UNDERSTANDING THE PROTEIN-PROTEIN INTERACTIONS OF HEME *A* SYNTHASE AND THEIR IMPLICATIONS FOR CYTOCHROME *C* OXIDASE ASSEMBLY

By

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ABSTRACT

UNDERSTANDING THE PROTEIN-PROTEIN INTERACTIONS OF HEME *A* SYNTHASE AND THEIR IMPLICATIONS FOR CYTOCHROME *C* OXIDASE ASSEMBLY

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Heme *a* is an obligatory cofactor in the terminal enzyme complex of the electron transport chain, cytochrome *c* oxidase. The heme *a* molecule is synthesized from heme *o* within the mitochondria by a multi-spanning inner membrane protein, heme *a* synthase (Cox15 in yeast). The insertion of heme *a* is critical for cytochrome *c* oxidase function and assembly, but this process has not been fully elucidated. In an effort to increase our understanding of heme *a* insertion into cytochrome *c* oxidase, we investigated the protein-protein interactions that occur with Cox15 in *Saccharomyces cerevisiae.*

Cox15 in *S. cerevisiae* exists in six protein complexes ranging in size from ~120 kDa – 1 MDa as observed via blue native PAGE (BN-PAGE). The two largest complexes at approximately 750 kDa and 1 MDa are reminiscent of the respiratory supercomplexes containing both complex III (cytochrome bc_1 complex) and complex IV (cytochrome *c* oxidase). The large 750 kDa and 1 MDa Cox15 complexes were not observed in yeast strains in which the supercomplexes are unable to form, thus supporting the hypothesis that Cox15 is present in the respiratory supercomplexes. In addition, Cox15 was found to interact with one of the catalytic subunits of the cytochrome bc_1 complex, Cyt1, and we propose that Cox15 and Cyt1 interact within the supercomplexes. No other proteins from the cytochrome bc_1 complex or cytochrome *c* oxidase were found to interact with Cox15, although if Cox15 is present in the respiratory supercomplexes, by definition, it would seem that Cox15 must also interact (at least indirectly) with the other components of the respiratory supercomplexes.

Of the lower four Cox15-containing complexes ranging from ~120 – 440 kDa, the complex at 120 kDa was the most prominent, indicating that the majority of the Cox15 observed by BN-PAGE is represented by this species. Although 120 kDa is ~1.5 times larger in molecular weight than monomeric

C-terminal tagged Cox15, we were unable to identify other proteins that interact with Cox15 in this 120 kDa band. Because it is accepted that molecular weights of proteins are over-estimated via BN-PAGE due to the effect of detergent, we hypothesize that this lowest complex represents monomeric Cox15.

Experiments to test the composition of the remaining Cox15-containing complexes revealed that approximately 30% of Cox15 interacts with itself in homo-oligomeric complexes. In addition, experiments to test if other proteins interacted with Cox15 revealed that cytochrome *c* oxidase assembly factors may exist with Cox15 in one of the Cox15-containing complexes. It does not appear, however, that assembly factors of cytochrome *c* oxidase represent predominant protein interactions with Cox15. Finally, Cox15 was shown to interact with the cytosolic heat shock proteins, Ssa1 and Hsc82. Deletions of Ssa1 and Hsc82, however, indicated that these proteins are not part of the Cox15-containing complexes observed via BN-PAGE. Based on previous studies implicating cytosolic heat shock proteins in mitochondrial protein uptake, we predict that Ssa1 and Hsc82 are involved in the import of Cox15 into the mitochondria.

This thesis is dedicated to Axel Charles Erickson whose life was taken by Leigh's disease. May God's grace strengthen his parents' hearts and may they find much joy from the time they had with him. Though this work is but a small piece in the greater body of research devoted to the understanding of proteins involved in Leigh's disease, may Axel's life inspire us as we seek to understand mitochondrial respiration.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Eric Hegg, for his support and guidance through the course of this project. His excitement, wisdom, and passion for teaching have allowed me to mature into a scientist. Most of all, I am thankful for his encouragement which often inspired me to persevere in my work. I could not have asked for a better mentor.

I also want to acknowledge the important role my parents have played in my life. Their sacrifices allowed me to come to Michigan State University to pursue my PhD. More importantly, their love and encouragement is one of the most significant reasons I have come to the completion of my PhD studies.

Finally, I would like to thank my husband, Matt Herwaldt, for all of his love and support. His steady perspective kept me grounded in the midst of difficult experiments and the uncertainties of graduate school. Furthermore, I am very grateful for all of the sacrifices he made at work and and at home to help care for our daughter, Anna Grace, while I finished my studies. I will always be thankful for his continual love and encouragement in my life.

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KEY TO ABBREVIATIONS

1. 3'-UTR: Three prime untranslated region
2. ADP: Adenosine diphosphate
3. ATP: Adenosine triphosphate
4. BN/SDS-PAGE: BN-PAGE of purified Cox15 is run in the first direction and a lane is excised from the gel and mounted to the top of an SDS-PAGE gel so that the lane from the blue native gel can be run in the second dimension.
5. BN-PAGE: Blue Native Polyacrylamide Gel Electrophoresis
6. CI: NADH dehydrogenase (complex I) of the electron transport chain
7. CII: Succinate dehyrogenase (complex II) of the electron transport chain
8. CIII: Cytochrome bc_1 complex (complex III) of the electron transport chain
9. CIV: Cytochrome *c* oxidase (complex IV) of the electron transport chain
10. Cox1: One of the mitochondrial encoded subunits of cytochrome *c* oxidase. Contains the heme *a* molecules.
11. *COX15::HIS*: Cox15 containing a C-terminal 6X-Histidine tag expressed on the pRS426 yeast expression vector between the Met25 promoter and Cyc1 terminator.
12. *COX15::MYC*: Genomic Cox15 containing a C-terminal (13X) c-Myc protein tag.
13. *COX15::TAP:* Cox15 containing a C-terminal tandem affinity purification tag expressed on the pRS426 yeast expression vector between the Met25 promoter and Cyc1 terminator.
14. CtaA: Heme *a* synthase (bacterial nomenclature)
15. CtaB: Heme *o* synthase (bacterial nomenclature)
16. Cyt *c*: Cytochrome *c*
17. *CYT1::HA*: Genomic Cyt1 protein containing a C-terminal (3X) HA tag
18. *CYT1::HA*: Genomic Cyt1 protein containing a C-terminal (3X) HA tag
19. ETC: Electron transport chain
20. FAD: Flavin adenine dinucleotide

21. FMN: Flavin mononucleotide

22. $FMNH_2$: Reduced flavin mononucleotide

23. HAS: Heme *a* synthase

24. HOS: Heme *o* synthase

25. *HSC82*::*HA*: Genomic Hsc82 protein containing a C-terminal HA tag.

26. IMS: Intermembrane space of the mitochondria

27. KanMX: G418 antibiotic resistance cassette

28. mRNA: messenger ribonucleic acid

29. MTS: Mitochondrial targeting sequence

30. NAD: Nicotinamide adenine dinucleotide

31. NADH: Reduced form of nicotinamide adenine dinucleotide

32. Ni-NTA: Nitrilotriacetic acid

33. PBS: Phosphate buffered saline

34. PVDF: Polyvinylidene fluoride

35. Q: Ubiquinone

36. *SCO1*::*HA*: Genomic Sco1 protein containing a C-terminal (3X) HA tag

37. SDS: sodium dodecyl sulfate

38. SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis

39. *SSA1*::*HA*: Genomic Ssa1 protein containing a C-terminal (3X) HA tag

40. *SSB1*::*HA*: Genomic Ssb1 protein containing a C-terminal (3X) HA tag

41. TBS: Tris buffered saline

42. TRP1: Encodes the indole-3-glycerolphosphate synthase-N-(5'-phosphoribosyl)anthranilate isomerase enzyme involved in the biosynthasis of tryptophan. Used as a selection marker in yeast genetics.

43. URA3: Encodes Orotidine 5'-phosphate decarboxylase, enzyme in the biosynthasis of uracil. Used as a selection marker in yeast genetics.

Chapter 1:

Cytochrome *c* oxidase and the electron transport chain

Background

The mitochondrial electron transport chain (ETC) is fundamental to life in all eukaryotic organisms. The reactions that occur in the ETC provide our cells with the energy currency they need to survive. These ETC reactions entail the continuous flow of electrons through the various electron acceptor proteins that make up the ETC (Figure 1). The electrons that are delivered to either Complex I (NADH dehydrogenase) or Complex II (succinate dehydrogenase) arise from different sources [1]. NADH dehydrogenase receives its electrons from NADH pools within the mitochondrial matrix while succinate dehydrogenase receives its electrons from succinate. Both NADH dehydrogenase and succinate dehydrogenase deliver electrons to ubiquinone [3,4]. Interestingly, yeast do not contain a NADH dehydrogenase complex like the one depicted in Figure 1, but they do have three smaller NADH dehydrogenases that do not pump protons across the inner membrane [5-9]. Once ubiquinone receives its electrons from either NADH dehydrogenase or succinate dehydrogenase, it is responsible for delivering electrons to complex III (cytochrome bc_1 complex) [10,11]. From there, the mobile electron carrier, cytochrome *c*, delivers electrons from the cytochrome bc_1 complex to complex IV (cytochrome *c* oxidase). NADH dehydrogenase, the cytochrome bc_1 complex, and cytochrome *c* oxidase use the energy that is generated from this electron flow to pump protons from the mitochondrial matrix to the intermembrane space. This generates a proton gradient across the inner mitochondrial membrane, and complex V (ATP synthase) then uses this gradient to drive the synthesis of ATP, the energy currency of our cells [12].

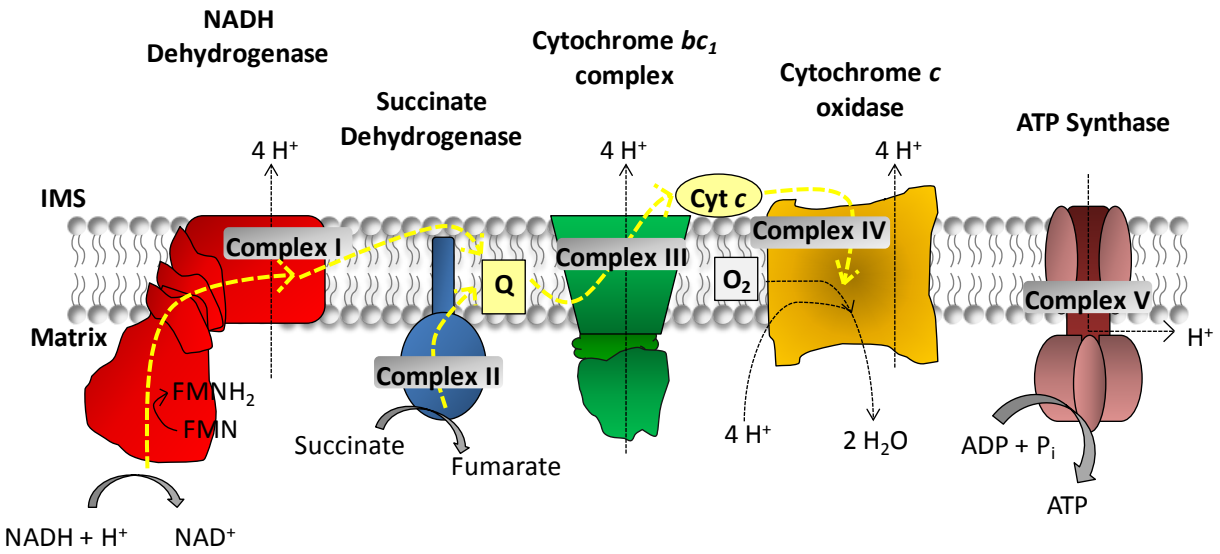

Figure 1: Representation of the electron transport chain: Complex I (NADH dehydrogenase) catalyzes the exergonic transfer of two electrons to ubiquinone. In addition, it catalyzes the endergonic transfer of four protons from the matrix to the intermembrane space (IMS) [3]. The yellow dotted line represents that path of electron flow between the components of the transport chain. Complex I as depicted in this figure is not present in yeast. Complex II (succinate dehydrogenase), also a member of the citric acid cycle, binds its substrate, succinate. It then transfers electrons to ubiquinone (Q) [4]. Complex II is not involved in pumping protons across the mitochondrial inner membrane [4]. Complex III (cytochrome bc_1 complex) transfers electrons from ubiquinol (QH_2) to cytochrome *c* (Cyt *c*) while simultaneously pumping a total of four protons into the IMS [10,11]. Complex IV (cytochrome *c* oxidase) receives two electrons from two molecules of CYT *c.* The electrons pass through the enzyme to the active site where they reduce O_2. Ultimately, it requires 4 electrons and 4 substrate protons to reduce O_2 to two molecules of H_2O. Cytochrome *c* oxidase is also responsible for the pumping of four protons from the matrix [13-16]. Ultimately, complex V (ATP synthase) uses the proton gradient that is generated during electron transport to synthesize ATP from ADP and inorganic phosphate [12].

The terminal electron acceptor in the ETC is an oxygen molecule that binds to the active site of cytochrome *c* oxidase. Because of the important role cytochrome *c* oxidase plays in the ETC, the significance of understanding the chemistry that occurs within this enzyme cannot be understated. The electrons that are delivered from cytochrome *c* are first delivered to the di-nuclear copper center, termed the Cu_A site, present in the Cox2 subunit (Figure 2). From the Cu_A site, the electrons are transferred to a low spin heme *a* molecule present in Cox1. Finally, from this heme *a* site the electrons are transferred to the active site of the enzyme where they reduce their substrate, molecular oxygen, to water (Figure 2). The active site where this chemistry occurs consists of both a heme a_3 and Cu_B molecule and is also located in the Cox1 subunit [13-16]. While the proteins that are involved in the insertion of copper into Cox1 are fairly well understood, it is still debated what proteins are involved in delivering the heme to Cox1. In addition, while it is thought that metallation of Cox1 occurs in an early assembly intermediate forming with Cox1, the exact intermediate where this occurs is still unclear. Despite the many gaps in our understanding of cofactor insertion into Cox1, the correct incorporation of the heme and copper molecules is critical for the maturation of Cox1, and hence, the maturation of the holo-enzyme [17].

Cytochrome *c* oxidase is a complex enzyme that utilizes an equally complex assembly pathway. Despite many years of research, there is still much to learn regarding the assembly of this complicated enzyme. Bovine and *S. cerevisiae* cytochrome *c* oxidase consists of 13 and 11 subunits, respectively. Three of these subunits, Cox1, Cox2, and Cox3 are encoded by the mitochondrial genome. All other subunits are nuclear encoded. It is thought that all of the subunits, both mitochondrial and nuclear encoded, form around Cox1 once it is inserted into the inner membrane. It is likely that the heme and copper cofactors are added to Cox1 before the incorporation of other subunits around the Cox1 core. Because of this, any mutations in the proteins involved in the translation, maturation, or metallation of Cox1 result in the inability for the rest of the enzyme to assemble [18-21]. As alluded to above, in the

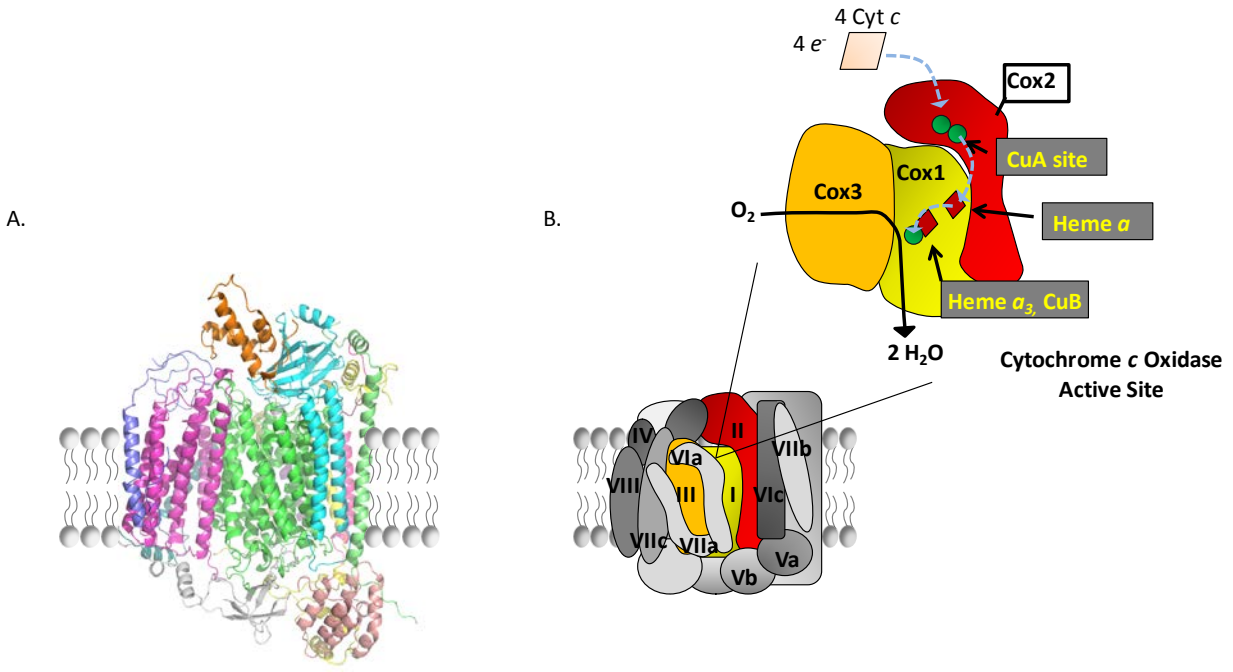

Figure 2: Structure of cytochrome *c* oxidase. A. Crystal structure of cytochrome *c* oxidase from *Rhodobacter sphaeroides*. Cytochrome *c* oxidase from *R. sphaeroides* only consists of four subunits, and its catalytic core has high structural similarity to the mammalian catalytic core. The catalytic core is represented by, Cox1 (light green), Cox2 (light blue), and Cox3 (magenta). Cox4 (purple) is the only structural subunit. Structure is courtesy of Dr. Leann Buhrow. B. Schematic of bovine cytochrome *c* oxidase. Bovine oxidase contains 13 subunits. Three of these subunits are mitochondrial encoded and compose the catalytic core. Cox1, Cox2, and Cox3 are buried within the nuclear encoded subunits (grey) and are thought to be assembled early in the process. The mitochondrial subunits are emphasized for clarification. Figure adapted from [2].

absence of fully assembled cytochrome *c* oxidase, aerobic respiration ceases. This results in devastating diseases that are often incompatible with life. For example, mutations in the Cox15 protein responsible for the synthesis of heme *a* have been demonstrated to result in severe infantile cardioencephalopathies and Leigh's syndrome [22-25].

In this chapter I will review what is known regarding the assembly of cytochrome *c* oxidase, paying particular attention to the insertion of the heme *a* molecules into Cox1. Much of our knowledge regarding cytochrome *c* oxidase assembly has come from studies in *Saccharomyces cerevisiae*.

Ultimately, it is our goal to take the knowledge we have gained from studies in yeast and apply it to the human enzyme. Therefore, I will compare similarities and differences between assembly of yeast and human cytochrome *c* oxidase. Finally, I will also review our understandings of the ETC as a whole and discuss current models for its organization. The implications ETC organization may have on whether cytochrome *c* oxidase assembly proceeds within monomeric units or within supercomplexes will also be discussed.

Cytochrome c *oxidase assembly*

It is known that the assembly of cytochrome *c* oxidase in yeast utilizes over three dozen proteins for proper assembly [13,17,26,27]. The identification of these proteins in yeast is facilitated by the ability to manipulate the genome easily to screen for nuclear genes that result in respiratory deficiency [17]. While mutant screens have been conducted to learn about the factors that assist in human cytochrome *c* oxidase assembly, these screens are limited to the characterization of the genetic defects that are presented in patient cell lines with cytochrome *c* oxidase deficiency [17,28]. Because of this, the number of proteins identified to be involved in the assembly of yeast cytochrome *c* oxidase is far higher than with the human enzyme, although many of the same assembly factors are presumably required in humans. While the assembly of yeast cytochrome *c* oxidase has been shown to proceed through a series of intermediates that form with newly translated Cox1, several recent studies are beginning to implicate a similar set of intermediates that form in human cells [29-32]. Mick et al, 2012 [29] identified the human equivalent of many of the yeast sub-assembly complexes that form with Cox1 during assembly. In addition, Szklarczyk et al. (2012) [32] specifically identified several assembly factors in humans that are orthologs to well-known yeast assembly factors [32]. The following section will outline cytochrome *c* oxidase assembly in yeast and then will compare that to what is known regarding assembly of human cytochrome *c* oxidase.

Sub-assembly complexes that form with newly translated Cox1: Studies in Saccharomyces cerevisiae

The assembly intermediates that form during the maturation of Cox1 and the incorporation of Cox1 into mature cytochrome *c* oxidase are difficult to study in yeast because the assembly intermediates are rapidly degraded in strains lacking fully assembled cytochrome *c* oxidase [33]. This is likely because the synthesis of Cox1 is down-regulated when cytochrome *c* oxidase fails to assemble [34]. Nevertheless, through techniques such as blue native PAGE (BN-PAGE), mass spectrometry, and co-immunoprecipitation, much progress has been made towards understanding the early assembly intermediates that form with Cox1 in *S. cerevisiae*.

COX1 mRNA synthesis in *S. cerevisiae* begins with the proteins, Mss51 and Pet309 [35,36]. Pet309 has been shown to be necessary for the stability of the *COX1* mRNA transcript, but it is unknown if it interacts directly with the mRNA [17,37]. In contrast, Mss51 has been shown to interact with both the *COX1* mRNA transcript and the translated protein, suggesting a dual role for Mss51 in Cox1 assembly [34,38,39]. The earliest characterized assembly intermediate that is reported to form with Cox1 contains Mss51, Ssc1 (the mitochondrial Hsp70), and Mdj1, the co-chaperone of Ssc1. This complex is stabilized by the integral membrane proteins Cox14 and Coa3 (Figure 3) [34,40-43]. In addition, while Cox14 and Coa3 likely play a role in stabilization, it has been reported that the C-terminal residues of Cox1 are also important for stabilization of this complex [43]. The formation of this assembly intermediate is thought to sequester Mss51 from further rounds of Cox1 translational activation, thus serving to down-regulate further rounds of cytochrome *c* oxidase assembly [42,44]. Downstream of the Cox1-Mss51-Ssc1-Mdj1-Cox14 assembly intermediate, the assembly factor Coa1 associates with the assembly complex (Figure 3). This causes Mss51, Ssc1, and Mdj1 to be released from the complex, freeing up Mss51 for future rounds of Cox1 translation [41,42,44-46].

The role of the mitochondrial Hsp70 in association with Mss51 is not clear. Ssc1 is known to be involved in protein folding and insertion into the mitochondrial inner membrane. It is possible that the

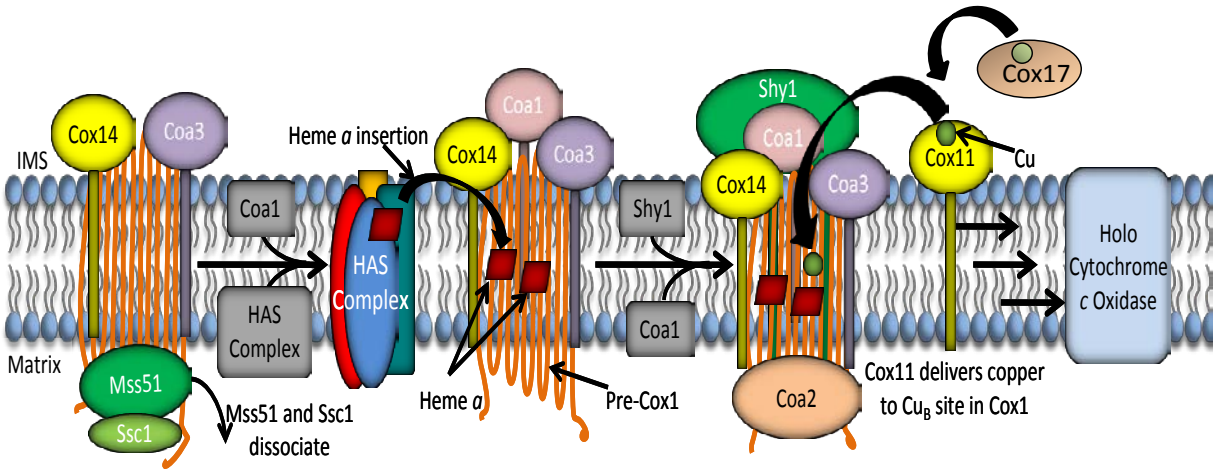

Figure 3: Schematic representation of the early assembly intermediates that associate with Cox1. Cox1 is depicted as the wavy orange line. Cox1 transverses the membrane 12 times. Assembly factors interact with Cox1 during its insertion into the membrane and the addition of its metal cofactors into the active sites.

role of Ssc1 in this early Cox1-containing assembly complex is to aid in the insertion of Cox1 into the membrane [47]. Evidence that argues against this hypothesis is the fact that Ssc1 stably binds to Mss51 both when Mss51 is bound to the Cox1-containing complex and when it is released [47]. This binding of Ssc1 to Mss51 seems to be different than the classical Ssc1-client protein interactions because Ssc1 remains bound to Mss51 regardless of the presence or absence of ATP [47]. It has been proposed that Ssc1 maintains a pool of Mss51 that is ready for Cox1 translation [47].

Following the release of Mss51 from the Cox1-containing assembly intermediate, Mss51 is free to act as a translational activator of Cox1 [42,47]. It is thought that the release of Mss51 and Ssc1 occurs at the same time point when Cox1 obtains its cofactors or interacts with other nuclear-encoded cytochrome *c* oxidase subunits [17,34,45,48]. It is proposed that Shy1 operates at this time and may interact with the Cox1, Cox14, Coa1, and Coa3 complexes [45,47] (Figure 3).

The early assembly intermediates that form with newly translated Cox1 are the most well understood of all of the intermediates that occur during the assembly of cytochrome *c* oxidase. Exactly how and when the heme and copper cofactors are inserted into Cox1 is still the topic of on-going

research. Cox17, a soluble protein of the IMS is thought to deliver copper to Cox11, an inner membrane protein that delivers the copper to the Cu_B site in Cox1 [49-52]. Because of the reactive nature of heme *a*, it is thought that heme insertion into Cox1 either occurs co-translationally or that a protein chaperone delivers heme from Cox15, the enzyme that synthesizes heme *a*, to cytochrome *c* oxidase [17]. The inner membrane protein, Shy1 (Surf1 in humans), has been proposed to be the chaperone that delivers heme to cytochrome *c* oxidase, but definite proof of this concept is lacking [53-55]. A more thorough discussion of the role of Shy1 in heme delivery to Cox1 is included on pages 15-17. Alternatively, heme *a* may be delivered directly from Cox15 to Cox1. As depicted in Figure 3, Cox15 has been proposed to exist in a high molecular weight protein complex [56], but the identity of what proteins may exist in this complex is lacking and will be the subject of this thesis. One possibility may be that the Cox15 protein complex is responsible for heme insertion into Cox1 (Figure 3).

Coa2, another assembly factor essential for cytochrome *c* oxidase assembly, has been implicated in heme *a* delivery to Cox1 because it was shown to interact transiently with Shy1 [54] (Figure 3). In addition, Coa2 was proposed to be involved in heme delivery to cytochrome *c* oxidase due to the finding that a mutant allele of Cox10 (N196K) was able to rescue a *coa2* knockout strain [56]. Whether Coa2 interacts with Shy1 during Cox1 hemylation remains an open question. Despite the inability to identify definitively a protein chaperone for heme *a*, compelling evidence indicates that heme insertion does not occur co-translationally as the heme *a* sites have been shown to form downstream of the Cox1 intermediates containing Coa1 [55].

Characteristics of Cox15, an enzyme involved in heme a *biosynthesis*

As mentioned above, Cox15 is one of the enzymes responsible for synthesizing heme *a.* Heme *b* is first converted to heme *o* by Cox10, and then Cox15 converts the heme *o* to heme *a*. A description of the reactions involved in the conversion of heme *b* into heme *a* is included in Chapter 2. The mechanism Cox15 utilizes to synthesize heme *a* and the structure of the Cox15 enzyme is still largely

unknown. Most of the structural studies have been conducted on the bacterial homologue, CtaA from *Bacillus subtilis*. Based on hydropathy plots, CtaA is predicted to contain eight transmembrane domains [57,58]. CtaA is also predicted to be a product of gene duplication and fusion since the N and C-terminal halves of CtaA are homologous. In further support of this hypothesis, the sequence of CtaA from the thermophilic archaeon, *Aeropyrum pernix*, was found to be half that of *B. subtilus* CtaA and had a similar sequence to both the N and C-terminal halves of *B. subtilius* CtaA [59].

There are four conserved histidine residues within the bacterial homologues of CtaA; two of these histidines are proposed to be involved in coordinating a heme *b* cofactor while the other two are thought to be at the site of heme *o* binding [58,60]. These same conserved histidine residues are also present in eukaryotic homologues of Cox15. The hydropathy plot obtained of Cox15 from *S. cerevisiae* using the TMred server (http://www.ch.embnet.org/software/TMPREDform.html) predicts that Cox15 contains eight transmembrane domains, similar to bacterial CtaA. Figure 4 depicts both the sequence of *S. cerevisiae* Cox15 as well as a cartoon of its predicted structure, highlighting the conserved histidine residues thought to be involved in heme coordination and catalysis.

Assembly of the cytochrome c oxidase holo-enzyme

Following the early stages of Cox1 incorporation into the inner mitochondrial membrane and the incorporation of its cofactors, the other core and nuclear encoded subunits assemble to form the cytochrome *c* oxidase holo-enzyme. Very little is known about this process other than Cox1, Cox2, and Cox3 have separate assembly pathways [13,61]. At some point downstream of the Cox1 assembly intermediate involving Coa1, Shy1, Cox14, and Coa3, it is thought that Cox1 associates with the nuclear-encoded Cox4 and Cox5a subunits [33,45]. After the addition of these two nuclear subunits, Cox2 and Cox3 are probably added to the complex followed by the rest of the nuclear subunits [13,45,61].

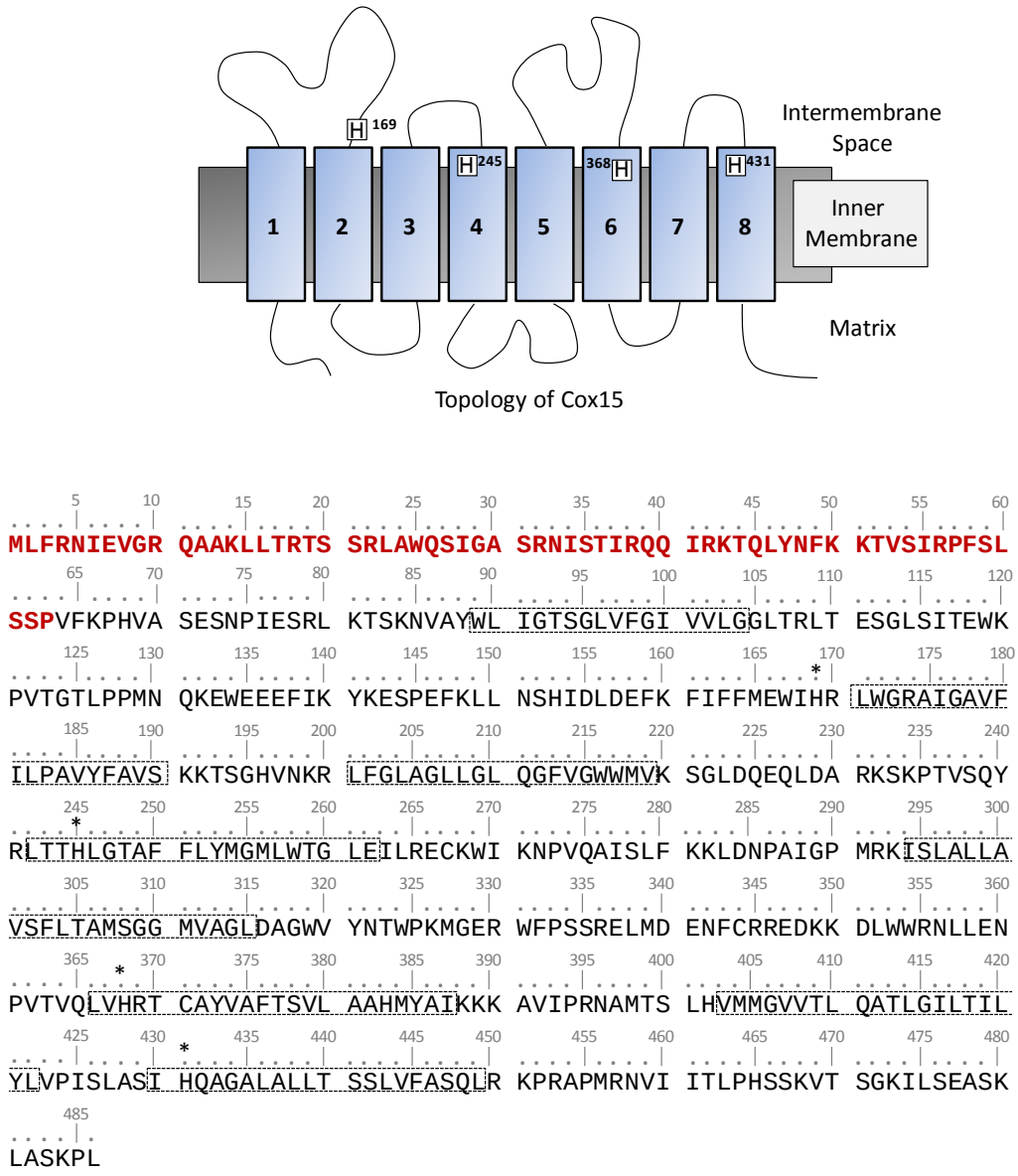

Figure 4: Topology and sequence of Cox15: The topology of Cox15 within the inner mitochondrial membrane. The conserved histidine residues believed to be involved in heme coordination and catalysis are depicted. As in CtaA, all conserved histidine residues from Cox15 are located within the transmembrane domains, with the exception of histidine 169 which is located immediately before the second transmembrane domain. The sequence shown below the topology model highlights the predicted mitochondrial targeting sequence in red, the conserved histidine residues with an asterisk, and the predicted transmembrane regions (highlighted by hashed boxes). The hydropathy plot of Cox15 was obtained using the TMpred server (http://www.ch.embnet.org/software/TMPRED_form.html), and the mitochondrial targeting sequence was predicted using the Mitoprot server (http://ihg.gsf.de/ihg/mitoprot.html).

Sub-assembly complexes that form with newly translated Cox1: Studies with the human enzyme

As mentioned above, at least thirty assembly factors have been identified as being involved in the assembly of *S. cerevisiae* cytochrome *c* oxidase. While the number of assembly factors described for human cytochrome *c* oxidase is much smaller, the concept that Cox1 utilizes a sequential and ordered assembly pathway actually began with studies of the human enzyme [62]. Studies of patient fibroblasts with mutations in either *COX10, SCO1, or SURF1* revealed cytochrome *c* oxidase stalled in at least five distinct assembly intermediates. When screening these intermediates for the presence of cytochrome *c* oxidase subunits, only the Cox1 subunit was found in the three lower molecular weight intermediates (along with other proteins). The other two higher molecular weight intermediates were shown to contain Cox1 with a variety of other cytochrome *c* oxidase subunits [63]. All of these intermediates, particularly those that only contained Cox1 out of all the other cytochrome *c* oxidase subunits, likely represent Cox1-containing complexes with various cytochrome *c* oxidase assembly factors. Until recently, only seven assembly factors were known to share homology between yeast and human. These included all of the copper machinery proteins (Cox17, Cox11, Sco1, and Sco2), the heme biosynthetic enzymes (Cox10 and Cox15), and the Surf1 protein (Shy1 in yeast), the function of which is still debated [64].

In 2012, several studies began to reveal that other homologs of yeast cytochrome *c* oxidase assembly factors exist in humans. A report by Szklarczyk and coworkers described several more homologs of yeast assembly factors. Using a novel iterative otology predication method developed in their laboratory, the authors described likely homologues of pet100, pet117, Cox20, Cox24, Coa1, Coa3, and Cox14 assembly factors in the human genome [32]. The authors went on to prove that C12orf62, the predicted homologue of yeast Cox14, does indeed function in cytochrome *c* oxidase assembly, thus confirming the reasonableness of their other predictions. This study indicates that as techniques to identify homology between genomes becomes more powerful, we may begin to identify additional

similarities. Table 1 contains a summary of the overlap between known yeast and human proteins involved either in cytochrome *c* oxidase or its assembly.

Finally, studies by Mick and coworkers (2012) provided further biochemical evidence indicating overlap between the early stages of cytochrome *c* oxidase assembly in yeast and human. This work indicated that CCDC56, proposed to be a homolog of yeast Coa3 by Szklarczyk et al., was definitely involved in the assembly of cytochrome *c* oxidase. In addition, this work determined that some of the "Coa" complexes known to associate with newly translated Cox1 in yeast also associated with human Cox1 [29]. Newly translated Cox1 was found in protein complexes ranging from 140-250 kDa and could be traced migrating from these complexes into mature cytochrome *c* oxidase. Furthermore, they tracked CCDC56 (homolog of yeast Coa3) and ascertained via blue native electrophoresis that this protein formed protein complexes that had a very similar distribution to protein complexes containing early intermediates of Cox1 [29]. This indicated that the Cox1-containing protein complexes ranging from 140-250 kDa presumably reflect Cox1 in association with various assembly factors. To support the hypothesis further that human Cox1 associates with assembly proteins during the early stages of its assembly, CCDC56 (Coa3) was found to co-purify with both Cox1 and Surf1. Additionally, through a series of mass spectrometry and co-purification experiments, Mick et al. showed that c12orf62 (yeast Cox14), CCDC56 (yeast Coa3), COX1, TIM21, SURF1, c7orf44 (yeast Coa1), COX4-1, COX5a, and COX6c form a series of dynamic complexes [29]. While more work is needed to outline with certainty the various sub-assembly complexes that form with human Cox1 and assembly factor proteins, these studies revealed that the assembly of human Cox1 has some overlap with yeast Cox1. Unlike in yeast, however, where the assembly factors down regulate Cox1 translation, the assembly factors seem to regulate positively Cox1 translation in humans [29]. Contrary to yeast, a knockdown of CCDC56 (yeast Coa3) and c12orf62 (yeast Cox14) reduces Cox1 translation rather than increasing it. In addition, it should be noted that it is reasonable to predict that the number of cytochrome *c* oxidase assembly factors

Yeast Protein Name	Human Protein Name	Function	
Cox1	COX1		Cytochrome *c* oxidase
Cox2	COX2	Catalytic Subunits; mitochondrially encoded	
Cox3	COX3		
Cox4	COX5b		
Cox5a	COX4-1		
Cox6	COX5a		
Cox7	COX7a	Required for assembly and function; nuclear encoded	
Cox8	COX7c		
Cox7a	COX6c		
	Cox7b		
	Cox8		
Cox9	COX6b	Non-essential subunits	
Cox13	COX6a		
Mss51	ZMYND17 (predicted)	Translational activator of Cox1 and involved in early Cox1 sub-assembly complex	Cox1 Assembly Factors
Cox14	C12orf62	Involved in early Cox1 sub-assembly complex	
COA1	C7orf44/ MITRAC14	Involved in early Cox1 sub-assembly complex	
COA3	CCDC56 / MITRAC12	Involved in early Cox1 sub-assembly complex	
Coa2		Present in Cox1-Shy1 sub-assembly complex	
Pet117	PET117/ LOC100303755 (predicted)	Involved in cytochrome *c* oxidase assembly	
Shy1	SURF1	Involved in Cox1 assembly; possible role in heme insertion	
Cox10	COX10	Heme *a* biosynthesis	
Cox15	COX15	Heme *a* biosynthesis	

Table 1: Comparison of cytochrome *c* oxidase subunits and assembly factors between *S. cerevisiae* and *Homo sapiens*. The proteins that are of unknown or only predicted function are shaded in grey. Information is adapted from (29, 32) and the *Saccharomyces Genome Database (www.yeastgenome.org)*. Table is continued on the following page.

(Table 1 cont'd)

Yeast Protein Name	Human Protein Name	Function	
Cox11	COX11	Required for copper delivery to Cox1	Copper Machinery
Cox17	COX17	IMS protein, delivers copper to Cox11	
Sco1	SCO1	Anchored to IM; delivers copper to Cox2	
Cox18	COX18	Likely copper binding protein in IMS	
Cox19	COX19	Likely copper binding protein in IMS	
Cox23	CHCHD7 (Predicted)	Likely copper binding protein in IMS	
Pet191	PET191	Integral IM protein, likely copper binding	
Cmc1	CMC1	Likely copper binding protein in IMS	
Cmc2	CMC2	Likely copper binding protein in IMS	
PET191	COA5/C2orf64	IM anchored; likely copper binding protein in IMS	
Cox24	AURKAIP1 (Predicted)	Splicing of Cox1 introns	Cox1 splicing
Mss116		Splicing of Cox1 introns	
Suv3		Splicing of Cox1 introns	
Mrs1		Splicing of Cox1 introns	
Mne1		Splicing of Cox1 introns	
Mss18		Splicing of Cox1 introns	
Nam2		Splicing of Cox1 introns	
Ccm1		Splicing of Cox1 introns	
Pet309	PTCD1 (Predicted)	Translational activator of COX1 mRNA	Translational Activators
Pet54		Translational activator of COX3 mRNA	
Pet122		Translational activator of COX3 mRNA	
Pet494		Translational activator of COX3 mRNA	
	TACO1	Human COX1 translational activator	
	LRPPRC	Human translational activator of COX1 mRNA / may be homoglous to Pet309	
COX20	FAM36A (Predicted)	Cox2 chaperone	Other Assembly Factors
Mss2		Involved in membrane insertion of Cox2 C-terminal tail	
Pnt1		Involved in membrane insertion of Cox2 C-terminal tail	
PET100	PET100/ LOC100131801 (Predicted)	Chaperone for assembly; interacts with a subcomoplex of Cox7, Cox8, and Cox9, but not with the holo-enzyme	

identified in yeast is greater than the amount identified in humans. This is because yeast *COX1* contains introns, unlike human *MTCOX1*. Many of the assembly proteins in yeast have been shown to have a role in the splicing of *COX1* mRNA [32]. An example such as this reminds us that while evidence for similarities between yeast and human cytochrome *c* oxidase assembly is mounting, clear differences do exist between the organisms.

The Surf1 debate: Examining the role of Surf1 in cytochrome c *oxidase assembly*

As mentioned above, despite numerous studies implicating that the Surf1 protein (Shy1 in yeast) is involved in heme insertion into Cox1, its exact role in this process is unclear. Surf1 homologues have been reported to be present in at least six prokaryotic and nine eukaryotic organisms, indicating that this protein must play an important role in the assembly of cytochrome *c* oxidase [65]. Several studies in *S. cerevisiae* have suggested that Shy1 plays some sort of role in heme insertion into Cox1. While Cox1 early assembly intermediates containing Mss51, Cox14, and Coa1 are still present in *S. cerevisiae* lacking heme *a* biosynthesis, the Cox1 complexes containing Shy1 are absent [55]. This seems to indicate that Shy1 only associates with Cox1 if heme *a* is present. In addition, it was reported that *S. cerevisiae* lacking the Cox11 assembly factor were sensitive to hydrogen peroxide due to the heme a_3 molecule [53]. When Shy1 was knocked out in Δ*cox11 S. cerevisiae*, however, the cells were no longer peroxide sensitive. This indicates that heme a_3 was either not inserted into Cox1 or fell out in the absence of Shy1. Finally, a recent study conducted by Bareth et al. (2012) [66] presented data that suggested Cox15 and Shy1 exist in complexes with one another separate from the "Coa" complexes discussed above. While this evidence does not indicate that Shy1 delivers heme *a* to Cox1, it does provide evidence that Shy1 may interact with Cox15. Taken together, all of this data generated from studies in *S. cerevisiae* might suggest that Shy1 acquires heme *a* from Cox15 and plays some role in the hemylation of Cox1.

While the studies in *S. cerevisiae* provide indirect evidence that Shy1 may function during the insertion of heme into Cox1, work on the bacterial homolog (Surf1) in *Paracoccus denitrificans* may provide even more direct support of this idea. Perhaps the strongest evidence arguing for a heme insertase role for Surf1 was the finding that *Paracoccus denitrificans* Surf1 bound heme *a* with a 1:1 stochiometry when heterologously expressed in *E. coli* along with CtaA and CtaB (heme *a* synthase and heme *o* synthase, respectively) [67]. If CtaA and CtaB were not expressed along with Surf1, it was found that Surf1 no longer bound heme. In addition, Surf1 was found to have conserved histidine residues that were likely involved in coordinating the heme. When these residues were mutated, Surf1 was no longer able to bind heme [67]. This work provides intriguing evidence supporting the hypothesis that Surf1 acts as a heme *a* chaperone.

While evidence pointing to the heme binding ability of Surf1 seems to support the heme insertase hypothesis for Surf1, other work on bacterial Surf1 brings up some uncertainty regarding this conclusion. Even though Surf1 in *P. denitrificans* clearly has heme binding ability, it does not appear to be solely responsible for heme insertion into cytochrome *c* oxidase. A deletion of Surf1 in *P. denitrificans* still allowed for 40% incorporation of heme into the heme a_3 site [68]. Correspondingly, about 40% of the heme a_3-Cu_B site was fully assembled [68]. Likewise, work in *Rhodobacter sphaeroides* demonstrated that when Surf1 was absent, 50% of the heme a_3 sites were still populated; 10-15% of the cytochrome *c* oxidase contained heme a_3 but not Cu_B and 35-40% of the enzymes contained a wild type heme a_3: CuB site [21]. These data from both *P. denitrificans and R. sphaeroides* indicate that while Surf1 may have a role in the hemylation of the heme a_3 site, it is not strictly required. In addition, it should be noted that while Surf1 appears to be involved in the formation of the heme a_3 site, it does not seem to be involved in the population of the heme *a* site [21,68].

Similar findings in eukaryotes indicate that Surf1/Shy1 is not solely responsible for the hemylation of Cox1. While deletion of Shy1 in *S. cerevisiae* leads to severely decreased levels of

cytochrome *c* oxidase, there is still residual, fully functional enzyme present [68]. Similarly, human cells in which Surf1 is non-functional still exhibit approximately 10-15% cytochrome *c* oxidase activity as wild-type cells. This data from eukaryotic cytochrome *c* oxidase supports the observations from bacteria that Surf1 is not the only protein involved in inserting heme *a* into cytochrome *c* oxidase. Furthermore, while Surf1 was convincingly shown to bind heme in *P. denitrificans,* eukaryotic Surf1 has not yet been demonstrated yet to bind heme. In fact, mutations in the corresponding heme-binding residues in yeast Shy1 do not alter the function of Shy1 [69]. Altogether, the data regarding the role of Surf1/Shy1 in hemylation of cytochrome *c* oxidase clearly leaves some gaps in our understanding of its role. Some groups argue that the evidence points to the hypothesis that Surf1/Shy1 acts to stabilize the heme a_3 site, but does not actually function as a heme insertase [17].

Cytochrome c *Oxidase Assembly: Respirasomes*

Our understanding of the ETC has evolved over time. Historically, the textbook model of the ETC consisted of the individual complexes freely floating in the mitochondrial inner membrane with the mobile electron carriers diffusing through the membrane to deliver electrons from one complex to the next (Figure 1). This model is termed the fluid or random collision model [70]. This model was challenged with the advent of BN-PAGE to study native protein complexes. In 2000 two independent studies characterized the existence of yeast cytochrome *c* oxidase in two supercomplexes identified via BN-PAGE [71,72]. The first supercomplex consisted of a dimer of both the cytochrome bc_1 complex (complex III) and of cytochrome *c* oxidase (complex IV). The second supercomplex consisted of a faster migrating complex consisting of a dimer of complex III and only a single copy of complex IV. These studies also suggested that most, if not all, of cytochrome *c* oxidase was involved in supercomplexes due to the fact that the monomeric protein complex was not readily observed on their native gels [71,72]. Following these studies, thoughts began to shift towards the notion that the individual complexes of the

electron transport chain did not exist in isolation, but in supercomplexes. In mammals, these supercomplexes were proposed to consist of (I,III_2,IV_{0-3}) (I,III_2), (I,IV) and (III_2,IV_{1-2}) [1,5,71,73]. Because yeast lack complex I, the supercomplexes in this organism were proposed to be (III_2), (III_2, IV), and (III_2,IV_2) [5,71,72,74-78]. Notably, while complex II is not usually detected in supercomplexes, a few reports have detected complex II in association with other respiratory chain complexes [5,79,80].

While more and more evidence pointed towards the existence of supercomplexes, there still was skepticism regarding the functional relevance of these supercomplexes. Much of the work that characterized respiratory supercomplexes involved co-migration of proteins on BN-PAGE and size exclusion chromatography [71,72,81,82]. These techniques often utilized the detergent digitonin to solubilize inner membrane proteins, and critics questioned whether supercomplexes observed via BN-PAGE simply reflected artifacts of digitonin solubilization [73]. To investigate if supercomplexes were an artifact of digitonin solubilization, Acin-Perez and coworkers conducted studies addressing if supercomplexes are observed in the presence of detergents other than digitonin. Ten commonly used detergents were used to solubilize mitochondria from mouse cultured fibroblasts. Supercomplexes were observed in all detergents used with the exception of dodecyl maltoside [73]. While it was shown that supercomplexes were not just an artifact of digitonin solubilization, the functional relevance of the supercomplexes still needed to be addressed.

To demonstrate a functional relevance of supercomplexes, the study by Acin-Perez discussed above also began to unravel the physiological relevance of supercomplexes in mouse epithelial cells. To do this, Acin-Perez and coworkers demonstrated that supercomplexes were capable of respiration by proving that supercomplexes containing complex I were capable of NADH reduction, that supercomplexes contained the mobile electron carriers cytochrome *c* and ubiquinone, and that supercomplexes isolated from BN-PAGE gels were able to consume oxygen in the presence of NADH [73]. Additionally, if the authors isolated only complex I, complex III, or complex IV, they did not observe

oxygen consumption when mixed together in the same reaction mixture with NADH as an electron source. They were, however, able to observe respiration with isolated complex IV in the presence of N,N,N',N'-tetramethyl-1,4-phenylenediamine as an electron source [73]. This work nicely indicates that although complex IV does not need to be part of the supercomplex to be functional, supercomplexes are not merely artifacts of aggregation on BN-PAGE; they are functional entities that are capable of respiration [73].

A second study published in 2013 by Lapuente-Brun et al. went beyond showing that isolated supercomplexes are functional and began to resolve the purpose of supercomplexes in mitochondria [1]. This study provided evidence that the association of the mammalian supercomplexes (CI, $CIII_2$) and ($CIII_2$, CIV_{1-2}) provides a mechanism to utilize electrons efficiently from different substrates. This would eliminate one substrate from saturating the electron transport enzymes. The authors first investigated the (CI, $CIII_2$) supercomplex and demonstrated that CIII exists in two populations: in supercomplex with CI and in a pool by itself, ready to receive electrons from CII or other sources that deliver electrons to FAD (such as glycerol-3-phosphate dehydrogenase). In addition, this study demonstrated that CIII preferentially associates with CI over CII. Because CI receives electrons from NADH and CII receives electrons from FAD, a preferential association of CIII with CI is significant, because it suggests that the mitochondria utilize supercomplexes to favor NADH as an electron source over FAD [1].

Next, the authors investigated the ($CIII_2$, CIV_{1-2}) supercomplex and showed that the formation of this supercomplex serves to partition CIV into two pools: one pool receiving electrons from NADH and one pool receiving electrons from FAD [1]. In the course of their studies they identified a protein found only among chordates that is responsible for ($CIII_2$, CIV_{1-2}) supercomplex formation. They named the protein SCAF1. They demonstrated that if SCAF1 is present (CIV is therefore bound in supercomplexes), rat liver mitochondria were able to utilize electrons from both NADH and FAD substrates better than mitochondria in which SCAF1 was absent (and CIV was not bound in supercomplexes). If mitochondria

were only supplied FAD, however, mitochondria lacking SCAF1 were able to utilize more of the electron donor than mitochondria containing SCAF1. This indicates that SCAF1 serves to partition CIV into supercomplexes so that different pools of CIV are created. One of these pools utilizes NADH as an electron source and another pool utilizes FAD as an electron source. Interestingly, rat liver mitochondria lacking SCAF1 (and therefore unable to form CIV-containing supercomplexes) displayed higher respiratory rates and ATP production than mitochondria containing SCAF1. While the implications of these results are not clear, it does seem from these studies that supercomplexes may serve to create separate pools of CIV that are ready to receive electrons from either CI or CII sources. The authors suggest that by partitioning CIV in different supercomplexes that are capable of receiving electrons from either the NADH or FAD pathway, the competitive inhibition by one pathway over the other is minimized.

Solid state versus plasticity model: Implications for cytochrome c *oxidase assembly*

While the physiological relevance of supercomplexes has been demonstrated, a topic that is currently under intense debate is whether respiratory complexes exist permanently bound within supercomplexes [71,72,74,75], or if there is flux between individual respiratory complexes and their incorporation into supercomplexes [79,83]. This later theory would describe a dynamic scenario that would account for the formation of sub complexes such as (CI,$CIII_2$) and ($CIII_2$,CIV). It could be envisioned, for example, that the (CI,$CIII_2$) sub complex would then associate with CIV to form a larger supercomplex [1]. While some groups prefer the "solid state" model describing a more permanent state of supercomplexes, an increasing number of groups are accepting the "plasticity model" to describe supercomplex formation [83].

As discussed above, the presence and functionality of supercomplexes has been established. In addition, however, it has also been well demonstrated that monomeric cytochrome *c* oxidase also exists *in vivo* [84]. During *in vivo* labeling experiments performed by Lazarou and coworkers, radiolabeled

human COX6a (analogous to yeast Cox13) was imported into mitochondria and observed at various time points via BN-PAGE. In wild type cells, most of COX6a seemed to incorporate primarily into monomeric CIV with a small amount detected over time in the supercomplexes [84]. In a cell line derived from a patient suffering from Leigh's syndrome, COX6a no longer was observed incorporating into monomeric CIV but into the $I/III_2/IV$ supercomplex [84]. This indicates that under the conditions in this study, there is more monomeric CIV present than CIV associated in supercomplexes. It also suggests that the incorporation of late-assembling COX subunits occurs primarily into monomeric CIV. A parallel experiment was performed in yeast in which the assembly of Cox13 into cytochrome *c* oxidase was monitored following *in vitro* import into the mitochondria [84]. Similar to the mammalian system, Cox13 was found to assemble primarily into the monomeric form of CIV [84].

Analogously, it has been shown that assembly of individual respiratory complexes occurs primarily in the monomeric respiratory complexes with the assembly of supercomplexes occurring at later time points. Acin-Perez et al. used metabolic labeling of mouse mitochondria to watch the time course of supercomplex assembly compared to monomeric complexes [73]. They were able to observe that the monomers of CIV formed within 0.5 hours, but that the full complement of supercomplexes containing CIV was not present until twelve hours later. Interestingly, any supercomplex that contained complex V was fully assembled within 0.5 hours indicating that CIV and CV display differences in their equilibrium between free enzyme complex and supercomplex. A similar metabolic labeling experiment was performed in yeast [46]. In this experiment chloroamphenicol was added to halt nuclear translation, and this was followed by a chase period. It was observed over the course of various time points, that labeled Cox1 was assembled into subassembly intermediates and monomeric COX at earlier time points while its detection in supercomplexes occurred at later time points [46]. These experiments all point to the notion that once monomeric cytochrome *c* oxidase is formed, it is able to incorporate into respiratory supercomplexes.

Assembly of cytochrome c *oxidase in supercomplexes versus monomeric units*

Based on these studies, it is reasonable to conclude that the assembly of cytochrome *c* oxidase primarily occurs in its monomeric form and that over time assembled monomers begin to associate into supercomplexes. When interpreting these results, however, it is important to remember the effect detergent may play. As mentioned above, mitochondria solubilization in DDM largely disrupts supercomplexes while solubilization in digitonin preserves them. Another study performed by Bianchi and coworkers [85] analyzed the distribution of labeled Cox13 into CIV into yeast mitochondria using digitonin as the solubilizing agent. Unlike the study discussed above by Lazarou and coworkers [84], Cox13 was found to incorporate only into CIV bound in supercomplexes. In fact, no monomeric complex IV was detected, highlighting the impact that experimental design can have on the results. While some studies may suggest that cytochrome *c* oxidase subunits are incorporated into the enzyme before supercomplex incorporation; other studies imply incorporation of the subunits occurs primarily at the supercomplex level. Keeping in mind the effect detergent plays on the detection of supercomplexes, the conclusion that is most compatible with all of the data is that cytochrome *c* oxidase subunits are capable of incorporation into the enzyme both at the monomeric and supercomplex levels.

To provide additional support, Peter Rheling's laboratory produced evidence that some of the cytochrome *c* oxidase assembly factors that have been identified in yeast are also present in supercomplexes [45]. Specifically, his lab revealed that both Shy1 and Cox14 are present in the yeast supercomplexes (III_2/IV) and (III_2/IV). Rheling et al. proposed that Shy1 and Cox14 remain bound to Cox1 in an assembly intermediate consisting of Cox1, Cox5a, and Cox4. In addition, their evidence indicates that when Shy1 and Cox14 are present in the respiratory supercomplexes, they actually form interactions with subunits of the cytochrome bc_1 complex [45]. While it is yet unclear what these findings mean, they may indicate that some pools of cytochrome *c* oxidase are assembled within

supercomplexes and that certain assembly factors must be recruited to the supercomplexes to assist with this process.

Present work

Much progress has been made towards understanding the processes involved in the assembly of cytochrome *c* oxidase. As discussed above, however, there are still many intriguing issues we do not fully understand. How and when heme *a* is inserted has been the topic of many investigations, yet we still have little idea how this critical process occurs. In addition, although many fascinating discoveries have been made regarding the structure of the ETC, numerous questions still need to be addressed. For example, does cytochrome *c* oxidase exclusively assemble first in its monomeric form and then associate into supercomplexes? Or, is cytochrome *c* oxidase capable of assembling both within its monomeric unit and within supercomplexes? If so, would this imply there are two separate pools of cytochrome *c* oxidase, and what would the physiological implications of this be?

The goal of this dissertation is to investigate the protein-protein interactions that occur with Cox15. In the following chapters, I will describe the protein complexes we have found that contain Cox15, and the strategies we utilized to identify the proteins that interact with Cox15 in these complexes. Notably, despite the fact that Cox15 is observed to form many protein complexes via BN-PAGE, we have found that the majority of Cox15 likely exists as a monomer, while some Cox15 is able to interact with itself. These findings may explain why Cox15 has not been identified previously in any of the early sub-assembly intermediates that form with Cox1 and other assembly factors. In addition we have revealed that Cox15 is present in respiratory supercomplexes containing the cytochrome bc_1 complex and cytochrome *c* oxidase. As discussed in this chapter, it is still debated if cytochrome *c* oxidase assembles and then is incorporated into supercomplexes, or if it is capable of associating with the cytochrome bc_1 complex before its assembly is complete. The identification of Cox15 in the

supercomplexes might support the later interpretation since we hypothesize that Cox15 is present within the supercomplexes to aid in cytochrome *c* oxidase assembly. If Cox15 is not present in the supercomplexes for heme insertion into Cox1, these findings may indicate a novel role for Cox15. Finally, our studies have found that Cox15 interacts with the cytosolic heat shock proteins, Ssa1 and Hsc82. While we do not think these interactions are represented by any of the complexes observed in BN-PAGE, we discuss the role Ssa1 and Hsc82 may play in the uptake of Cox15 into the mitochondria.

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Chapter 2:

Heme *a* synthase from *Saccharomyces cerevisiae* exists in a high molecular weight protein complex

Introduction

Cytochrome *c* oxidase, a large multi-subunit enzyme in the inner membrane of the mitochondria, plays a vital role in aerobic respiration in eukaryotic organisms. Serving as the terminal electron acceptor in the electron transport chain, an oxygen molecule is reduced to two water molecules while four protons are pumped across the inner mitochondrial membrane. For this chemistry to occur, two heme *a* molecules must be properly inserted into the active site of this enzyme. Heme *a* is synthesized via a series of reactions catalyzed by two integral membrane proteins, heme *o* synthase and heme *a* synthase, which are located in the mitochondrial inner membrane. The commonly known heme *b* is first converted to heme *o* by heme *o* synthase. This reaction is characterized by the addition of a farnesyl moiety to the vinyl side chain at position 2 [1]. Heme *o* is then subsequently converted to heme *a* by heme *a* synthase via a reaction in which the methyl group at position 8 is oxidized to a formyl group [1] (Figure 5). The only known destination for the resulting heme *a* is cytochrome *c* oxidase. While much progress has been made in understanding the assembly of cytochrome *c* oxidase, very little is known about how heme *a* is inserted into Cox1, the catalytic heme-containing subunit of cytochrome *c* oxidase. With the help of techniques such as blue native polyacrylamide electrophoresis (BN-PAGE), we know that many proteins termed cytochrome *c* oxidase assembly factors are involved in forming sequential, sub-assembly complexes with Cox1 during its translation, membrane insertion, and assembly into the holo-enzyme [2-6]. Heme *a* synthase, however, has yet to be found within any of these complexes.

We have found that heme *a* synthase exists in high molecular weight protein complexes. This chapter will focus on proving the existence of these complexes within the mitochondria, and the proteins involved in these protein complexes will be the focus of this thesis. By unraveling the protein-protein interactions of heme *a* synthase, we will begin to fill in the many gaps in our understanding of heme *a* insertion into cytochrome *c* oxidase.

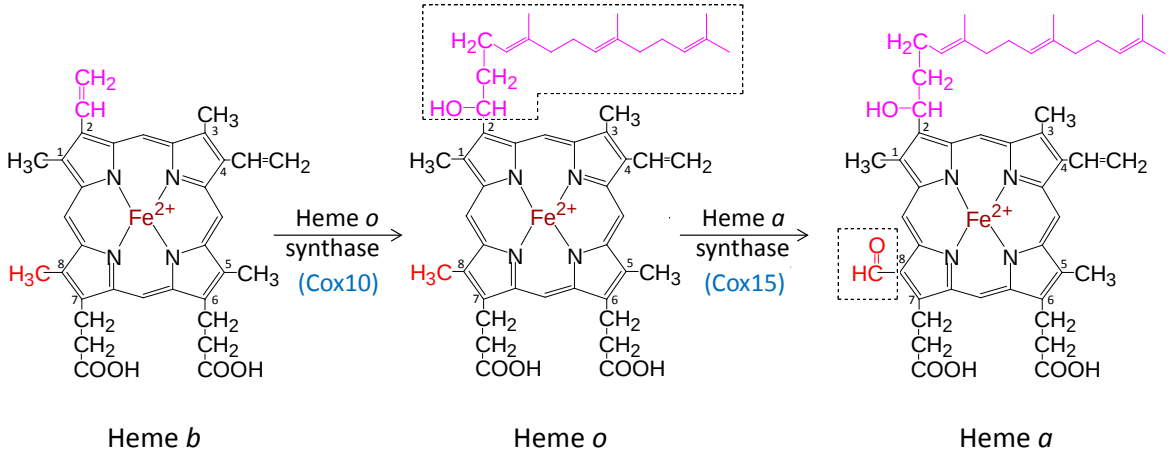

Figure 5: The conversion of heme *b* to heme *a*. Heme *b* is first converted to heme *o* by the addition of a hydroxyfarnesyl moiety at position C2 on the porphyrin ring. This reaction is catalyzed by heme *o* synthase (also known as Cox10 in eukaryotes). The methyl group at position 8 on heme *o* is then oxidized to a formyl group, resulting in the heme *a* molecule. This reaction is catalyzed by heme *a* synthase, or Cox15 as referred to in eukaryotes. While the exact mechanism of Cox15 is still under debate, it has been proposed that Cox15 may act as an oxygenase along with its partners ferredoxin and ferredoxin reductase to convert the methyl group to a formyl group [7]. In addition, it has also been found that the oxygen incorporated into heme *a* is derived from water [8]. This may occur either through oxidation of heme *o* via outer sphere electron transfer using a heme *b* cofactor to activate O_2 or it may occur through the autoxidation of heme *o,* activating O_2 directly on the heme *o* substrate [8].

Results

Determine if Cox15 exists in high molecular weight protein complexes

The foundation for this thesis is the observation that Cox15 from *Saccharomyces cerevisiae* forms high molecular weight protein complexes when run on a blue native gel (Figure 6). The Cox15 complexes that are observed on blue native PAGE (BN-PAGE) range in size from about 120 kDa – 1 MDa. Consistently, Cox15 distributes primarily in the lowest complex which migrates at ~120 kDa and its distribution in the upper complexes appears to be less abundant. When run on a denaturing gel, C-terminal tagged *COX15::MYC* runs at 75 kDa. The upper complexes ranging from ~232 kDa – 1 MDa clearly represent Cox15-containing high molecular weight protein complexes as these complexes are about three to thirteen times greater in size than monomeric Cox15. These complexes are represented by bands B-F in Figure 6. While the lowest Cox15-containing complex at 120 kDa may also represent a higher molecular weight complex, it is difficult to rule out the possibility that this complex represents monomeric Cox15. While BN-PAGE is useful in analyzing protein complexes, the molecular weight that is ascertained via BN-PAGE must be taken as an approximation. Because these are hydrophobic proteins solubilized by detergent, it is highly probable that the estimated molecular weights are a bit higher than the actual weight of the complex [9,10]. In particular, the detergent micelle that forms around the solubilized protein complex undoubtedly will cause the protein complex to run higher than the sum of the molecular weights of the individual proteins involved in the complex [9]. In addition, protein modifications such as glycosylation and phosphorylation are maintained in BN-PAGE and may contribute to alterations in observed molecular weights [9]. Therefore, it is quite possible that the Cox15 complex observed at ~120 kDa in Figure 1 represents monomeric Cox15.

The observation that Cox15 forms high molecular weight protein complexes ranging from at least 232 kDa – 1 MDa lead us to hypothesize that Cox15 interacts with either itself or other proteins. Our goal was to identify the proteins that were part of these different Cox15 complexes and to

determine if these proteins, together with Cox15, play a role in inserting heme into cytochrome *c* oxidase.

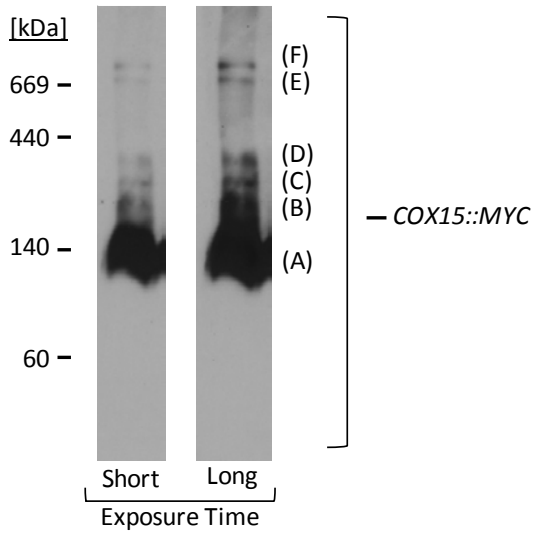

Figure 6: Distribution of *COX15::MYC* in high molecular weight complexes observed on BN-PAGE. Mitochondria solubilized in 1% digitonin and run on BN-PAGE. For this experiment, mitochondria were prepared from *S. cerevisiae* in which Cox15 contained a C-terminal c-MYC tag. Western blotting from the blue native was used to detect the distribution of *COX15::MYC*. Discrete bands are marked by Roman numerals. The same blot is shown exposed either for 30 seconds (Short) or 2 minutes (Long).

Ensure that the Cox15 complex we observe is not an artifact of BN-PAGE

Size Exclusion Chromatography

To ensure that the Cox15 complex we observe on BN-PAGE is a physiologically relevant entity, we utilized size exclusion chromatography to verify the Cox15 distribution using a different experimental approach. Size exclusion chromatography confirmed that Cox15 could be detected in high molecular weight protein complexes ranging from ~120 – 800 kDa (Figure 7A). We next sought to break apart the Cox15 complex using the denaturants urea and sodium dodecyl sulfate (SDS). The Cox15 complex was predominantly shifted down in size to ~200 kDa using these denaturants, with SDS breaking apart the Cox15 complex more noticeably than urea (Figures 7B and 7C). Interestingly, however, the Cox15

protein complex was not broken apart to its monomeric molecular weight as easily as the control protein, porin (Figures 7E and 7F). Porin, a mitochondrial outer membrane protein was used as a control because it has been reported to associate in high molecular weight protein complexes around 440 kDa [11,12]. In addition, porin is also a highly hydrophobic protein and various reports have determined that it contains at least 13 transmembrane helices [13-15]. It is possible that Cox15 may not respond well to agents that disrupt the hydrophobic interactions of the protein, causing non-physiologically relevant aggregation on a size exclusion column. Furthermore, it is well documented that the resolution of size exclusion chromatography is far less than that of BN-PAGE [16-18]. Size exclusion chromatography confirmed that Cox15 does associate in high molecular weight complexes, but this technique was not able to completely break up the Cox15 complexes into monomers.

Purification of Cox15 and two-dimensional blue native/SDS-PAGE electrophoresis

In addition to verifying that Cox15 distributes in high molecular weight complexes using size exclusion chromatography, we also wanted to determine if the Cox15 high molecular weight complexes were maintained following purification. Genomically expressed *COX15::MYC* and *COX15::HIS* overexpresed on a yeast expression plasmid were purified via c-Myc and Ni-NTA (Nitrilotriacetic acid) column chromatography, respectively. Purified Cox15 was then run on both BN-PAGE and two dimensional BN/SDS-PAGE. On both 1D and 2D gels, Cox15 is observed in high molecular weight protein complexes (Figure 8). This indicates that the Cox15 complex is maintained during non-denaturing purification and can be observed on BN-PAGE. It is also important to note that Cox15 is observed in high molecular weight protein complexes regardless of the identity of the purification tag (Figure 8).

Finally, to observe whether Cox15 could be broken apart into its monomer and observed on BN-PAGE, we treated purified Cox15 with SDS and analyzed the SDS-treated Cox15 using two-dimensional BN/SDS-PAGE gel. Unlike in the size exclusion experiments, Cox15 was clearly shifted to its monomeric molecular weight (Figures 8C and 8F). This supports the hypothesis that the hydrophobic protein Cox15

does not distribute well on a size exclusion column, and that blue native electrophoresis may achieve better resolution of the Cox15 complex. In addition, the fact that the Cox15 complex can be broken apart by the addition of SDS indicates that these high molecular weight complexes are not just an artifact of aggregation.

Discussion

The sum of the experiments discussed in this chapter suggests that Cox15 exists in high molecular weight protein complexes. Not only does Cox15 distribute in discrete protein complexes ranging up to 1 MDa, when analyzed via BN-PAGE, Cox15 also exhibits a high molecular weight distribution on a size exclusion column. Cox15 high molecular weight complexes can be broken up with SDS and observed in its monomeric form in both size exclusion chromatography and very convincingly in two-dimensional BN/SDS-PAGE.

From these experiments it is difficult to unequivocally determine if the band at 120 kDa on a blue native gel represents monomeric Cox15 or Cox15 associating in a higher mass complex. The apparent molecular weight of 120 kDa would seem to suggest that Cox15 is present in a high mass complex, but due to the limitations discussed above, it cannot be ruled out that this species represents monomeric Cox15. More experiments will be needed to determine what this band represents. In addition, the Cox15-containing complexes at 750 kDa and 1 MDa are reminiscent of the complex III and complex IV-containing supercomplexes reported by Cruciat and Brunner et al. [19] and Schagger and Pfeiffer [20]. Chapter 3 will discuss the identity of these Cox15-containing complexes in more detail.

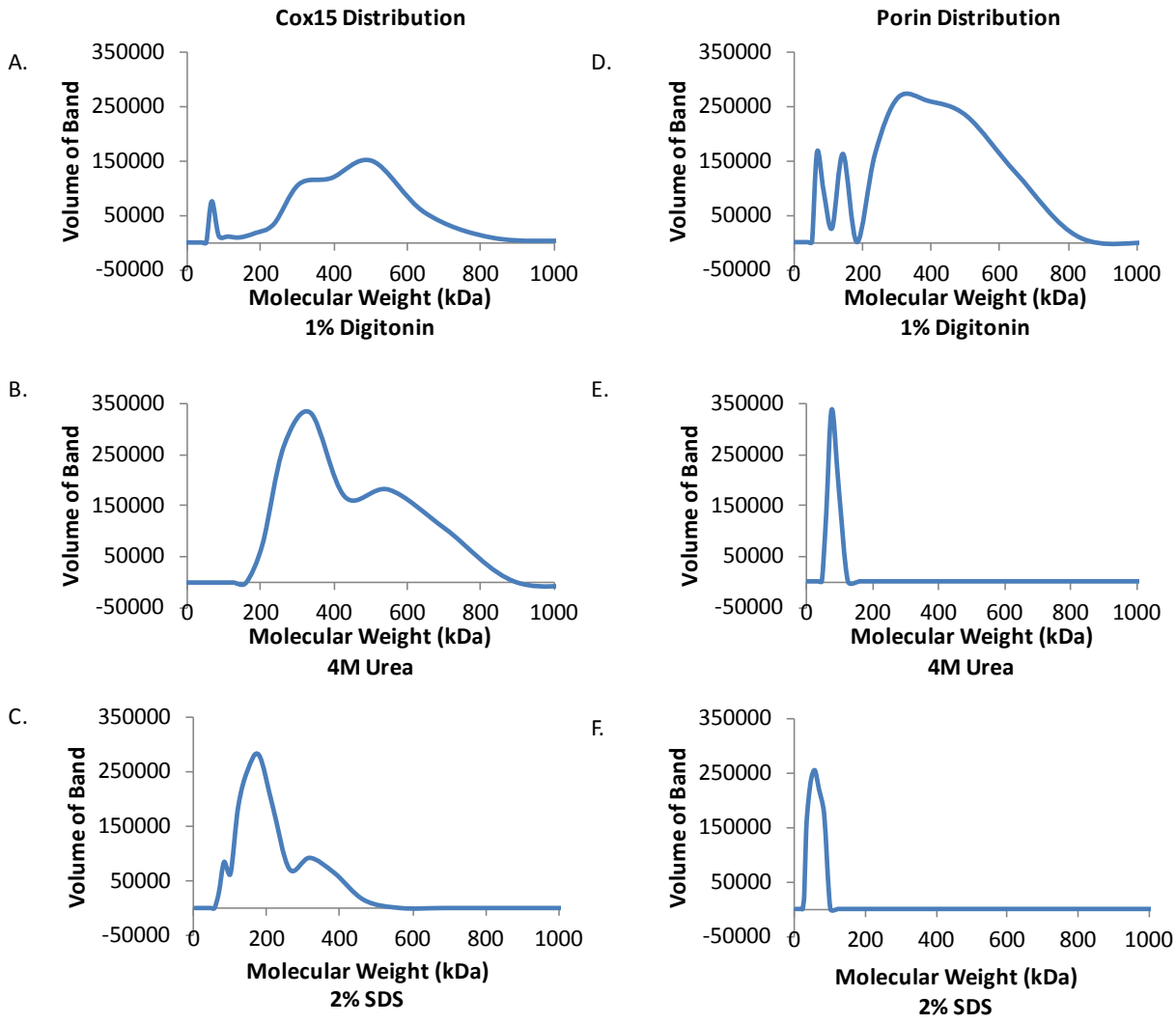

Figure 7: Size exclusion chromatography distribution of Cox15 and porin. Whole mitochondria were solubilized in 1% digitonin and loaded on a Superdex 200 column. After separating solubilized mitochondria, size exclusion standards were utilized to estimate the molecular weight of Cox15 and porin by generating a standard curve of protein mass to time of elution from the column. Western blotting was used to determine which fractions contained Cox15 and porin. A. Cox15, 1% digitonin. B. Cox15, 4M urea. C. Cox15, 2% SDS. D. Porin, 1% digitonin. E. Porin, 4M urea. F. Porin, 2% SDS.

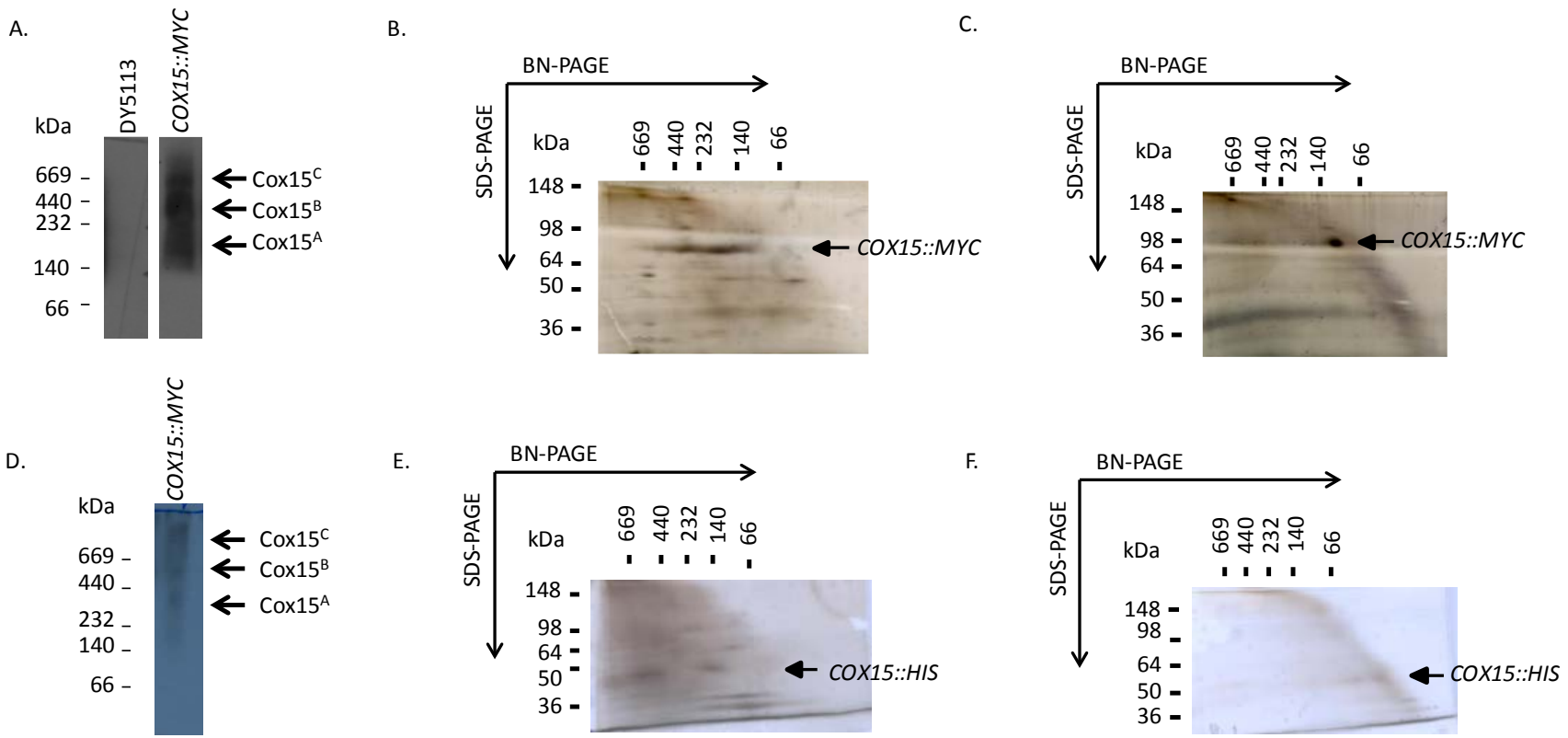

Figure 8: One-dimensional and two-dimensional BN-PAGE of purified Cox15. A. Genomic *COX15::MYC* was purified via anti c-Myc chromatography and analyzed via one-dimensional BN-PAGE. B. BN/SDS-PAGE was utilized by excising a lane from the-one dimensional BN-PAGE in A. and mounting to the top of an SDS-PAGE gel. C. Purified Cox15-cMyc was incubated with 1% SDS before separating on BN/SDS-PAGE. D. Cox15 containing a C-terminal 6X-Histidine tag was expressed on a yeast expression plasmid and purified via Ni-NTA chromatography. Purified Cox15-His was run on one-dimensional BN-PAGE. E. BN/SDS-PAGE utilized by excising a lane from the one-dimensional BN-PAGE in E. and mounting to the top of an SDS-PAGE gel. F. Purified *COX15::HIS* was incubated with 1% SDS before running on BN/SDS-PAGE.

Experimental Procedures

Yeast strains and growth conditions

Saccharomyces cerevisiae containing a genomic copy of *COX15::MYC* (C-terminally tagged) was generated previously [21]. All cell growth of *COX15::MYC* was in YPD. The *COX15::HIS* construct (C-terminally tagged) was generated by Julia Cricco. Cox15 was inserted in the pRS426 yeast expression vector between the Met25 promoter and the Cyc1 terminator. Cox15 with a 6X-histidine tag was inserted between the BamHI and HindIII restriction sites. The *COX15::HIS* construct allowed for overexpression of Cox15 as the pRS426 vector is expressed at approximately 20 copies per cell. In addition, the methionine levels were reduced during cell growth to modestly induce Cox15 expression via the Met25 promoter. Cells were grown in 5 mL buffered synthetic complete media with 2% glucose for 48 hours. The synthetic complete media recipe was obtained from *Molecular Cloning A Laboratory Manual* [22]. To buffer the media, 0.126 M Na_2HPO_4 and 0.036 M citric acid was used, and the pH was adjusted to 6.5 with 1 M NaOH. This 5 mL starter culture was then used to inoculate a 500 mL culture of the same media. This culture was grown for 48 hours until late log phase (OD_{600} of approximately 10). Finally, 5 mL of the secondary culture was used to inoculate four 500 mL cultures of buffered synthetic complete media (0.126 M Na_2HPO_4, 0.036 M citric acid – pH 6.5) containing 0.2% glucose, 3% ethanol, and 3% glycerol as the carbon sources [22]. These cultures were grown for 48 hours until an OD_{600} of approximately 5 was reached. To induce *COX15::HIS* expression utilizing the Met25 promoter, the primary and secondary cultures contained 0.67 mM methionine while in the final growth the methionine concentration was lowered to 0.5 mM. This reduction in methionine concentration was sufficient to induce *COX15::HIS.* Appropriate amino acids were used to select for the Myc and Histidine tags on Cox15. Cells were harvested, washed twice with tap water, and stored at -80 ^{o}C.

Mitochondria isolation

COX15::MYC mitochondria from 100 mL of *S. cerevisiae* were prepared by rupturing cells with glass beads in 600 mM sorbitol, 20 mM HEPES-KOH (pH=7.4), and 1 mM Phenylmethylsulfonyl fluoride (SHP buffer). To rupture cells, 5 mL of buffer was added to every 5 grams of cell pellet in a corning tube. No more than 20 mL of resuspended mitochondria was added to each corning tube. Approximately 5 mL of glass beads were added to the resuspended mitochondria. The mitochondria were vortexed at 3000 rpm for one minute followed by one minute incubation on ice. This was repeated eight times. The beads were removed from solution via a five minute spin at 1,500 x *g*. Cell debris was further separated by two subsequent 15 minute spins at 1,500 x *g*. Mitochondria were isolated by a 20 minute spin at 12,000 *x g* and were resuspended in SHP buffer and stored at -80^{o}C.

Blue native of COX15::MYC

BN-PAGE was performed as described previously [23]. Briefly, 10 µg of mitochondria were solubilized on ice for 30 minutes in 10 µL of solubilization buffer (20mM Tris (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% v/v glycerol, 4mM PMSF, and 1% wt/vol digitonin). Solubilized lystate was clarified via centrifugation at 17,000 x *g* for 15 minutes at 4 ^{o}C. Following centrifugation, 1 µL of sample buffer (5% Coomassie Brilliant Blue G-250, 500 mM 6-amino caproic acid, 100 mM bis-tris) was added to the supernatant and samples were loaded on a 4-15% gradient gel (Biorad). The dimensions of the gel were 8.6 x 6.7 cm (W x L). Electrophoresis was performed using the Biorad mini-PROTEAN-TGX system. The gel was run at 120 volts for 4 hours. Following electrophoresis, the gel was blotted for 3 hours at 60 volts using 50 mM tricine, 7.5 mM imidazole (pH=7.0) as the transfer buffer. Following protein transfer, the blot was washed for 5 minutes with methanol, rinsed in TBS buffer (50 mM Tris, 150 mM NaCl, pH=7.0), blocked overnight in 5% milk/TBS solution, and was blotted with the anti-cMyc antibody (Invitrogen).

Size exclusion chromatography distribution of COX15::MYC

C*OX15::MYC* mitochondria were isolated as described above. For detection of *COX15::MYC*, 4 mg of mitochondria were solubilized in 1 mL lysis buffer containing 150 mM NaCl, 50 mM imidazole (pH=7), 5 mM 6-aminohexanoic acid, Roche protease inhibitor, and 1% digitonin. For detection of Cox15 in the presence of 4M urea or 2% SDS, either urea or SDS was added to the appropriate final concentration in the same lysis buffer. Solubilization was carried out for 15 minutes at 4 ^{o}C for native solubilization or room temperature in the case of urea and SDS solubilization to prevent crystallization of urea and SDS in the solubilization reaction. Solubilized material was centrifuged at 20,000 *x g* for 15 minutes and loaded on a Superdex 200 gel filtration column that was pre-equilibrated with the same buffer used for mitochondria solubilization. The flow rate through the column was set at 1 mL/minute. Fifty-five elution fractions were collected, and the presence of Cox15 within these elutions was detected by western blotting. To generate graphs in Figure 2, ImageQuant 5.2 software was used to quantify band intensity of *COX15::MYC* on western blots. The numbers obtained from this analysis were plotted to determine the relative amount of *COX15::MYC* present in each fraction that contained C*OX15::MYC* signal. The fractions that lacked *COX15::MYC* signal were determined not to contain Cox15.

Two dimensional blue native/SDS-PAGE of purified COX15::MYC

Mitochondria were prepared as before from 1.8 L of *COX15::MYC S. cerevisiae* grown in YPD. Mitochondria were solubilized for two hours in 600 mM sorbitol, 20 mM HEPES, 4.1% digitonin, 150 mM NaCl, and Roche protease inhibitor in 5 mL total volume. Solubilized lysate was clarified via centrifugation at 12,000 *x g* for 30 minutes and added to 300 µL of anti c-Myc resin (Sigma). Lysate and resin were incubated overnight at 4 ^{o}C, washed 8 times with 1 mL PBS, and eluted in ten 1-mL fractions of PBS containing 0.1 mg/mL c-Myc peptide and 0.1% digitonin. Each elution fraction was incubated with resin for 5 minutes before collecting. Elution fractions containing *COX15::MYC* were pooled and concentrated using an Amicon 10 MWCO membrane until the total volume was reduced to 80 µL. The

buffer was exchanged by adding 500 mM 6-aminohexanoic acid and 200 mM NaCl to a final volume of 200 µL. The centrifugal filter device was spun at 14,000 *x g* in a fixed angle rotor until the total volume was about 80 µL. This was repeated once again until 150 µL total volume was recovered. Following buffer exchange, 0.02% Ponceau and 10% glycerol (final concentrations) were added to the purified protein to allow for better loading on the BN-PAGE gel. BN-PAGE was run as described previously. After BN-PAGE, a gel lane was excised and mounted to the top of an SDS-PAGE gel. Following electrophoresis, the gel was silver stained using the Proteosilver kit (Sigma). To disrupt the Cox15 high molecular weight complexes prior to BN-PAGE, 10 µL of purified Cox15 was mixed with 10 µL 2X SDS-PAGE buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) and incubated at 45 ^{o}C for 15 minutes.

Two dimensional blue native/SDS-PAGE of COX15::HIS

Mitochondria were prepared from 4 L of culture grown in buffered, synthetic media as previously described. For these experiments, *S. cerevisiae* containing the *COX15::HIS* construct on the pRS426 yeast expression vector were used. Mitochondria were solubilized for 2 hours in a 5 mL volume of 600 mM sorbitol, 20 mM HEPES, 4.1% digitonin (w/v), 500 mM NaCl, and Roche protease inhibitor. Solubilized lystate was clarified at 12,000 *x g* for 20 minutes and 20 mM imidazole and 5% glycerol (final concentrations) were added. The lysate was incubated with 500 µL of Ni-NTA resin overnight. Following incubation, the resin was washed once with 8 mL of 25 mM Tris (pH 7.5), 10% glycerol, 500 mM NaCl, 20 mM imidazole, and 0.5% triton X-100. Two more washes followed with 2 mL of the same buffer that contained 35 mM imidazole. Finally, the purified Cox15 was eluted in five 500 µL fractions containing the same buffer with 500 mM imidazole.

Elutions containing Cox15 were concentrated using an Amicon 10 MWCO membrane. To concentrate, 250 µL of purified protein was centrifuged at 14,000 *x g* in a fixed angle rotor for 5 minutes. A final volume of 80 µL was obtained. The buffer was exchanged with a final volume of 200 µL of 500

mM 6-aminohexanoic acid and 200 mM NaCl until the protein was resuspended in a volume of 100 µL. This was repeated two times. Proteins were run on BN-PAGE, and a gel lane from the BN-PAGE was excised and mounted at the top of an SDS-PAGE gel. The SDS-PAGE gel was silver stained using the Proteosilver kit (Sigma). To dissociate the high molecular weight Cox15 complexes, 10 µL of purified Cox15 was mixed with 10 µL 2X SDS-PAGE buffer and incubated at 45 ^{o}C for 15 minutes before performing BN-PAGE.

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Chapter 3:

Mass spectral analysis of purified Cox15 to analyze protein-protein interactions

Introduction

This chapter will discuss the use of mass spectrometry to identify protein-protein interactions with Cox15. As reported in Chapter 2, Cox15 exists in high molecular weight protein complexes. Because the Cox15-containing complexes range in size up to 1 MDa, we hypothesize that not all of these complexes merely represent interactions of Cox15 with itself. While some of the Cox15 complexes may reflect homo-oligomeric associations of Cox15, it is likely that other proteins also associate with Cox15, particularly in some of the higher mass complexes. Mass spectrometry is an excellent tool to generate potential targets of proteins that may interact with Cox15 [1]. While the power of mass spectrometry-based techniques is undeniable, it is also important to approach data generated by mass spectrometry with caution. Mass spectrometry-based strategies often result in poor reproducibility from one laboratory to the next and even from one sample to the next due to variations in sample preparation [2-4]. The following quotation sums up some of the challenges associated with assessing potential protein-protein interactions based solely on mass spectral data:

> "In an ideal world, interaction discovery methods would find all interactions within an organism, and one could estimate the total number of unique interaction types in nature by simply clustering similar interactions and extrapolating the resulting number to all species. However, it is known that methods miss real interactions (false negatives), or predict them wrongly (false positives) [5]."
>
> – Patrick Aloy (Institute for Research in Biomedicine, Barcelona, Spain) and Robert B. Russell (SmithKline Beecham Pharmaceuticals)

Consequently, mass spectral data cannot be used to prove the presence or absence of a given protein-protein interaction. They are exceedingly valuable data nonetheless, because they can be used to generate large numbers of testable hypotheses.

The work presented in this chapter will serve to generate hypotheses of potential interaction partners with Cox15. Not every protein discussed in this chapter is likely to reflect a real interaction partner with Cox15. Instead, this work will provide a platform for future studies of Cox15 protein-protein interactions. Importantly, as will be reflected in subsequent chapters, some of the results presented in this chapter do correctly identify proteins that interact with Cox15.

Protein-protein interactions with Cox15: Cox15 in the literature

Little is known about protein-protein interactions with Cox15. Currently, the two most utilized techniques for screening protein-protein interactions are yeast two-hybrid and protein purification coupled to mass spectrometry [6]. These techniques can be utilized for small-scale approaches and also for large scale screens of entire proteomes. While techniques such as mass spectrometry and yeast two-hybrid are vital in advancing our knowledge of protein interactions, there are inherent difficulties with these strategies when studying membrane proteins. In the case of protein purification followed by mass spectrometry, often the protein purification methods utilized in large scale screens are not suitable for membrane proteins [6,7]. If too much detergent is used, membrane protein interactions may be compromised. If too little detergent is used, protein complexes embedded in the membrane may not be released [8]. In addition, the classic yeast two-hybrid method to screen protein-protein interactions fails to detect membrane protein interactions accurately because this technique depends on the ability of the interacting proteins to localize to the nucleus [6]. While an adaption of the classic yeast two-hybrid technique has been developed (split-ubiquitin system) for membrane proteins, problems such as the identification of false positives and negatives are still a concern [6,7]. Because of these caveats, the identification of protein interactions with membrane proteins using standard biochemical techniques is

limited. While protein interactions have been reported with Cox15 in the literature, caution must be taken when analyzing these results due to the complications discussed above. The next section will present a review of what is known regarding protein-protein interactions with Cox15 using mass spectrometry and yeast two-hybrid approaches.

Cox15 in yeast-two-hybrid studies of Srs2 and Cln3

A few putative protein-protein interactions involving Cox15 have been reported arising from yeast two-hybrid screens. A yeast two-hybrid screen looking for interactions with yeast Srs2 reported the presence Cox15 in their screen [9]. Srs2 is an ATP-dependent DNA helicase located in the nucleus and is thought to play a role in DNA damage and repair. In this study, 800 prey plasmids interacted with the Srs2 bait and 350 of these were sequenced. In total, 67 genes occurred more than once in their screen, with *Cox15* being present on three of these plasmids. However, given that Srs2 is reported to be localized to the nucleus and this is the only report of a putative Cox15-Srs2 interaction, it is likely that this is one of the many false positives expected in this high throughput screen.

Two independent groups studying two-hybrid screens with the human protein Cln3 (a protein associated in an unknown way with Batten disease) reported that Cox15 was one of several proteins that interacted with Cln3, although the physiological relevance of this potential interaction remains unclear [10,11]. While Cln3 is known to be present in the lysosome membrane in both yeast and humans, it has also been reported to be present in the mitochondria and has been implicated in unfolding of subunit c of ATP synthase [12]. Given the occurrence of Cox15 in two independent studies of Cln3, this putative interaction may very well warrant further exploration.

Cox15 in large scale protein interaction network studies

In 2006 Gavin et al. [13] attempted to map all the protein complexes that exist in *Saccharomyces cerevisiae.* To accomplish this, they attached a TAP epitope tag to all 6,466 known open reading frames reported by Kumar et al. (2002) [14]. They successfully purified 1,993 proteins out of the

6,466 tagged proteins. All purified proteins were analyzed via mass spectrometry to identify potential interaction partners. Cox15 was among the proteins purified and analyzed by mass spectrometry. It was found that Fsk1, Imd3, Ssa1, Ssa2, and proteins from

the 60S ribosome co-purified with Cox15. The potential relevance of these proteins will be discussed later in this chapter.

A similar study was conducted to map the protein interactome in *Caenorhabditis elegans.* Yeast two-hybrid studies were conducted on 3024 *C. elegans* proteins. Cox15 was reported to bind the Pqn-11 protein [15]. A blast of Pqn-11 does not indicate homology with any yeast proteins, although there is homology with an uncharacterized human protein. Due to the putative nature of the Pqn-11 protein, it is likely that future studies investigating an interaction with Pqn-11 and Cox15 will wait until more evidence is gathered regarding this interaction.

Results

Mass spectrometry analysis of purified Cox15

To identify proteins that may interact with Cox15 in the Cox15 high molecular weight protein complexes, Cox15 expressed on pRS426 (a low copy-number plasmid) was purified using the tandem affinity purification (TAP) strategy [16,17]. The C-terminal TAP tag, which consisted of both a protein A domain and the calmodulin binding peptide domain, allowed us to use two rounds of purification to obtain very pure Cox15. Following the final round of purification, an on-bead digest of Cox15 was performed, and the proteins that had co-purified with Cox15 were analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results are presented in Table 2. Mass spectrometry of a control purification of untagged *S. cerevisae* only detected trace amounts of actin.

Over half of the proteins identified in the *COX15::TAP* purification experiment were proteins from the cytochrome bc_1 complex of the respiratory chain and from ATP synthase. These proteins held

particular interest for us because a preliminary mass spectrometry experiment in which histidine-tagged Cox15 was purified on a Ni-NTA column also identified numerous proteins from both the cytochrome bc_1 complex and ATP synthase (unpublished data from Behzad Khodaverdian). In addition to these proteins, we also identified seven other proteins from our *COX15::TAP* screen. While the significance of some of these proteins is currently unclear, the identification of proteins from the 60S ribosome and from the mitochondrial Hsp70, Ssc1, is intriguing. The 60S ribosomal subunits were also identified in the mass spectrometry experiment of purified Cox15 performed by Gavin and coworkers discussed in the introduction for this chapter [13]. The identification of Ssc1 may similarly be of significance given that Ssc1 has been implicated in forming a high mass protein complex with newly translated Cox1 as well as the cytochrome *c* oxidase assembly factors, Mss51, Cox14, and Coa3 [18-20]. In addition, Ssc1 has been shown to interact with Cox4, aiding in the assembly of cytochrome *c* oxidase [21]. Due to the importance of Ssc1 in cytochrome *c* oxidase assembly, presumably near the time of heme insertion, an interaction of Cox15 and Ssc1 may be worth further study. Finally, although the detection of proteins from the cytochrome bc_1 complex and ATP synthase in multiple co-purification experiments is intriguing, it is important to note that proteins from the respiratory complexes are very abundant proteins in the mitochondrial inner membrane. To ensure that these proteins do not represent false positives, we sought an alternative purification and mass spectral strategy to determine the likelihood that Cox15 does interact with these proteins.

Protein	Molecular Weight	Unique Peptides	Number of Peptides	Sequence Coverage	
Cytochrome *c* oxidase assembly protein **(Cox15)**	55 kDa	3	5	6%	
ATP Synthase, subunit alpha **(Atpα)**	59 kDa	5	8	11%	ATP Synthase
ATP Synthase, subunit beta **(Atpβ)**	55 kDa	6	14	16%	
ATP Synthase, subunit d **(Atp4)**	27 kDa	3	7	16%	
ATP Synthase, subunit 7 **(Atp7)**	20 kDa	4	7	27%	
Cytochrome bc_1 subunit 1 **(Cor1)**	50 kDa	2	2	5.7%	Cytochrome bc_1 complex
Cytochrome bc_1 subunit 2 **(Qcr2)**	40 kDa	4	7	12%	
Cytochrome c_1, heme protein **(Cyt1)**	34 kDa	2	4	10%	
Cytochrome bc_1 subunit 7 **(Qcr7)**	15 kDa	2	4	17%	
Cytochrome bc_1 subunit 10 **(Qcr10)**	9 kDa	2	3	36%	
Mitochondrial outer membrane protein, porin **(VDAC1)**	30 kDa	4	7	17%	Other proteins
Heat shock protein, mitochondrial **(Ssc1)**	71 kDa	3	5	6%	
60 S Ribosomal Protein **(RL13A)**	23 kDa	4	5	21%	
60 S Ribosomal Protein **(RL19A)**	22 kDa	3	4	20%	
Glutaredoxin-2, mitochondrial **(Grx2)**	16 kDa	2	3	17%	
Mitochondrial phosphate protein **(Mir1)**	33 kDa	2	3	8.7%	
ADP, ATP carrier portein **(Adt2)**	34 kDa	2	2	5.7%	

Table 2: List of all proteins identified in the *COX15::TAP* purification strategy via LC-MS/MS.

In a second strategy, genomically expressed Cox15 containing a C-terminal c-Myc tag (*COX15::MYC)* was purified using an anti-Myc antibody column. The advantage of this strategy was that Cox15 was not overexpressed, thereby reducing false positives that might result from an over-abundance of Cox15. Following anti-Myc purification, quantitative mass spectrometry (LC-MS/MS) was used to analyze protein interaction partners with Cox15. This experiment was performed on both *S. cerevisiae* containing *COX15::MYC* as well as *S. cerevisiae* containing untagged *COX15* as a control. For every protein that was identified from these two purifications, the number of spectral counts detected in the mass spectrometer was compared between the control and purified *COX15::MYC* fractions. This provided an advantage over the *COX15::TAP* experiments in that the results were more quantitative. These experiments were performed on both digitonin and Triton X-100 solubilized mitochondria to assess any differences in the detected protein interactions resulting from the identity of the detergent.

The quantitative mass spectrometry experiments indicated that some of the proteins from the cytochrome bc_1 complex as well as the alpha and beta subunits of ATP synthase may represent artifacts of the purification procedure (Table 3). For the represented proteins, the number of spectra identified was either nearly the same between the control and *COX15::MYC* purifications or enriched in the control relative to the *COX15::MYC* purification. These results suggest that although this subset of proteins from the cytochrome bc_1 complex and the alpha and beta subunits from ATP synthase may be prevalent in Cox15 purifications, they may not represent real interaction partners with Cox15. In addition, many other proteins were identified as being either equally or more enriched in the control purification compared to the *COX15::MYC* purification (data not shown). Of significance, 22 ribosomal subunits of the 60S ribosome and 5 subunits of the 40S ribosome were represented equally between the control and *COX15::MYC* purifications. This is significant because, as discussed above, in the large scale screen of the yeast proteome performed by Gavin et al. [13], ribosomal subunits were identified in their mass

spectral experiments of purified TAP-tagged Cox15. The results from this study indicate that these proteins likely represent artifacts of purification rather than true interaction partners with Cox15. Similarly, Fsk1, a protein involved in cell wall remodeling, was also found to associate with Cox15 in these studies [13]. Our work indicates that Fsk1 is two-fold enriched in the control purifications relative to the *COX15::MYC* purifications. As demonstrated, the results of these studies allowed us to rule out certain proteins from being interaction partners Cox15.

			Number of Spectra		
Protein	**Standard Name**	**Protein Size**	**Control**	***COX15::MYC* Purification**	**Solubilization**
ATP synthase subunit alpha	Atpα	59 kDa	32	28	Triton X-100
ATP synthase subunit alpha	Atpα	59 kDa	22	15	Digitonin
ATP synthase subunit beta	Atpβ	55 kDa	32	38	Triton X-100
ATP synthase subunit beta	Atpβ	55 kDa	21	23	Digitonin
Cytochrome *c* oxidase subunit 2	Cox2	29 kDa	4	2	Digitonin
Cytochrome bc_1 complex subunit 1	Qcr1	50 kDa	9	0	Triton X-100
Cytochrome bc_1 complex subunit 1	Qcr1	50 kDa	12	14	Digitonin
Cytochrome bc_1 complex subunit 2	Qcr2	40 kDa	18	2	Triton X-100
Cytochrome bc_1 complex subunit 2	Qcr2	40 kDa	7	8	Digitonin
Cytochrome bc_1 complex subunit Rieske	Rip1	23 kDa	2	2	Triton X-100
Cytochrome c_1, heme protein	Cyt1	34 kDa	3	2	Digitonin
Cytochrome bc_1 assembly	Cbp3	39 kDa	16	7	Triton X-100
Cytochrome bc_1 assembly	Cbp6	19 kDa	8	1	Triton X-100

Table 3: Proteins from respiratory complexes that were not enriched in *COX15::MYC* purifications. The number of spectra obtained in LC-MS/MS is compared between the *COX15::MYC* and control purifications. The table represents proteins that were either enriched in the control purification compared to the *COX15::MYC* purification or were represented equally in both purifications. Proteins were considered equally enriched between purifications if their spectral counts were less than 2-fold enriched in the *COX15::MYC* purification relative to the control. Only those proteins from the respiratory complexes that were recognized in these experiments are represented in this table. Other proteins not belonging to the respiratory complexes also fit these criteria but are not shown.

Not only did these experiments allow us to rule out certain proteins as likely interaction partners with Cox15, they also identified new proteins that may interact with Cox15. Table 4 represents all of the proteins that were enriched four times or more in the *COX15::MYC* purifications compared to the control purifications. When Triton X-100 was used to solubilize the mitochondria, seven proteins were enriched at least four times in the purified protein fractions (Table 4). When digitonin was used to solubilize the mitochondria, however, only two proteins were identified to be enriched at least four times in the purification fraction (Table 4). The protein that most strongly associated with Cox15 in both the digitonin and Triton X-100 solubilized mitochondria was the cytosolic heat shock protein of the Hsp70 family, Ssa1 (Table 4). In these experiments, Ssa1 was enriched 9.5 and 8.7 fold over the control purification in the Triton X-100 and digitonin solubilization experiments, respectively. Interestingly, in the Triton X-100 solubilization experiments, the cytosolic heat shock protein belonging to the Hsp90 family, Hsc82, was also detected as being 8-fold enriched compared to the control purification. While it may seem unlikely for cytosolic proteins to interact with Cox15, both Ssa1 and Hsc82 have been implicated in playing a role in importing proteins into the mitochondria [22-25]. The role Ssa1 and Hsc82 play in this process will be the topic of Chapter 5. Finally as discussed above, in their mass spectral analysis of TAP-tagged Cox15, Gavin et al. also detected Ssa1 as a possible interaction partner with Cox15 [13]. Our work verifies this finding.

In addition to Ssa1 and Hsc82, Table 4 also highlights other proteins that may represent interaction partners with Cox15. Two proteins of particular interest are Cyt1, one of the catalytic subunits of the cytochrome bc_1 complex, and Mdj1, the co-chaperone of the mitochondrial Hsp70, Ssc1. Both of these proteins may be worthy of future study due to the overlap with the *COX15::TAP* experiments. While Mdj1 was not detected in the TAP experiments, its interaction partner, Ssc1 was detected. It is important to highlight this overlap as it may provide further verification that Cox15 does interact with Ssc1/Mdj1. In addition, Cyt1 was also detected in the TAP experiments, so a second

detection of this protein may implicate it as being a real interaction partner. It should be noted, however, that while Cyt1 was positively identified in both Table 2 and Table 4, Cyt1 was not identified as a significant interaction partner when digitonin was used for solubilization of *COX15::MYC* (Table 3). This observation is a good reminder that while quantifying the mass spectra between the control and *COX15::MYC* purifications is a helpful tool to generate hypotheses of real Cox15 interaction partners, some of these results may be misleading. Therefore, it will be crucial to follow-up this work with other methods to verify protein interactions and experiments to determine the physiological relevance of these interactions. Only then will we be able to verify that the proteins identified in this work do indeed interact with Cox15. In addition, it is important to remember that false negatives may occur in these studies. Even if a protein is NOT identified by these studies as interacting with Cox15, it does not rule out the possibility that an interaction exists.

Protein	Standard Name	Protein Size	Enrichment in Cox15 Purification	Solubilization
Heme *a* synthase	Cox15	55 kDa	44	Triton X-100
Heat shock protein, Ssa1	Hsp70	70 kDa	9.7	Triton X-100
Heat shock protein, Hsc82	Hsp90	81 kDa	8	Triton X-100
ATP synthase subunit gamma	Atp3	34 kDa	6	Triton X-100
Eukaryotic translation initiation factor 4E	IF4E	24 kDa	6	Triton X-100
Cytochrome c_1, heme protein	Cyt1	34 kDa	5	Triton X-100
40S ribosomal protein S9-A	RS9A	22 kDa	4	Triton X-100
DnaJ homolog 1, mitochondrial	Mdj1	56 kDa	4	Triton X-100
Heme *a* synthase	Cox15	55 kDa	56	Digitionin
Heat Shock protein, Ssa1	Hsp70	70 kDa	8.5	Digitionin
Enoyl-[acyl-carrier protein] reductase, mitochondria	Etr1	42 kDa	4	Digitionin

Table 4: Proteins that were enriched at least four fold in *COX15::MYC* purifications compared to control purifications. All proteins that had spectral counts four times greater in the *COX15::MYC* purification relative to the control are shown. As an example, 44 spectra were detected in the *COX15::MYC* purification. The control purification only recognized 1 peptide fragment of Cox15. This resulted in a 44 fold enrichment of Cox15.

Discussion

As discussed in the introduction of this chapter, a few studies have reported Cox15 interactions either by way of studying a particular protein or as part of high-throughput studies looking at protein-protein interactions in *S. cerevisiae* or *C. elegans.* Only further studies will verify the isolated reports that Cln3, Srs2, and the *C. elegans* protein Pqn-11 interact with Cox15. The significance of these interactions is not apparent. The work presented in this chapter has shed further light on the high-throughput screen of protein-protein interactions in *S. cerevisiae* reported by Gavin et al. [13]. We have shown that the association of Cox15 with ribosomal proteins and the Fsk1 protein are likely artifacts. Conversely, our data support their report that the cytosolic Hsp70, Ssa1, may interact with Cox15. In addition to Ssa1, our work has also identified a possible interaction of the cytosolic heat shock 90 protein, Hsc82, with Cox15. While follow-up studies will be needed to provide additional evidence for these interactions, our findings suggest that Ssa1 and Hsc82 may somehow be important for Cox15. Based on past reports in the literature, it is reasonable to hypothesize that Ssa1 and Hsc82 are involved in importing Cox15 into the mitochondria [22-25]. Interestingly, previous reports have suggested that Ssa1 in *S. cerevisiae* mediates protein import by way of the Tom70 receptor [24]. More work will be needed to determine if Ssa1 hands off Cox15 to Tom70 and what role Hsc82 may play in this process. Initial experiments to address this topic will be the discussion of Chapter 5.

In addition to implicating Ssa1 and Hsc82 as interacting with Cox15, we have also detected a possible interaction of Cox15 with the mitochondrial Hsp70 machinery, Ssc1 and Mdj1. Ssc1 is most notably known for its role in protein translocation through the Tim23 complex of the mitochondrial inner membrane [26-29]. One possibility is that Ssc1 interacts with Cox15 during its insertion into the inner mitochondrial membrane. As discussed above, however, based on our detection of Ssa1, Hsc82, and Tom70 in these studies, we hypothesize that Cox15 is imported into the mitochondria in a Ssa1/Tom70-dependent fashion [22-25]. It is thought that hydrophobic inner membrane proteins that

are recognized by the Tom70 machinery are then laterally imported into the inner membrane via the Tim22 complex [30-32]. From these past reports, it is likely that if Cox15 is recognized by the Tom70 receptor, it may be inserted into the inner membrane via Tim22. While the mechanism of protein uptake through the Tim22 is largely unknown, Ssc1 has not yet been implicated in this process.

If Ssc1 is not involved in protein uptake in the mitochondria, there is another intriguing possibility relating to cytochrome *c* oxidase assembly that could explain why Ssc1 may interact with Cox15. It is known that Ssc1 forms a complex with newly translated Cox1. The cytochrome *c* oxidase assembly factors, Mss51, Cox14, and Coa3 are also thought to be part of this complex [18-20,33]. In addition, it is reported that Ssc1 and its co-chaperone, Mdj1, help to stabilize the interaction of Mss51 with Cox1 [19], and it is hypothesized that when Mss51 and Ssc1/Mdj1 dissociate from the Cox1-containing complex, the heme and copper co-factors are inserted into Cox1 [18,34,35]. Thus, an attractive possibility is that the interaction between Ssc1 and Cox15 is crucial for heme *a* insertion into Cox1 during cytochrome *c* oxidase assembly.

In addition to the involvement of Ssc1 in early cytochrome *c* oxidase assembly, Ssc1 has also been implicated in later stages of assembly. Bottinger and coworkers (2003) identified a stable complex consisting of Cox4, Ssc1, and Mge1 [21]. They further demonstrated that an interaction between Cox4 and Ssc1/Mge1 seemed to facilitate the incorporation of Cox4 and Cox5a into respiratory supercomplexes under stressed conditions. In addition, they reported that Cox4 arrests at the Ssc1/Mge1/Cox4 complex when it cannot assemble into mature cytochrome *c* oxidase. Because Ssc1 was only found to interact with Cox4, and not to other components of cytochrome *c* oxidase or the cytochrome bc_1 complex, its putative role in supercomplex assembly is intriguing. As mentioned in Chapter 2 and discussed in depth in Chapter 4, Cox15 also seems to be present in the respiratory supercomplexes. Thus, another intriguing possibility is that is that Ssc1 plays a role in Cox15 interacting with the supercomplexes.

As discussed, we have three hypotheses to explain why Cox15 might interact with Ssc1. (1) Ssc1 is involved in Cox15 import into the mitochondria. (2) Ssc1 interacts with Cox15 in early Cox1 intermediates to aid in heme insertion. (3) Ssc1 plays some role in recruiting Cox15 to respiratory supercomplexes. It is important to note that these hypotheses are not mutually exclusive. Perhaps a Cox15-Ssc1 interaction plays a role in two or more of these possibilities. The next step will be to support further an interaction of Cox15 with Ssc1 using co-immunoprecipitation studies, and if an interaction does exist between Cox15 and Ssc1, the implications of this interaction will result in exciting future studies.

Finally, Cyt1, one of the catalytic subunits of the cytochrome bc_1 complex, was also identified in our mass spectral studies as possibly interacting with Cox15. As discussed in Chapter 2, based on the BN-PAGE distribution of Cox15, we hypothesize that Cox15 may be present in the respiratory supercomplexes. It is possible that Cox15 and Cyt1 might interact within the supercomplexes. We have also noted in these studies, however, that we were unable to detect significant interaction of Cox15 with other subunits of the cytochrome bc_1 complex or cytochrome *c* oxidase. If Cox15 is present in the respiratory supercomplexes, it would seem that Cox15 interacts with additional subunits from these complexes. As discussed earlier in this chapter, it is important to consider both false positives and negatives when analyzing mass spectrometry data from purified membrane proteins. For instance, perhaps the interaction of Cyt1 and Cox15 represents a false positive. If this is true, proteins other than those from the cytochrome bc_1 complex and cytochrome *c* oxidase may mediate the presence of Cox15 within the supercomplexes. Conversely, if the absence of other Cox15 interaction partners from the cytochrome bc_1 complex and cytochrome *c* oxidase represents a false negative, then future experiments will be needed to identify which proteins from these complexes interact with Cox15. More discussion on this topic is included in Chapter 4.

Experimental Procedures

Mass spectrometry of purified COX15::TAP

Mitochondria from a 2-L culture of *S. cerevisiae* were isolated as described in the experimental section of Chapter 2. Following mitochondrial isolation, mitochondria were resuspended in 10 mL of 600 mM sorbitol, 20 mM HEPES-KOH, and 1 mM Phenylmethylsulfonyl fluoride (SHP buffer). In addition, 150 mM NaCl and 3% ANAPOE-$C_{12}E_9$ was added for solubilization of mitochondrial membranes. Solubilization proceeded for 4 hours at 4 ^{o}C. Solubilized lysate was flash frozen, thawed, and clarified by centrifugation for 45 minutes at 12,000 x *g*. To the clarified lysate, 10 mM Tris-Cl (pH 8), 10 mM β-mercaptoethanol, 1 mM Mg-Acetate, 1 mM imidazole, and 2 mM $CaCl_2$ were added resulting in these final concentrations. The adjusted protein lysate was added to 300 µL calmodulin binding peptide resin (Agilent). This was rotated for 4 hours at 4 ^{o}C. The protein lysate was eluted from beads and the beads were washed with 30 mL of calmodulin binding buffer (10 mM Tris-Cl (pH 8), 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM Mg-Acetate, 1 mM imidazole, 2 mM $CaCl_2$). The bound *COX15::TAP* was eluted in 5 fractions of 200 µL calmodulin elution buffer (10 mM Tris-Cl (pH 8), 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM Mg-Acetate, 1 mM imidazole, 2 mM ethylene glycol tetraacetic acid (EGTA)). Eluted protein was then added to IgG beads (Sigma) and rotated at 4 ^{o}C overnight. Unbound protein was collected from the column, and the resin was washed with 30 mL of TEV cleavage buffer (10 mM Tris-Cl (pH 8), 150 mM NaCl, 0.025% ANAPOE-$C_{12}E_9$). This was followed by a wash with 30 mL of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4)**.**

On-bead digest and mass spectrometry (LC-MS/MS)

Proteins bound to IgG resin were digested on-bead by washing 3 times with 100 µL of 50 mM ammonium bicarbonate so that the resin was completely submerged. This was followed by the addition of 5 ng/µL trypsin so that the beads were just submerged in the digestion buffer and allowed to incubate for 6 hours at 37 ^{o}C. The solution was acidified to 5% formic acid (pH < 2.0) and centrifuged at

14,000 *x g* at room temperature. Peptide supernatant was removed and the peptides were concentrated by C18 reverse-phase chromatography. Purified peptides were then re-suspended in 2% acetonitrile/0.1% trichloroacetic acid to 20 µL. From this, 10 µL was were automatically injected by a Waters nanoAcquity Sample Manager autoinjector (www.waters.com) and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/min in 2% acetonitrile/0.1% formic acid. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% acetonitrile/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3u, 200A, 100U x 150mm, www.michrom.com) and eluted over 35 minutes with a gradient of 5% B to 30% B in 21 minutes at a flow rate of 1 µl/min.

Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (www.thermo.com) using a Michrom ADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at *m/z* 400) and the top ten ions in each survey scan are then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting MS/MS spectra are converted to peak lists using BioWorks Browser v3.3.1 (ThermoFisher) using the default parameters and searched against all yeast protein sequences downloaded from Uniprot (www.uniprot.org, downloaded 11-11-2011), using the Mascot searching algorithm, v 2.3 (www.matrixscience.com, [36]). The Mascot output was then analyzed using Scaffold, v3.4.7 (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet[2] computer algorithm [37]. Assignments validated above the Scaffold 95% confidence filter are considered true. (The Mascot parameters for all databases were the following: allowance of up to 2 missed tryptic sites, variable modification of oxidation of methionine, peptide tolerance of +/- 10 ppm, and MS/MS tolerance of 0.6 Da).

Mass spectrometry of purified COX15::MYC

Mitochondria were prepared from a 2-L culture of either untagged *S. cerevisiae* or *S. cerevisiae* containing *COX15::MYC* as described in Chapter 2. The isolated mitochondria were solubilized in SHP buffer containing 150 mM NaCl, Roche Protease Inhibitor, and either 4.1% digitonin or 1% Triton X-100. Solubilization proceeded for 2 hours at 4 ^{o}C and lysate was clarified at 12,000 x *g* for 30 minutes. Clarified lysate was added to washed anti-Myc resin (one column volume of PBS followed by three 5-mL washes of 0.1 M ammonium hydroxide (pH 11-12), 0.1% Triton X-100, and 150 mM PBS) and incubated overnight at 4 ^{o}C. Following incubation with the beads, the unbound protein lysate was collected and the column was washed with PBS until the OD_{280} of the washes was less than 0.01. Bound protein was eluted in ten 1-mL aliquots of 0.1 M ammonium hydroxide (pH 11-12), 0.1% Triton X-100, and 150 mM NaCl into vials that contained 50 µL 1 M acetic acid. Each protein elution fraction was run on SDS-PAGE and analyzed by western blotting to determine which fraction contained *COX15::MYC*. The fractions containing *COX15::MYC* were concentrated to approximately 150 µL using Amicon-Ultra 10K centrifugal filter units by centrifuging at 5000 x *g* for 20 minutes at 4 °C. Following concentration, 15 µL of the concentrated protein fraction was added to 15 µL of 2X SDS-PAGE buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) and was incubated at 45 ^{o}C for 15 minutes and loaded on a SDS-PAGE gel. (Solubilization at 45 ^{o}C is optimal for Cox15). The gel was run for 15 minutes until the sample just entered the gel. The gel was then stained overnight with Coomassie Brilliant Blue G-250 and destained in 10% acetic acid until the background was colorless.

The protein band containing the entire protein fraction was excised and subjected to in-gel trypsin digestion [38] with the following modifications. Briefly, the gel bands were dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate (pH 8.0) so that the gel bands were completely submerged. This was carried out at 56 ^{o}C for 45 minutes. This dehydration was repeated and gel bands were incubated in 50 mM iodoacetamide, 100 mM ammonium

bicarbonate for 20 minutes in the dark to ensure the peptides were completely denatured (reaction was performed in the dark to prevent the decomposition of iodoacetamide). The gel bands were washed with enough 100 mM ammonium bicarbonate to completely submerge the bands and were dehydrated again. Sequencing grade modified trypsin was prepared to 0.01 µg/µL 50 mM ammonium bicarbonate and approximately 50 µL of this was added to each gel band so that each was completely submerged. The bands were incubated at 37 ^{o}C overnight. The peptides were extracted from the gel in a solution of 60% acetonitrile/1% trichloroacetic acid by incubation in a water bath sonicator at room temperature. This solution was concentrated via vacuum to 2 µL. The peptides were re-suspended in 20 µL of 2% acetonitrile / 1% trichloroacetic acid, and 10 µL of this solution was injected by a Waters nanoAcquity Sample Manager autoinjector (www.waters.com) and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/min in 5% acetonitrile / 0.1% formic acid. The bound peptides were then eluted onto a Waters BEH C18 nanoAcquity column (1.7 µm, 100 µm x 100 mm) over 16 minutes with a gradient of 5% buffer B to 30% buffer B in 9 minutes, ramping to 90% buffer B at 10min, holding for 30 seconds and returning to 5% buffer B at 10.6 minutes using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water / 0.1% Formic Acid, Buffer B = 99.9% acetonitrile / 0.1% Formic Acid) with an initial flow rate of 0.8uL/min.

Eluted peptides were sprayed into a ThermoFisher LTQ Linear Ion trap mass spectrometer outfitted with a MICHROM Bioresources ADVANCE nano-spray source. The top five ions in each survey scan were then subjected to data-dependent zoom scans followed by low energy collision induced dissociation (CID) and the resulting MS/MS spectra were converted to peak lists using BioWorks Browser v 3.3.1 (ThermoFisher) using the default LTQ instrument parameters. Peak lists were searched against the UniProt-SwissProt protein database, downloaded (7/2012) from www.uniprot.org, using the Mascot searching algorithm, v2.3 (www.matrixscience.com, [37]). The Mascot output was then analyzed using Scaffold, v3.6.2 (www.proteomesoftware.com) to probabilistically validate protein identifications using

the ProteinProphet[2] computer algorithm. Assignments validated above the Scaffold 95% confidence filter are considered true. (The Mascot parameters for all the databases were the following: allowance of up to two missed tryptic sites, fixed modification of carbamidomethyl cysteine, variable modification of oxidation of methionine, peptide tolerance of +/- 200 ppm, MS/MS tolerance of 0.6 Da, peptide charge state limited to +2/+3).

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Chapter 4:

Proteins that are part of the Cox15 complex

"But instead of a cell dominated by randomly colliding individual protein molecules, we now know that nearly every major process in a cell is carried out by assemblies of 10 or more protein molecules. And, as it carries out its biological functions, each of these protein assemblies interacts with several other large complexes of proteins. Indeed, the entire cell can be viewed as a factory that contains an elaborate network of interlocking assembly lines, each of which is composed of a set of large protein machines."

-Bruce Alberts

"The Cell as a Collection of Protein Machines: Preparing the Next Generation of Molecular Biologists" [1]

Introduction

As alluded to in the quotation above, a significant amount of work in molecular biology is directed towards identifying protein-protein interactions and elucidating their physiological relevance [1,2]. As research uncovers the myriad of protein interaction networks that exist, the remarkable complexity underlying cellular function is becoming readily apparent [3]. The focus of this chapter will be to present the work I have completed to identify the proteins that interact with Cox15, and to use this information to advance our understanding of the complexities of cytochrome *c* oxidase assembly.

Cytochrome *c* oxidase assembly is a sequence of events that also involves the interaction of many proteins called assembly factors (for a more detailed description of these interactions, see Chapter 1). The catalytic subunit of cytochrome *c* oxidase, Cox1, interacts sequentially with these assembly factors within protein complexes. The predominance of evidence suggests that discrete protein complexes may exist at certain time points during cytochrome *c* oxidase assembly [4-26]. Because very little is known about when and how heme *a* is inserted into cytochrome *c* oxidase, it is our

objective to understand what comprises the Cox15 protein complexes in an effort to elucidate heme *a* delivery to Cox1.

When determining the proteins that are part of the Cox15 complexes observed on a blue native gel, we considered three distinct possibilities (Figure 9). First, some or all of the Cox15 complexes may be homo-oligomeric, consisting strictly of multiple copies of Cox15. Second, all of the Cox15 complexes may be hetero-oligomeric, consisting of one copy of Cox15 in association with other proteins. The third and final possibility is a combination of these two scenarios, in which Cox15 interacts with other proteins in addition to itself. Furthermore, these three possibilities are not mutually exclusive. For instance, Cox15 may interact with itself and form homo-oligomeric complexes which are reflected by the lower bands observed in the blue native gel, while at the same time the higher molecular weight complexes might reflect the association of Cox15 with other proteins. Perhaps the various Cox15-containing complexes exist to perform different functions.

Results

Determine if the Cox15 complex contains multiple copies of Cox15

We first sought to determine if Cox15 associates with itself. Perhaps some of the Cox15 complexes observed on a blue native represent homo-oligomeric complexes. To determine if Cox15 interacts with itself, we conducted co-purification experiments with differentially tagged Cox15. First, the pRS426 yeast expression plasmid containing C-terminally histidine-tagged Cox15 (*COX15::HIS)* was expressed in a *S. cerevisae* strain with genomically Myc-tagged Cox15. This design resulted in a slight overexpression of *COX15::HIS* as the pRS426 plasmid is expressed at approximately 20 copies per cell. In addition, Cox15 was expressed behind the Met25 promoter. Methionine levels were altered during growth so that Cox15 expression was only modestly induced [27,28]. *COX15::HIS* was purified via

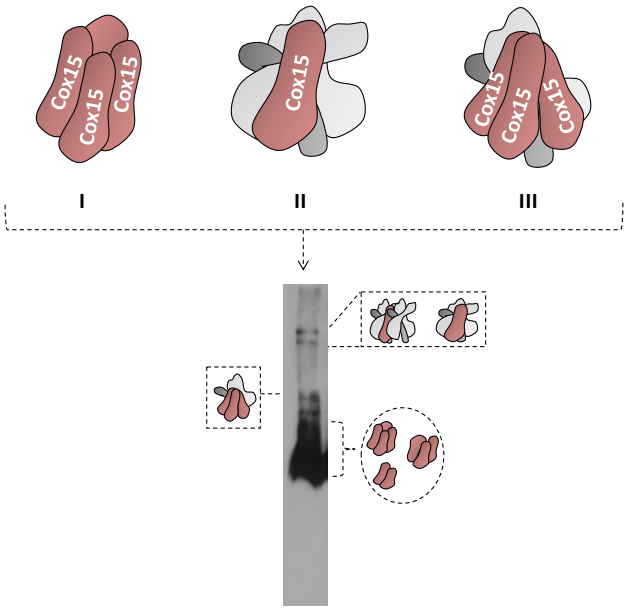

Figure 9: Possibilities of the composition of the Cox15 complexes. This model depicts three possible ways to describe the Cox15 complexes. 1) Cox15 may associate with itself in homo-oligomeric complexes, 2) Cox15 may associate with other proteins, 3) Cox15 may associate with itself and other proteins. These three hypotheses are not mutually exclusive. Cox15 may form different complexes for different purposes. This figure shows a hypothetical model for how Cox15 may be incorporated into different protein complexes and how this may correspond to the banding pattern on a blue native.

affinity chromatography using a nickel-nitrilotriacetic (Ni-NTA) column, and the elution fractions were probed for the presence of both *COX15::HIS* and *COX15::MYC*. If Cox15 interacts with itself, one would expect to observe both *COX15::HIS* and *COX15::MYC* eluting together from the Ni-NTA resin. As shown in Figure 10A, *COX15::MYC* eluted with *COX15::HIS.* When this interaction was quantified, it was estimated that only ~20% of the *COX15::MYC* bound to the Ni-NTA column (Table 5). It should be noted, however, that approximately 30% of the *COX15::HIS* also did not bind to the Ni-NTA column, and we must assume that a certain amount of *COX15::MYC* is also associated with the *COX15::HIS* that flowed through the column. Taking this into account, we estimated that approximately 30% of the *COX15::MYC* interacts with *COX15::HIS.* Although this interaction is not quantitative, control experiments indicate that only 2.2% of genomic *COX15::MYC* bound to the Ni-NTA column when the *S. cerevisiae* strain

contained only an empty plasmid (Figure 10B and Table 5). This indicates that the majority of the observed co-purification of *COX15::MYC* with *COX15::HIS* depicted in Figure 10A is the result of a specific interaction between *COX15::MYC* and *COX15::HIS.*

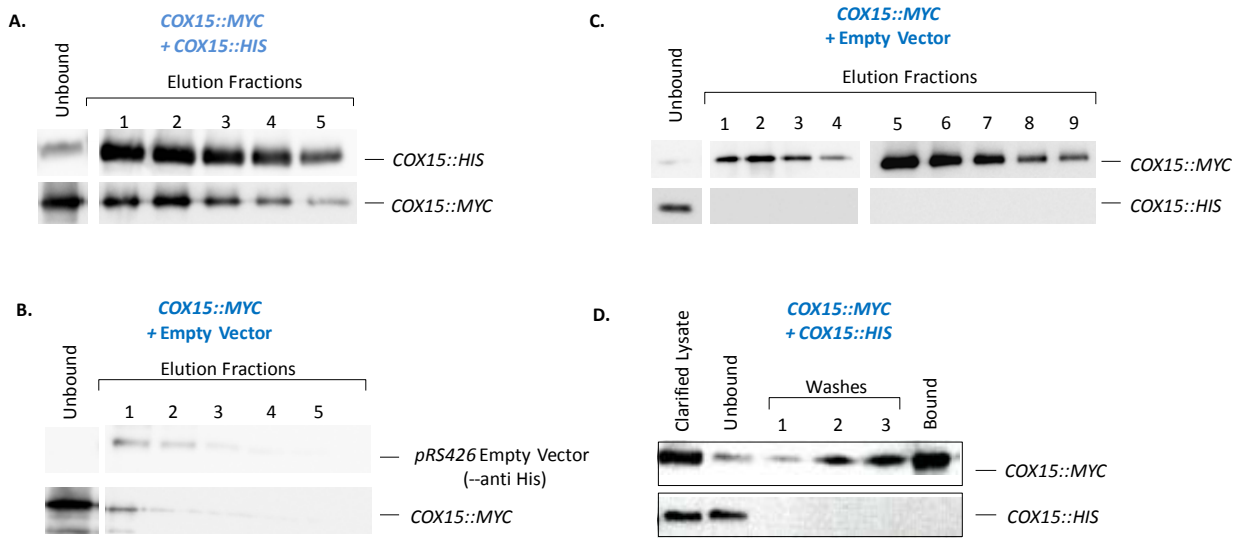

Figure 10: Co-purification and co-immunoprecipitation experiments of *COX15::MYC* and *COX15::HIS.* (A.) Ni-NTA chromatography of *S. cerevisiae* containing genomically tagged *COX15::MYC* expressing *COX15::HIS* on the *pRS426* expression plasmid. *COX15::HIS* was purified and elution fractions were analyzed for the co-purification of *COX15::MYC*. (Unbound fraction; 0.2% of the total fraction loaded, Eluate fraction; 2% of each fraction loaded). (B.) Ni-NTA chromatography of *S. cerevisiae* containing genomically tagged *COX15::MYC* expressing an empty *pRS426* expression plasmid. Mitochondrial extract was purified via Ni-NTA chromatography and elution fractions were analyzed to ensure *COX15::MYC* did not co-purify. Some non-specific signal was detected in the elution fraction lanes when probed with an anti-histidine antibody. No non-specific signal was detected in the flow through. The non-specific signal was quantified and was determined to only be 5% of the signal detected when *COX15::HIS* is expressed and purified. The amount of *COX15::MYC* quantified in these blots was 2.2% of the total (Unbound fraction; 0.2% of total loaded, Eluate fraction; 2% of total loaded) (C.) Anti-Myc column chromatography of *S. cerevisiae* containing genomically tagged *COX15::MYC* expressing *COX15::HIS* on the *pRS426* expression plasmid. *COX15::MYC* was purified and *COX15::HIS* was NOT observed to co-purify with *COX15::MYC*. (Unbound fraction; 0.2% of total loaded, Eluate fraction; 2% of total loaded) (D.) Anti c-Myc co-immunoprecipitation of *S. cerevisiae* containing genomically tagged *COX15::MYC* expressing *COX15::HIS* on the *pRS426* expression plasmid. *COX15::MYC* was bound to anti-Myc resin and *COX15::HIS* was NOT observed to interact with the *COX15::MYC*.

To confirm the results from the experiment described above. We repeated this strategy in the opposite direction. The co-purification of *COX15::HIS* with *COX15::MYC* was monitored following purification of *COX15::MYC* via anti c-Myc chromatography. Interestingly, no co-purification of *COX15::HIS* was observed in the elution fractions containing *COX15::MYC*; none of the His-tagged Cox15 bound to the column, and all of it was entirely accounted for in the unbound fraction (Figure 10C). These surprising results were repeated several times. In addition, *COX15::HIS* was not found to co-immunoprecipitate with *COX15::MYC* using anti c-Myc resin (Figure 10D). While these results are perplexing given the co-purification observed following Ni-NTA chromatography, one possible explanation is that an experimental condition in the anti c-Myc chromatography procedure impedes the observation of *COX15::HIS* co-purification, although it is not obvious what that experimental condition might be since the protein solubilization conditions were identical between the two experiments. Another possibility is that some unknown property of the anti-Myc resin might inhibit the *COX15::MYC*-*COX15::HIS* interaction during purification. Currently, however, we cannot provide a definitive explanation for these results.

In support of the Ni-NTA chromatography experiments which suggest some amount of Cox15 interacting with itself, data collected by a previous student in the lab, Behzad Khodaverdian, also indicated that Cox15 is capable of interacting with itself. Inactive mutants of *COX15::HIS* were expressed in *S. cerevisiae* containing an untagged wild-type copy of Cox15. The inactive mutants were purified and analyzed by high performance liquid chromatography (HPLC), and heme *a* was found to co-purify with the inactive mutants of Cox15. These results suggest either that the native Cox15 (containing heme *a*) associated with the mutant Cox15, or that heme *a* dissociated from native Cox15 and then bound to the mutant Cox15. Given that the diffusion of free heme *a* seems unlikely, the observation that heme *a* is purified with inactive mutants of Cox15 provides additional evidence for a Cox15-Cox15 interacation. Given that only 30% of *COX15::MYC* was found to co-purify with *COX15::HIS*,

my hypothesis is that some, but not all, of the Cox15 complexes observed on the blue native represent homo-oligomeric complexes.

Cox15-cMyc + *pRS426* + Cox15-His	Percent protein recovered in elutions	Percent protein recovered in flow through
Cox15-Histidine(6X)	67.4%	32.6%
Cox15-cMyc	19.7%	80.3%
Cox15-cMyc + *pRS426* + Empty Vector		
Cox15-cMyc	2.2%	97.8%

Table 5: Mass balance analysis of the amount of genomically tagged *COX15::MYC* associating with plasmid expressed *COX15::HIS* in Ni-NTA experiments. ImageQuant software (5.2) was used to quantify the pixel intensity in western blots from co-purification experiments. Pixel intensities were compared between elution fractions, flow through, and total protein. The sum of pixel intensities for either the flow through or all the combined elutions were compared to that of the total estimated protein. The numbers obtained using these methods were normalized to 100% to estimate the percentages presented in the above table.

Utilization of 2D blue native/SDS-PAGE to ascertain if other proteins associate with Cox15

A strategy we undertook to identify other proteins that may be part of the Cox15 complex was 2D blue native-SDS PAGE (BN/SDS-PAGE). Cox15 with a C-terminal Myc tag was purified from *S. cerevisiae* using non-denaturing anti-Myc chromatography and run on BN-PAGE. A lane from the blue native gel was excised and mounted to the top of an SDS-PAGE gel. Following SDS-PAGE, the gel was silver stained. This allowed us to observe the distribution of purified Cox15 in the high molecular weight complexes as well as any other proteins that are part of the complex. These other proteins will appear either above or below Cox15 on the SDS-PAGE gel. Figure 11A is a silver stain of a 2D BN/SDS-PAGE gel. Cox15 can be observed in the 2D gel in high molecular weight complexes ranging from ~140-600 kDa. In particular, the 2D gel showed Cox15 associating in two distinct areas. Cox15 is most enriched around

440 kDa and in a broader range between ~140-250 kDa. Both the higher molecular weight band at 440 kDa and the broad band from 140-250 kDa were excised and analyzed by mass spectrometry to determine if any other proteins with the same molecular weight as *COX15::MYC* were also present in these bands. Mass spectrometry indicated that Cox15 was the only protein present in both of the bands analyzed.

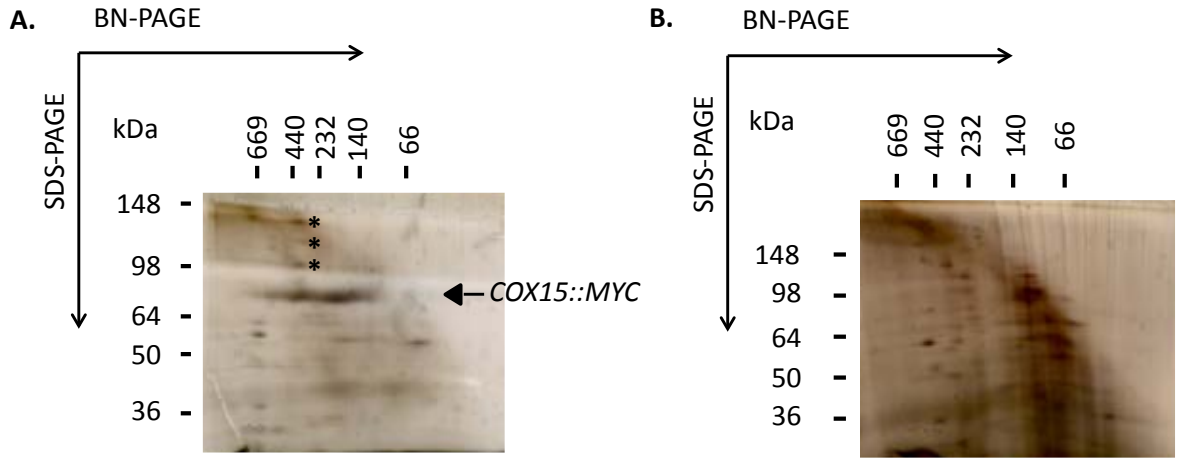

Figure 11: Two-dimensional BN/SDS-PAGE of purified *COX15::MYC*. (A.) Mitochondria were isolated from *S. cerevisiae* containing a genomic copy of *COX15::MYC*. *COX15::MYC* was purified using anti-Myc chromatography and run on a blue native gel. A gel lane containing purified *COX15::MYC* was excised from the native gel and mounted to the top of an SDS-PAGE gel. Following SDS-PAGE, the gel was silver stained to detect the presence of proteins. *COX15::MYC* is denoted by the arrow. Asterisks are used to denote new protein bands that do not appear to be present in the control gel. (B.) Control experiment in which mitochondria were isolated from *S. cerevisiae* with an untagged genomic copy of Cox15. The same purification strategy as in (A.) was used, and the purified fractions were analyzed via 2D BN/SDS-PAGE. Proteins were detected by silver staining. No purified Cox15 was detected.

In addition, the 2D BN/SDS-PAGE gel indicated that there were no other proteins that form in a stoichiometric complex with Cox15. A comparison of the 2D BN/SDS-PAGE gels run of purified *COX15::MYC* and purified untagged mitochondrial extract, indicates that there are not many obvious

differences in the protein bands above and below Cox15 (Figures 11A and B). There are, however, three bands we detected that do not appear to be present in the control gel (Figure 11B). These bands are marked with asterisks in Figure 11A and are present above the Cox15 complex at 440 kDa. All three bands were excised and analyzed via mass spectrometry. Unfortunately, the protein concentration was not high enough to identify the proteins. Regardless of their identity, however, it is important to note that these gels indicate that under these experimental conditions, no other protein is present in the Cox15 complex to the same level as Cox15.

Determine if cytochrome c oxidase assembly factors are part of the Cox15 complex.

Since the 2D BN/SDS-PAGE experiments did not reveal any obvious protein candidate that may associate with Cox15 in complexes, we utilized a different experimental approach to identify potential interaction partners. Because we can only account for approximately 30% of Cox15 interacting with itself, the Cox15 complexes we observe on a blue native gel presumably represent Cox15 interacting with an unidentified protein or proteins. It would seem logical that some of these other proteins may be cytochrome *c* oxidase assembly factors since heme *a* incorporation into Cox1 likely occurs when the other assembly factors are interacting with Cox1. To test this hypothesis, the Cox15 complexes were analyzed in knockouts of cytochrome *c* oxidase assembly factors which are thought to interact with Cox1 around the time of heme insertion: Shy1, Coa1, Coa2, Coa3, Sco1, Cox14, and Mss51. If one or more of these assembly factors are a part of a particular Cox15-containing complex, we would expect that the complex would be unstable in the absence of the assembly factor. Alternatively, if the Cox15-containing complex is stable in the absence of an assembly factor that is part of the complex, we would expect to observe a size shift of the respective band in the absence of that assembly factor. Figure 12 depicts the distribution of the Cox15 complexes in the various knockouts. The majority of the lower Cox15

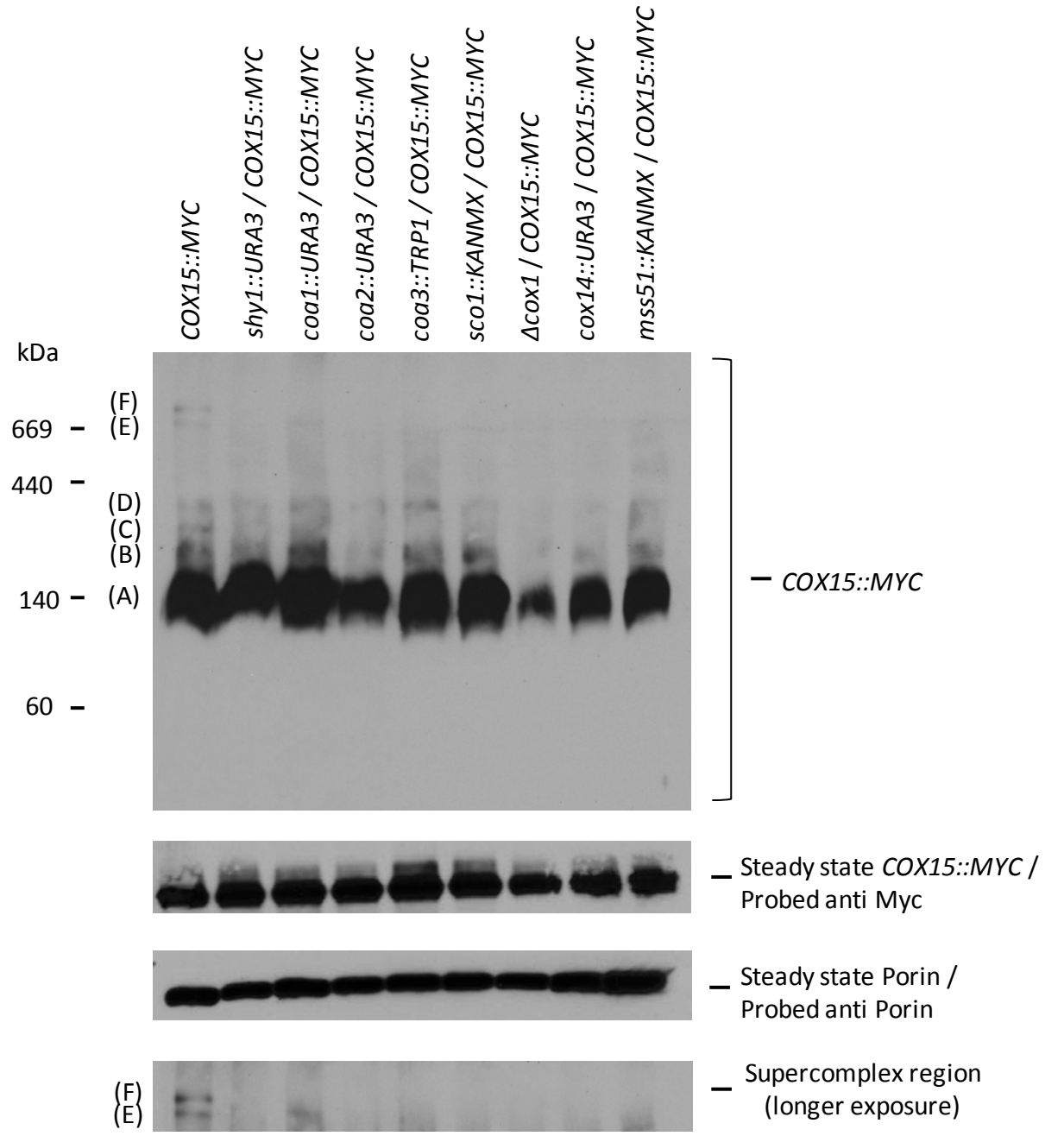

Figure 12: BN-PAGE of Cox15 complexes in wild-type, *Δcox1,* and various cytochrome *c* oxidase assembly factor mutants. BN-PAGE was used to analyze mitochondria isolated from either wild-type *S. cerevisiae* containing genomic *COX15::MYC* or strains containing genomic *COX15::MYC* in the various knockouts/mutants depicted. Steady state protein levels of *COX15::MYC* and porin were also evaluated via SDS-PAGE to ensure proper protein quantification and loading on the BN-PAGE. A longer exposure of the supercomplex region from the BN-PAGE gel is depicted below to highlight the absence of supercomplexes in all of the mutants tested.

complexes remain intact in all of the strains tested, and they are not shifted in molecular weight (Bands A, B, and D in Figure 12). However, Coa2, Coa3, and Cox14 are all small proteins with a molecular weight less than 10 kDa (Table 6). It is likely that the sensitivity of BN-PAGE is not good enough to detect the absence of one of these proteins. Shy1, Coa1, Sco1, and Mss51 all represent assembly proteins with a molecular weight of greater than 20 kDa (Table 6). While it may be possible to detect the absence of one of these larger proteins via BN-PAGE, it is probable that the resolution of these gels still may not be good enough to observe a loss of these proteins from the Cox15 complexes. This is likely to be particularly true for the higher molecular weight Cox15 complexes. On first approximation, Figure 12 suggests that Shy1, Coa1, Sco1, and Mss51 are not integral components of any of the Cox15 complexes. As discussed, however, we cannot make this conclusion with absolute certainty. In spite of this, because the Cox15 complexes representing bands A, B, and D are still apparent on BN-PAGE we *can* conclude that the formation of these Cox15 complexes is not dependent on the presence Shy1, Coa1, Coa2, Coa3, Cox14, or Mss51.

Protein	Molecular Weight (kDa)
Cox1	59
Shy1	43
Coa1	22
Coa2	7.6
Coa3	9.5
Sco1	33
Cox14	7.8
Mss51	48

Table 6: Molecular weights of selected cytochrome *c* oxidase assembly factors (and Cox1). These proteins were deleted in *COX15::MYC S. cerevisiae* to analyze the distribution of the Cox15 complexes.

In addition to analyzing the Cox15 complexes in the absence of the above assembly factors, we investigated if Cox1 was present in any of the Cox15 complexes. Because Cox1 is approximately 60 kDa we might expect to see a shift in size of the Cox15 complex if Cox1 was absent. As observed in Figure 12, a deletion of Cox1 does not cause a shift in any of the Cox15 complexes. While all of the Cox15-containing complexes appear slightly attenuated in the *Δcox1* strain, we believe this represents an artifact of this mitochondria preparation as this has not been observed with other preparations of *Δcox1* mitochondria. Furthermore, the Cox15 complexes do not appear attenuated in the *mss51Δ* strain. It has been demonstrated that *COX1* is not translated when Mss51 is absent, thereby representing a similar condition as the *cox1Δ* strain [9]. Consequently, it appears that the Cox15 complexes are not altered in the absence of Cox1. In addition, because Cox1 is rapidly degraded in all of the other cytochrome *c* oxidase assembly mutants tested [8,11,13,15,16,18,29,30], the persistence of the lower Cox15 complexes in these mutants suggest that the lower Cox15 complexes are not dependent on Cox1, the final destination for heme *a*.

By analyzing the Cox15 complexes in the absence of Cox1 and the various assembly factors discussed above, we can make several inferences regarding the identity of these Cox15 complexes. First, we can conclude that the *formation* of the Cox15 complexes at A, B, and D (Figure 12) are not dependent on any of the cytochrome *c* oxidase assembly factors tested. While we cannot rule out the possibility that these assembly factors are present in the Cox15 complexes for the reasons discussed above, it seems likely that Shy1, Coa1, Sco1, and Mss51 are not integral components of the complexes. We cannot rule out the possibility, however, that Shy1, Coa1, Coa1, Coa3, Sco1, Cox14, and Mss51 are responsible for the formation of Cox15 complex C or that they are part of the complex. In addition, the presence of the Cox15 complexes in these mutants reveals that Cox15 still forms complexes in the absence of Cox1. Finally, these studies have also implicated the presence of Cox15 in respiratory supercomplexes containing complex III and IV. We know that the respiratory supercomplexes do not

form when cytochrome *c* oxidase is unable to assemble. Our observation that the Cox15-containing complexes at ~750 kDa and 1 MDa are absent in Figure 12 is likely because the respiratory complexes are no longer present. While knowing that Cox15 is present in the supercomplexes does not inform us what proteins interact with Cox15 within the supercomplexes, we can now target the proteins known to be part of the supercomplexes as possible interaction partners with Cox15.

Determine if Cox15 and Cyt1 from respiratory complex III interact

As discussed in Chapter 2, mass spectrometry analysis of purified Cox15 identified Cyt1, one of the catalytic subunits of the cytochrome bc_1 complex, as a candidate for interacting with Cox15. Because we hypothesize that Cox15 is present in the respiratory complexes (III_2/IV) and (III_2/IV_2), it logically follows that Cox15 must interact with proteins from either the cytochrome bc_1 complex or cytochrome *c* oxidase. Other than Cyt1, all of the experiments discussed thus far have not implicated any protein from either the cytochrome bc_1 complex or cytochrome *c* oxidase as interacting with Cox15. For these reasons, we chose Cyt1 as our first target to investigate what proteins Cox15 may specifically interact with in the respiratory complexes. To probe for an interaction between Cox15 and Cyt1, co-immunoprecipitation experiments were conducted in a yeast strain containing a genomic copy of C-terminally tagged *COX15::MYC* and a genomic copy of C-terminally tagged *CYT1::HA*. *CYT1::HA* was bound to anti HA resin and, after several washes, the resin was probed for both the presence of *CYT1::HA* and *COX15::MYC*. Figure 13A confirms that *CYT1::HA* was indeed bound to the anti HA resin. When probed with an anti-Myc antibody, western blots of the bound fraction revealed that a small fraction of total *COX15::MYC* was also bound to the HA resin (Figure 13A). *COX15::MYC* was not found to co-immunoprecipitate with the control protein, *SCO1::HA* (Figure 13B). These data provide additional evidence on top of the mass spectrometry experiments that Cox15 may interact with Cyt1 (see Chapter 3, Table 4).

As reflected in Figure 13, only a small but reproducible fraction of Cox15 is bound to Cyt1. If Cox15 only associates with Cyt1 in the respiratory supercomplexes, BN-PAGE data would indicate that only a small amount of Cox15 interacts with Cyt1. As depicted by the distribution of the Cox15 complexes on BN-PAGE (Figure 12), Cox15 is predominantly present in the lower molecular weight complexes and only a small fraction of Cox15 is present in the respiratory supercomplexes. While it is likely that Cox15 is bound to Cyt1 only in the respiratory supercomplexes III_2/IV_2 and III_2/IV, experiments will have to be conducted to prove definitively that this is where the interaction occurs.

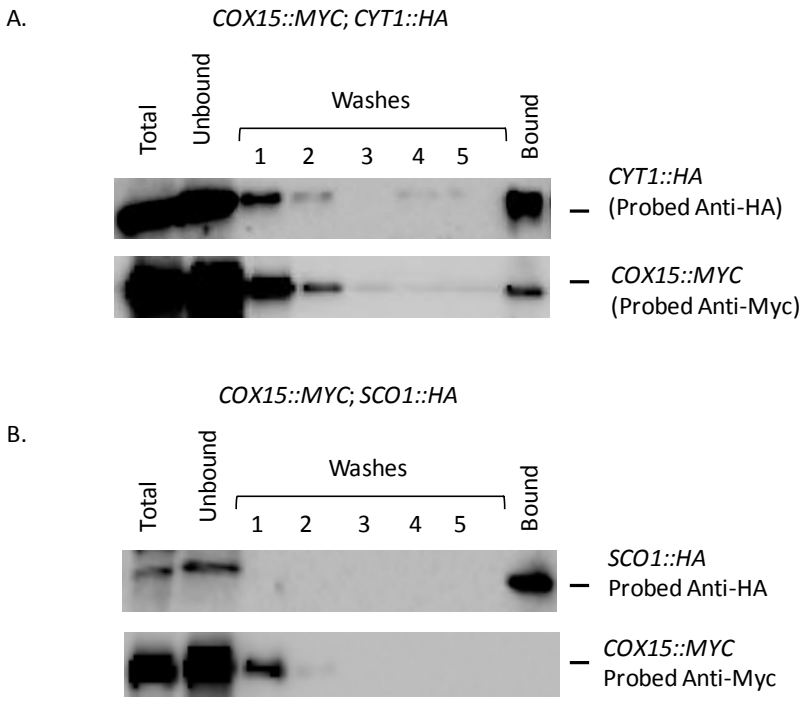

Figure 13: Co-immunoprecipitation of Cyt1 with Cox15. (A.) Mitochondria from *S. cerevisiae* expressing both genomically tagged *COX15::MYC* and *CYT1::HA* were isolated and solubilized. *CYT1::HA* was bound to anti-HA resin and western blots were run to analyze the bound material for the presence of *CYT1::HA* and *COX15::MYC*. (B.) Mitochondria from *S. cerevisiae* expressing both genomically tagged *COX15::MYC* and *SCO1::HA* were isolated and solubilized. *CYT1::HA* was bound to anti-HA resin and western blots were run to analyze the bound material for the presence of *CYT1::HA* and *COX15::MYC*.

Discussion

The work presented in this chapter indicates that some of the Cox15 complexes observed on a blue native gel represent homo-oligomers of Cox15. It does not appear that all of the Cox15 present in the mitochondria is present in homo-oligomers, however, because we can only account for about 30% of Cox15 interacting with itself. This begs the question as to where is the rest of Cox15? The answer to that question depends partially on whether Cox15 exists as a monomer in the mitochondria and if that monomeric species is represented by the Cox15 complex at 120 kDa on the blue native gel. Monomeric *COX15::MYC* is only 75 kDa, so an approximation of 120 kDa on a blue native may not indicate that this is monomeric Cox15. As discussed in Chapter 2, however, the molecular weights estimated by BN-PAGE often result in an overestimation due to the detergent used for solubilization [31], so it is not unreasonable to hypothesize that this band represents monomeric *COX15::MYC*. Because the band at 120 kDa represents the majority of the Cox15 detected on BN-PAGE, if this band is monomeric Cox15, then it would suggest that the majority of Cox15 exists alone and not in association with any other protein. It would then follow that some of the lower complexes (bands B-D on the blue native gel) could represent homo-oligomeric species. If the band at 120 kDa does *not* represent monomeric Cox15, however, it seems unlikely that this band represents strictly homo-oligomeric Cox15 since this band accounts for far more than 30% of the total Cox15 represented on the gel.

In an effort to identify other proteins that associate with *COX15::MYC,* we utilized 2D BN/SDS-PAGE, but if other proteins do associate with Cox15, we were not able to observe them. This may indicate that the Cox15 complexes largely represent homo-oligomeric species. Alternatively, it can also indicate that under our experimental conditions, Cox15 is artificially enriched, masking the presence of other associating proteins. As discussed above, we can only account for about ~30% of Cox15 interacting with itself, suggesting that the Cox15 complexes do not primarily represent homo-oligomeric complexes.

Blue native gels of the Cox15 complexes in *S. cerevisiae* strains in which some of the assembly proteins are deleted that either interact with Cox1 during its early stages of assembly or are involved in co-factor insertion, suggest that these proteins are not a part of the Cox15 complexes represented by bands A, B, and D. It is important to remember the caveats discussed above when drawing this conclusion, however. We also cannot rule out the possibility that some of these assembly factors associate with Cox15 in the complex represented by band C on the BN-PAGE gel. In addition, it is also important to consider the report by Bareth et al. (2003) that Cox15 is present in sub-stoichiometric amounts in some of the complexes that form with the early assembly factors of Cox1 [32]. Specifically, Bareth and coworkers report that Cox15 co-immunoprecipitates with both Coa1 and Coa3. Significantly, this interaction with Cox15 was not detected when Cox15 had a C-terminal tag; Cox15 is only found to associate with Coa1 and Coa3 if Cox15 was untagged. This important observation may explain why we have been unable to detect an interaction of Cox15 with cytochrome *c* oxidase assembly factors in our mass spectral studies reported in Chapter 3. Perhaps even more intriguing was their report that Cox15 is present with Shy1 in two distinct protein complexes around 170 kDa and 220 kDa. The authors suggest that these complexes represent hetero-oligomeric complexes only containing Shy1 and Cox15 since they were unable to detect any other proteins within these complexes. Notably, in these studies the Shy1 and Cox15 interaction was not compromised with C-terminally tagged Cox15.

It is important to discuss the implications the study performed by Bareth and co-workers has on our understanding of the distribution of *COX15::MYC* on BN-PAGE. Based on their work it seems reasonable to conclude that Cox15 is present, at least to a small extent, in some of the early sub-assembly complexes that form with Cox1. To explain this observation in light of our data, we hypothesize that the C-terminal tag on Cox15 still allows protein-protein interactions between Cox15 and these assembly proteins during BN-PAGE. The tag may create enough instability, however, that the association of Cox15 with these proteins is abolished during the purification of Cox15. This would

explain why we did not detect any of these assembly proteins in our mass spectral studies of purified Cox15 (Chapter 3). If the Cox15-containing bands (A-D) on our BN-PAGE gels represent an interaction of Cox15 with these assembly proteins, then we must conclude that BN-PAGE does not provide the resolution necessary to detect size shifts in the absence of any one of these proteins. Alternatively, it is attractive to assign band C to representing Cox15 in association with some of the cytochrome *c* oxidase assembly proteins (since it is missing in these knockouts) and bands B and D to representing homo-oligomeric Cox15. Since Bareth et al. report that the association of Cox15 with assembly proteins is sub-stoichiometric, it is likely that the abundant band A on our BN-PAGE gels does not represent an association of Cox15 with these cytochrome *c* oxidase assembly proteins. While it might be tempting to assign Shy1 to band A, this would not make sense in light of our findings that this band is still present when Shy1 is knocked out**.** In light of all of the data, the most parsimonious explanation is that this band represents monomeric Cox15.

It may be worth exploring an interaction of Cox15 with the mitochondrial heat shock protein, Ssc1 and its co-chaperone (Mdj1). These proteins were identified as potential Cox15 interaction partners in Chapter 3. If these proteins do interact with Cox15, they may interact as part of the proposed interaction of Cox15 with early Cox1 sub-assembly complexes [32]. As discussed in Chapter 3, Ssc1 has been implicated in forming a high mass protein complex with newly translated Cox1 as well as the cytochrome *c* oxidase assembly factors, Mss51, Cox14, and Coa3 [8,9,18]. Because of this relationship, it will be important to assess the Cox15 complexes in deletion strains of Ssc1 and Mdj1.

Finally, BN-PAGE of *COX15::MYC* has indicated that Cox15 is present in the respiratory supercomplexes. While it may seem unlikely that a protein involved in the assembly of cytochrome *c* oxidase would associate with proteins from the cytochrome bc_1 complex in respiratory supercomplexes, a previous report by Mick and coworkers (2007) indicated that this occurs with the assembly proteins Shy1 and Cox14 [16]. In addition, this study reports that Shy1 interacts with both Rip1 and Cor1 of the

cytochrome bc_1 complex as well as proteins from cytochrome *c* oxidase. Interestingly, Mick et al. also reported that they detected Shy1, Cox14, Cyt1, and Cox4 in respiratory supercomplexes even in the absence of Cox2. They did not, however, detect Shy1, Cox14, or Cyt1 in respiratory supercomplexes in the absence of Cox4. This is significant because Cox4 is likely the first nuclear subunit to assemble with Cox1. If Cox4 does not associate with Cox1, cytochrome *c* oxidase fails to assemble. Cox2, on the other hand, is thought to assemble downstream of the Cox1-Cox4-Cox5a subassembly. In the absence of Cox2, the Cox1-Cox4-Cox5a subassembly is stable. Because Shy1 and Cox14 are found in respiratory supercomplexes with Cyt1 and Cox4 when Cox2 is absent, this indicates that supercomplexes may form with partially assembled cytochrome *c* oxidase. Because of the precedent that Shy1 and Cox14 are present in both fully formed respiratory supercomplexes and supercomplexes containing partially assembled cytochrome *c* oxidase, the authors of this study hypothesize that Shy1 and Cox14 are present in the supercomplexes to assist with the incorporation of later subunits into the Cox1-Cox4-Cox5a subassembly [16]. Whether this is the reason Shy1 and Cox14 are present in the supercomplexes remains to be determined, but it does suggest that it is not unreasonable to conjecture that Cox15 is also present in these supercomplexes.

Thus far the only protein we have identified as potentially interacting with Cox15 in respiratory supercomplexes is Cyt1. If Cox15 is present in the supercomplexes, it should also interact (at least indirectly) with components of cytochrome *c* oxidase as well as other proteins of the cytochrome bc_1 complex. Mass spectrometry studies of purified Cox15 have not detected any proteins from these respiratory complexes co-purifying with Cox15. In addition, the BN-PAGE data of the Cox15 complexes in various knockouts of cytochrome *c* oxidase assembly factors suggests that Cox1 is not a part of the Cox15 complexes. This may highlight the difficulty of conducting studies such as these with membrane proteins. Perhaps the solubilization and purification procedures used for mass spectrometry analysis does not allow some of these interactions to persist. Because of this, future experiments will be needed

to probe for other interactions of Cox15 with various subunits of both the cytochrome bc_1 complex and cytochrome *c* oxidase. Co-immunoprecipitation experiments will provide one approach to accomplish this. In addition, it will be important to investigate interactions of Cyt1 with Cox15 in Δ*cox4* and *Δcox13 S. cerevisiae* strains. (In *Δcox13* mitochondria, much of the cytochrome *c* oxidase holo-enzyme is able to assemble. This is contrary to Δ*cox4* mitochondria in which no intermediates of the holo-enzyme assemble). If the Cyt1-Cox15 interaction persists when Cox13 is absent, but does not persist when Cox4 is absent, this will indicate that Cox15 follows the same trend as observed by Mick et al. [16] for both Shy1 and Cox14.

Ultimately, it will be necessary to determine why cytochrome *c* oxidase assembly factors such as Shy1, Cox14, and Cox15 are present in the respiratory supercomplexes and if other assembly proteins are also a part of the supercomplexes. Perhaps the presence of assembly proteins within supercomplexes indicates that there are two pools of cytochrome *c* oxidase being assembled in the inner membrane of the mitochondria. One could hypothesize that assembly factors associate with Cox1 destined to form monomeric cytochrome *c* oxidase and that they also associate with a second Cox1 pool that will soon become incorporated into supercomplexes before the entire holo-enzyme is formed. These intriguing ideas and their consequences for aerobic respiration will certainly lead to exciting studies.

Experimental Procedures

Co-purification of COX15::HIS and COX15::MYC via Ni-NTA chromatography

Protein purification

Two liters of *S. cerevisae* containing a genomic copy of C-terminal tagged *COX15::MYC* containing either the empty yeast expression vector *pRS426* or *pRS426* containing *COX15::HIS* were utilized for these experiments. Cell growth conditions and the generation of the *COX15::MYC* and *COX15::HIS* constructs are described in Chapter 2. Mitochondria were isolated as outlined in the experimental section of Chapter 2 and all of the mitochondria isolated from the 2 L of culture were solubilized for two hours in 600 mM sorbitol, 20 mM HEPES, 500 mM NaCl, 1% Triton X-100, and Roche protease inhibitor in a 5 mL total volume. Ni-NTA resin (500 µL) was washed with 6 mL of 25 mM Tris (pH 7.5), 10% glycerol, 500 mM NaCl, 20 mM imidazole, and 0.5% Triton X-100. Solubilized protein lysate was incubated with the Ni-NTA resin for two hours in the presence of 500 mM NaCl, 1% Triton X-100, Roche protease inhibitor, 20 mM imidazole, and 5% glycerol. Following incubation of protein lysate with resin, 4 mL of the washing buffer from above was used to wash the column. This was followed by a 2 mL wash of the same buffer containing 35 mM imidazole and a 2-mL wash of this buffer containing 50 mM imidazole. The protein was eluted from the column using this same buffer containing 100 mM imidazole in five 500-µL fractions.

To verify that purification of *COX15::HIS* was successful and ascertain if *COX15::MYC* co-purified, western blotting was used to monitor the distribution of these proteins in the unbound and elution fractions. SDS-PAGE gels (10% acrylamide) were used for electrophoresis, and proteins were blotted to PVDF membranes. For protein loading, a total of 10 µL was loaded on each gel. This resulted in a loading of approximately 0.2% of the flow through fraction and 2% of each of the elution fractions.

Mass balance analysis

ImageQuant (5.2) software was utilized to estimate the amount of protein recovered in the elution and unbound fractions compared to total protein. For background subtraction, the local average pixel intensity was calculated by placing the same rectangle used to estimate band pixel intensity on a portion of the blot that did not contain signal from protein.

Co-purification of COX15::HIS and COX15::MYC via anti c-Myc chromatography

Protein purification

As in the previous experiment, 2-L of culture of *S. cerevisae* containing a genomic copy of C-terminal tagged *COX15::MYC* containing either the empty yeast expression vector *pRS426* or *pRS426* containing *COX15::HIS* were utilized for these experiments. Cell growth, mitochondrial isolation, and solubilization were carried out as above for the Ni-NTA purification of *COX15::HIS* (600 mM sorbitol, 20 mM HEPES, 500 mM NaCl, 1% Triton X-100, and Roche protease inhibitor in a total volume of 5 mL). Anti c-Myc resin (Sigma) was prepared by washing 300 µL of the resin with three washes each of 5 mL of 0.1 M NH_4OH (pH 11-12), 0.1% Triton X-100, and 500 mM NaCl. This was followed by three washes each of 5 mL phosphate buffered saline (pH = 7.4) (PBS). Protein lysate (5 mL) was incubated with column resin overnight at 4 °C on a rocking platform. Following collection of the unbound fraction, the column was washed eight times with 1 mL PBS, and purified protein was eluted in ten 1-mL fractions of 0.1M NH_4OH, 0.5% Triton X-100, and 500 mM NaCl into vials that contained 30 µL of 1 M acetic acid. SDS-PAGE and western blotting were performed as above. The protein loading on the gel was also the same as the experiment above.

Co-immunoprecipitation of COX15::HIS and COX15::MYC

Anti c-Myc resin (100 µL) was washed five times with 1 mL of PBS and 200 µL of protein lysate prepared in the co-purification experiments described above was added to washed resin. Resin was incubated for 1.5 hours at 4 ^{o}C on a rocking platform. The unbound fraction was removed and the resin was washed 4X with 1 mL of PBS. After the final wash, 10 µL of PBS was left above the resin and 50 µL of 2X SDS-PAGE buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) was incubated with the resin for 5 minutes at 95 ^{o}C. SDS-PAGE and western blotting was performed as described above.

Co-immunoprecipitation of CYT1::HA and COX15::MYC

Mitochondria from 100 mL of culture of *S. cerevisiae* expressing genomic copies of *CYT1::HA* and *COX15::MYC* were prepared as described in Chapter 2. Cultures were grown in YPD from a 5-mL overnight culture. Mitochondria were solubilized in 4.1% digitonin, 150 mM NaCl, and Roche protease inhibitor in a total volume of 200 µL for two hours at 4 ^{o}C on a rocking platform. Solubilized mitochondria were spun for 30 minutes at 12,000 *x g* to remove unsolubilized material, and the resulting supernatant was added to 50 µL of Pierce Anti-HA resin. Resin and 200 µL of mitochondrial lysate were incubated overnight at 4 ^{o}C on a rocking platform, washed five times with 500 µL Tris Buffered Saline (50 mM Tris-Cl (pH=7.5), 150 mM NaCl) with 1% Tween-20 (TBST) and protein was eluted with 50 µL of 2X SDS-PAGE loading dye. SDS-PAGE and western blotting were performed as described above. For protein loading, 10 µL of each sample was run on the gel resulting in 0.05% of the unbound fraction and 80% of each eluate.

Blue native of COX15::MYC in cytochrome c *oxidase assembly factor mutants*

Purified mitochondria containing genomic *COX15::MYC* in *S. cerevisae* containing deletions of *shy1, coa1, coa2, coa3, sco1, mss51, cox14, and cox1* were isolated as described previously (Chapter 2). All assembly factor proteins except for *coa3* were knocked out by Behzad Khodaverdian using homologous recombination of KanMX or *Candida albicans URA3* into the respective locus. The *Δcox1* strain was also prepared by Behzad Khodaverdian as performed in [33,34]. The pYGT21 plasmid containing wild-type intronless Cox1 was obtained from J. Lazowska, CNRS, Gif-sur-Yvette, France. Mutagenesis of Cox1 in the pYGT21 plasmid was performed using the QuikChange site-directed mutagenesis kit (Strategene) and the plasmid was transformed into a rho^{o} strain kindly provided by Thomas Fox. To knock out Coa3 via homologous recombination of the *TRP1* cassette from pBS1479, the primers used were 5'-ATACTACGTGAGCAGCAACGAAAGCACATATATAGACGACAAAGTAGTGGAACGATCAT TCAC-3' and 5'-GCGCAAAGCCTATTGATGGAAGACCACAGCGTACCTCCACATTAACGGTCTTTATGTTTGATACA TGATTG-3'. BN-PAGE was performed as described in Chapter 2.

Two-dimensional blue native/SDS-PAGE of COX15::MYC

2-Dimensional BN/SDS-PAGE was performed exactly as described in Chapter 2. As discussed earlier, the silver stained 2D gel revealed that Cox15 associated in two distinct areas. Cox15 was most enriched around 440 kDa and also in a broader range between ~140-250 kDa. Both the band at 440 kDa and the broad band from 140-250 kDa were excised from the silver stained 2D gel and analyzed by mass spectrometry (LC-MS/MS) following an in gel tryptic digest. Mass spectrometry and the tryptic digest were performed as described for the mass spectrometry of purified *COX15::MYC* in Chapter 3.

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Chapter 5:

Cytosolic chaperones, Hsc82 and Ssa1, interact with Cox15 but are not part of the Cox15 complexes

Introduction: protein import into the mitochondria

Mitochondria are essential components of the cell. While they may be most notable for their role as the energy producers of the cell, we are now beginning to understand the many roles mitochondria play within the cell. We now know that mitochondria are also essential in such processes as signaling, cell cycle progression, and apoptosis [2]. Mitochondria are also involved in the biosynthesis of amino acids, lipids, iron-sulfur centers, and heme [3-5]. In order to execute the functions listed above, mitochondria contain 15-20% of the total proteins within the cell [6]. Mitochondria are unique organelles of the cell in that the proteins they contain are from dual genetic origin. While most of the proteins within this organelle are encoded by the nucleus and transported into the mitochondria, mitochondria also contain their own genomes which encode for about 1% of total mitochondrial proteins [1]. These mitochondrial-encoded proteins form some of the most critical components of the respiratory chain. The majority of mitochondrial proteins, however, are encoded by the nucleus. In fact, it is estimated that the mitochondria must import several hundred polypeptides [7].

Much research over the last 50 years has been dedicated to understanding how mitochondria import nuclear encoded proteins. The paradigm is that proteins destined for the mitochondria contain mitochondrial targeting sequences. These sequences often exist at the N-terminus of the protein and are generally cleaved following import. Some mitochondrial proteins, however, particularly hydrophobic proteins, contain internal targeting sequences that often occur prior to the hydrophobic residues within the protein [1].

The protein machinery that is involved in translocating mitochondria-bound proteins is generally referred to as the "TIM/TOM" complexes. The TOM complex is a multi-component complex spanning the outer mitochondrial membrane and is involved in importing proteins into the mitochondrial intermembrane space (IMS). Its largest components are the protein translocating pore, Tom40, and three receptor proteins, Tom20, Tom22, and Tom70 (Figure 14). Tom20 is involved in recognizing proteins with the classical N-terminal targeting sequence while Tom70 is often involved in recognizing proteins that contain internal targeting sequences [1]. Tom22 is capable of recognizing both N-terminal presequences and internal targeting sequences [8]. Two separate TIM complexes (Tim22 and Tim23) span the inner mitochondrial membrane and are involved in import of proteins either into the matrix or the inner membrane. Proteins that are recognized by Tom20 (and contain the classical N-terminal targeting sequence) are delivered to the Tim23 complex for transport into the matrix while proteins that are recognized by Tom70 (and often contain the internal targeting signals) are delivered to the Tim22 complex for lateral transport into the inner mitochondrial membrane. Proteins targeted through the Tom70/Tim22 pathway often utilize the small intermembrane space TIM proteins as chaperones (Figure 14).

Proteins destined to the mitochondrial outer membrane belong to one of two classes: β-barrel proteins or proteins that contain α-helical transmembrane segments [1]. While little is known how the α-helical proteins are inserted into the outer membrane, considerable progress has been made in our understanding of how the β-barrel proteins are inserted. The β-barrel proteins are first imported into the membrane through interactions with the TOM complex and the small TIM proteins located in the IMS then act as chaperones for the β-barrel proteins. Finally, the sorting and assembly machinery (SAM complex) is responsible for insertion of the β-barrel proteins into the outer membrane (Figure 14). Proteins destined for the IMS are imported via the TOM complex and are modified by the MIA machinery so that two or more disulfide bonds are inserted (Figure 14). This prevents the IMS proteins

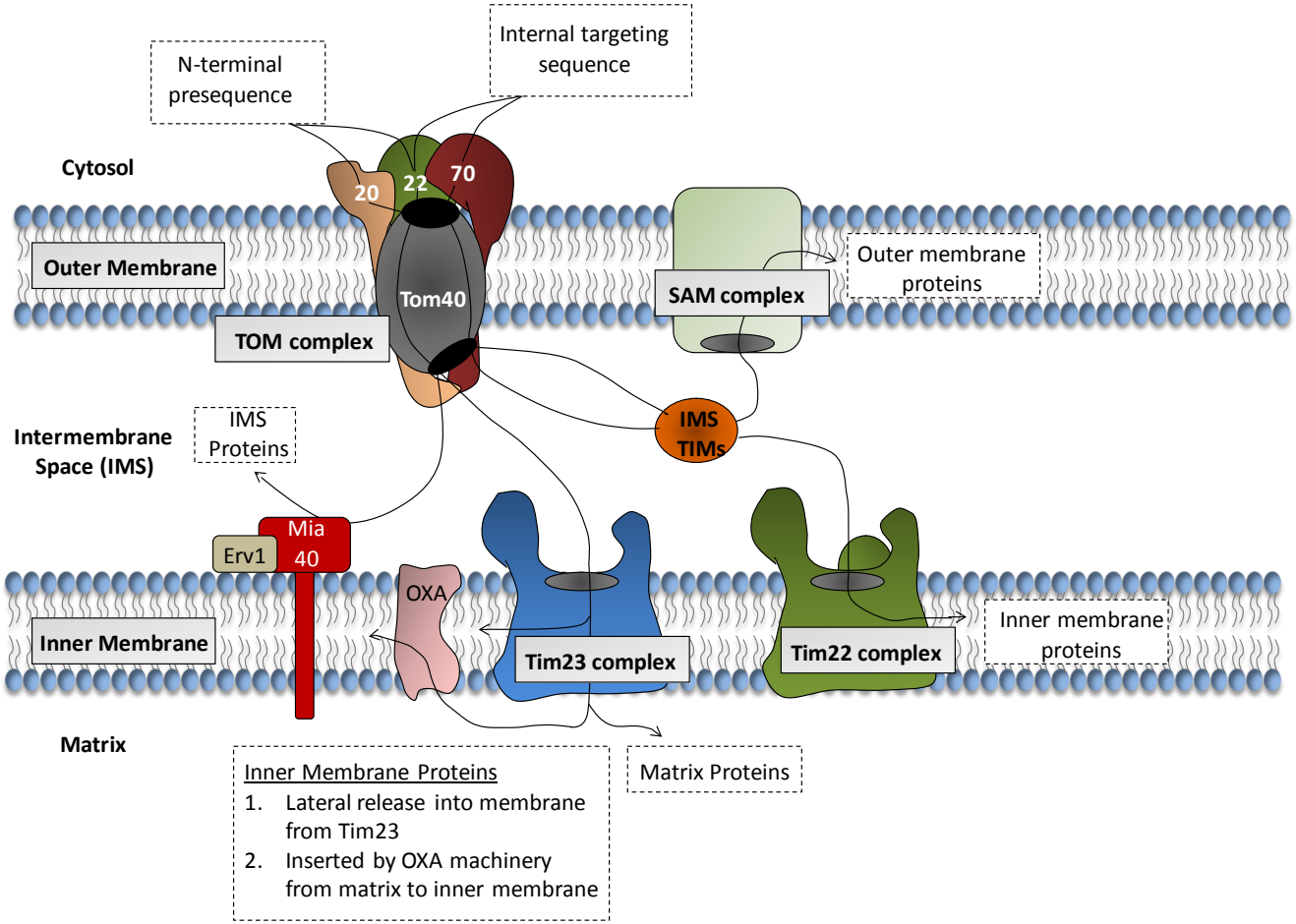

Figure 14: Protein import into the mitochondria. The TOM complex is responsible for importing proteins into the mitochondria. It has three major receptors, Tom20, Tom22, and Tom70. Tom20 recognizes proteins with N-terminal cleavable presequences while Tom70 recognizes proteins with internal targeting sequences. Tom22 is capable of recognizing both types of presequences. Proteins destined for the matrix or inner membrane utilize the TIM complexes. Tim23 is the target for proteins imported via Tom20/Tom22 while the Tim22 complex is the target for proteins imported via Tom70. Inner membrane bound proteins passing through the Tim23 complex can either be laterally released into the membrane or enter the matrix and are inserted by the OXA machinery. IMS proteins utilize the Mia40 machinery while β-barrel proteins destined for the outer membrane make use of the SAM complex. See text for more detail. Figure adapted from [1].

from translocating back into the cytosol. Like the matrix and inner membrane proteins, outer membrane and IMS proteins may either utilize the classic N-terminal presequences or internal targeting sequences for mitochondrial uptake [1].

Previously it was generally assumed that protein import into the mitochondria occurs following translation on cytoplasmic ribosomes and specific targeting to the mitochondria by targeting sequences [9-11]. Since the 1970's, however, evidence has been accumulating that suggests that untranslated mRNAs may localize to the mitochondria prior to translation [11-16]. In fact, some studies even suggest that protein import can occur co-translationally for a certain subset of mitochondrial proteins [17-19]. This idea first began with the observation that certain cytoplasmic ribosomes are bound to the mitochondria [16,20,21]. More recently, it was demonstrated by Garcia and coworkers (2010) that *ATP2* mRNA from *Saccharomyces cerevisiae* localized to the mitochondria both through its N-terminal mitochondrial targeting sequence (MTS) and through two distinct regions in the open reading frame region of its mRNA [22]. By fusing various pieces of the *ATP2* mRNA to the *LacZ* reporter gene and visualizing the localization of the *LacZ* mRNA using fluorescent in situ hybridization, Garcia et al. demonstrated the importance of the *ATP2* mRNA for mitochondrial localization. Unexpectedly, if they replaced the *ATP2* MTS with a MTS from a non-mitochondrial associating mRNA, the *ATP2-LacZ* construct still localized to the mitochondria. This mitochondrial localization was traced to the two distinct regions in the *ATP2* open reading frame-localized mRNA mentioned above. The authors from this study suggested that mRNA contains messages for its localization to various cellular compartments as well as information required for protein synthesis [22].

Not only was *ATP2* mRNA shown to localize to the mitochondria but additional studies also revealed that numerous other mitochondrial mRNAs also localized to the mitochondria. As the identity of the mRNAs that localized to the mitochondria increased, it became apparent that mitochondrial localizing mRNAs encode certain types of proteins. Studies using DNA microarrays and fluorescent in

situ hybridization estimated that approximately 50% of mRNAs encoding mitochondrial proteins localized to the outside of the mitochondria [19,23]. In addition, the distribution of 112 nuclear encoded mRNAs involved in seven different mitochondrial complexes was studied by quantitative real time PCR of purified mitochondria-bound polysomes [19]. The mRNAs encoding for the subunit proteins of ATP synthase (Atp1, 2, 3, 4, 5, and 10*)* and the cytochrome bc_1 complex (Cor1, Cor2, Rip1, and Cyt1) were found to be translated in the vicinity of the mitochondria. Intriguingly, the mRNA of the assembly factors for ATP synthase and the cytochrome bc_1complex were found to be enriched in cytoplasmic polysome fractions. Cytochrome *c* oxidase is unique among the respiratory complexes in that none of the mRNAs encoding for its nuclear encoded subunits were translated on mitochondria-bound ribosomes, but were found primarily in the cytoplasmic fractions. Conversely, the cytochrome *c* oxidase assembly factors, Shy1, Sco1, Oxa1, Cox10, Cox15, and Cox11 were all translated in the vicinity of the mitochondria [19].

While it has become apparent that some mRNAs of mitochondrial proteins localize to the mitochondria, the question remains: what factors mediate an association of mRNA with the mitochondria? It is thought that mRNA binding proteins such as those belonging to the Pumilio-Fem-3 binding factor (Puf3) bind to *cis* elements in the 3'-UTR of approximately half of the mitochondrial localizing mRNAs [18,24-26]**.** A computational study conducted by Anderson et al. [25] suggested that a specific mRNA sequence (CYUGUAAAUA) in the 3'-UTR was necessary for mRNA mitochondrial localization, and Gerber and coworkers further demonstrated that this mRNA motif was recognized by Puf3 proteins [24]. While Puf3 proteins appear to play a role in the localization of selected mRNAs, it has been demonstrated that other mRNAs are directed to the mitochondria by other means. For instance, mRNAs for both mitochondrial ABC transporter (Atm1) and the β subunit of ATP synthase (Atp2) exclusively locate to the mitochondrial membrane, but they do so in a Puf-independent manner [23,27]. In addition, the findings of Garcia et al. discussed above provides evidence that *ATP2* mRNA

localizes to the mitochondria (at least in part) as a result of specific sequences in its open reading frame-localized mRNA [22]. Thus, it is becoming increasingly clear that other mRNA binding proteins (or other yet to be identified factors) must also be involved in mediating mRNA localization to the mitochondria.

One such factor is the cytosolic heat shock protein belonging to the hsp70 family, Ssa1. Evidence for this arose when genetic screens of yeast mutants detected that mutations in Ssa1 impaired protein import into both the mitochondria and the endoplasmic reticulum [28,29]. When the concentration of Ssa1 was depleted in yeast, it was noted that a build-up of unprocessed ATP2 occurred, indicating that this protein was not imported into the mitochondria [28]. In 2003, Young et al. established that both yeast heat shock protein 70 (Hsp70) and human Hsp70 mediates protein import into the mitochondria through an interaction with the Tom70 receptor [30]. This was demonstrated for the ADP/ATP carrier protein and the mitochondrial peptide transporter, both of which are known to be imported in a Tom70 dependent manner. When they examined the import of two Tom20 mediated proteins, the Rieske iron-sulfur protein (ISP) and the matrix processing peptidase α subunit (Mppα), they found that the mitochondrial import of these proteins was not affected by inhibitors of Hsp70 or Tom70. These studies revealed that for the subset of mitochondrial proteins that are imported via the Tom70 receptor, import is likely mediated by Hsp70/Tom70 interactions. Those proteins that are imported through the Tom20 receptor, however, do not appear to utilize Hsp70.

Finally, a study conducted by Eliyahu and coworkers in 2012 [31] provided further evidence that Ssa1 is involved in mediating mRNA association with the mitochondria. Using microarray analysis to monitor mRNA expression changes in yeast containing a temperature sensitive mutant of Ssa1, they observed that when Ssa1 levels were diminished there was a concomitant reduction in mRNA localization to the mitochondria. Using northern blotting, the mitochondrial mRNA association of several mitochondrial genes, including Atp2, was found to decrease 2-3 times upon Ssa1 depletion. Likewise, they observed an increase in mitochondrial mRNA association of these genes when Ssa1 was

overexpressed. This increase in mitochondrial association was not observed, however, when Tom70 was absent, thus supporting the notion that Hsp70 and Tom70 work together. Finally, the authors proposed that Ssa1 does not mediate mitochondrial mRNA association through direct interactions with mRNA. They compared various characteristics of the mRNA sequences that were affected by Ssa1 with the mRNA sequences that showed no Ssa1 effect in localization. The only factor that seemed to be specific to mRNA exhibiting SSA1-dependent localization was the hydrophobic nature of the translated protein. Thus, the authors concluded that Ssa1 binds to newly translated protein. While it is yet to be resolved how Ssa1 mediates mRNA localization to the mitochondria, it is clear that Ssa1 somehow facilitates the association of some mitochondria-bound mRNAs with the mitochondria, and it does so in a Tom70-dependent manner.

Putative interaction of Cox15 with the cytosolic chaperones, heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90)

In Chapter 2 it was reported that the Hsp70 protein, Ssa1, was the strongest potential interaction partner with Cox15 observed via mass spectrometry. As previously mentioned, Garcia and coworkers reported that translation of *COX15* mRNA appears to occur in the vicinity of the mitochondria [19]. Furthermore, Eliyahu et al. found that Ssa1 mediates mRNA localization to the mitochondria through binding to the target protein: not the mRNA [31]. Due to the accumulation of evidence suggesting that *COX15* mRNA localizes to the mitochondria and that Ssa1 may be involved in this process through interaction with the nascent Cox15 protein, we sought to verify that Ssa1 interacts with Cox15.

In addition to Ssa1, mass spectrometry experiments identified Hsc82, a member of the Hsp90 family, as interacting with Cox15. While Hsp90 proteins have been implicated in playing a similar role as Ssa1 in protein import into the mitochondria in mammalian cells, it is less clear what role Hsp90 (Hsc82) plays in yeast [30]. Currently Hsc82 is thought to act as a cytosolic chaperone to prevent hydrophobic

inner membrane proteins from aggregating in the cytosol, perhaps ensuring that mitochondrial targeted proteins are properly delivered to Ssa1.

We hypothesized that the putative interactions we detected via mass spectrometry between Cox15 and both Ssa1 and Hsc82 relate to the mitochondrial import machinery discussed above. Assuming that Cox15 does interact with Ssa1 and Hsc82, this may lead to future studies to determine if Cox15 utilizes a Ssa1/Tom70 dependent pathway into the mitochondria. This chapter will describe the work we have completed to verify that Cox15 interacts with the cytosolic chaperones, Ssa1 and Hsc82.

Results

Co-immunoprecipitation of Ssa1 and Hsc82 with Cox15

To provide additional evidence to the mass spectrometry experiments reported in Chapter 2, co-immunoprecipitation experiments were conducted probing for an interaction of Cox15 with either Hsc82 or Ssa1. First, *COX15::MYC* was bound to anti c-Myc resin, and co-immunoprecipitation of Hsc82 and Ssa1 was verified using antibodies directed against these proteins. As a control, the co-immunoprecipitation of *COX15::MYC* and GAPDH was also monitored. These experiments confirmed that Hsc82 and Ssa1 co-immunoprecipitate to some extent with Cox15 while no GAPDH was detected interacting with Cox15 (Figure 15A). As depicted in Figure 15A, only a very small amount of Ssa1 was found to co-immunoprecipitate. The relevance of this will be discussed below.

These co-immunoprecipitation experiments were also conducted in the opposite direction. Hemmagglutinin (HA)-tagged Ssa1 and Hsc82 were each expressed in *S. cerevisiae* expressing *COX15::MYC*. The HA-tagged proteins were bound to anti-HA resin, washed, and analyzed for the presence of *COX15::MYC. COX15::MYC* was bound to both *SSA1::HA* and *HSC82::HA* (Figure 15B). As a control, Ssb1, a cytoplasmic heat shock protein belonging to the Hsp70 family was also HA-tagged and tested for co-immunoprecipitation with *COX15::MYC*. Unlike Ssa1, which seems to associate with the

mitochondria, Ssb1 has been shown to be associated with cytoplasmic ribosomes. Figure 15B demonstrates that *COX15::MYC* does *not* associate with *SSB1::HA*. It is important to note that for both co-immunoprecipitation experiments depicted in Figure 15A and 15B, all proteins were expressed genomically to avoid artifacts from overexpression. In addition, while the co-immunoprecipitation of native Ssa1 was very minimal with purified *COX15::MYC* in Figure 15A, the co-immunoprecipitation of *COX15::MYC* with purified *SSA1::HA* in Figure 15B appears more significant. As a result, it seems reasonable to conclude that co-immunoprecipitation experiments do confirm that Ssa1 and Hsc82 interact with Cox15. Finally, it is not expected that much of the total cellular Hsc82 or Ssa1 would be bound to Cox15 as these proteins are known to interact with a myriad of other proteins in addition to Cox15. If Hsc82 and Ssa1 play a role in importing Cox15 into the mitochondria, it is likely that an interaction between Cox15 and these proteins is very transient.

In sum, co-immunoprecipitation experiments confirm what was observed by mass spectrometry experiments – Cox15 associates with Ssa1 and Hsc82.

Determine whether Ssa1 and Hsc82 are part of the Cox15 complexes

While it is likely that Ssa1 and Hsc82 are involved in the import of Cox15 into the mitochondria, we also wanted to ascertain if these proteins are part of any of the Cox15 complexes observed by BN-PAGE. To accomplish this, knockouts of Ssa1 and Hsc82 were generated and the Cox15 complexes that formed were evaluated by BN-PAGE. A GAPDH knockout was used as the control. While the Cox15 complex appears to be a bit attenuated in both the *hsc82::TRP1* and *tdh1::TRP1* (GAPDH knockout) strains, all of the complexes are present and an overexposure of the film reveals no differences between the complexes in any of the strains (Figure 14C). Overexposure of the film also reveals the presence of

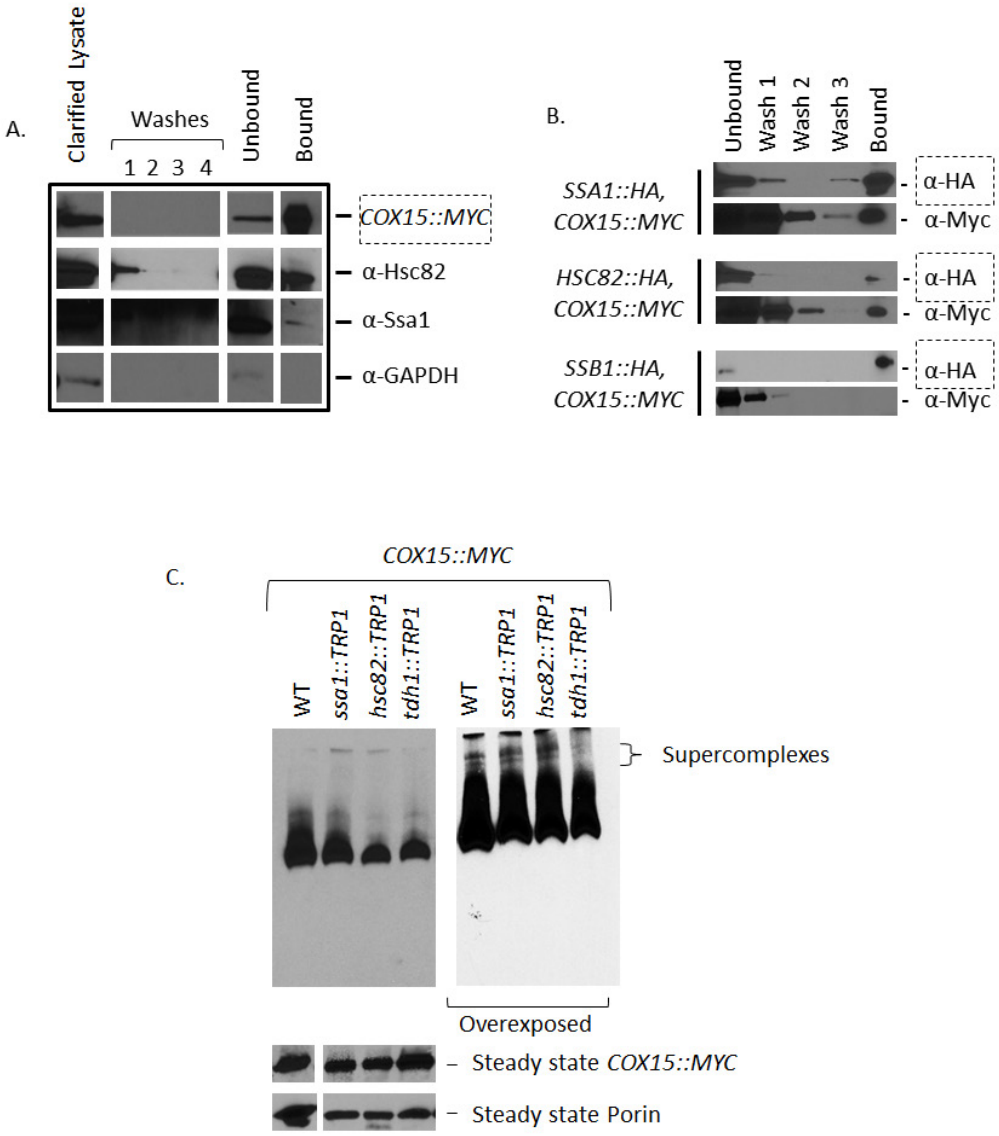

Figure 15: Association of cytosolic heat shock proteins with Cox15. A. C*OX15::MYC* was bound to anti c-Myc resin and bound fractions were probed with antibodies for Hsc82, Ssa1, and GAPDH. The dashed box around *COX15::MYC* is used to indicate that this was the protein pulled down in these experiments. B. HA-tagged Ssa1, Hsc82, and Ssb1 were bound to anti HA resin. Bound fractions (indicated by the dashed boxes) were analyzed for the presence of the respective HA-tagged protein and for the presence of *COX15::MYC*. The strain containing *SSB1::HA* was used as a control. *COX15::MYC* was not found to associate with *SSB1::HA.* C. BN-PAGE was used to analyze the Cox15 complexes from isolated mitochondria containing deletions of Ssa1, Hsc82, and Tdh1. Tdh1 is one of four isoforms of GAPDH in yeast.

the respiratory supercomplexes in all knockouts (Figure 15C). With the caveat that various heat shock proteins can compensate for one another, to a first approximation it appears that neither Ssa1 nor Hsc82 are part of the Cox15 complex observed in BN-PAGE.

Discussion

Potential involvement of Ssa1 and Hsc82 in the import of Cox15 into the mitochondria

We have demonstrated that Cox15 interacts with the cytosolic heat shock proteins Ssa1 and Hsc82. In yeast, Ssa1 has previously been shown to be important for transporting the inner mitochondrial membrane proteins ADP/ATP carrier (AAC) and the mitochondrial peptide transporter into the mitochondria by interacting with the TOM70 receptor of the TOM complex [30]. While members of the hsp90 family have not been found to interact with TOM70 in yeast, the hsp90 proteins have been found to interact with Tom70 during the import of mammalian mitochondrial proteins [30]. In addition, it is reported that members of the hsp90 family interact with hsp70 proteins in higher eukaryotes and that this interaction is conserved from yeast to humans [32-34]. Hsc82 (the yeast hsp90) was found to form stable interactions with the Ssa subgroup of hsp70 proteins in yeast [35]. Based on this, a reasonable hypothesis is that Hsc82 binds to Cox15 to prevent it from aggregating in the cytosol and then delivers Cox15 to Ssa1 by forming an interaction with Ssa1. Cox15 may then be imported into the mitochondria in an Ssa1/Tom70 dependent manner.

While we have detected an interaction of Cox15 with Ssa1 and Hsc82, our data does not suggest that these protein-protein interactions are represented by any of the Cox15 complexes observed on BN-PAGE. Cox15 is still present in both of the supercomplexes as well as in the lower molecular weight complexes in knockouts of Hsc82 and Ssa1. Since Ssa1 and Hsc82 are fairly large proteins (~70 and 80 kDa, respectively), we would expect to observe a size shift of the bands observed on the blue native gel if either Ssa1 or Hsc82 were present in any of the Cox15 complexes. It is likely that the interactions

Cox15 has with Ssa1 and Hsc82 represent transient interactions during Cox15 import into the mitochondria.

Future experiments will be needed to determine if Ssa1 interacts with Cox15 to mediate protein import into the mitochondria, and if so, if the import process occurs in a Tom70-dependent manner. In addition, it will be interesting to investigate whether Hsc82 interacts with Cox15 prior to association with Ssa1, or if Hsc82 mediates Cox15 import into the mitochondria via an alternative pathway than Ssa1/Tom70. Finally, in light of work reporting that Ssa1 is capable of mediating mRNA localization to the mitochondria as well as experiments indicating that Cox15 mRNA localizes to the mitochondria prior to translation, it would be intriguing to investigate if Ssa1 is responsible for localizing Cox15 mRNA to the mitochondria.

Experimental Procedures

Cloning

A 3x hemagglutinin (HA) tag was added to the C-terminus of Ssa1, Hsc82, and Ssb1 via homologous recombination of the 3xHA-TRP1 cassette from the pFA6a-*3HA-TRP1* plasmid. The primers were obtained from Integrated DNA Technologies. For *SSA1::HA*, the primers were 5'-AGCTCCAG AGG C TG AAGGTCCAACCGTTGAAGAAGTTGATCGGATCCCCGGGTTAATTAA-3' and 5'-CATTAAAAGACATT TTCG TTATTATCAATTGCCGCACCAATTGGCGAATTCGAGCTCGTTTAAAC-3'. The primers that were used for *HSC82::HA* were 5'-AGTTGAAGAGGTTCCAGCTGACACCGAGATGGAAGAAGTTGAT CGGATCCCCGGGTTAAT TAA-3' and 3'-GTAAACAAATTTATATAATATATAA AACATGAAGGCGAAAAAAGAGAG AA TTCGAGCTCGTTTA AAC-5'. The primers that were used for *SSB1-HA* and were 5'- AGTTGGTTTGAAGAGAGTTGTCACCAAGGC CATGTCTTCTCGTCGGATCCCCGGGTTAATTAA-3' and 5'-ATATAAGTAATATTCATATATATGTGATGAATG CAG TCGAATTCGAGCTCGTTTAAAC-3'. HA-tags were appended to Ssa1, Hsc82, and Ssb1 in the *COX15::MYC* strain. The *COX15::MYC* strain was prepared as described in [36].

Co-immunoprecipitation experiments

For co-immunoprecipitation of Ssa1, Hsc82, and GAPDH with *COX15::MYC,* mitochondria were prepared as described in Chapter 2 from 80 mL of COX15*::MYC S. cerevisiae.* Mitochondria were solubilized for 2 hours at 4 ^{o}C on a rocking platform in 200 µL of solubilization buffer (600 mM sorbitol, 20 mM HEPES, 150 mM NaCl, 4% digitonin, and Roche protease inhibitor). Solubilized lysate was clarified via centrifugation at 12,000 x *g* and incubated with 50 µL of anti-Myc resin (Sigma) that had been washed with five 1-mL aliquots of phosphate buffered saline (PBS) , (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Resin and lysate were incubated overnight at 4 ^{o}C on a rocking platform. Following incubation, resin was centrifuged for 10 seconds at 12,000 x *g*, and unbound material was removed. The resin was washed four times with 1 mL of PBS. Bound material was eluted by incubating the resin in 50 µL 2X SDS-PAGE buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) for five minutes at 95 ^{o}C. The resin was vortexed, centrifuged 10 seconds at 12,000 x *g,* and the supernatant was loaded on SDS-PAGE gel.

For co-immunoprecipitation of *COX15::MYC with SSA1::HA, HSC82::HA, and SSB1::HA* mitochondria were prepared as described in Chapter 2 from 100 mL of *S. cerevisiae* containing genomic *COX15::MYC* expressing genomic copies of *SSA1::HA, HSC82::HA,* or *SSB1::HA*. Solubilized lysate was clarified via centrifugation at 12,000 x *g* and added to 50 µL of anti-HA resin (Pierce) that had been washed with 50 µL of tris buffered saline (TBS), (50 mM Tris, 150 mM NaCl). The mixture was incubated overnight at 4 ^{o}C on a rocking platform. Following incubation, resin was centrifuged at 12,000 x *g* and unbound material was removed by pipetting. The resin was washed three times with 500 µL of TBS containing 0.05%Tween-20. The bound material was eluted by incubating the resin with 50 µL of non-reducing 2X SDS-PAGE buffer for 5 minutes at 95 ^{o}C. The resin was vortexed and the supernatant was loaded on an SDS-PAGE gel.

Western analysis

Fractions from the co-immunoprecipitation experiments were separated on a 10% SDS-PAGE gel, blotted to polyvinylidene fluoride (PVDF) membrane, and probed with the respective antibodies. Anti-cMyc antibody (Invitrogen) was used for detection of *COX15::MYC* by diluting 1:10000 in 5% milk/TBST followed by a 1:10000 dilution in 5% milk/TBST of the secondary antibody, goat anti mouse (Thermo). Anti-HA antibody (Pierce) was used for detection of all HA-tagged proteins by diluting 1:10000 in 1% bovine serum albumin (BSA)/TBST followed by a 1:10000 dilution in 1% BSA/TBST of the secondary antibody, goat anti mouse. To detect native *S. cerevisiae* Ssa1, the Ssa1/2 goat polyclonal antibody (Santa Cruz, sc-23752) was diluted 1:100 in 1% BSA/TBST followed by a 1:2000 dilution in 1% BSA/TBST of the secondary antibody, donkey anti goat (Abcam). For detection for native *S. cerevisiae* Hsc82, the rabbit polyclonal anti-Hsc82 antibody (Abcam, ab-30920) was diluted 1:5000 in 5% milk/TBST followed by a 1:10000 dilution in 5% milk/TBST of the secondary antibody, goat anti rabbit (Abcam). The rabbit polyclonal anti-GAPDH loading control antibody (Abcam #ab9485) was used for detection of *S. cerevisiae* GAPDH by diluting 1:833 in 1% BSA/TBST followed by a 1:10000 dilution in 1% BSA/TBST of the secondary antibody, goat anti mouse. TBST used for all blots contained 1% Tween-20. All secondary antibodies were conjugated to horse radish peroxidase, incubated for 5 minutes with SuperSignal West Pico Chemiluminescent Substrate (Pierce), and detected with film.

Blue native PAGE

BN-PAGE was performed as described in Chapter 2.

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Chapter 6:

Conclusions and future directions

We have demonstrated that Cox15 exists in high molecular weight protein complexes ranging from 120 kDa – 1 MDa (Chapter 2, Figure 6). BN-PAGE indicates that Cox15 primarily associates in the 120 kDa molecular weight complex. In addition, we noted that the protein complexes at 750 kDa and 1 MDa are reminiscent of the respiratory supercomplexes reported by Cruciat and Brunner et al. [1] and Schagger and Pfeiffer [2], suggesting that Cox15 may be present within these supercomplexes containing the cytochrome bc_1 complex and cytochrome *c* oxidase.

To determine what proteins Cox15 associates with in the high molecular weight protein complexes depicted via BN-PAGE, we utilized mass spectrometry to analyze purified Cox15. We were expecting to identify other cytochrome *c* oxidase assembly factors co-purifying with Cox15 since we predicted Cox15 may interact with other assembly machinery during heme insertion into Cox1. In addition, we were predicting to observe components of the cytochrome bc_1 complex and cytochrome *c* oxidase due to our hypothesis that Cox15 is present within complex III and IV-containing respiratory supercomplexes. Surprisingly, our mass spectral studies did not predominantly identify these proteins as potential interaction partners with Cox15.

The most enriched proteins detected with purified Cox15 in our mass spectrometry studies were the cytosolic heat shock proteins, Ssa1 and Hsc82 (Chapter 3, Table 4). Co-immunoprecipitation experiments further confirmed that these proteins do interact with Cox15 (Chapter 5, Figure 14). An interaction of Cox15 with Ssa1 and Hsc82 may hold important information revealing the import mechanism for Cox15 into the mitochondria. In particular, Ssa1 has been implicated in importing a subset of proteins into the mitochondria through interactions with the Tom70 receptor [3,4]. The role

Hsc82 has during protein import into the mitochondria in yeast remains to be elucidated, but it may be reasonable to conjecture that Hsc82 delivers Cox15 to Ssa1 prior to import. Further work will need to verify that Ssa1 delivers Cox15 to Tom70 and to determine what role Hsc82 plays in this process. When conducting these studies, it will be critical to take into account the possible functional redundancy of these heat shock proteins. Ssa1 belongs to a sub-family of Hsp70 proteins that involves Ssa1-4. Ssa1 and Ssa2 share 99% sequence identity with one another, and Hsc82, shares 92% sequence identity with Hsp82, a second member of the Hsp90 family. This high degree of sequence identity between cytosolic heat shock proteins may suggest, for example, that Ssa2-4 may interact with Cox15 in the absence of Ssa1. This possibility will be important to consider when investigating the Cox15 high molecular weight complexes when Ssa1 is deleted. Because Ssa2-4 may interact with Cox15 in high molecular weight complexes in the absence of Ssa1, the persistence of the Cox15 high molecular weight complexes in the absence of Ssa1 may not preclude that cytosolic heat shock proteins are part of the Cox15 complexes.

In addition to cytosolic heat shock proteins, mass spectrometry of purified Cox15 revealed a possible interaction of Cox15 with the mitochondrial heat shock protein-70 machinery (Tables 2 & 4, Chapter 3). Mass spectrometry of purified TAP-tagged Cox15 detected Ssc1, the mitochondrial heat shock protein while mass spectrometry of purified Myc-tagged Cox15 detected Mdj1, the co-chaperone of Ssc1. The presence of the mitochondrial heat shock protein machinery in two independent experiments may indicate a true interaction with Cox15. We are particularly interested in a possible interaction of Ssc1 with Cox15 due to the role Ssc1 is known to have during cytochrome *c* oxidase assembly. As discussed in Chapter 2, Ssc1 forms an early assembly intermediate with newly translated Cox1 and the assembly factors Mss51, Cox14, and Coa3. Furthermore, Ssc1 and Mss51 have been proposed to dissociate from the Cox1-containing complex around the time of heme insertion into Cox1. Perhaps Ssc1 plays a regulatory role with Cox15 to aid in heme *a* insertion into Cox1. To investigate if Ssc1 and Cox15 interact, co-immunoprecipitation experiments will be utilized.

The final protein of note detected in our mass spectrometry studies of purified Cox15 was Cyt1, one of the catalytic subunits of the cytochrome bc_1 complex (Tables 2 & 4, Chapter 2). We were interested to investigate whether Cox15 and Cyt1 interact because of our observation that Cox15 was present in respiratory supercomplexes depicted by BN-PAGE (Figure 6, Chapter 2). Co-immunoprecipitation experiments verified an interaction between Cyt1 and Cox15 (Figure 13, Chapter 4). To verify that the bands at 750 kDa and 1 MDa observed via BN-PAGE reflect the presence of Cox15 in respiratory supercomplexes, we observed the Cox15 high molecular weight complexes in yeast mutants in which Cox1 was either not transcribed or rapidly degraded (Figure 12, Chapter 4). In these yeast mutants, cytochrome *c* oxidase fails to assemble and the respiratory supercomplexes also fail to form. As predicted, Cox15 was no longer observed in the Cox15-containing complexes present at 750 kDa and 1 MDa in yeast mutants lacking supercomplexes. This indicates that at least a fraction of the Cox15-Cyt1 interaction likely occurs within the supercomplexes. It is important to note, however, that if Cox15 is present in the respiratory supercomplexes, it likely interacts (either directly or indirectly) with other components of the cytochrome bc_1 complex and cytochrome *c* oxidase. Although other components of these complexes were not detected in our mass spectrometry studies of purified Cox15, it is important to mention that the sensitivity of mass spectrometry can be several orders of magnitude less than that of western blotting with a strong monoclonal antibody. Because of this, co-immunoprecipitation experiments with antibodies directed against other components of the cytochrome bc_1 complex and cytochrome *c* oxidase will be necessary to determine if proteins in addition to Cyt1 interact with Cox15 within the respiratory supercomplexes. It is intriguing to consider why only Cyt1 was detected in these studies. Cyt1 has a large soluble domain in the IMS side of the inner mitochondrial membrane, and it is reasonable to hypothesize that the structure of Cyt1 makes it more conducive for mass spectrometry detection than other components of the respiratory supercomplexes.

Once we determine if Cox15 interacts with other proteins from both the cytochrome bc_1 complex and cytochrome *c* oxidase, it will be necessary to determine whether Cox15 interacts with these proteins strictly within supercomplexes. To reveal where interactions with proteins from these respiratory complexes occur, co-immunoprecipitation experiments of Cox15 with various proteins from the cytochrome bc_1 complex and cytochrome *c* oxidase will be performed in Δ*qcr8* and *Δcox4* mutants. Both of these mutants fail to form fully assembled cytochrome bc_1 complex and cytochrome *c* oxidase, respectively [5]. In addition, in the absence of either fully assembled complex III or complex IV, supercomplexes do not form. If Cox15 still interacts with Cyt1 and other proteins from the cytochrome bc_1 complex in a Δ*qcr8* mutant, this will suggest that Cox15 interacts with proteins from complex III prior to their incorporation into a fully formed complex III and prior to formation of a supercomplex. Likewise, if Cox15 still interacts with proteins from cytochrome *c* oxidase when the mature complex fails to form and when supercomplexes are absent, we will know that Cox15 interacts with components of cytochrome *c* oxidase in early assembly intermediates.

Regardless of whether Cox15 interacts with components of the cytochrome bc_1 complex and cytochrome *c* oxidase only within supercomplexes or also within early assembly intermediates, it is important to consider why Cox15 is present within respiratory supercomplexes. If Cox15 is found to interact with early assembly intermediates of cytochrome *c* oxidase, it would be reasonable to conjecture that these interactions aid in proper heme *a* insertion into holo-cytochrome *c* oxidase. More intriguing, however, will be to determine why these interactions persist within supercomplexes. Does this point to a novel role for Cox15 other than heme *a* insertion into Cox1? Alternatively, the presence of Cox15 within supercomplexes may indicate a dynamic nature of the respiratory supercomplexes. It is generally assumed that holo-complex IV forms prior to its interaction with complex III within supercomplexes. The presence of Cox15 within supercomplexes, however, may challenge this notion.

Perhaps heme *a* insertion and assembly of holo-cytochrome *c* oxidase occurs simultaneously with supercomplex formation.

In addition to considering why Cox15 is present within supercomplexes, we must also ask why Cox15 interacts with components of complex III. As discussed above, it may seem more obvious why Cox15 would interact with proteins from cytochrome *c* oxidase in both early assembly intermediates and within the supercomplexes. It is less obvious why Cox15 would interact with proteins from the cytochrome bc_1 complex. If interactions between Cox15 and the cytochrome bc_1 complex only occur within respiratory supercomplexes, it may be reasonable to hypothesize that Cox15 interacts with complex III proteins simply because of its presence within supercomplexes. (This would assume that Cox15 is present within supercomplexes because of a function specific to cytochrome *c* oxidase). If Cox15 interacts with components of the cytochrome bc_1 complex when supercomplexes do *not* form, however, this will point to a specific function of the complex III – Cox15 interaction, such as complex III proteins helping to regulate Cox15 function.

Finally, while we have determined that Cox15 exists within respiratory supercomplexes depicted by the high molecular weight complexes at 750 kDa and 1 MDa on BN-PAGE, it is important to consider what the lower complexes observed via BN-PAGE represent. Further work will be needed to decisively determine whether interactions of Cox15 with Ssc1 and the cytosolic heat shock proteins, Ssa1 and Hsc82, are represented by these bands. As discussed in Chapter 4, the Cox15 complex depicted by band C on BN-PAGE likely reflects sub-stoichiometric interactions of Cox15 with cytochrome *c* oxidase assembly factors. This finding would support the findings of Bareth et al. (2014) who report a sub-stoichiometric association of Cox15 with cytochrome *c* oxidase assembly factors [6]. We also have determined that at least 30% of Cox15 interacts with itself, indicating that some of the bands observed on BN-PAGE represent homo-oligomers of Cox15. The final observation that remains to be resolved is whether the very strong band that represents the majority of Cox15 at 120 kDa is monomeric Cox15 or

dimeric Cox15. Because we have estimated that only about 30% of Cox15 interacts with itself, our current hypothesis is that this abundant band represents monomeric Cox15. In further support of this, it has been reported that the molecular weights of protein complexes are often overestimated by BN-PAGE [7,8]. If Cox15 does exist largely as a monomer within the mitochondria, our finding that the majority of Cox15 exists alone implies a surprisingly independent nature for Cox15 during heme *a* insertion into cytochrome *c* oxidase.

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