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In Vitro Characterization Of Serine 47 Phosphorylated Cytochrome C

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IN VITRO* CHARACTERIZATION OF SERINE 47 PHOSPHORYLATED CYTOCHROME *c

by

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DEDICATION

To,

All who are dear to my heart

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LIST OF ABBREVIATIONS

1. C*c*O – Cytochrome *c* oxidase
2. ROS – Reactive oxygen species
3. Apaf-1– Apoptotic protease activating factor 1
4. IMM – Inner mitochondrial membrane
5. OMM – Outer mitochondrial membrane
6. ETC – Electron transport chain
7. OxPhos – Oxidative phosphorylation

Chapter 1

An Introduction to cytochrome *c*

1.1 Discovery of cytochrome *c*

In 1886, Charles A. MacMunn first described respiratory pigments and named them myohematin or histohematin[1]. In 1925, David Keilin rediscovered these cellular pigments and named them 'cytochromes'. He found that these heme group containing proteins were found not only in plants and animals but also in yeast and bacteria. He further classified cytochromes according to their lowest energy absorption band in their reduced state and named them cytochrome *a* (604nm), *b* (564nm) and *c* (550nm)[2]. Keilin laid out the basic picture of the respiratory chain in the 1920's. Due to the presence of an iron (Fe) atom in heme it was believed that cytochrome *c* could act as an electron carrier by undergoing oxidation reduction reactions.

Until nearly two decades ago cytochrome *c* protein was known to assume the role of a passive electron carrier in the electron transport chain shuttling electrons from cytochrome bc_1 (Complex III) to cytochrome *c* oxidase (Complex IV). Wang *et al.* were the first to publish that cytochrome *c* is an essential component of the programmed cell death process or type II apoptosis[3]. The paper pointed to cytochrome *c* as one of several factors to influence the dATP dependent activation of CPP32 (caspase 3). They also performed immuno-depletion of cytochrome *c* from HeLa, HEK293 as well as U937 cells and observed that such cells lost their ability to activate caspase 3 to initiate apoptosis. However, this phenomenon was reverted when cytochrome *c* was added to the immuno-depleted extracts.

Cytochrome *c* is a positively charged globular protein, 12.4kDa in size. It contains a heme group covalently attached via thioether bonds with cysteines 14 and 17 of the peptide

chain. The Fe in the heme group is coordinated by His18 and Met80 residues. Cytochrome *c* is a nuclear gene encoded protein which is translated in the cytoplasm as apocytochrome *c*. The heme group is attached to apocytochrome *c* upon its translocation to mitochondria. Under normal (i.e., non-apoptotic) conditions cytochrome *c* is located in the intermembrane space of mitochondria and is mostly associated with the outer surface of the inner mitochondrial membrane by electrostatic and hydrophobic interaction with the negatively charged phospholipid, cardiolipin[4].

1.2 Functions of cytochrome *c*

Cytochrome *c* protein is involved in many diverse and important functions mainly oxidative phosphorylation, cell apoptosis, ROS scavenging, cardiolipin peroxidation, and ROS formation via $p66^{shc}$ (Fig. 1).

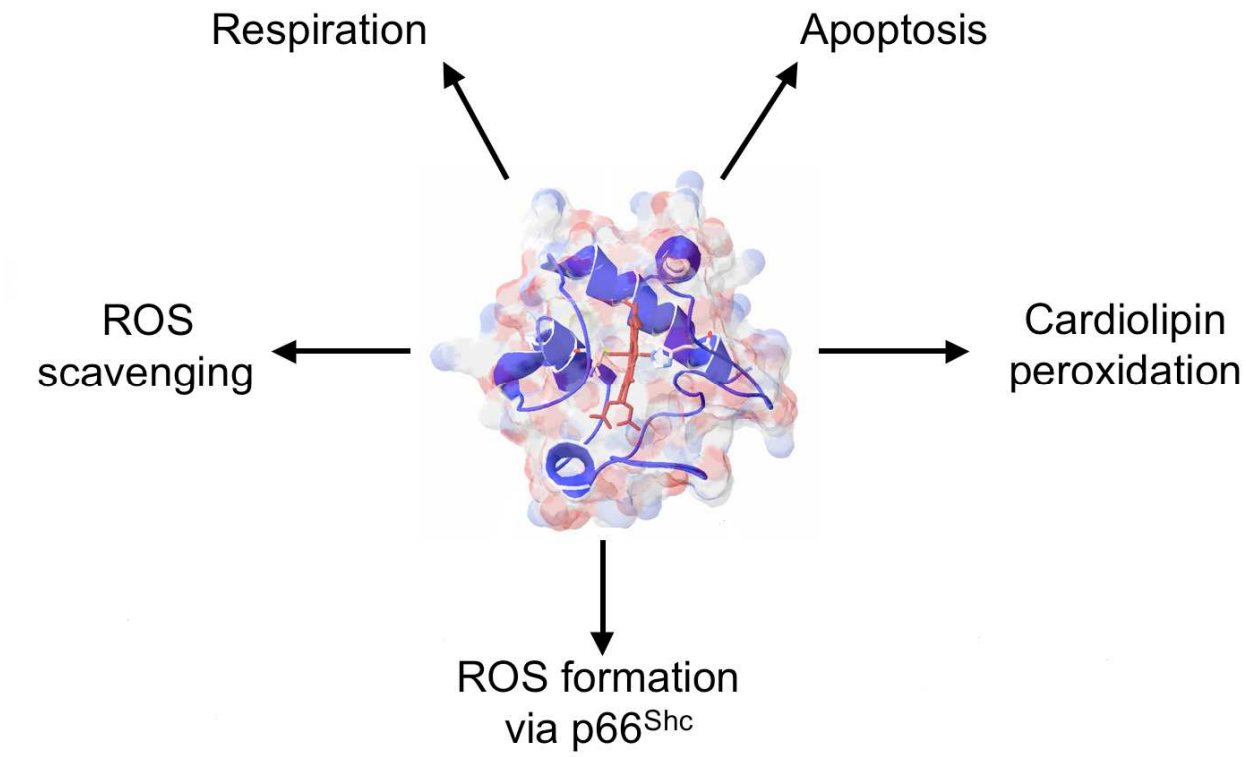

Figure 1. Important roles of cytochrome *c* in a cell[5].

1.2.1 Role of cytochrome *c* in oxidative phosphorylation

The oxidative phosphorylation machinery comprises of electron transport chain (ETC) and ATP synthase (Complex V). It is located in the inner mitochondrial membrane. Electrons from NADH and $FADH_2$ are fed into the ETC, and after passing through the different complexes in the ETC reach oxygen, the final electron acceptor, which is then reduced to water. The ETC has four complexes: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), *bc1* complex (Complex III) and cytochrome *c* oxidase (CcO; Complex IV). Cytochrome *c* functions as an electron carrier by transferring electrons from Complex III to Complex IV. As electrons are passing through the complexes, protons are pumped from the mitochondrial matrix into the mitochondrial intermembrane space via complexes I, III and IV. This results in a proton gradient with the inter membrane space having a higher hydrogen ion concentration than in the matrix. This proton gradient leads to a resultant proton motive force (consisting of the mitochondrial membrane potential $\Delta\Psi$, which is the major contributing factor in eukaryotes, and ΔpH) that is used by the ATP synthase to generate ATP from ADP and phosphate. This ATP generation is coupled to the transport of protons back into the mitochondrial matrix.

Cytochrome *c,* as mentioned earlier, acts as an electron carrier from Complex III to Complex IV transferring one electron at a time. It takes four such electron transfers for oxygen to be reduced to water. ATP, the end product of OxPhos, acts as a feedback inhibitor of the process in an uncompetitive manner[6]. Arginine 91 of cytochrome *c* serves as a binding site for ATP thereby converting the otherwise high affinity cytochrome *c*-C*c*O binding site into a low affinity one[7].

1.2.2 Role of cytochrome *c* in apoptosis

It is now accepted that under stress conditions, cytochrome *c* is released from mitochondria into the cytosol. The released cytochrome *c* binds to Apaf-1 protein in the cytosol, which in the presence of dATP undergoes heptamerization to form the apoptosome[8]. The apoptosome then recruits procaspase 9. This binding leads to activation of the zymogen to active caspase 9. Caspase 9 in turn activates caspases 3 and 7 by cleavage. Caspases 3 and 7 are known to be the executioner caspases of apoptosis.

Genetic studies have brought to light, the importance of cytochrome *c* in the intrinsic (mitochondrial or type II) pathway of apoptosis. In their 2005 paper, Hao *et al* reported that lysine 72 of the cytochrome *c* protein is critical for its role in apoptosis[10]. They generated a mouse model with a mutation of lysine 72 to alanine (Lys72Ala) which retained normal respiration but lacked normal apoptotic function. This was due to a failure of mutant cytochrome *c* to initiate oligomerization of Apaf-1. The Lys72Ala mutant mice were embryonic lethal and exhibited developmental defects of brain. In 2000, Newmeyer *et al* reported that trimethylation of Lys72 reduces the ability of cytochrome *c* protein to activate caspases in cell free systems while maintaining normal oxidative phosphorylation levels.[11] In addition, De La Rosa *et al* published that specific nitration of solvent exposed residue, Tyr74 on cytochrome *c* rendered it incapable of performing its pro-apoptotic function while retaining its peroxidase activity.[12] These studies demonstrate the necessity of cytochrome *c* protein for maintaining proper tissue development and homeostasis.

1.2.3 Cytochrome *c* as a ROS scavenger

Reactive oxygen species (ROS) are highly reactive byproducts of oxidative metabolism and it is known that mitochondria are the main generator of ROS in a cell[13]. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ($^{\cdot}OH$) free radicals. When superoxide

is formed it is quickly converted to hydrogen peroxide by mitochondrial superoxide dismutase (Mn-SOD), which is further detoxified by catalase to water. The complexes I and III of electron transport chain and the reduced ubiquinol pool are the major contributors to ROS formation in mitochondria[5]. In addition, in their 2012 paper, Quinlan *et al* report that under low succinate levels and when complexes I and III are inhibited, complex II produces superoxide at high rates[14].

Within the mitochondrial intermembrane space, oxidized cytochrome *c* can act as a ROS scavenger by its ability to convert superoxide back to oxygen. This reduced cytochrome *c* then passes on the electron to cytochrome *c* oxidase thereby regenerating the oxidized cytochrome *c* which can carry on with superoxide scavenging.

1.2.4 Cytochrome *c* as a cardiolipin peroxidase

Cytochrome *c* is attached to the inner mitochondrial membrane by anionic phospholipids, mainly mitochondria-specific cardiolipin. Its structure is unique with four acyl chain groups. This phospholipid composes nearly 20% of the mitochondrial lipid pool. It is believed that 15-20% of mitochondrial cytochrome *c* is bound to cardiolipin[15]. Cytochrome *c* can either be loosely or tightly attached to cardiolipin. The loose attachment is by means of electrostatic interactions between positively charged lysine residues of cytochrome *c* and negatively charged phosphate groups of cardiolipin[4]. Hydrophobic interactions are at play when cytochrome *c* is tightly attached to cardiolipin. This tight conformation is the result of the insertion of an acyl chain of cardiolipin into the hydrophobic pocket of cytochrome *c* while keeping the other acyl chains embedded in the inner membrane.

Under apoptotic stress, 40% of cardiolipin translocates from the inner mitochondrial membrane to the outer membrane where it forms complexes with cytochrome *c*, activating the peroxidase activity of the protein[16]. The peroxidation of cardiolipin results in decreased affinity of

cytochrome *c* for oxidized cardiolipin thus increasing the availability of the soluble form of cytochrome *c*. Also, peroxidation of cardiolipin affects the integrity of the outer membrane and facilitates the formation of the mitochondrial permeability transition pore through which cytochrome *c* and other proapoptotic factors are released into the cytosol[5].

1.2.5 Cytochrome *c* and $p66^{shc}$

$p66^{shc}$ is a splice variant of two cytoplasmic adaptor proteins, $p52^{shc}$/$p46^{shc}$ which transfer intracellular signals from activated tyrosine kinases to Ras[17]. Instead of Ras regulation, $p66^{shc}$ finds its function in intracellular pathways that regulate ROS metabolism and apoptosis. Like cytochrome *c*, $p66^{shc}$ localizes to the intermembrane space of mitochondria. It is a redox enzyme that generates mitochondrial ROS, thereby signaling the cell to undergo apoptosis. Thus $p66^{shc}$ is proapoptotic in function. In the presence of apoptotic signals, $p66^{shc}$ oxidizes cytochrome *c* and generates H_2O_2. Thus, a fraction of electrons from the electron transport chain are diverted from cytochrome *c* to $p66^{shc}$ under conditions of stress leading to the generation of H_2O_2.

$p66^{shc}$ is a life span determinant in mammals. $p66^{shc}$ null mice have shown decreased aging related diseases like atherosclerosis[17]. Intracellular ROS levels are significantly reduced in $p66^{shc-/-}$ cells and these cells are resistant to apoptotic agents such as staurosporine and ultraviolent light[17]. In the presence of apoptotic signals, $p66^{shc}$ undergoes different modifications. The cytosolic $p66^{shc}$ is serine-phosphorylated and cytosolic levels go up. It was observed that phosphorylation of $p66^{shc}$ is essential for its apoptotic function. However, translocation of serine-phosphorylated $p66^{shc}$ from cytosol to mitochondria is not in significant measure, hence serine phosphorylation is believed to have non-mitochondrial functions[17]. The mitochondrial $p66^{shc}$ which under basal conditions is part of a high molecular weight complex that includes members of the TIM-TOM import complex gets detached from the complex and is released as a

monomer[17]. This monomer increases mitochondrial H_2O_2 levels and results in mitochondrial swelling and apoptosis.

1.3 Regulation of cytochrome *c* release

When a cell encounters and succumbs to apoptotic signals, one of the first steps of apoptosis is the release of cytochrome *c* and other proapoptotic factors into the cytosol[8]. For this to be achieved, cytochrome *c* has to first mobilize from the inner mitochondrial membrane (IMM) and then translocate to the outer mitochondrial membrane (OMM) and into the cytosol[8].

1.3.1 Detachment of cytochrome *c* from inner mitochondrial membrane

Several mechanisms of cytochrome *c* mobilization from the IMM have been proposed so far. The predominant hypothesis, however, is that oxidized cardiolipin has a low affinity for cytochrome *c* and thus results in detachment of cytochrome *c*. Cardiolipin can be oxidized by cytochrome *c* when in the cytochrome *c*-cardiolipin complex, and by ROS[8].

Another mechanism of cytochrome *c* mobilization is by increased cytosolic Ca^{2+}, which weakens the electrostatic interactions between cytochrome *c* and cardiolipin thereby triggering cytochrome *c* release.

1.3.2 Release of cytochrome *c* from outer mitochondrial membrane

After cytochrome *c* is mobilized from the IMM it is translocated into the cytosol through the OMM. Since the OMM is usually impermeable to proteins, mitochondrial outer membrane permeabilization (MOMP) precedes the release of cytochrome *c* into the cytosol[8]. Though the nature of the formation of these openings is not fully understood, many direct and indirect mechanisms have been proposed to date.

1.3.2.1 Role of BCL2 family in pore formation

The BCL2 family of proteins are important regulators of apoptosis. The members of the family can be classified into two sub-groups based on their function: pro-apoptotic and anti-apoptotic. The BCL2 family of proteins possesses four conserved α-helical segments called BH domains: BH1, BH2, BH3, BH4[9]. While most of the anti-apoptotic members of the BCL2 family display sequence conservation in all the four domains, the pro-apoptotic members have less sequence conservation of the BH4 domain[9]. The pro-apoptotic members are further divided into two classes: one contains BH1-3 domains, e.g., Bax and Bak. These are also called effector proteins. The second group includes proteins that contain only the BH3 domain, e.g., BID and BAD.

When inactive, Bax is in the cytosol as a monomer. Sometimes Bax is seen loosely associated to the mitochondrial outer membrane. In the presence of apoptotic stimuli, Bax gets activated and translocates to the mitochondrial outer membrane where it inserts itself into it. Here it undergoes oligomerization and contributes to the formation of pores in the membrane. Unlike Bax, in a non-apoptotic cell Bak resides in the mitochondrial outer membrane in its inactive form. However, when it encounters apoptotic signals it undergoes oligomerization[8]. When both Bax and Bak are activated and present on the OMM they form large openings in the OMM, thus facilitating cytochrome *c* release into the cytosol along with other pro-apoptotic proteins.

The BH3 only proteins Bad and Bid are pro-apoptotic and activate Bax and Bak proteins. When active, Bid known as tBID induces oligomerization of Bax and Bak triggering the formation of pores in the outer mitochondrial membrane to release IMS proteins into the cytosol.

The anti-apoptotic members of the BCL2 family of proteins, e.g., BCL2 and Bcl-xL, prevent MOMP so that cytochrome *c* is not released into the cytosol. These antagonists either

bind to Bax and Bak to inhibit their oligomerization or they bind to BH3 only proteins and stop their pro-apoptotic functions.

1.3.2.2 Role of Ca^{2+} in the release of cytochrome *c*

Ca^{2+} acts as an important regulator of cytochrome *c* release by regulating phosphorylation of pro-apoptotic members of the BCl2 family. For example, elevated cytosolic Ca^{2+} levels activate calcineurin, which in turn dephosphorylates Bad promoting its heterodimerization with Bcl-xL and thus apoptosis[18]. In addition, elevated Ca^{2+} levels regulate Bim phosphorylation to promote its pro-apoptotic function[8].

Mitochondria and endoplasmic reticulum are the major Ca^{2+} stores in a cell. The Ca^{2+} in the mitochondrial matrix favors mitochondrial permeability transition (MPT) by aiding permeability transition pores (PTP) to open[20]. The Ca^{2+} induced opening of PTP results in loss of membrane potential which results in the swelling of mitochondrial matrix and finally leads to the release of cytochrome *c* from the IMS into the cytosol. Low levels of cytochrome *c*, once released, interact with inositol1,4,5 phosphate (IP3) receptors of the endoplasmic reticulum and triggers Ca^{2+} release from the ER to the cytosol[20]. This Ca^{2+} further induces PTP to open thus releasing more cytochrome *c* into the cytosol[8].

1.3.2.3 Effect of cytochrome *c* phosphorylation on respiration kinetics and apoptotic function

Recent studies from our group and others have shown that mitochondria are targeted by cell signaling pathways. These studies have shown that cytochrome *c* undergoes reversible phosphorylations and thus could be part of a cell signaling network. The kinases and phosphatases involved in these cell signaling pathways remain elusive and research on this aspect has gained much interest. To date, four phosphorylation sites have been mapped on

cytochrome *c* by mass spectrometry: Tyr97 in cow heart[20], Tyr48 in cow liver[21], and Thr28 and Ser47[22] in human skeletal muscle.

In 2006, Lee *et al* isolated cytochrome *c* from cow heart tissue under conditions that preserve physiological phosphorylation and found that the protein was tyrosine phosphorylated[20]. Mass spectrometry assigned Tyr97 as the phosphorylated residue. Spectral analysis of purified oxidized heart cytochrome *c* showed a shift of the Met80 absorption band at 695nm to 687nm.This indicated that phosphorylation on Tyr97 had affected the functionality of the protein, specifically the heme moiety. The authors also tested the effect of this phosphorylation on oxygen consumption of purified cow liver C*c*O and found that the phosphorylated cytochrome *c* followed sigmoidal kinetics as opposed to cytochrome *c* treated with alkaline phosphatase, which followed hyperbolic kinetics.

In 2008, Yu *et al* analyzed phosphorylation of cytochrome *c* isolated from cow liver, and by mass spectrometry found out that it was phosphorylated on a novel site, Tyr48[21]. Analysis of oxygen consumption was performed using isolated cow liver C*c*O with cow liver cytochrome *c* that was phosphorylated on Tyr48, and cow liver cytochrome *c* treated with alkaline phosphatase. Since Tyr48 is structurally facing towards the inside of the protein, phosphorylation on this residue could alter the structure of the protein thereby inhibiting its function. This was corroborated by the turnover number of Tyr48 phosphorylated vs phosphatase treated cow liver cytochrome *c*, which was $3.7s^{-1}$ and $8.2s^{-1}$, respectively.

However, since tissue isolated cytochrome *c* usually is a mixture of phosphorylated as well as unphosphorylated forms it is difficult to determine the true implications of these phosphorylations. To this end, Pecina *et al* used a prokaryotic over-expression system to generate phosphomimetic mutants of Tyr48 phosphorylated cytochrome *c*[23]. They mutated the Tyr48 residue to Glu so that the negative charge of Glu may mimic that of the phosphate group

of phosphorylated Tyr48. They also introduced two controls: a wild type cytochrome *c* protein and a non phosphorylatable cytochrome *c* control by mutating Tyr48 to Phe. They next analyzed oxidation of these wild type and mutant forms of cytochrome *c* in the reaction with C*c*O. They observed that the phosphomimetic substitution inhibits oxidation by liver C*c*O where Km values increase from 1.1µM for wild type to 3.7µM for the Tyr48Glu mutant. They next analyzed the capability of these three cytochrome *c* proteins to initiate apoptosis by downstream activation of caspase 3[24]. To measure activation of caspase 3 they used an artificial caspase 3 substrate which gave a fluorescent signal upon cleavage by caspase 3. They observed that both the wild type and Tyr48Phe mutants displayed similar caspase 3 activities while the Tyr48Glu mutant did not give any measurable signal for caspase 3 activity. These data suggest that phosphorylation of cytochrome *c* controls the execution of apoptosis, a process that determines the cell's fate.

1.4 Structure of cytochrome *c*

Horse heart cytochrome *c* was one of the first proteins to be crystallized, in the late 1960's[25]. Cytochrome *c* has a heme group with an iron atom at the center which is involved in the electron transfer activity of the protein. High resolution crystal structure of cytochrome *c* revealed that this heme group is covalently attached to the peptide chain through thioether linkages with cysteine 14 and 17 residues[5]. Cytochrome *c* is a highly basic protein due to high lysine content and has an isoelectric point of 9.6. The Fe atom of heme is in hexacoordinate configuration with Met80 and His18 residues. This increases the stability of the protein. Cytochrome *c* in its oxidized state displays a weak absorption band at 695nm due to the Fe-Met80 bond. Only functional cytochrome *c* displays this band and hence it is a marker for the protein's integrity and correct folding.

Cytochrome *c* is a nuclear encoded protein which is translated in the cytosol to form apocytochrome *c*. This apocytochrome *c* is then translocated to the mitochondrial

intermembrane space where the enzyme heme lyase catalyses the covalent attachment of a heme group to cysteines 14 and 17 through thioether linkages[5]. Attachment of heme results in cytochrome *c* taking its native compact conformation. Mitochondrial import of cytochrome *c* neither requires the presence of ATP nor does it depend on mitochondrial membrane potential.

Sequence analysis of all the 285 known cytochrome *c* sequences shows that the number of amino acids ranges from 104-114 in most mitochondrial cytochrome *c* species of which only a few amino acids are highly conserved throughout evolution[24]. These key residues play an important role in maintaining the structure, function, folding, and stability of the protein. The key residues are Cys14, Cys17, His18, Gly29, Pro30, Gly41, Asn52, Trp59, Tyr67, Leu68, Pro71, Pro76, Thr78, Met80, and Phe82[24].

Cys14 and Cys17 of cytochrome *c* are part of a pentapeptide motif, CXXCH, and covalent attachment of the heme group to this motif is an important step in cytochrome *c* maturation. Mutational studies show that these cysteine residues are required for the translocation of apocytochrome *c* from cytoplasm to mitochondria[26].

His18 and Met80 are the two axial ligands of cytochrome *c*, where His18 binds heme from the proximal front while Met-80 is on the distal side in the conventional view (Fig.1). His18 is known to stabilize the heme of cytochrome *c*[24]. It is found that Met80 favors a high reduction potential of cytochrome *c* by maintaining it in the reduced state. Met80 mutants have shown their inability to form axial coordination with heme. For example, Met80Ala mutants do not form axial co-ordination with heme thus rendering it in penta-coordinate state.[24, 27]

The side chain of Trp59 is hydrogen bonded to heme propionate side chain and gives a hydrophobic environment to the heme crevice. This hydrophobic interaction contributes to the stability of the cytochrome *c* protein. Studies with Ser, Cys, and Gly mutants of Trp 59 showed that they were thermo-labile and non-functional[24].

Another well conserved residue is Tyr67 which is located within the helix. The hydroxyl group of Tyr67 has influence on its redox properties. For example, in its reduced state the hydroxyl group forms hydrogen bonds with the side chains of Asn52, Thr78, and Met80 and the internally bound conserved water molecule, Wat166[24]. However, in its oxidized state Wat166 gets reoriented and this disrupts all of these hydrogen bonds. Hence it affects both redox potential and stability of the protein.

Phe82 is located on the surface of the protein and is in close proximity to the heme. Studies have shown that this residue stabilizes the heme environment and the side chain of Phe82 maintaining the overall conformation of the protein[24, 28]. Thus, this residue holds an important role in maintaining the functionality of cytochrome *c*.

There are three prolines in the cytochrome *c* amino acid sequence and they are highly conserved throughout evolution. They are at positions 30, 71 and 76. Their rigid cyclic structure contributes to the overall conformational integrity of the protein. For example, Pro30 holds His18 in place within the heme crevice[24, 26] and forms the closed crevice structure of cytochrome *c*.

Lys72 is an important residue for cytochrome *c* binding to C*c*O as well as bc_1 complex. Studies have shown that in yeast trimethylation of this residue favors transport of apocytochrome *c* to the mitochondria. However, it is unmethylated in higher organisms. Lys 72 plays a key role in the cytochrome *c*-Apaf1 interaction.[24, 29]

As with other proteins that are synthesized in the cytosol, cytochrome *c* is also subject to posttranslational modifications. This may include modifications like cleavage of the N-terminal methionine residue and N-terminal acetylation.[30] *In vitro* studies have shown that N-terminal acetylation of cytochrome *c* prevents protein degradation. Besides methylation[31] and phosphorylation, cytochrome *c* can also undergo nitration. *In vivo* nitration of human cytochrome *c* has been observed in 3 tyrosine residues, namely Tyr67, Tyr74[32], and Tyr97[33]. Being solvent

exposed, Tyr74 and Tyr97 are preferred for nitration. *In vitro* studies show that nitration of these residues impairs both respiration and apoptosis, while it enhances the peroxidase activity of cytochrome *c*.

Though *in vivo* nitration of Tyr46 and Tyr48 has not been reported, *in vitro* studies have been performed to understand potential effects of nitration of these residues and it has been shown that nitration of these residues result in formation of a non-functional apoptosome thus hindering apoptosis[34].

Owing to the high lysine content in cytochrome *c* it could be possible that the protein undergoes yet another modification, acetylation[30]. Besides neutralizing the positive charge of the protein rendered by the lysine content, acetylation could also increase its hydrophobicity as well as have effects on the protein's conformational integrity. Though the functional relevance of acetylation is not fully understood it has been shown that cytochrome *c*, when chemically acetylated, could impact its redox properties.

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Chapter 2

Characterization of bovine cytochrome *c* from kidney tissue

2.1 Background of the study

Regulation of the proteins of OxPhos machinery by cell signaling is a recent research area. So far, over 20 phosphorylation sites have been mapped on mammalian OxPhos proteins. For almost all of those phosphorylations the effect on enzyme function and the kinases and phosphatases mediating these (de-)phosphorylations remain unknown.

In the recent past the Hüttemann lab reported two novel phosphorylation sites, Tyr97 and Tyr48 of bovine heart and liver, respectively. Since mass spectrometry unambiguously assigned two distinct phosphorylation sites in the two tissues, it was proposed that cell signaling takes place in a tissue specific manner. To further test this hypothesis additional functional experiments were designed. The effects of these Tyr phosphorylations on the functions of cytochrome *c* protein, particularly respiration and apoptosis, were assessed. Results revealed that both phosphorylations led to an inhibition of cytochrome *c* in the reaction with C*c*O. This fortified the working model of the Hüttemann lab that under healthy conditions cytochrome *c* is phosphorylated and maintains the cell in a “controlled” respiration state to avoid high mitochondrial membrane potentials that are known to cause excessive formation of ROS. Tyr48 phosphomimetic mutational studies of cytochrome *c* showed that the mutants were incapable of initiating apoptosis by caspase activation.

Also reported are the phosphorylations of Thr28 and Ser47 of human skeletal muscle suggesting that a different cell signaling pathway might be responsible for it.

Based on the above reports our group hypothesized that phosphorylation sites were tissue specific. To obtain further support for this concept, bovine kidney tissue, which has not been

previously analyzed, was chosen to test for any novel phosphorylation sites and their possible effects on its function.

2.2 Isolation of cytochrome *c* from bovine kidney tissue

Fresh cow kidneys were purchased from a slaughter house, transported on dry-ice to the lab, and stored in the -80^{o}C freezer until used. Cytochrome *c* was isolated from the kidney tissues using the acid extraction method as described in detail in reference 1 (see Fig. 2 for a schematic representation). All steps were performed on ice. The tissues were ground and homogenized in 100 mM phosphate buffer (pH7.4), whose pH was lowered to 4.5 with acetic acid[1]. The homogenate was incubated in this low pH buffer overnight at 4ºC.Most cellular proteins aggregate and precipitate at low pH, while cytochrome *c* stays in solution. Moreover, at low pH cellular phosphatases are inactive.

The following day the homogenate was centrifuged at 8500rpm for 35min (Sorvall SLC-6000 rotor). The pellets were discarded (cell debris and precipitated proteins) while the supernatants were pooled and pH readjusted to 7.4 with NaOH/KOH and protease and phosphatase inhibitors were added immediately to the solution to inhibit the activity of proteases and phosphatases that might be reactivated at physiological pH. This mixture was incubated at 4ºC for 20min before the last centrifugation step. Cytochrome *c* was purified from this supernatant by ion exchange chromatography. First, the solution was applied to a DE52 anion exchange column, which was equilibrated with phosphate buffer, pH7.4, 3.6mS/cm conductance. Cytochrome *c* being positively charged, was collected in the flow-through and the pH was adjusted to 6.5. The flow-through was loaded onto a CM52 cation exchange column, which was equilibrated in 20 mM phosphate buffer, pH6.5 and conductance 5.5mS/cm[1]. The pH and conductivity of the flow-through was readjusted to that of a CM52 cation exchange column before applying it to the column. The positively charged cytochrome *c* protein bound to the CM52 column. In order to obtain a single cytochrome c fraction, the protein was oxidized on the

column using 2mM $K_3Fe(CN)_6$ and was then eluted out by a step elution method using 30, 50, 80, 120 and150mM phosphate buffers.

For better purity, the DE52 and CM52 column steps were repeated. This was followed by HPLC size-exclusion chromatography, equilibrated with 150mM phosphate buffer, pH6.5 to obtain a highly pure and homogeneous fraction of cytochrome *c* usually containing a mixture of phosphorylated and dephosphorylated forms of the protein. Cytochrome *c* protein was concentrated under vacuum to a 2mL fraction followed by desalting by centrifugation using Amicon Ultra-15 3k units (Millipore, Billerica, MA). The desalted protein was aliquoted and stored at -80^{o}C till used.

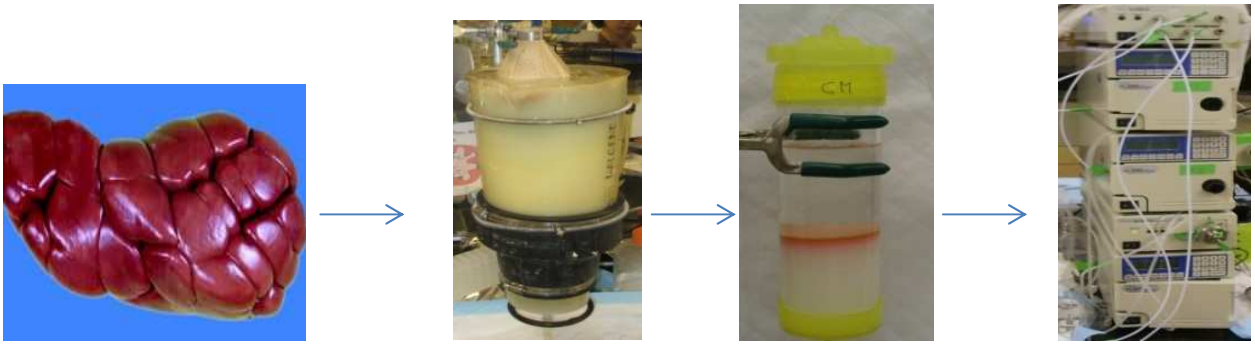

Figure 2. Schematic diagram of cytochrome *c* isolation.

2.3 Protein concentration and purity

The isolated protein was reduced with 100mM sodium dithionite ($Na_2S_2O_4$) following desalting using NAP-10 columns (GE Healthcare, Piscataway, NJ) and analyzed on a Jasco V-570 double beam spectrophotometer (2nm band width)[1]. The concentration of reduced cytochrome *c* was determined by the difference spectra at 550 nm of the reduced minus oxidized form, and calculated using □(red.–ox.)550 nm=19.6 mM^{-1} $cm.^{-1}$

To check for the purity of the isolated protein, samples were loaded onto a 12% Tris tricine SDS PAGE gel and visualized by Coomassie staining.

2.4 Western blotting

Next, cytochrome *c* was subjected to SDS gel electrophoresis. 12% Tris tricine gels were prepared and denatured proteins were loaded alongside with suitable controls. After the run was complete the proteins were transferred to PVDF membranes (0.2µm, Bio Rad) using a semi-dry blot apparatus (BioRad). Care was taken to pre-soak the membranes as well as the gel in the transfer buffer (25mM Tris, 192mM glycine, 20% methanol) used for blotting[1]. Transfer was performed at 15V for 1hr. After transfer, the membranes were blocked in their respective blocking buffers (Table1). Blocking was done under gentle shaking at room temperature for 2h. After blocking, the blots were incubated with their respective primary antibodies (Table1) and left under shaking overnight, at 4ºC. Each incubation was followed by washing the blots with 1X TBS-T 6 times at 10 min intervals. The blots were then incubated with their respective secondary antibodies (Table1). After washing with 1X TBS-T, the blots were used to detect signals using the ECL detection kit.

Name of the blot	**Blocking agent**	**Primary Ab**	**Secondary Ab**	**Detection kit**
Anti-cytochrome *c*	5%NFDM in 1XTBS-T	Anti-Cytochrome *c*, 1:10000	IgG HRP-anti mouse(1:10000)	ECL
Anti-phosphoSerine	2% BSA in 1X TBS-T	Anti-phospho serine antibody cocktail, 1:5000	IgM HRP-anti mouse(1:10000)	ECL

Table 1. Antibodies for Western blots

2.5 Mass spectrometry of purified cytochrome *c* to detect site- specific phosphorylation(s)

Purified cytochrome *c* was sent to our collaborator, Dr. Arthur Salomon (Brown University, Rhode Island), who is an expert in protein phosphorylation, for assignment of phosphate groups by mass spectrometry on specific sites of cytochrome *c*.

2.6 Bacterial overexpression of mutant cytochrome *c* proteins

The pLW01 vector is a suitable prokaryotic expression system to generate the cytochrome *c* constructs since the vector also has the cDNA encoding the heme lyase (CYC3) gene which is necessary for the covalent attachment of the heme group to the cytochrome *c* apoenzyme but missing in bacteria. The vector, originally containing the horse cytochrome *c* cDNA sequence, was a kind gift from Dr. Lucy Waskell (University of Michigan) and was earlier used in our lab to create rodent cytochrome *c* constructs.[2] Site directed mutagenesis was performed using 5' GCT GGA TTC GAG TAC ACA GAT 3' and 5' GGA TTC GCC TAC ACA GAT GC 3' to mutate Ser 47 of cytochrome *c* to phosphomimetic glutamic acid and unphosphorylatable alanine, respectively. The outer primers that amplified the entire cytochrome *c* cDNA contained NcoI and BamHI restriction sites and were 5' AAT TTA CCA TGG GTG ATG TTG AAA AAG 3' and 5' AAT AAA GGA TCC AGT GGA ATT ATT CAT 3' respectively (refer Table 2) .The mutated cytochrome *c* cDNA sequences were later subcloned into the pLW01 bacterial expression plasmid after restriction digested by NcoI and BamHI. The generation of the mutant constructs was confirmed by sequencing and the correct mutants were used to transform competent *E. coli* cells to overexpress the protein. The clones were inoculated into 20 mL TB medium (Difco, BD, Franklin Lakes, NJ) containing 100 µg/mL carbenicillin and grown overnight at 37ºC under shaking. These cultures were used to inoculate 4 L of 100 µg/mL carbenicillin-containing TB medium that was grown until it reached an OD_{600} of

2-3[2]. At this time, the expression of cytochrome *c* was induced by addition of 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein was overexpressed for 6 h at 37ºC. The cells were harvested by centrifugation for 40 min at 4500 rpm, 4ºC and the pellets were immediately stored at -80ºC until used.

Name	Primer Sequences	Tm (^{o}C)
S47A, Forward primer	5' GGA TTC GCC TAC ACA GAT GC 3'	55.9
S47A, Reverse primer	5' GCA TCT GTG TAG GCG AAT CC 3'	55.9
S47E, Forward primer	5' GCT GGA TTC GAG TAC ACA GAT 3'	54.3
S47E, Reverse primer	5' ATC TGT GTA CTC GAA TCC AGC 3'	54.3
Outer forward primer	5' AAT TTA CCA TGG GTG ATG TTG AAA AAG 3'	56.7
Outer Reverse primer	5' AAT AAA GGA TCC AGT GGA ATT ATT CAT 3'	54.7

Table2. Primers used in site directed mutagenesis and overlap extension PCR

2.7 Purification of cytochrome *c* from bacterial pellets

The bacterial pellets were thawed overnight at 4ºC. For every 10gm pellet 100ml of lysis buffer was used. The lysis buffer was 20mM phosphate buffer, pH7.4 supplemented with protease inhibitors. The pellet was resuspended in the lysis buffer using a hand homogenizer. This was followed by lysis via French Pressure Cell Press (American Company, Aminco).

Figure 3. French Pressure Cell Press.

The lysed cells were centrifuged at 15000rpm for 45min to remove the pellet with cell debris. The pH of the supernatant was adjusted to 7.4 and conductivity matched with that of the DE52 column before it was loaded onto the equilibrated column. The flow-through was collected and pH readjusted to 6.5 and conductivity matched with that of the equilibrated CM52 column. The flow through was then loaded onto the column where the positively charged cytochrome *c* binds. The column was washed with equilibration buffer (30mM phosphate buffer, pH6.5). Cytochrome *c* was eluted from the column using a high salt buffer (0.5M NaCl in 30mM phosphate buffer, pH 6.5). The eluted protein was desalted by centrifugation using Amicon

Ultra-15 3k units (Millipore, Billerica, MA). Buffer exchange was performed in deionized water and the protein aliquoted and stored in -80^{0}C.

2.8 *In vitro* functional characterization of cytochrome *c* mutant proteins

2.8.1 C*c*O activity measurements using the Clark type oxygen electrode system

Our lab has a stock of regulatory competent C*c*O which was isolated from bovine liver under conditions preserving its phosphorylation status[3]. Liver type C*c*O is the same isozyme as expressed in kidney. An aliquot of C*c*O was diluted to 3μM final concentration in the presence of a 40-fold molar excess of cardiolipin and 0.2 mM ATP in C*c*O measuring buffer (10mM K-HEPES (pH 7.4), 40mM KCl, 2mM EGTA, 10mM KF, 1% Tween 20) and dialyzed overnight at 4ºC to remove cholate bound to C*c*O during enzyme purification. Respiration of C*c*O (54.54nM) was analyzed in a closed chamber equipped with a micro Clark-type oxygen electrode (Oxygraph system; Hansatech, Pentney, UK) at 25ºC in 220μL of C*c*O measuring buffer and 20 mM ascorbate as electron donor[2]. Increasing amounts of purified cytochrome *c* variants (0-15 μM) were added, and oxygen consumption was recorded and analyzed with the Oxygraph software (Hansatech).[2] The activity was expressed as turnover number (TN) (min^{-1}).

2.8.2 Caspase 3 activity induction by cytochrome *c* mutant proteins

An *in vitro* approach with cell free extracts was used to assess the capability of cytochrome *c* mutants to induce caspase 3 activity. Cytoplasmic extracts were prepared from a HeLa cell line. Cells were harvested from eight 150mm plates[4]. After washing the plates twice with 1X PBS the cells were harvested following centrifugation at 4ºC, 800rpm, 5 min. This was repeated once after which the pellet was resuspended in 5mL of ice cold cell extraction buffer (20mM HEPES, pH7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 1mM PMSF) followed by centrifugation at 4ºC, 800 rpm, 5 min. The cell pellet was then gently resuspended in the extraction buffer and immediately transferred to a dounce homogenizer and

kept on ice for 15 min for the cells to swell[2]. After swelling, the cells were broken open using a B-type pestle. Cell breakage was confirmed using a microscope and lysates transferred to Eppendorf tubes and centrifuged at 4ºC, at 15000g for 15min to remove nuclei and other organelles. The resultant supernatant is the cytosolic fraction. The protein concentration of the lysate was measured using the D_c assay kit (Bio Rad, Hercules, CA). The cytosol extract was diluted to a concentration of 2mg/mL for the caspase assay. The kit used was the EnzChek Caspase-3 assay kit (Invitrogen, Carlsbad, CA) with rhodamine 110-linked DEVD tetrapeptide as an artificial substrate of caspase-3, which fluoresces upon cleavage. The cytosolic extract from HeLa cells (2mg/ml) was incubated with individual cytochrome *c* mutants (15µg/ml) at 37ºC for 2.5h. After incubation, 10µl from the activated extracts were aliquoted and assayed for caspase 3 activity in triplicate. Also, in parallel, a similar assay was performed in the presence of caspase 3 inhibitor.

Fluorescence was detected using a Fluoroskan Ascent FL plate reader (Labsystems, Thermo Scientific, Waltham, MA), excitation filter 485nm/14nm bandwidth, emission filter 527nm/10nm bandwidth. Fluorescence values were acquired in 30min intervals for 3h. Amount of cleaved substrate was calculated from the rhodamine 110 calibration curve, and data were expressed in pmol of DEVD3 min^{-1} (mg of protein)$^{-1}$.

2.8.3 Measurement of cytochrome *c* redox potential

The midpoint redox potential ($E^{0'}$) was analyzed spectrophotometrically using 2,6 dichloroindophenol (DCIP,$E^{0'}$=237 mV) as a reference compound, which has an absorption band at 600 nm in its oxidized state[5]. One milliliter of cytochrome *c* solution (2mg/mL) was mixed in a large spectrophotometric cuvette with 2mL of 50mM citrate buffer, pH6.5, 0.1mL of 1mM DCIP, and 50µL of 1mM $K_3Fe(CN)_6$ to fully oxidize cytochrome *c*. Absorbances corresponding to fully oxidized cytochrome *c* (A_{550}-A_{570}) and DCIP (A_{600}) were recorded using a

Jasco V-570 double beam spectrophotometer. The mixture was then sequentially reduced by 1µL additions of 5mM ascorbate (pH 6.5), and absorbance values were acquired at each step. When readings became constant, a few grains of $Na_2S_2O_4$ were added to fully reduce cytochrome *c* and DCIP. For each step, ratios of oxidized and reduced forms of both compounds were calculated. Data obtained were plotted as $\log(DCIP_{OX}/DCIP_{RED})$ versus $\log(Cyt_{OX}/Cyt_{RED})$, yielding a linear graph with a slope of n-DCIP/n-cytochrome *c* and a y-axis intercept of n-cytochrome *c* / 59.2(E cytochrome *c* – E DCIP). These values were used to calculate the $E^{0'}$ of cytochrome *c* from the Nernst equation.

2.9 Results

2.9.1 Western blotting

Cytochrome *c* was purified from bovine kidney tissues under conditions that preserved its physiological phosphorylation status. After eluting cytochrome *c* from the CM52 cation exchange column it was passed through an HPLC gel filtration column to increase the purity of the protein. The protein was found to be clean on a Coomassie gel (Fig. 4). Note that the weaker higher band (24kDa) represents the cytochrome *c* dimer, which is sometimes observed.

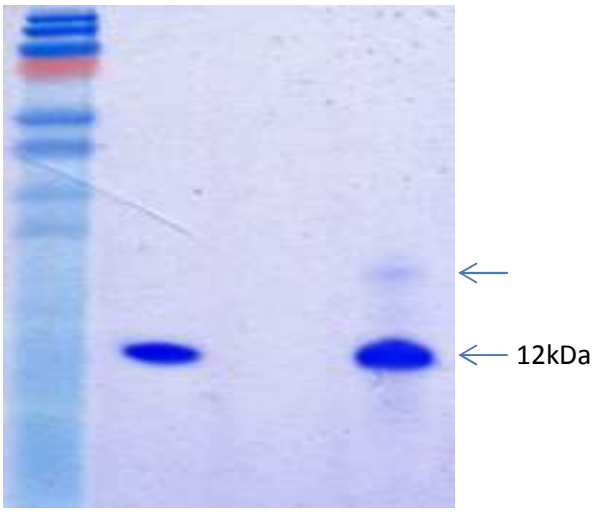

Figure 4. Coomassie gel of cytochrome *c* purified from kidney. Lane 1: Page Ruler prestained protein ladder,10-170 kDa, Lane 2: cow heart cytochrome *c* from Sigma(2µg) Lane 3: 1XLDS and Lane 4: isolated cow kidney cytochrome *c* (2µg).

Western blotting with anti-phospho Serine antibodies was performed to analyze the cytochrome *c* phosphorylation status. Both gave strong signals for phosphorylation as shown below.

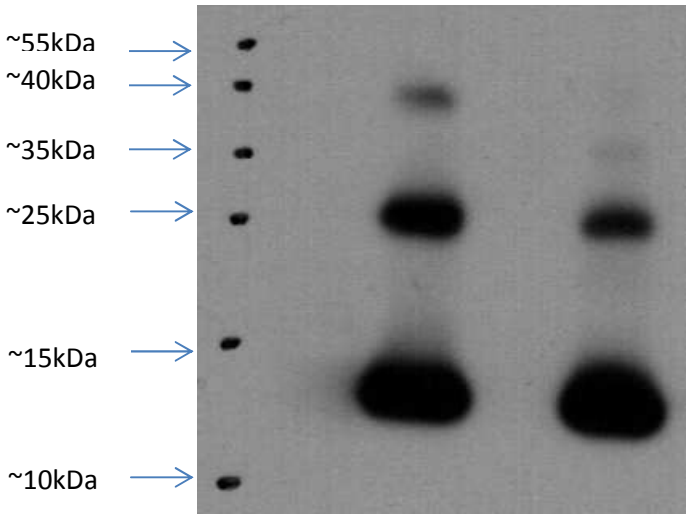

Figure 5. Western blot with anti-cytochrome *c* antibodies. Lane 1: Page Ruler pre-stained protein ladder, 10-170 kDa, Lane 2: cow heart cytochrome *c* from Sigma (2µg), Lane 3: empty, Lane 4: cytochrome *c* purified from cow kidney tissue (2µg).

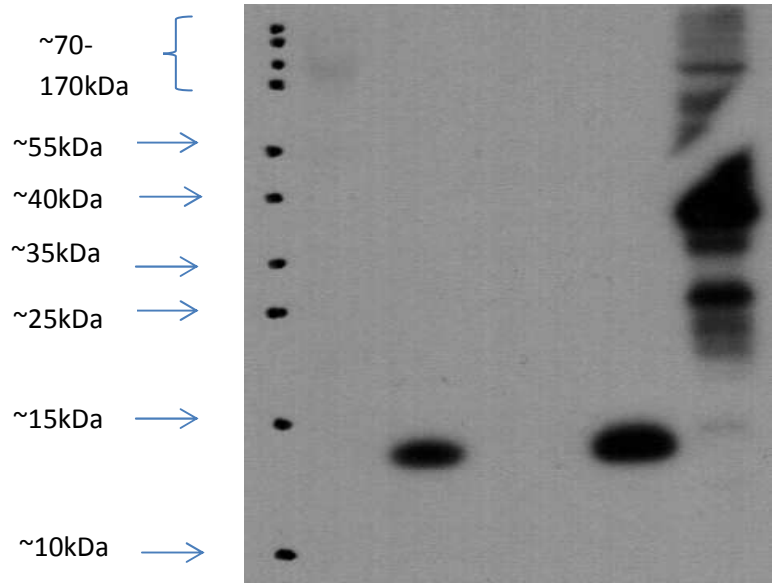

Figure 6. Western blot with anti-phospho serine antibodies. Lane 1: Page Ruler prestained protein ladder, 10-170 kDa, Lane 2 & Lane 4: cytochrome *c* purified from cow kidney tissue (1µg and 2µg respectively), Lane 3: empty, Lane 5: positive control, ovalbumin.

2.9.2 Mass spectrometry of cytochrome *c* to detect site-specific phosphorylation(s)

Because the anti-phospho-Ser/Thr antibodies are not highly specific due to the small epitope they recognize and in order to assign the specific phosphorylation sites, isolated cytochrome *c* was digested with trypsin and analyzed by immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry (IMAC/ nano LC/ESI-MS/MS). In this method the phosphorylated peptides are enriched before being subjected to tandem mass spectrometry for fragment examination.

The analysis revealed Ser47 phosphorylation of cytochrome *c* on the peptide, **KTGQAPGFpSYTDANK**. Depending on the individual kidney cytochrome *c* isolation two additional phosphorylation sites were revealed, Thr28 and Thr 51 (data not shown).

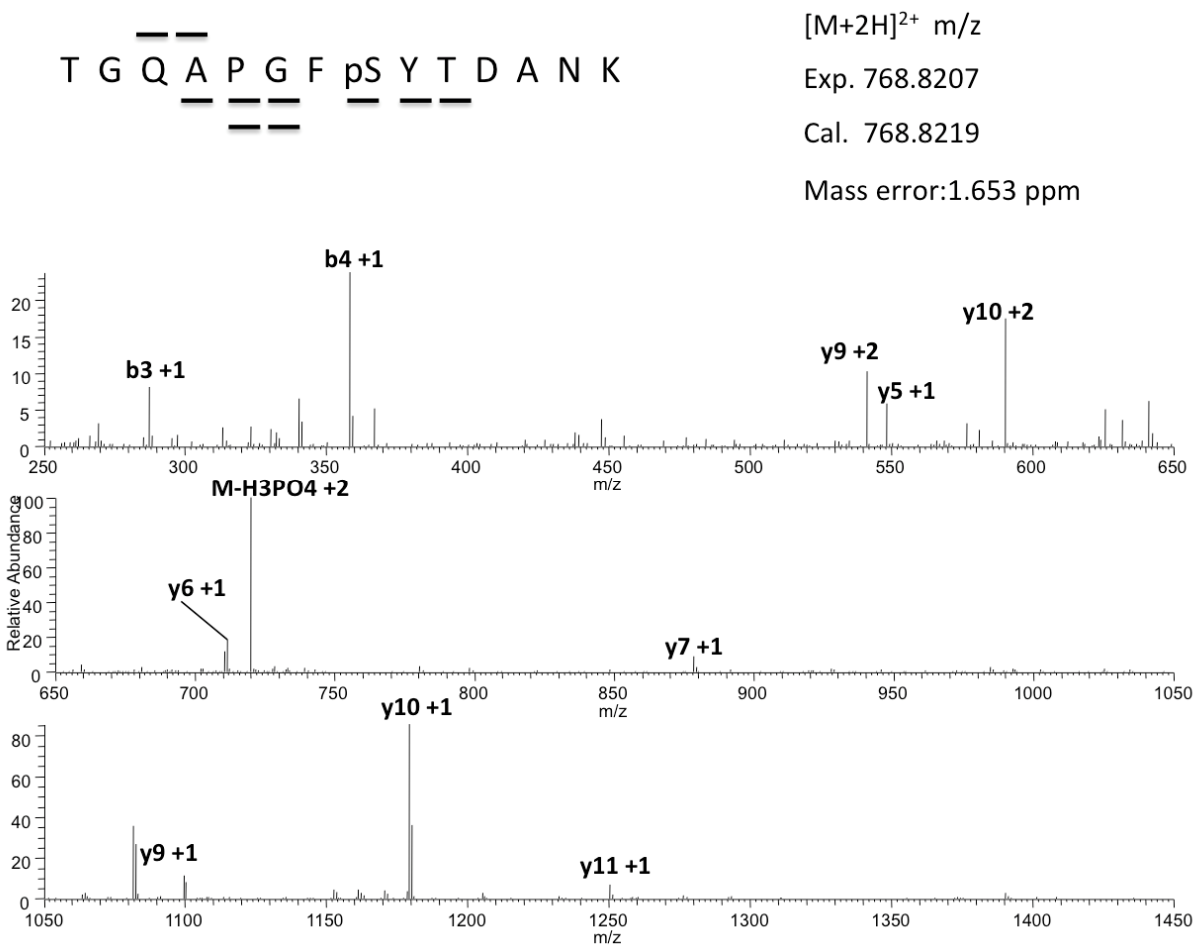

Figure 7. Nano-LC/ESI/MS/MS spectrum of KTGQAPGFpSYTDANK. Peptides were eluted into the mass spectrometer with an HPLC gradient (0-50% acetonitrile, 0.1M acetic acid in 30min). The mass spectrometer acquired top 3 data dependent ESI MS/MS spectra. The phosphorylation site was revealed by fragment ions y6 and y7 where y7-y6 gave the mass of phosphoserine.

2.9.3 Purification of cytochrome *c* from bacterial pellets

Phosphomimetic substitution is a commonly used method to study the *in vitro* effects of protein phosphorylation. The commonly used amino acids as phosphomimetic substitutes are negatively charged glutamate and aspartate.

This study was aimed at studying the effects of Ser47 phosphorylation of cytochrome *c*. The bacterial pLW01 expression vector was used to generate the rat cytochrome *c* constructs. The vector particularly suits the study since it also encodes the heme lyase gene which is

required but not present in bacteria for covalent attachment of the heme group to apo-cytochrome *c*. Besides wild-type cytochrome *c*, site directed mutagenesis was performed to generate Ser47Glu and Ser47Ala mutants. The correct mutants were confirmed by sequencing and further used to transform One shot BL21 (DE3) E.coli cells for cytochrome *c* overexpression. The bacterial pellets were lysed and French-pressed before purification through ion exchange chromatography. Five to ten mg of purified cytochrome *c* protein per liter of bacterial culture was obtained. Purity of the proteins was checked on SDS-PAGE gel followed by Coomassie blue staining. Absorption spectra revealed that all proteins are fully reducible.

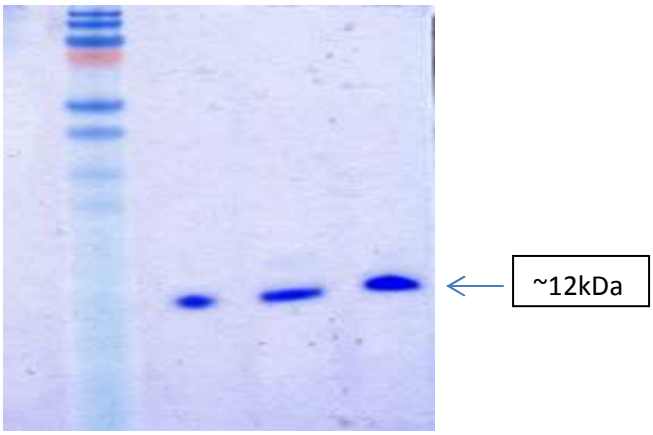

Figure 8. Coomassie gel showing purity of the harvested mutant proteins.

Lane 1: Page Ruler pre-stained protein ladder, 10-170 kDa, Lane 2: Wild type cytochrome *c*, Lane 3: S47A mutant protein, Lane 4: S47E mutant protein.

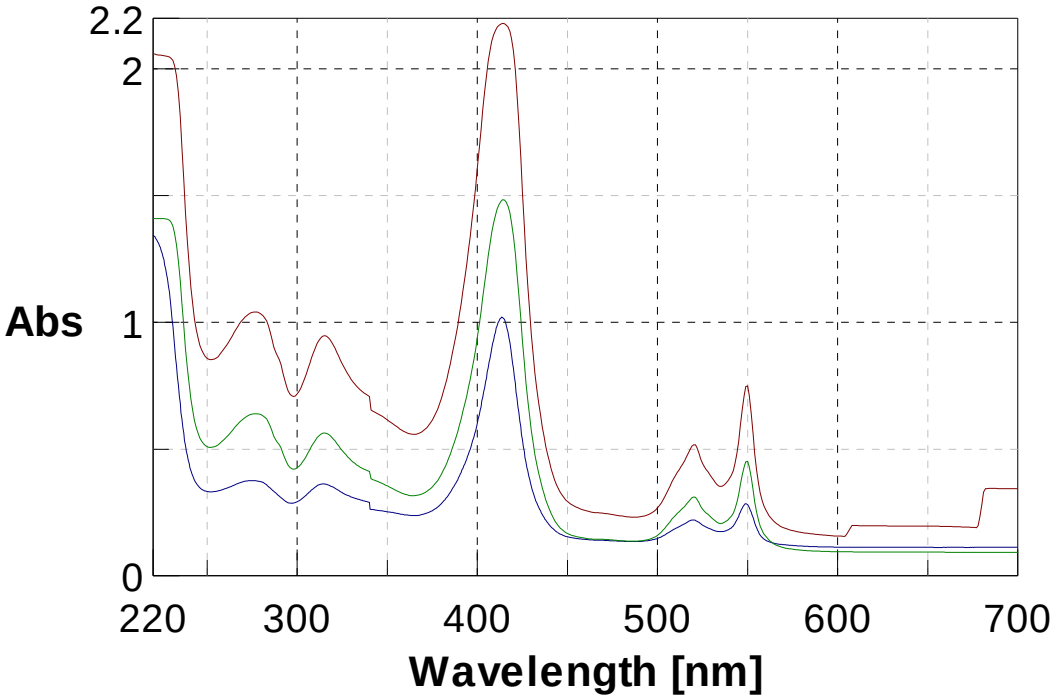

Figure 9. Absorption spectra of purified cytochrome *c* mutant proteins. Spectral analysis of the cytochrome *c* variants(blue- WT, green- S47A, brown- S47E) indicated that they are correctly folded, including the presence of the weak 695nm absorption band in the oxidized spectrum (not shown).

2.9.4 C*c*O activity measurements using Clark type oxygen electrode system

A kinetic study of purified cytochrome *c* oxidation with bovine liver C*c*O was performed. Purified mutant cytochrome *c* proteins were used, with WT cytochrome *c* as control. Interestingly, results revealed that C*c*O oxidizes Ser47Glu mutant cytochrome *c* at significantly lower rates as compared to WT and Ser47Ala controls. V_{max} of the Ser47Glu mutant was decreased by 40-50% compared to WT and Ser47Ala controls suggesting that this phosphorylation down-regulates mitochondrial ETC electron flux.

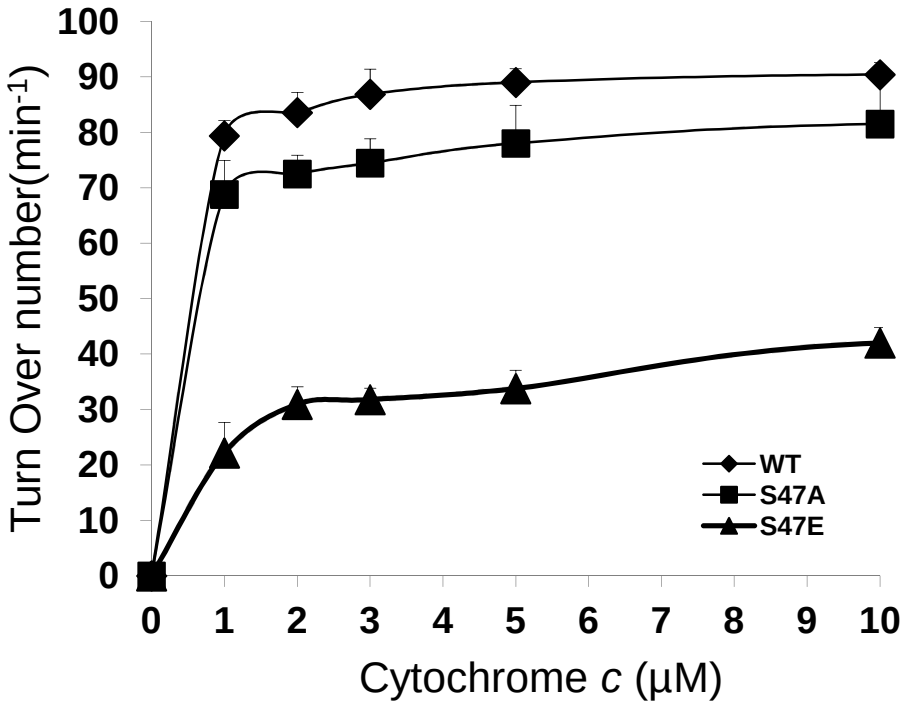

Figure 10. C*c*O activity of purified cytochrome *c* mutant proteins. V_{max} of S47E mutant is reduced by ~40- 50% compared to WT and S47A controls.

2.9.5 Caspase 3 activity induction by cytochrome *c* mutant proteins

To assess the effect of mutation on the ability of the protein to participate in apoptosis, caspase 3 activity was measured. For this purpose wild type cytochrome *c*, Ser47Glu, and Ser47Ala mutants each were incubated with cytosolic extracts from HeLa cells. Strikingly, the results reveal that mutation to the Ser47 residue leads to a loss of the protein's ability to initiate apoptosis. Both mutations result in negligible caspase activity with cytosolic extracts from HeLa cells. This suggests that Ser47 might be a key residue to regulate the protein's ability to trigger apoptosis.

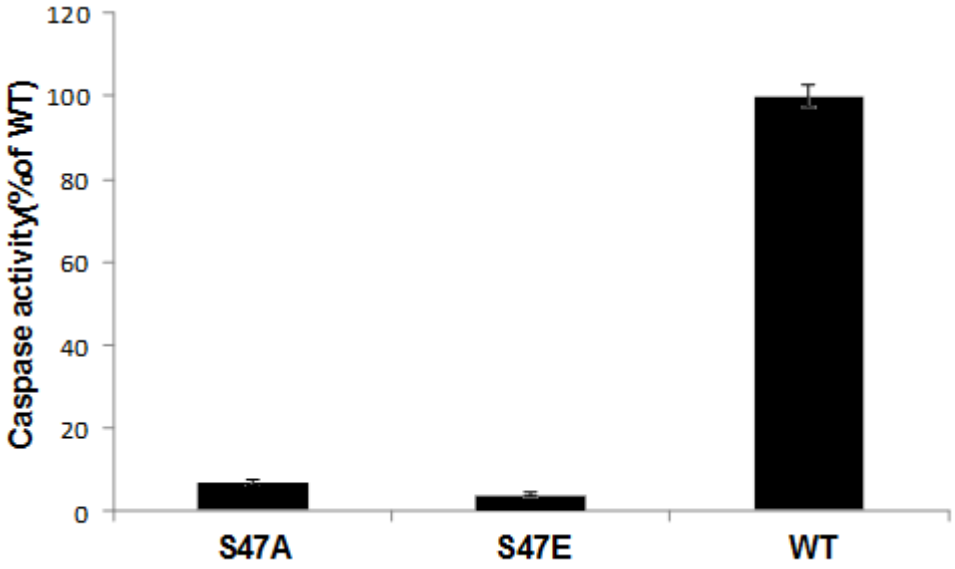

Figure 11. Caspase-3 activity after incubation with purified cytochrome *c* mutant proteins. The ability to trigger apoptosis is abolished in both S47E as well as S47A mutants.

2.9.6 Measurement of cytochrome *c* redox potential

To effectively function as a mobile electron carrier in the electron transport chain cytochrome *c* must have a redox potential value between those of Complexes III and IV. As part of the initial characterization, the redox potential of the cytochrome *c* mutants and the WT was measured using the equilibration method. The wild type cytochrome *c* redox potential value was 272 ± 2mV while the mutants displayed slightly lower values 270 ± 1mV and 256 ± 12mV for Ser47Ala and Ser47Glu mutants, respectively.

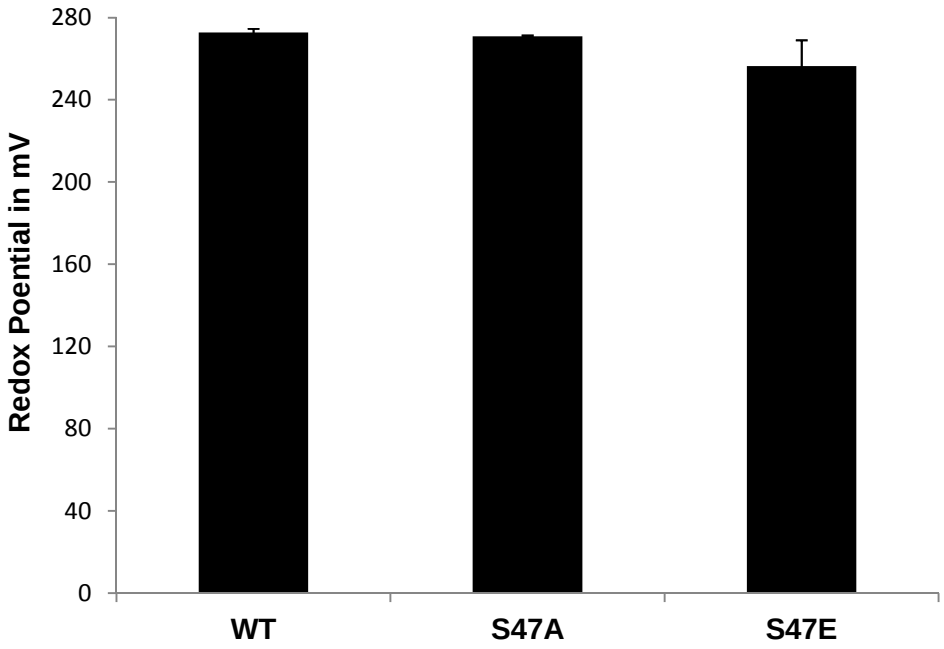

Figure 12. Redox potential assay of cytochrome *c* mutant proteins

2.10 Future directions

Cytochrome *c* plays diverse roles in the life and death activities of a cell. Keeping this mind one would expect tight regulatory mechanisms governing its functions. However, regulation of cytochrome *c* by cell signaling was not given serious consideration until recently when our group found and published that cytochrome *c* was phosphorylated on Tyr97 and Tyr48 in bovine heart and liver and that these phosphorylations resulted in a partially inhibited ("healthy') mitochondrial respiration status of a cell while preventing apoptosis, in a tissue specific manner. In the present study on bovine kidney cytochrome *c* it was found that Ser47 was phosphorylated. Phosphomimetic mutants were generated and used to carry out *in vitro* assays to characterize this protein. In this study, C*c*O activity, caspase3 activity, and redox potential measurements of the phosphomimetic mutants were analyzed alongside controls.

It is known that under stress conditions cytochrome *c* can act as cardiolipin peroxidase thereby affecting mitochondrial membrane integrity leading to its release into the cytosol. Also

known is that under apoptotic stress unphoshorylated cytochrome *c* can generate ROS together with $p66^{shc}$ by channeling electrons from electron transfer chain to $p66^{shc}$ which will in turn generate hydrogen peroxide. Future experiments will be done to measure the cardiolipin peroxidase activity of the phosphomimetic mutants vs unphosphorylated controls. Also measured will be the ROS generation via $p66^{shc}$.

Next in line would be crystallographic studies to study the effects of these mutations on the structure of the protein.

Also of interest would be to perform a similar set of experiments to functionally characterize Ser47 phosphorylated cytochrome *c* in a mammalian cell culture system to further confirm our results. Another important research direction would be to identify kinase(s) and phosphatase(s) responsible for the regulation of cytochrome *c*, which would be helpful in designing novel therapeutic strategies for treatment of diseases such as cancer where the cells exhibit mechanisms to evade apoptosis perhaps mediated by hyper-phosphorylation of cytochrome *c*, as well as neurodegenerative diseases, where dysfunctional mitochondrial respiration is a hallmark.

2.11 References

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Chapter 3

Summary

3.1 Characterization of bovine cytochrome *c* from kidney tissue

The long standing working model of our lab is that under healthy conditions cytochrome *c* is phosphorylated leading to a "controlled" respiration state by maintaining mitochondrial membrane potential, $\Delta\psi_{m,}$ at physiologically low levels (80-140mV) thereby avoiding excessive ROS production. However, under stressed conditions, cytochrome *c* gets dephosphorylated which leads to high electron transfer rates and membrane potential (>140mV). This hyperpolarization leads to excessive ROS production which triggers apoptosis.

The aim of this study was to characterize the cytochrome *c* protein isolated from bovine kidney. Phosphorylation on Ser47 residue was determined by mass spectrometry. To further analyze the effect of this phosphorylation, phosphomimetic mutants were designed and generated. Preliminary studies to measure basic activities such as respiration, apoptosis, and redox potential were performed.

The redox potential of the phosphomimetic mutant was slightly lower compared to WT, Importantly, phosphomimetic cytochrome *c* displayed significantly reduced C*c*O activity when compared to controls supporting our overall model of healthy (i.e., lower) respiration rates under normal conditions. In addition, the ability to induce apoptosis as measured by caspase 3 activity revealed that mutation of Ser47 almost fully diminishes the ability of cytochrome *c* to initiate apoptosis. This suggests that this Ser47 residue might be key to its proper functioning in the apoptotic cascade. Thus, the results of this study are squarely fitting our working model.

LIST OF PUBLICATIONS

Papers:

1. Hüttemann, M., Helling, S., Sanderson, T. H., Sinkler, C., Samavati, L., Mahapatra, G., Varughese, A., Lu, G., Liu, J., Ramzan, R., Vogt, S., Grossman, L. I., Doan, J. W., Marcus, K., and Lee, I. (2012) Regulation of mitochondrial respiration and apoptosis through cell signaling: Cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. Invited Review. Biochim Biophys Acta, 1817(4):598-609.
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ABSTRACT

IN VITRO CHARACTERIZATION OF SERINE 47 PHOSPHORYLATED CYTOCHROME *c*

by

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Cytochrome *c* is a 12.4kDa ubiquitously expressed protein known to be involved in many physiological processes of the cell such as respiration and apoptosis. The goal of our lab is to increase our knowledge of the regulation of cytochrome *c* in these opposite activities, and our working model posits that cytochrome *c* is decisively regulated by phosphorylation. When phosphorylated, cytochrome *c* leads to an "optimal" functioning in the electron transport chain by lowering electron flux, preventing harmful high mitochondrial membrane potentials and thus ROS production under healthy conditions. However, under cellular stress cytochrome *c* might be dephosphorylated favoring high mitochondrial membrane potentials and ROS and its participation in apoptosis. Our lab has previously published two phosphorylation sites in cow, namely Y48 in the liver and Y97 in the heart. The aim of my thesis was to identify phosphorylation site(s) on kidney cytochrome *c* and to perform functional characterization of the cow kidney protein. Kidney cytochrome *c* was found to be phosphorylated on S47 and based on this, suitable cytochrome *c* variants were over expressed in a prokaryotic system. These cytochrome *c* variants were used to study the effect of phosphorylation on the most common activities of cytochrome *c* protein i.e., cellular respiration and apoptosis. The results of the *in vitro* study revealed that the phosphomimetic mutant Ser47Glu has lower rates of respiration

compared to wild type as well as S47A mutant which is in line with the working model of our lab. In addition, any mutation of the Ser47 residue resulted in almost fully diminished caspase activity when compared to wild type, suggesting that this residue might be key to the regulation of the apoptotic activity of cytochrome *c*.

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