most active at 4% O$_2$, although
increased activities were also seen at lower oxygen levels (Hüttemann et al., 2007). The human prostate cancer cell line used in the experiment may have posed issues for the non-mammalian constructs. As the transcription factors present have evolved with the human genome, they may be incompatible with the elements from the COX4-2 gene of anole and Xenopus.

The above concerns are specific to my experiment, but there is a general issue with transfections which may also affect the validity of the results. Much of the activity of elements present within promoters is likely caused by the genetic and epigenetic environment surrounding them. When elements are isolated within a reporter plasmid, the effects of its surrounding environment are lost, though this issue affects all such studies.

4.2.2 Polypeptide structure of COX4-2 in vertebrates

The functional significance of COX4-2 to the COX complex in mammals is well known to be the increase in turnover rate of the enzyme. This appears to be independent of the cytosolic ATP levels, in contrast to COX complexes containing COX4-1 (Fukuda et al., 2007; Hüttemann et al., 2007). The reason for this difference is hypothesized to be the presence of two cysteine residues in the polypeptide sequence of COX4-2. These cysteines are thought to form a disulfide bond in the matrix ATP binding site of the subunit which prevents the binding of adenylates (Hüttemann et al., 2007). This has not been proved experimentally, so I examined the COX4-2 polypeptide sequences of an array of lineages to determine the conservation of the cysteine pair across vertebrates.

I found that the three cysteine residues are only found in mammals, and even within mammals they are not universally conserved. Residues Cys-16, Cys-30, Cys-84 are conserved within most mammalian species, the first two of which are thought to create the necessary
disulfide bond. While the middle cysteines are conserved across all mammalian species I examined, the first and last cysteines are not present in all mammals, suggesting that the disulfide bond is not the important structural difference between the COX4 paralogs (Figure 7). It cannot be ruled out that the increase in turnover rate for COX complexes containing COX4-2 is not seen in those species lacking the first cysteine, or that the latter two cysteines are able to produce the functional difference in COX4-2. Of non-mammalian species, only anole possesses the middle of the three cysteines, while some teleosts possess a single cysteine residue further towards the C-terminus. Similar to the mammals lacking the cysteine pair, it is unknown if COX4-2 performs the same function in these species as in humans. Measurements of COX activities for tissues possessing more COX4-2 compared to those containing more COX4-1 must be carried out in lower vertebrate species to determine if this isoform possesses the same function across vertebrates, as the conservation of the polypeptide structure does not provide a clear answer.

4.3 General Conclusions

Although the induction of the COX4-2 gene during hypoxic stress has been reported in multiple widely-cited studies, questions about this response remain. The oxygen sensitivity of COX4-2 has only been seen in a select few experimental models in human and mouse, and studies have found conflicting results concerning the regulation of the transcriptional increase under low oxygen levels. In my thesis, I sought to gain a better understanding of the oxygen-responsiveness of COX4-2 across vertebrate lineages.

I found that COX4-2 gave minimal or no transcriptional response to hypoxia in any of the vertebrate models I employed. The putative oxygen-responsive elements likewise were not activated during exposure to hypoxia with the exception of the HRE2 from human. These results
lead to the argument that COX4-2 is not of importance in the cellular hypoxic response. However, the muted response of the human HRE2 element and the predominance of COX4-2 transcripts present in turtle muscle and liver tissues run contrary to my assertion, and demonstrate that this interesting gene response must be further investigated.

Of the three putative oxygen sensitive elements present in the COX4-2 promoter, the ORE was most conserved across mammals, yet only the HRE2 in human showed an increased activity during hypoxia treatments. These conflicting results make conclusions about the regulation of COX4-2 in mammals difficult to make, but I have found no evidence to suggest that COX4-2 is regulated by oxygen in lower vertebrates, particularly fish. The three cysteine residues found in human COX4-2 are also not universally conserved in mammals, and only one is found outside of Mammalia. This suggests that the cysteines are either unimportant in the specialized function of the COX4-2 isoform, or that the hypothesized cysteine bond which prevents the binding of adenylates to COX4 is only present in mammals.

Collectively, my results call into question the importance of COX4-2 in the cellular response to hypoxia. Outside of Mammalia, neither the transcriptional switch from COX4-1 to COX4-2 during hypoxia nor the conservation of the putative elements and key polypeptides responsible for this oxygen sensitivity are seen. Thus, the hypoxia-responsiveness of COX4-2 may be restricted to select tissues within some mammalian species, or the response seen in other studies may be coincidental. The importance of COX4-2 may instead be related to its uniquely higher expression levels in brain and respiratory tissue, seen in both mammals and fish (Hüttemann et al., 2001; Little et al., 2010). The fluctuation of oxygen within these tissues may necessitate the use of COX complexes with higher turnover rates to prevent cellular damage by ROS, or fulfill energy requirements. The predominance of COX4-2 transcripts seen in the tissues of the anoxia-tolerant western painted turtle may exist for this reason. My results also
demonstrate the importance of examining a variety of animal models from an array of taxa to assess the potential function of a gene. While the findings of the Fukuda (2007) and Hüttemann (2007) groups are widely cited in studies ranging from the anoxia tolerance of high-altitude mammals to potential oncogenes, my results suggest that COX4-2 may not be of importance in these fields of research.
References


Appendix

Table A1. Species used for assessment of conservation of COX4-2 promoter elements (DNA sequences) and cysteine residues (amino acid sequences, AA).

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