The role of the PHB complex in mitochondrial biogenesis

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Citation for published version (APA):
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Mitochondria are essential organelles whose primary function is the synthesis of ATP by oxidative phosphorylation. The biogenesis of the oxidative phosphorylation system (OXPHOS), composed of respiratory chain complexes and the ATP synthase complex, requires gene products from both the nuclear and the mitochondrial genomes. Although the complete 3D structures of several OXPHOS complexes are resolved, still very little is known about the processes involved in their assembly. The importance of understanding the assembly of OXPHOS complexes increased when several assembly factors were found to be responsible for a wide range of human neurodegenerative diseases [1, 2]. A protein that plays an important role in the biogenesis and assembly of respiratory chain complexes is the prohibitin (PHB) complex. This thesis deals with various aspects of the mitochondrial PHB complex and its putative role in the process of respiratory chain complex assembly. The relatively simple and well-characterised yeast Saccharomyces cerevisiae was used for structural and functional studies at the molecular level. Next, I used the nematode Caenorhabditis elegans to investigate the effect of depletion of the PHB complex during organismal development. As an introduction, the first chapter briefly reviews some aspects of the regulation of mitochondrial biogenesis, covering from mitochondrial gene expression to the final assembly of OXPHOS complexes. This review will be followed by an outline of the thesis.

1. Mitochondrial biogenesis

1.1 An overview of mitochondria

About 1.5 billion years ago, a successful symbiosis occurred between the ancestor of today's eukaryotic cell and respiring Gram-negative purple bacteria [3]. The endosymbiont provided the host with about 15 times more ATP by oxidative phosphorylation than the host was able to obtain from the same molecule of glucose by glycolysis. In return, the host probably offered the endosymbiont a safer environment or even a more efficient way to replicate its DNA. Nowadays, mitochondria are responsible for the production, by oxidative phosphorylation, of most of the ATP required for all cellular processes. Moreover, many essential metabolic processes take place within mitochondria, including the tricarboxylic acid cycle (TCA cycle), iron/sulfur cluster assembly, and biosynthesis of amino acids. In metazoans, mitochondria also play a crucial role in controlling apoptosis by integrating and triggering numerous death signals [4-7]. The involvement of mitochondria in central cellular processes, in cell death and in ageing, has generated the interest of researchers to study mitochondrial defects that contribute to the pathogenesis of a wide range of human degenerative diseases [8-10].

Mitochondria are bounded by two membranes, the outer and the inner membrane, which define two separate compartments, a narrow intermembrane space and an internal matrix space. The matrix space contains hundreds of enzymes, among which are those required for the oxidation of acetyl CoA in the TCA cycle. The oxidation of acetyl CoA to CO₂ results in the production of high-energy electrons that are held by NADH and FADH₂. Those electrons are then transported through respiratory enzyme complexes embedded in the mitochondrial inner membrane (Complexes I, II, III and IV). The last electron transfer reaction takes place at complex IV with the reduction of oxygen to water. The energy released by the electron passage is used by complexes I, III and IV, to pump protons from the matrix to the intermem-
brane space. This creates an electrochemical proton gradient that is used by ATP synthase (Complex V) to phosphorylate ADP, thereby producing ATP and completing the process of oxidative phosphorylation (OXPHOS) (Figure 1).

The electron transport chain and the ATP synthase are organised as a cluster in the mitochondrial cristae. Mitochondrial cristae have been regarded as infoldings of the inner membrane. However, in the last years, high-resolution 3D images of electron tomography studies show cristae as chambers of variable shapes that are connected to the mitochondrial inner boundary membrane by thin tubules of typically ~30 nm of diameter [11-13]. Mitochondrial cristae vary in abundance and shape, depending on the cell type and on the physiological state of the cell [14-16] (Figure 2). The structure of mitochondria also reflects their cellular role. Indeed, the number of mitochondria, their morphology and intracellular distribution vary in response to energetic demands and physiological conditions of the cell. Mitochondria are very dynamic organelles, and events of growth, fusion, and fission continuously change mitochondrial morphology and distribution within the cell [17, 18]. Mitochondria can therefore appear as single elaborate networks or as highly fragmented structures.

As a remnant of the primitive endosymbiont, the mitochondrion contains circular double-stranded DNA molecules, being the only cytoplasmic organelle in fungal and animal cells that harbours its own genome. In mammals, the mitochondrial DNA (mtDNA) encodes 13 subunits of the oxidative phosphorylation system, 22 tRNAs and 2 rRNAs [20]. In the (unicellular) yeast, *Saccharomyces cerevisiae*, the mtDNA codes for 24 tRNAs, 2 rRNAs, 1 RNA molecule component of RNaseP and 8 structural proteins [21]. With the exception of the ribosomal protein Var1p, the other seven proteins are subunits of the core catalytic centres of the respiratory chain.

![Figure 1](image_url). The mitochondrial respiratory chain and ATP synthase. Complexes I to V are localised in the mitochondrial inner membrane. Complexes I, III and IV translocate protons (H⁺) from the matrix to the intermembrane space. Complex V couples the transport of these protons back to the matrix to the synthesis of ATP.
Figure 2. Three-dimensional reconstruction of isolated, frozen-hydrated rat-liver mitochondria by electron tomography. (A) Cristae are shown in green. The outer and inner membrane are shown in red and yellow respectively [11]. (B) The inner membrane is shown in brown. Cristae branching off the inner membrane are shown in various colours [19]. Courtesy of Dr. C. A. Mannella, Resource for the Visualization of Biological Complexity (NIH/NCRR), Wadsworth Center, Albany, NY, USA.

It has been demonstrated that several molecules of mtDNA associate with proteins to form nucleoids in yeast [22-24] and more recently in human cells [25, 26]. Nucleoids are believed to be linked to the mitochondrial inner membrane and constitute the units of mtDNA inheritance [27].

The coding capacity of the mtDNA is limited to a few structural proteins of the OXPHOS system and to some ribosomal and transfer RNAs. Therefore, mitochondria rely on the nuclear genome for the remaining respiratory subunits (around 100), the matrix enzymes involved in metabolic and biosynthetic reactions, the machinery for maintenance, replication and transcription of mtDNA, the translational activators of mitochondrial mRNAs, components of the translational machinery and proteins involved in assembly of the respiratory system. Furthermore, mitochondrial proteins synthesised on cytoplasmic ribosomes need to be imported into mitochondria by nuclear-encoded translocases embedded in both the inner and the outer mitochondrial membrane [28, 29]. Therefore, mitochondrial biogenesis and function depend on a precise crosstalk between two physically separated genetic systems, the nuclear and the mitochondrial genome [30-32]. Extremely complex and well-coordinated processes are needed to accurately regulate this crosstalk in response to the changeable physiological circumstances of the cell.

Why mitochondria have kept a portion of their original genome is still an open question. Most of the original symbiont's genetic material has been transferred to the nucleus or lost during the evolutionary process. However, genes coding for key hydrophobic components of the respiratory chain seem to have remained. Transfer of genes may still go on, or may have stopped when changes in the endosymbiont's genetic code presented a barrier to further use of the information transferred (see below, section 1.4.3). On the one hand, DNA is extremely susceptible to damage by oxygen radicals coming from the respiratory chain. Therefore, putting distance between the DNA and the mitochondrial membrane seems the safest. Moreover, the
machinery required to maintain and regulate two separated genomes is enormously costly for the cell. On the other hand, there seem to be physical and regulatory reasons for keeping hydrophobic core components of the respiratory chain encoded within mitochondria [33, 34].

1.2 The drawback of respiration

Most of the oxygen consumed by the cell is reduced in the final step of mitochondrial electron transport in the mitochondrial oxidative phosphorylation system. Although respiration is an extremely efficient way to produce energy, the incomplete reduction of molecular oxygen and the leakage of electrons from the electron transport chain result in the production of oxygen radicals. Reactive oxygen species (ROS) are natural byproducts of oxidative metabolism and they act as effector molecules in several cellular pathways [35, 36]. However, they are also mediators of cell damage. A single free electron transfer to molecular oxygen results in the production of superoxide (O$_2^-$). Complexes I and III of the electron transport chain are the main producers of superoxide, which is likely to damage mtDNA [37]. Manganese superoxide dismutase (MnSOD) reduces O$_2^-$ to H$_2$O$_2$, which is not a free radical by itself. However, H$_2$O$_2$ can easily diffuse and react with free reduced transition metals producing -OH (known as the Fenton reaction), which is extremely reactive and can extensively damage DNA, lipids and proteins.

Although cells have developed anti-oxidant systems to protect themselves from oxidative damage, the rate of ROS production seems to be higher than the scavenging capacity of the cell and oxidative stress has been associated with a variety of diseases [38] and the process of ageing [39-41]. Defects in OXPHOS increase superoxide production due to impaired electron flow, thereby increasing oxidative stress and damage to DNA and proteins of the electron transport chain. Thus, damaged mitochondria can easily embark on a vicious circle, with increased DNA damage leading to increased ROS production, which in turn will lead to more DNA damage. Moreover, ROS inhibits a number of mitochondrial enzymes that play an important role in energy production, such as α-ketoglutarate dehydrogenase, which will limit the formation of NADH [42, 43]. ROS also inactivates Fe-S clusters, causing iron release and inactivation of Fe-S containing complexes. The released iron will be prone to enter the Fenton reaction, thereby increasing the production of ROS.

Reactive oxygen species can disrupt normal physiological pathways and lead to apoptosis. Worthy of mention is the fact that two mitochondrial death effectors, AIF (apoptosis-inducing factor) and cytochrome c, appear to work as free radical scavengers inside mitochondria. However, they trigger cell death when translocated outside mitochondria [44, 45], suggesting a complex relationship between cell death and oxidative stress.

1.3 Inter-organelle crosstalk

As mentioned above, biogenesis of mitochondria requires the coordinated expression of the nuclear and the mitochondrial genome. This coordination takes place at multiple levels [31, 46]. In mammals, several nuclear transcription factors of nuclear OXPHOS genes, such as NRF1 and NRF2 (for Nuclear Respiratory Factors), have been identified. Interestingly, NRF1 and/or NRF2 also regulate the expression of the mitochondrial transcription factor TFAM,
which is involved in mtDNA replication/transcription, nicely coupling nuclear and mitochondrial gene expression (reviewed [47, 48]). In the yeast *Saccharomyces cerevisiae*, transcription of nuclear encoded mitochondrial genes is regulated by carbon source [49] and oxygen [50]. The Hap2/3/4/5 transcriptional activator complex, for example, plays an important role in reprogramming gene transcription during the diauxic shift from fermentative to respiratory growth [51], with Hap4 playing a key role. Overexpression of Hap4 alone is sufficient to upregulate mitochondrial biogenesis and activity even in the presence of glucose when respiratory activity is normally repressed [52]. Conversely, nuclear gene expression responds to alterations in mitochondrial activity, a process called retrograde communication [32]. Three proteins involved in this phenomenon, two transcription factors (Rtg1 and Rtg3) and a heat shock protein (Rtg2) have been characterised in yeast [53-57]. *RTG* genes regulate the expression of several TCA cycle enzymes in cells with compromised mitochondrial respiratory activity [58], whereas expression of those TCA cycle enzymes in cells with robust respiratory function is under the control of the HAP transcription complex [59]. In vertebrates, no gene homologues for *RTGs* have been identified. However, in myocytes, decreased ATP synthesis and disrupted mitochondrial membrane potential result in increased levels of cytosolic Ca$^{2+}$, which seem to be responsible for a cellular response to mitochondrial dysfunction [60-62].

1.4 Mitochondrial gene expression

1.4.1 Replication

An important issue in mitochondrial biogenesis is the regulation of mtDNA copy number. In *S. cerevisiae*, mtDNA replication mechanisms are less understood than in mammals, probably because for many years the topology of yeast mtDNA was believed to be circular, by analogy with animal mtDNA [63, 64], despite mostly only linear molecules being found. This led researchers to dogmatically believe in the broken-circle theory, which has hindered progress in learning how yeast mtDNA is replicated. Later, it was revealed that the *S. cerevisiae* mitochondrial genome consists mostly of linear tandem arrays of the 75-kb mitochondrial genome, and a tiny amount of large circular forms that seem to replicate by means of the rolling-circle process [65]. In *S. cerevisiae*, despite the fact that extensive genetic and biochemical studies have revealed a number of proteins involved, the biochemical mechanism of replication and maintenance of mtDNA is still poorly understood. Recent studies suggest that yeast mtDNA replication system diverges from that observed in mammals, and may involve recombination coupled to rolling-circle replication mechanisms [66, 67].

The replication of mtDNA has been more extensively studied in mammalian mitochondria. There are currently two models for animal mtDNA replication. The generally believed strand-asymmetric model of mammalian mtDNA replication postulates two sites of initiation of DNA synthesis, one for each strand, heavy (H) and light (L) strand. These two origins are physically separated and DNA synthesis temporally distinct [68, 69]. Recently, it has been proposed that mammalian mtDNA replication proceeds predominantly, perhaps exclusively, by a strand-coupled replication mechanism [70, 71]. Despite advances in our understanding of mtDNA replication mechanisms in animal mitochondria, and the fact that many factors
have been proposed to influence mtDNA copy number, very little is known on how mtDNA replication is regulated [72].

1.4.2 Transcription

In mammals, the TFAM protein is essential for mtDNA transcription and maintenance [73]. Recently, two new transcription factors have been discovered in mammalian mitochondria, TFBM1 and TFBM2 [74, 75]. TFAM, TFBM1, TFBM2 and the mitochondrial RNA polymerase can drive transcription in vitro. Although the basal transcriptional machinery seems to have been identified, the control of mitochondrial transcription in mammals is still a mystery (for review see [76]).

In yeast, mitochondrial transcription requires two components, a mitochondrial RNA polymerase (Rpo41) and a transcription factor sc-mtTFB (Mtfl), which is the orthologue of TFBM1 and TFBM2 [77, 78]. The yeast orthologue of TFAM, Abf2p, is involved in mtDNA packaging but is not essential for transcription. Yeast mitochondrial transcripts contain introns and are polycistronic. This implies that a number of RNA-processing events are required before mRNAs are ready for translation. Removal of introns is especially intricate because it requires previous translation of intron-encoded maturases that are required for splicing [79]. Still much needs to be resolved regarding mRNA stability and processing, although new insights regarding the mitochondrial degradosome and its role in mRNA degradation are emerging [80].

Recent data obtained in \textit{S. cerevisiae} nicely show that transcription and translation processes are strongly linked. Mitochondrial RNA polymerase, Nam1p (a matrix protein involved in transcript processing and translation events [81]), and Slslp (a membrane-bound regulator of transcription-coupled processes [82]), work together to localize transcription complexes to the inner membrane, where ribosomes are located, to ensure efficient mitochondrial translation [83] (Figure 3).

1.4.3 Translation

The first characteristic feature regarding mitochondrial translation is that the mitochondria use an alternative genetic code. In one dialect used by mitochondria in many organisms, the universal codon UGA does not mean "stop" but codes for Trp. In vertebrates, AGA means "stop" while universally means Arg, and in yeast and metazoans AUA encodes Met instead of Ile.

mtDNA encodes some transfer and ribosomal RNAs of the mitochondrial translational machinery with the rest being imported from the cytosol. In recent years, new protein components of the mitochondrial ribosomes have been identified in yeast and mammals with the help of mass spectrometry and affinity purification techniques [84-87]. The translational machinery of mitochondria shows a low degree of sequence conservation. With only a minority of mitochondrial ribosomal proteins (MRP) showing significant sequence similarity to other ribosomal proteins, the majority are unique [88, 89]. It has been speculated that the conserved proteins might be responsible for central steps of protein synthesis, whereas the unique pro-
teins or domains may have specialised in coupling translation to the inner membrane [90] or even to transcriptional processes.

Another characteristic feature of the translation of most if not all mitochondrially-encoded respiratory chain subunits in yeast is the requirement for mRNA-specific translational activators. Translational activators recognise targets in the mRNA 5'-untranslated leaders and seem to mediate interactions with mitochondrial ribosomes near the inner membrane, facilitating the insertion of newly synthesized hydrophobic polypeptides into the membrane [91, 92]. Recent work demonstrates physical associations among COX1, COX2 and COX3 mRNA-specific translational activators at the matrix side of the inner membrane. Moreover, interactions between Nam1p (see above) and the translational activators are also found, again suggesting a controlled delivery of mitochondrial mRNAs to the translation system [93] (Figure 3).

Besides the nuclear control of mitochondrial translation, an intra-mitochondrial regulatory mechanism has been postulated for a more immediate response to mitochondrial metabolic changes. The TCA cycle enzyme (NAD⁺)-dependent isocitrate dehydrogenase (Idh) binds to mitochondrial mRNAs [94]. Recently, a mitochondrial mRNA was found to negatively regulate Idh activity in an AMP dependent manner, suggesting a direct mechanism to regulate mitochondrial translation in response to energetic demands [95]. The fact that disruption of Idh results in increased mitochondrial translation efficiency also supports a physiological relevance for the binding of mRNAs by Idh [96].

![Figure 3](image)

**Figure 3.** Mitochondrial transcription and translation are coordinated processes at the inner mitochondrial membrane. Nam1p is proposed to bind mtRNA polymerase [81] to facilitate the interaction of Sis1p at the mitochondrial inner membrane with mtDNA-bound mtRNA polymerase [83]. Nam1p interacts with translational activators of cytochrome oxidase subunit mRNAs [93], probably helping to establish interactions of the 5'-UTR of the mRNA with its translational activator.
The complexities of the post-translational events required for the correct assembly of functional respiratory complexes are discussed in more detail below, further illustrating the intricacies and expense of the maintenance and regulation of mitochondrial gene expression.

1.5 Assembly of mitochondrial respiratory complexes

Our current understanding of the many processes involved in mitochondrial assembly is largely based on studies in the yeast *S. cerevisiae*, due to the availability of extensive genetic and biochemical data on mitochondrial biogenesis in this organism. Analysis of yeast mutants disturbed in assembly has led to the identification of a number of nuclear gene products involved in the process of assembly. Assembly factors, in contrast to factors involved in translational control, often display evolutionary conservation [97]. Factors involved in assembly include proteins involved in co-factor addition, protein folding, stabilisation, membrane translocation and degradation. However, the exact mechanisms of action of many of those factors are still obscure.

The majority of subunits that build up the oxidative phosphorylation system are nuclear-encoded, however, most complexes contain subunits encoded by the mitochondrial genome. Mitochondria encode for hydrophobic subunits that need to be stoichiometrically assembled with the nuclear-encoded ones. Incorrectly folded or assembled subunits have a high potential of disturbing the integrity of the membrane [98], which will result in proton leakage, escape of ROS and ultimately compromise the metabolic efficiency of the cell. Therefore, a tight regulation must exist to ensure correct assembly of mitochondrial inner membrane complexes.

Mitochondrial proteins synthesised in the cytosol, often driven by a targeting sequence, traverse mitochondrial membranes in an unfolded conformation, helped by mitochondrial translocases located in the outer and inner membrane (TOM and TIM complexes, for translocase of the outer and inner membrane, respectively)(for reviews, see [28, 29]). The import and sorting events of the imported protein depend on the destination within mitochondria and on the membrane topology of the protein itself [99]. Imported unfolded proteins attain their native conformation at their site of function. Mitochondrially encoded proteins follow a shorter route, since they are synthesised near the mitochondrial inner membrane, which is their final destination. Once subunits coming from either compartment have attained their proper membrane topology, interaction with partner subunits will lead to full assembly into functional complexes. During the process of folding and before final assembly, many subunits go through maturation processes such as incorporation of prosthetic groups and proteolytic processing of N- and/or C-terminal ends. The coordinated functions of molecular chaperones, assembly factors and membrane-bound translocases are required for the process of folding and assembly. All these factors are briefly described below (see sections 1.5.1 to 1.5.5).

Remarkably, a distinct quality control system is present in the mitochondrial membrane to assist with folding and to selectively remove non-assembled polypeptides and prevent their potentially harmful accumulation in the membrane. The role of the quality control apparatus in respiratory complex assembly will be discussed in more detail within the context of the potential involvement of the mitochondrial prohibitin complex as an additional component of this system (see section 1.6).
1.5.1 Incorporation of prosthetic groups

Every complex of the electron transport chain contains prosthetic groups that function as electron donors/acceptors and need to be incorporated at some point during the process of complex assembly. The different prosthetic groups present in complexes I-IV are: Fe/S clusters (complex I, II, and III), heme (complex II, III, and IV), copper (complex IV) FMN (flavin mononucleotide) (complex I), FAD (flavin adenine dinucleotide) (complex II) [100]. Mutations in factors involved in the synthesis or incorporation of prosthetic groups disturb the process of complex assembly. Some examples of factors that influence cytochrome c oxidase (complex IV) assembly are Cox10p and Cox15p; enzymes involved in heme a biosynthesis, the precursor of cytochrome a and a, [101-103]. Sco1p, Sco2p, Cox11p, Cox17p and the newly identified Cox19p, proteins functioning in copper transport from the cytosol to mitochondria, are responsible for the attachment of copper atoms to Cox1p and Cox2p subunits of complex IV [104-111].

1.5.2 Molecular chaperones

Molecular chaperones play an essential role in the import and sorting of cytosolic mitochondrial proteins [112]. Recently, it has been demonstrated that in mammals the mitochondrial import receptor Tom70, at the outer membrane, recruits Hsp70 and Hsp90 for the delivery of preproteins and that translocation depends on the Hsp90 ATPase. In yeast, Hsp70 rather than Hsp90 is required for preprotein/Tom70 complex interactions [113]. In the intermembrane space Tim9p and Tim10p [114] bind to hydrophobic patches of precursor proteins and release them from the outer membrane Tom receptor [115]. In the mitochondrial matrix several chaperones stabilise and protect polypeptides by preventing aberrant interactions leading to aggregation and denaturation [116]. Mitochondrial Hsp60 forms an oligomeric complex that offers a protected environment for the folding of cytosolic imported proteins, like the ATP synthase complex [117]. The mitochondrial heat shock protein mtHsp70 (Ssc1) is required, together with Tim44p and Mge1, for the translocation of cytosolic precursor proteins [118]. Once in the matrix, a soluble chaperone system, mtHsp70 and Mdj1, assist further folding of mitochondrially synthesised and imported proteins [119, 120]. MThsp70 was found to bind mitochondrially encoded Atp9p oligomer and to promote its association with Atp6p [121]. A second Hsp70 homologue, Ssq1 is involved in the assembly of mitochondrial Fe/S clusters together with the DnaJ family member Jacl [122, 123]. Members of the Hsp100 family of chaperones have also been identified in mitochondria but their function is yet not well defined [124].

1.5.3 Assembly factors

Apart from the conventional molecular chaperones, there is a whole set of nuclear genes that affect assembly of the respiratory chain and show no similarity to known chaperone proteins. For many of the still growing family of assembly factors, there are as yet no specific functions known. In the case of cytochrome c oxidase (complex IV), mutations in nuclear genes cause lack or decreased levels of the studied subunits or of the whole complex, resulting in respiratory incompetence, whereas synthesis de novo is not impaired. This is the case for Pet100p, Pet117p, Pet191p, Cox14p, Cox15p, Cox16p and Cox20p [125] [126-130]. Assembly of F,F,
ATPase (complex V) requires Atp10p, Atp11p and Atp12p proteins. The membrane-localised Atp10p is involved in the assembly of the F\textsubscript{s} sector, while Atp11p and Atp12p are matrix proteins necessary for the assembly of the F\textsubscript{\iota} sector [131, 132] [133-136]. Assembly of the bc\textsubscript{i} complex (complex III) requires the action of Abc1p, Cbp3p, Cbp4p, and Bcs1p. Mutants affected in Abc1p are defective in conformation and function of the cytochrome bc\textsubscript{i} complex and complexes II and IV of the respiratory chain are also affected [137]. Cbp3p and Cbp4p are membrane-associated proteins specifically required for biogenesis of complex III, with mutations in both having similar effects, that is undetectable cytochrome b, reduced amounts of apocytochrome b, FeS, Qcr7p and Qcr8p [138-140]. Alteration of Bcs1p, a member of the AAA family of proteases, results in a decrease in FeS protein content indicating a role in the biogenesis of Rieske iron-sulfur protein [141].

1.5.4 Protein insertion

Mitochondrial inner membrane proteins exhibit a wide variation in membrane topologies. A number of mitochondrial- and nuclear-encoded membrane proteins have to be inserted from the mitochondrial matrix into the inner membrane. This sorting involves insertion of hydrophobic transmembrane segments and translocation of hydrophilic regions across the membrane. In contrast to the extensive studies on the mechanisms of import into mitochondria, not much is known about proteins responsible for the export of proteins from the matrix. Oxa1p belongs to a ubiquitous protein family that is involved in protein insertion into membranes of bacteria, mitochondria, and chloroplasts [142, 143]. Oxa1p is a polytopic inner membrane protein required for the export of Cox2p (complex IV), which spans the inner membrane twice \((N_{\text{int}}-C_{\text{out}})\) [144, 145]. Oxa1p also interacts with newly synthesised Cox1p, Cox3p (complex IV) and cytochrome b (complex III), which span the membrane 12, 7, and 8 times, respectively, and is required for their efficient integration into the membrane. These interactions are stabilised in the presence of ribosomes, suggesting a co-translational insertion from the matrix [146]. In addition to the export of mitochondrial-encoded proteins, Oxa1p also plays a role in translocation of imported nuclear-encoded proteins from the matrix, including Oxa1p itself [147]. Mba1p is another component of the mitochondrial protein export machinery identified in yeast. Mba1p, as Oxa1p, interacts with mitochondrial translation products and with sorted nuclear-encoded proteins during their integration from the matrix into the inner membrane [148]. Although Oxa1p and Mba1p seem to overlap in function and substrate specificity, they can function independently of each other. Cox18p is specifically required for the export of the C-tail of Cox2p, and interacts with Pnt1p and Mss2p in the inner membrane, suggesting that the three proteins work together in translocation of Cox2p domains [149]. These factors are probably just a subset of a growing list of factors required for mitochondrial protein export. Yet, they already illustrate the intricate processes involved in membrane insertion of respiratory chain subunits.

1.5.5 Polypeptide processing

Many mitochondrial proteins encoded in the nucleus are directed into the organelle by N-terminal targeting sequences [150] that are removed by signal peptidases. Three different mitochondrial signal peptidases have so far been described in yeast: (1) the matrix processing
peptidase (MPP) is a matrix metallo-protease involved in proteolytic processing of most N-terminal mitochondrial targeting sequences of imported precursor proteins [151-153]; (2) the mitochondrial intermediate peptidase (MIP), another metallo-protease localized in the matrix, removes octapeptides from signal sequences of several proteins, including iron-utilising proteins, TCA cycle enzymes and respiratory chain components [154-156]; and (3) the inner membrane peptidase (IMP), a serine-protease consisting of a small protein Somlp and two catalytic subunits Impl and Imp2 facing the intermembrane space. IMP processes nuclear- and mitochondrial-encoded proteins that are exported from the matrix into the inner membrane or the intermembrane space [157-160]. See Figure 4 for an overview of the process of assembly.

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**Figure 4.** Schematic representation of the assembly of the Oxidative Phosphorylation (OXPHOS) system. Nuclear-encoded subunits are imported from the cytosol through the translocases TOM and TIM (down). Mitochondrial-encoded subunits (Cox1p, Cox2p and Cox3p) translated at the inner membrane are inserted into the membrane with the help of Oxa1p and Mba1p (top). Mitochondrial processing peptidases (IMP, MPP and MIP) are shown. Assembly factors are schematically depicted.
1.6 Quality control: ATP-dependent proteases

Membrane bound organelles harbour a quality control system that ensures the selective removal of non-native, non-assembled or damaged polypeptides. In some cases and in addition to its proteolytic function, a chaperone-like role has also been proposed. Components of the quality control are ATP-dependent proteases derived from bacterial ancestors and are highly conserved in eukaryotic cells [161, 162].

Mitochondrial ATP-dependent proteases have been extensively studied in the yeast *S. cerevisiae*. Yeast mitochondria contain compartmentalised ATP-dependent proteases. In the mitochondrial matrix, an ATP-dependent serine protease, termed Pim1p, belongs to the highly conserved family of Lon-like proteases and is required for respiratory growth [163, 164]. Lon-like proteases form homo-oligomeric complexes and a heptameric stoichiometry has been described for Pim1p [165]. Substrates of Pim1p include subunits α, β and γ of the F_{0}F_{1}-ATPase complex, subunit β of the matrix processing peptidase, and ribosomal proteins [163, 164, 166]. The mitochondrial Hsp70 system was found to cooperate with Pim1p in the proteolytic degradation of misfolded peptides [166]. Disruption of the *PIM1* gene results in the accumulation of extensive mutations in the mtDNA, resulting in rapid loss of mtDNA [163, 164]. The fact that bacterial and human Lon proteases bind DNA in a site-specific manner, suggests a direct role of Pim1p in mtDNA metabolism [167, 168]. *PIM1* protease also controls the maturation of *COX1* and *COB* mRNA (whose introns code for mRNA maturases), and the subsequent translation of *COX1* mRNA [169]. *PIM1* protease has been reported to specifically and preferentially degrade oxidised aconitate, suggesting a protective role against ROS-mediated damage [170]. PIM1 protease is thus part of the quality control system in the mitochondrial matrix. In the mitochondrial inner membrane, quality control is ensured by two ATP-dependent proteases, termed AAA proteases (for ATPases associated with a number of cellular activities). With their catalytic domains facing opposite membrane surfaces and a native molecular mass of 850-1000 kD, these two membrane-embedded ATP-dependent proteolytic complexes play crucial roles in the biogenesis of mitochondria. The first complex, the *m*-AAA protease, is active at the matrix side of the inner membrane and contains Afg3p (YtalOp) and Rcalp (Ytal2p). The second complex, the *i*-AAA protease, is an oligomeric complex composed of Yme1p subunits and functions at the intermembrane space. Depletion of either of the *m*-AAA subunits impairs the respiratory capacity of the cells, and yeast cells lacking both *m*- and *i*-AAA proteases are not viable. The structure of these complexes is not known, though structural studies of bacterial homologues (FtsH) reveal a hexameric ring structure [171, 172].

1.6.1 Roles of membrane-bound AAA proteases

Membrane-bound AAA proteases belong to the metalloprotease sub-group of ATP-dependent proteases and are capable of degradation of incomplete or unassembled newly synthesized mitochondrial translation products [173, 174]. AAA proteases degrade protein domains initially present at the opposite membrane surface, implying that the proteases extract their substrate from the membrane bilayer for proteolysis [175]. The involvement of either one or both AAA proteases in the degradation of a specific substrate might be determined by the topology of the substrate itself and overlapping substrate specificity has been demonstrated between
the two AAA proteases. The AAA domain, located after the transmembrane domain, binds to unfolded solvent-exposed domains of membrane proteins [176], and ATP-dependent conformational changes of this domain might drive substrate unfolding and dislocation from the membrane. Yet other mitochondrial membrane translocases could be involved in the dislocation process in analogy to what happens in the endoplasmic reticulum [177-179].

Additionally, the m-AAA protease is required for at least two other processes in the biogenesis of mitochondria. First, the Afg3/Rca1 complex controls the splicing of COX1 and COB transcripts, two respiratory chain subunits encoded by intron-containing genes [180]. It has been suggested that the function of the protease is confined to introns encoding mRNA maturases. Since these proteins are synthesized as inactive proenzymes, the Afg3/Rca1 complex could mediate the proteolytic processing of the precursor forms [181]. Second, it has been postulated that the Afg3/Rca1 complex plays a chaperone-like role in respiratory complex assembly. Assembly of F1,F0-ATP synthase was found to depend on the m-AAA protease [182]. The fact that expression of COX1 and COB was restored after intron removal while cells remained respiratory incompetent suggested that the m-AAA protease affects the post-translational assembly of respiratory complexes [180]. However, evidence for the proposed role in assembly is indirect, and mainly based on (i) the reduced levels of respiratory complexes [182] [183] and (ii) the increased sensitivity of nuclear-encoded respiratory subunits to trypsin degradation, suggesting that these subunits remain unassembled [184] [180]. Recently, more extensive studies on the fate of newly synthesised mitochondrial proteins using pulse-chase protein labelling have been performed and these experiments do not support a direct role for the m-AAA protease in assembly. Instead, these results show that in m-AAA protease mutants, newly translated products are efficiently assembled, although newly assembled complexes are rapidly degraded. Moreover, accumulation of newly synthesised mitochondrial products does not seem to occur, suggesting that it may not be the accumulation of polypeptides in the membrane that causes the observed phenotypes. Instead, preliminary data point to altered membrane composition as a possible cause of the observed phenotypes [185].

Additionally, m-AAA protease is responsible, together with a putative rhomboid protease Pcpip, for the removal of the mitochondrial signal sequence of cytochrome c peroxidase (Ccpl) [186]. Ccpl is a nuclearly encoded protein that is imported into the mitochondrial intermembrane space and is involved in degradation of reactive oxygen species.

In yeast, proteolysis of mitochondrialy encoded respiratory chain subunits by AAA proteases results in the formation of free amino acid residues and a heterogeneous spectrum of peptides [187]. Interestingly, peptides of above 10 amino acids converge in the mitochondrial intermembrane space from where they are released to the cytosol. Peptides generated in the matrix by the m-AAA protease are actively transported across the inner membrane by an ABC (ATP-binding cassette) transporter (Mdl1), that shows high similarity to the transporter associated with antigen presentation (TAP) in higher eukaryotic cells [187]. Although the physiological role of the export of mitochondrial peptides is not known, authors speculate that it might allow the coordination of mitochondrial and nuclear gene expression in yeast [187]. It is noticeable that peptides derived from mitochondrial encoded membrane proteins have been detected at the cell surface of mammalian cells, where they are presented by class I MHC (Major
Histocompatibility Complex) molecules [188], suggesting that mitochondrial peptides could be used by the immune system for immune surveillance in mammals.

1.6.2 Quality control and disease

The importance of understanding the role of AAA proteases in mitochondrial biogenesis increased after the identification of the gene responsible for an autosomal recessive form of hereditary spastic paraplegia (HSP) [189]. This gene (SPG7) encodes paraplegin, a triple-A protein that shows greatest similarity to yeast Afg3p and Rca1p. HSP patients with paraplegin mutations have defects in mitochondrial oxidative phosphorylation, as evidenced by characteristic structural and functional abnormalities found in muscle biopsies [189]. HSP is a neurodegenerative disorder characterised by progressive lower limb spastic paralysis due to either failure in development or degeneration of the cortical axons. Why loss of paraplegin causes axonal degeneration is not clear. It may be that the terminal ends of long axons are especially vulnerable to mitochondrial defects in turnover or assembly.

1.6.3 Regulation of the quality control system

In yeast as in human, the molecular mechanisms underlying the cellular defects observed after inactivation of AAA proteases are as yet unclear. Defects associated with mutations in AAA proteases could be due to the impaired turnover/processing of a regulatory protein, though substrate proteins with potential regulatory functions have not yet been identified. Alternatively, both defective proteolysis and assembly functions will lead to the accumulation of misfolded proteins that will compromise mitochondrial function. In this context, the progressive nature of HSP could be explained by a cumulative defect in either turnover or accumulation of respiratory chain subunits. However, recent studies do not support defects in assembly or accumulation of newly synthesised mitochondrial proteins [185].

It is thus of great interest to understand how degradative and putative assembly functions of the quality control system are regulated. The functional homologue of AAA proteases in Escherichia coli (FtsH) has been the most extensively studied member of this family of proteases. In E. coli, a complex of two related proteins, HflC and HflK was found to negatively regulate the degradative activity of FtsH [190]. Yeast contains two proteins that share significant sequence similarity to HflC and HflK. These two proteins, Phb1 and Phb2, also called prohibitins, are highly conserved from yeast to human. Moreover, both yeast and mammalian prohibitins localise to mitochondria [191, 192] where they form a complex (see Chapter 3), suggesting that prohibitins might play a role in the regulation of the triple-A proteases. Based on this hypothesis, we set out to study functional and structural aspects of prohibitins. Figure 5 shows a schematic representation of the possible involvement of prohibitins in the biogenesis of OXPHOS complexes and its relationship with the quality control apparatus.
Figure 5. Involvement of the quality control system and the prohibitin complex (PHB complex) in the biogenesis of OXPHOS complexes. The quality control system of the mitochondrial inner membrane (m-AAA and i-AAA proteases) is depicted (left). The role of the PHB complex in the biogenesis of OXPHOS and its link to the proteases will be discussed in this thesis.

2. Outline of this thesis

This thesis focuses on mitochondrial prohibitin proteins (Phblp and Phb2p). Prohibitins have been ascribed many different functions since they were originally identified as potential negative regulators of the cell cycle. These proposed functions are reviewed and discussed in Chapter 2. This review is based on data presented in Chapter 3, where we show that prohibitins act as a membrane-bound chaperone that stabilizes newly synthesized mitochondrial proteins. In addition to the role of PHB proteins in respiratory complex assembly, putative roles in ageing and degenerative disorders are discussed in Chapter 2.

Chapter 3 reports results from biochemical studies in the yeast Saccharomyces cerevisiae showing that the PHB proteins form a high molecular weight complex in the mitochondrial inner membrane that stabilizes newly synthesized mitochondrial translation products by transiently interacting with them. This suggests a chaperone function that is further supported by sequence similarity to members of the hsp60 family.

Since knowing the structure of a protein complex may help to better understand its molecular mechanism of action, we performed structural studies on the prohibitin complex. Due to their location in the mitochondrial membrane and their size, prohibitins are difficult to purify for X-ray crystallography or NMR studies. In addition, low sequence similarity to known protein folds made them difficult to model. Using chemical crosslinking and mass spectrometry, we predicted a partial structure for the yeast PHB complex, which is described in Chapter 4.
Disruption of PHB proteins in yeast results in a reduced replicative lifespan of the cells [192] but not in an observable growth phenotype under laboratory conditions [193], suggesting that prohibitins are not essential for respiration. Without a clear phenotype, prohibitin function becomes difficult to investigate in the single yeast cell. Disruption of the Drosophila melanogaster homologue gene appeared to be lethal in the passage from larva to pupa [194], suggesting a stronger effect on the cellular metabolism of higher species. To further investigate this, in Chapter 5 we used Caenorhabditis elegans as a model organism to monitor the effect of PHB depletion during C. elegans development.

Finally, the data presented in this thesis will be summarised and discussed in Chapter 6 (General discussion). In addition, I will discuss some recent published data of interest not covered in the review presented in Chapter 2.

3. References


185. de Jong, L. University of Amsterdam (2003).


