The Energetic State of Mitochondria Modulates Complex III Biogenesis through the ATP-Dependent Activity of Bcs1

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SUMMARY

Our understanding of the mechanisms involved in mitochondrial biogenesis has continuously expanded during the last decades, yet little is known about how they are modulated to optimize the functioning of mitochondria. Here, we show that mutations in the ATP binding domain of Bcs1, a chaperone involved in the assembly of complex III, can be rescued by mutations that decrease the ATP hydrolytic activity of the ATP synthase. Our results reveal a Bcs1-mediated control loop in which the biogenesis of complex III is modulated by the energy-transducing activity of mitochondria. Although ATP is well known as a regulator of a number of cellular activities, we show here that ATP can be also used to modulate the biogenesis of an enzyme by controlling a specific chaperone involved in its assembly. Our study further highlights the intramitochondrial adenine nucleotide pool as a potential target for the treatment of Bcs1-based disorders.

INTRODUCTION

Mitochondrial oxidative phosphorylation (OXPHOS), which provides most of the ATP in animal cells, relies upon five multi-subunit complexes (I–V) embedded within the inner membrane of mitochondria. The respiratory complexes (I–IV) transfer electrons to the final acceptor, oxygen. This transfer is coupled to proton translocation across the inner membrane, and the resulting transmembrane proton gradient is used by the ATP synthase (complex V) to synthesize ATP from ADP and inorganic phosphate. Due to its dual genetic origin, nuclear and mitochondrial, the biogenesis of the OXPHOS system is an intricate process involving numerous factors that execute highly specific functions ranging from the synthesis of the individual subunits to their assembly into the respiratory complexes. In addition, the respiratory complexes are organized into supramolecular structures or “supercomplexes,” also called respirasomes, containing complexes I, III, and IV in higher eukaryotes and complexes III and IV in yeast (Schägger and Pfeiffer, 2000; Cruciat et al., 2000; Heinemeyer et al., 2007; Dudkina et al., 2011).

Complex III has a central position in the respiratory chain, allowing ubiquinol oxidation and cytochrome c reduction. It is an important site of proton translocation and production of reactive oxygen species. Complex III consists of 11 or 10 different subunits in mammals and yeast, respectively, three of which are catalytic: cytochrome b (Cytb), cytochrome c1 (Cyt1) and the Rieske-FeS protein Rip1 (Iwata et al., 1998; Hunte et al., 2000). In all eukaryotes, Cytb is encoded by the mitochondrial DNA, whereas the other complex III subunits have a nuclear origin. The complex is assembled through a dynamic modular pathway, starting with an early core containing Cytb and the subunits Qcr7 and Qcr8 and finishing with the incorporation of Rip1 (Figure 1A; for reviews, see Zara et al., 2009; Smith et al., 2012). Two proteins, Mzm1 and Bcs1, are required during the late stages of complex III assembly in yeast. Mzm1 appears to stabilize Rip1 (Cui et al., 2012). Deficiencies of Bcs1 lead to the accumulation of an inactive pre-complex III (pre-III) lacking Rip1 (Nobrega et al., 1992; Cruciat et al., 1999; 2000; Conte et al., 2011). Bcs1 mediates the translocation of Rip1 from the matrix to the intermembrane space, and the release of Rip1 depends on the hydrolysis of Bcs1-bound ATP (Wagener et al., 2011).

Bcs1 is detected in a high-molecular-weight complex that is anchored to the inner membrane and protruding into the matrix. An internal signal within the N-terminal domain targets Bcs1 to mitochondria (Fölsch et al., 1996). Bcs1 contains a Bcs1-specific domain and a highly conserved AAA region typical of the AAA-protein family (ATPase associated with diverse cellular activities; Figure 1B). This region contains the Walker A and B motifs of P loop ATPases involved in ATP binding and hydrolysis as well as a number of additional conserved structural elements such as the SRH (second region of homology). AAA proteins drive ATP-dependent dissociation, unfolding, or folding of nucleic acids and proteins (for review, see Hanson and Whiteheart, 2005). In mitochondria, the AAA proteins play a central role in the biogenesis and quality control of proteins (Gerdes et al., 2012).

Mutations in the human gene BCS1L (BCS1-like) are the most frequent nuclear mutations resulting in complex III-related
pathologies; very different clinical phenotypes are associated with these mutations, ranging from the mild Bjornstad syndrome to the lethal GRACILE syndrome (e.g., de Lonlay et al., 2001; Visapa¨ a¨ et al., 2002; De Meirleir et al., 2003; Hinson et al., 2007; Fernandez Vizarra et al., 2007; Mora´ n et al., 2010; Kotarsky et al., 2010; Leve´ en et al., 2011 ). An extensive mutational study of yeast Bcs1 has revealed the importance of the residues located at the junction between the Bcs1-specific and the AAA domains for the activity and stability of the protein ( Nouet et al., 2009 ). Interestingly, several human pathogenic mutations are located at this junction.

In this paper, we report the identification of extragenic compensatory mutations of respiratory-deficient bcs1 mutations located in the ATP binding domain of the yeast protein, among which one is the equivalent of a mutation found in a human patient. Remarkably, the compensatory mutations preferentially target the mitochondrial ATP synthase and lead to a strong decrease in the mitochondrial ATP hydrolytic activity while maintaining a sufficient level of ATP synthesis. We further show that increasing the ATP concentration in an in vitro assay also compensates for the Bcs1 deficiency. Based on these findings, we propose a model in which the ATP dependency of the protein Bcs1 is not just a requirement for its chaperon activity but also a way to couple the rate of complex III biogenesis to the energy-transducing activity of mitochondria.

RESULTS

Characterization of the bcs1-F342C Mutant

Previously, we isolated a yeast mutant with the single amino acid substitution F342C that modifies a highly conserved region of the AAA domain of Bcs1 ( Nouet et al., 2009; Figure S1 available online). According to the theoretical three-dimensional (3D) model of the yeast Bcs1 protein (Figure 1C), the residue...
F342 is located in the vicinity of the conserved SRH motif (see also Discussion). The bcs1-F342C mutant was unable to grow on respiratory substrates (Figure 1D), and it did not affect the steady-state level and oligomerization of Bcs1 (Figure 1E), suggesting that it probably decreased the activity of the protein.

As the OXPHOS complexes are organized into supramolecular structures, we have analyzed the effect of the bcs1-F342C mutation on supercomplexes III/IV and on ATP synthase oligomers. Under the blue native (BN)-PAGE conditions we used, mutation on supercomplexes III/IV and on ATP synthase oligobcs1 gesting that it probably decreased the activity of the protein.

An additional Atp2-containing complex of smaller size (sub-V) has been observed in a 2D experiment (Figure 1F). Due to its low amount, this complex could no longer be detected in 2D (Figure 1F). Thus, the oxygen consumption rate in mitochondria was substantially improved, with values estimated at ~70% in R3 and ~30% in R18 with respect to WT, versus <10% in the bcs1-F342C mutant (Figure 2E and Table S2). The residual oxygen consumption activity in the bcs1-F342C mutant did not induce any significant mitochondrial ATP synthesis, whereas a substantial ATP production of ~35% and ~15% with respect to WT was observed in R3 and R18, respectively. Thus, the atp1-V68G and atp2-A48D mutations improve the assembly of functional complex III and its insertion into supercomplexes and restore mitochondrial ATP synthase in the bcs1-F342C mutant.

The F1 Mutations atp1-V68G and atp2-A48D Lead to a Strong Decrease in the ATP Synthase Assembly and Hydrolitic Activity

We next determined how the atp1-V68G and atp2-A48D mutations impact ATP synthase. Being recessive, these mutations were expected to partially impair the function of the two F1 subunits. This possibility was examined first by measurements of the ATP hydrolitic activity of ATP synthase. Normally, when properly assembled into F1 oligomers, the Atp1 and Atp2 proteins are responsible for ~80%–90% of the ATP hydrolitic activity of mitochondria. While this activity was mostly unaffected in the bcs1-F342C mutant, it was drastically reduced by 95% in R3 and 80% in R18 with respect to WT (Figure 3A and Table S3). Similar deficits in ATP hydrolitic activity were observed in the single mutants atp1-V68G and atp2-A48D. As revealed by BN-PAGE, both R3 and R18, as well as the single atp1 and atp2 mutants, had a reduced content of fully assembled F1Fo complexes, and there was no indication of accumulation of free F1 (Figure 3B). However, despite the reduced content in F1, the steady levels of the Atp1 and Atp2 were essentially unaffected (Figure 3C). Previous work has shown that these proteins show a high tendency to form large inclusion bodies in the mitochondrial matrix when they cannot associate with each other (Ackerman and Tzagoloff, 1990; Lefebvre-Legendre et al., 2005). Aggregates strongly enriched in Atp1 and Atp2 proteins
were indeed observed on electronic micrographs of \textit{atp1-V68G} and \textit{atp2-A48D} cells (Figure S3). Thus, the lowering in mitochondrial ATP hydrolytic activity induced by the \textit{atp1-V68G} and \textit{atp2-A48D} mutations is mainly caused by a decreased ability of the Atp1 and Atp2 proteins to assemble with each other or by a diminished stability of the F\textsubscript{1} oligomers. As a result of this lower yield in F\textsubscript{1}, the ATP synthase proton-translocating domain F\textsubscript{o}, whose assembly is dependent on that of F\textsubscript{1} (Rak et al., 2009), also accumulated less efficiently in the \textit{atp1-V68G} and \textit{atp2-A48D} mutants, as shown by their low steady levels in the Atp6, a main component of the F\textsubscript{o} (Figure 3C).

The \textit{F\textsubscript{1}} Mutations \textit{atp1-V68G} and \textit{atp2-A48D} Lower the Energization of the Mitochondrial Inner Membrane by ATP

The influence of the \textit{bcs1-F342C}, \textit{atp1-V68G} and \textit{atp2-A48D} mutations was further investigated by mitochondrial membrane potential (\(\Delta\Psi\)) in vitro analyses. \(\Delta\Psi\) mainly results from the proton translocation by the respiratory complexes and the ATP synthase. Consistent with its very low respiratory activity, the membrane was poorly energized by ethanol in the \textit{bcs1-F342C} mutant in comparison to WT, whereas \(\Delta\Psi\) was restored in the revertants R3 and R18 due to their improved capacity to assemble complex III. In a first series of experiments (Figure 4A), we tested the effect of the addition of a small amount of ADP, which induces a \(\Delta\Psi\) consumption while imported into mitochondria. Return to the potential established before the addition of ADP (see Figure 1F legend).

A Specific Mutation in the F\textsubscript{o} Subunit Atp6 also Rescues the \textit{bcs1-F342C} Mutant

In order to better understand the functional links between ATP synthase and Bcs1, we have tested other mutations in ATP synthase for their capacity to rescue the \textit{bcs1-F342C} mutant. We selected three mutations (\textit{atp6-W136R}, \textit{atp6-L183P}, and \textit{atp6-V499F}) as shown by their low steady levels in \textit{atp1-V68G} and \textit{atp2-A48D} cells (Figure S3). Thus, the lowering in mitochondrial ATP hydrolytic activity induced by the \textit{bcs1-F342C}, \textit{atp1-V68G} and \textit{atp2-A48D} mutations is mainly caused by a decreased ability of the Atp1 and Atp2 proteins to assemble with each other or by a diminished stability of the F\textsubscript{1} oligomers. As a result of this lower yield in F\textsubscript{1}, the ATP synthase proton-translocating domain F\textsubscript{o}, whose assembly is dependent on that of F\textsubscript{1} (Rak et al., 2009), also accumulated less efficiently in the \textit{atp1-V68G} and \textit{atp2-A48D} mutants, as shown by their low steady levels in the Atp6, a main component of the F\textsubscript{o} (Figure 3C).
Hydrolytic Activity of F1 Assembly of ATP Synthase and Lead to a Strong Decrease in the Fo proton-translocating domain encoded by the mitochondrial atp6 gene.

Mitochondria were purified from WT, bcs1 atp1 atp2 atp6 V68G, R18 atp1 A48D, R3 bcs1 F342C, R18 atp1 A48D, and the loading control Nam1. See also Figure S3.

Figure 3. The ATP Dependency of Bcs1 and Complex III Biogenesis

Increasing the ATP Concentration Compensates for the In Vitro ATPase Deficiency of the bcs1-F342C Mutant

In order to test if and how the bcs1-F342C mutation affected the activity of Bcs1, we have set up an in vitro assay allowing the determination of its ATPase activity. We have purified WT and mutated Bcs1 proteins carrying a hexahistidine tag fused atp6-L247P affecting Atp6, an essential component of the Fo proton-translocating domain encoded by the mitochondrial genome. These mutations correspond to mutations found in human patients suffering from NARP (neuropathy, ataxia, and retinitis pigmentosa) or MILS (maternally inherited Leigh syndrome) (for review, see Houstek et al., 2009). Both the rate of mitochondrial ATP synthesis and hydrolysis are strongly reduced by 70% and 90%, respectively, in the atp6-W136R mutant, while the assembly/stability of the ATP synthase is normal (Kucharczyk et al., 2012, Figure 5A). The atp6-L183P and atp6-L247P partially compromise the assembly and/or stability of Atp6 within the ATP synthase, leading to mitochondrial ATP production deficits of 40%–60%, whereas the ATP hydrolytic activity of mitochondria is only modestly decreased (Kucharczyk et al., 2009; 2010). Each atp6 mutation was combined with the bcs1-F342C mutation (see Table S1), and only the atp6-W136R mutation was able to restore the growth on glycerol (Figure 5B). In the atp6-W136R bcs1-F342C double mutant, complex III activity and the insertion of this complex into supercomplexes were partially recovered, whereas they were not in the atp6-L183P bcs1-F342C (Figures 5C and 5D). Thus, the F1 mutations (atp1-V68G or atp2-A48D) and the Fo mutation (atp6-W136R) that all lead to a strong decrease in the rate of mitochondrial ATP hydrolysis can compensate for the defect in complex III assembly due to the bcs1-F342C mutation.

ATP Synthase Mutations Can also Rescue a bcs1 Mutation Found to be Pathogenic in Humans

The results described above might hold promise for developing therapeutic pathways for human diseases caused by Bcs1 deficiencies. In this respect, since the bcs1-F342C mutation has no known equivalent in human patients, we wanted to know whether the ATP synthase-mediated compensation could also rescue, in yeast, a bcs1 mutation that was known to be pathogenic in humans. We tested the mutation bcs1-F368I found in a patient with an early-onset encephalopathy (Fernandez-Vizarra et al., 2007). We constructed, in yeast, the bcs1-F401I mutation that is the equivalent of the human bcs1-F368I mutation (Figure S1). According to theoretical three-dimensional (3D) models of the human and yeast Bcs1 proteins, this mutation is located near the ATP binding site of Bcs1 (Figure 5E). The bcs1-F401I mutation led to a stringent respiratory growth deficiency (Figure 5F) and very severely compromised the activity of complex III (Figure 5G). As with the bcs1-F342C mutation, the steady-state levels and oligomerization of Bcs1 were not affected in the bcs1-F401I mutant (Figure 5E). Thus, the two mutations seem to have a very similar impact on Bcs1. Two of the ATP synthase mutations that rescue the bcs1-F342C mutant, one in F1 (atp1-V68G) and one in Fo (atp6-W136R), were tested for their capacity to compensate the bcs1-F401I mutant. The two double mutants (bcs1-F401I, atp1-V68G and bcs1-F401I, atp6-W136R) were able to grow on respiratory substrates (Figure 5F) and showed an improved complex III activity (Figure 5G). These results suggest that Bcs1 mutations responsible for human diseases might be treatable by modulation of the ATP synthase activity.

Increasing the ATP Concentration Compensates for the In Vitro ATPase Deficiency of the bcs1-F342C Mutant

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In order to test if and how the bcs1-F342C mutation affected the activity of Bcs1, we have set up an in vitro assay allowing the determination of its ATPase activity. We have purified WT and mutated Bcs1 proteins carrying a hexahistidine tag fused
Rip1 variants (Cui et al., 2012). These interactions are consistent with the absence of Bcs1 and Rip1, as previously proposed with immunoprecipitation (IP) data suggesting that pre-III and complex IV can interact inresulting from a block in Rip1 assembly. The immunoprecipitation data, in conjunction with the results from the ATP synthase activity assays, indicate that modulation of the ATP synthase activity can indeed improve the activity of a mutated Bcs1 protein via its ATP dependency.

We report that modulation of the ATP synthase activity can improve complex III assembly in the revertant strains (bcs1-F342C + atp1-V68G or atp2-A48D), the assembly of Rip1 within the complex III was substantially improved, as compared to the single bcs1-F342C mutant.

To further understand how ATP synthase defects could improve complex III assembly in the bcs1-F342C mutant, we tested its compensation by other mutations of this enzyme. Substantial rescue was observed with the mutation W136R tested its compensation by other mutations of this enzyme. Unexpectedly, a main target for compensatory mutations rescuing the bcs1-F342C mutant was the ATP synthase. Four spontaneous compensatory mutations were identified as single amino acid changes in the two subunits, Atp1 and Atp2, that form the ATP synthase catalytic head. The functional consequences of two of these mutations, V68G in Atp1 and A48D in Atp2, were characterized. Both changes severely compromise the capacity of the Atp1 and Atp2 subunits to bind to each other, leading to their accumulation in the mitochondrial matrix as large aggregates. As a result, the content in fully assembled complexes was substantially lowered, leading to a decrease in the enzyme’s synthetic and hydrolytic activities. Nevertheless, in the revertant strains (bcs1-F342C + atp1-V68G or atp2-A48D), the assembly of Rip1 within the complex III was substantially improved, as compared to the single bcs1-F342C mutant.

To further understand how ATP synthase defects could improve complex III assembly in the bcs1-F342C mutant, we tested its compensation by other mutations of this enzyme. Substantial rescue was observed with the mutation W136R in the subunit Atp6 of the ATP synthase proton channel. This mutation had no effect on the assembly of ATP synthase but seriously impaired its functioning, as shown by strong deficits in the subunit Atp6 of the ATP synthase proton channel. This mutation had no effect on the assembly of ATP synthase but seriously impaired its functioning, as shown by strong deficits in both the ATP hydrolytic and synthetic activities of mitochondrial (Kucharczyk et al., 2012). The bcs1-F342C mutant was not rescued by two other mutations (L183P and L247P) that partially compromise incorporation and/or stability of Atp6 within the ATP synthase, and lead to similar decreases in the rate of ATP synthesis but with only minimal effect on the ATP hydrolytic activity.

It is difficult to understand how reducing the capacity of the ATP synthase to produce ATP could rescue Bcs1-mediated

to their C terminus (Figure S4). The tag had no influence on the chaperone activity of Bcs1, and both proteins kept their capacity to form oligomers both in vivo and after purification from mitochondrial digitonin extracts (Figure 6A). The ATP hydrolytic activity of the Bcs1 proteins was measured at different concentrations of ATP; at 2.5 and 5 mM, the bcs1-F342C protein had a rate of ATP hydrolysis 2- to 3-fold lower than that of the WT protein (Figure 6B). However, at higher ATP concentrations (10 or 20 mM), no significant difference was observed between the mutant and the WT (Figure 6C). Similar ATPase activities were obtained in the absence of oligomycin, which rules out the contamination by complex V during Bcs1 purification. Thus, it can be inferred that the reduced hydrolytic activity of bcs1-F342C protein is probably due to a lower affinity of the mutated protein for the nucleotide, and increasing its concentration in the assay compensates this deficiency.

**DISCUSSION**

Previous work has established that incorporation of the Rip1 protein into the yeast complex III involves a protein, Bcs1, belonging to the AAA protein family (Nobrega et al., 1992; Cruciat et al., 1999; 2000; Conte et al., 2011; Wagener et al., 2011). Here, we report that modulation of the ATP synthase activity can improve the activity of a mutated Bcs1 protein via its ATP dependency.

The mutant bcs1-F342C displayed large amounts of pre-III resulting from a block in Rip1 assembly. The immunoprecipitation (IP) data suggest that pre-III and complex IV can interact in the absence of Bcs1 and Rip1, as previously proposed with Rip1 variants (Cui et al., 2012). These interactions are consistent with current structural models that predict that Rip1 is not located at the interface between the two complexes (Heinemeyer et al., 2007). However, according to the BN-PAGE data, the integration of complex IV into supercomplexes is compromised, suggesting that Bcs1 and Rip1 are essential to maintaining the integrity of respiratory chain supercomplexes. Combined defects of OXPHOS complexes were also reported in BCS1L-deficient patients (Fernandez-Vizarra et al., 2007; Morán et al., 2010).

Unexpectedly, a main target for compensatory mutations rescuing the bcs1-F342C mutant was the ATP synthase. Four spontaneous compensatory mutations were identified as single amino acid changes in the two subunits, Atp1 and Atp2, that form the ATP synthase catalytic head. The functional consequences of two of these mutations, V68G in Atp1 and A48D in Atp2, were characterized. Both changes severely compromise the capacity of the Atp1 and Atp2 subunits to bind to each other, leading to their accumulation in the mitochondrial matrix as large aggregates. As a result, the content in fully assembled ATP synthase was substantially lowered, leading to a decrease in the enzyme’s synthetic and hydrolytic activities. Nevertheless, in the revertant strains (bcs1-F342C + atp1-V68G or atp2-A48D), the assembly of Rip1 within the complex III was substantially improved, as compared to the single bcs1-F342C mutant.
defects in complex III assembly. It is important to keep in mind that ATP synthase is a reversible enzyme that can hydrolyse ATP coupled to the pumping of protons out of the mitochondrial matrix through the F_0 membrane domain (for review, see Ackerman and Tzagoloff, 2005). In the \( bcs1 \text{-} F342C \) mutant, the electron flow and proton gradient generation are severely impaired due to a drastic effect on complex III assembly; the resulting level of ATP synthesized in this mutant is under the detection threshold. However, the ATP synthase is normally assembled and exhibits a wild-type hydrolytic activity that can be modulated by the compensatory mutations. Thus, rather than a reduced capacity of the ATP synthase to produce ATP, it is the low \( F_1 \)-mediated ATP hydrolysis that is responsible for improving the assembly of complex III in the \( bcs1 \text{-} F342C \) mutant.

As the steady-state levels and oligomerization of Bcs1 were not affected by the F342C mutation, it is probable that the less-efficient capacity to assemble Rip1 is due to an altered activity of the protein. The mutated residue is within the AAA domain of Bcs1, close to the SHR motif that is known to be required for ATP hydrolysis in other AAA proteins (Karata et al., 1999; Hanson and Whiteheart, 2005). Modeling of the phenylalanine-to-cysteine substitution in the theoretical structure of Bcs1 suggests that the interactions between the position 342 and conserved amino acids of the SRH motif are indeed modified (Figure S1). Thus, the activity of Bcs1 might be compromised by less-efficient ATP hydrolysis. This hypothesis is supported by the lower ATPase activity of the mutated, compared to the WT purified, Bcs1 protein at ATP concentrations of 2.5–5 mM, but nearly the same activity at higher concentrations. Thus, it can be inferred that the compensatory activity, conferred by a strong decrease in \( F_1 \)-mediated ATP hydrolytic activity, results from a higher availability of ATP within mitochondria increasing the ATPase activity of the mutated Bcs1 protein and concomitant insertion of Rip1 to give fully assembled complex III. This would allow the reestablishment of a proton gradient and the synthesis of ATP by the remaining \( F_1 \text{-} F_0 \) complexes. The resulting ATP synthesis would further increase the matrix ATP content and stimulate Bcs1 activity. According to this suppressor mechanism, a \( bcs1 \) mutation not affecting the AAA domain should not be suppressed by the ATP synthase mutations. This was indeed observed (data not shown).

The genetic interaction between Bcs1 and ATP synthase revealed by the present study leads us to propose a model in which the complex III biogenesis would be modulated by the energetic state of mitochondria (see Figure 7). When yeast cells rely on oxidative phosphorylation, the level of ATP inside mitochondria is high and exchanged against cytosolic ADP to provide the extramitochondrial compartment of the cell with energy. Large amounts of complex III are required. In cells producing ATP by fermentation, the intramitochondrial concentration of ATP is low, the glycolytic ATP is imported into mitochondria by the ADP/ATP translocator, and there is no

Figure 5. Mutations in Subunits of \( F_1 \) and \( F_0 \) Rescue \( bcs1 \) Mutations, One of which Is a Human Disease-Related Mutation Modeled in Yeast

(A) Comparison of ATP hydrolysis of \( atp1 \text{-} V68G \), \( atp2 \text{-} V48D \), and the three \( atp6 \) mutants. Mutants that compensate for \( bcs1 \text{-} F342C \) are in bold. See legend of Figure 3A and Table S3.

(B) Dilution series of cells of the WT, \( bcs1 \text{-} F342C \), \( atp6 \text{-} W136R \), \( atp6 \text{-} L183P \), and the double mutants \( bcs1 \text{-} F342C \) and \( atp6 \text{-} W136R \) or \( atp6 \text{-} L183P \) were grown for 3 days at 28°C.

(C) Complex III activity as in legend of Figure 2D. Data are represented as mean ± SEM.

(D) SDS-PAGE and BN-PAGE analysis of mitochondrial proteins as in Figures 1E and 1G.

(E) Theoretical structural model of the AAA domain of the yeast Bcs1 with the positions of the amino acids F342, F401, and ADP. Mitochondrial complexes of WT and \( bcs1 \text{-} F401I \) were analyzed by BN-PAGE and immunoblotted with anti-Bcs1 antibody as in Figure 1E.

(F) Dilution series of cells of WT, \( bcs1 \text{-} F401I \), and of the double mutants \( atp6 \text{-} W136R bcs1 \text{-} F401I \) and \( atp1 \text{-} V68G bcs1 \text{-} F401I \) were grown for 4 days at 28°C.

(G) Complex III activity as in legend of Figure 2D. Data are represented as mean ± SEM.

modified (Figure S1). Thus, the activity of Bcs1 might be compromised by less-efficient ATP hydrolysis. This hypothesis is supported by the lower ATPase activity of the mutated, compared to the WT purified, Bcs1 protein at ATP concentrations of 2.5–5 mM, but nearly the same activity
need to produce large amounts of complex III. Thus, we propose that the ATP-dependent activity of Bcs1 is not just a requirement to exercise its chaperon activity, but also a way to couple the rate of complex III biogenesis to the energy-transducing activity of mitochondria.

The importance of ATP in the control of cellular activities is well established. There are numerous examples of such control in catabolic and anabolic pathways, like glycolysis, the Krebs cycle, and the electron transport chain of mitochondria. However, in all of these examples, ATP regulates the activity of an enzyme (e.g., cytochrome oxidase; Beauvoit and Rigoulet, 2001; Ramzan et al., 2010), whereas in the case of Bcs1, ATP could be used to modulate a late step in the assembly of an enzyme, complex III. This work shows that ATP might influence the biogenesis of an enzyme by controlling a protein specifically involved in its assembly. In the future, it would be interesting to determine whether other major ATP-dependent systems involved in mitochondrial quality control, like the m- and i-AAA proteases, are similarly modulated by the energetic activity of mitochondria.

The present study further defined the intramitochondrial adenine nucleotide pool as a potential target to treat Bcs1-based diseases, since this ATP-dependent compensatory mechanism is active on another yeast-modeled bcs1 mutation found in a human patient. We recently showed that yeast models of ATP synthase disorders could be used for the screening of drugs active against human diseases caused by defects in this enzyme (Couplan et al., 2011). Our results indicate that such an approach might also be fruitful in the case of Bcs1-based disorders.
**Cell Metabolism**

**ATP Dependency of Bcs1 and Complex III Biogenesis**

**EXPERIMENTAL PROCEDURES**

**Strains and Media**

*S. cerevisiae* strains are listed in Table S1. The nonfermentable media contain 2% glycerol, and the fermentable media contain either 2% glucose or 2% galactose with 0.1% glucose. Tetrad dissection was performed using a Singer MSM micromanipulator.

**Genetic Identification of the Compensatory Mutations**

Respiratory-competent revertants were isolated after plating independent subclones of the *bcs1-F342C* mutant on glycerol medium. Genetic crosses showed that the compensatory mutations were nuclear and extragenic and allowed the selection of strains carrying only the compensatory mutation associated to the WT *BCS1* gene. Further crosses suggested that the compensatory mutations of revertants R2, R12, and R18 are located in the same gene. We have constructed the double mutant carrying the compensatory mutations of R3 and R18 associated to the WT *BCS1* gene and shown that it exhibits a complete respiratory deficiency. After transformation of this double mutant with the wild-type genomic library, two classes of respiratory-competent transformants carrying ATP1 or ATP2 were isolated. Sequencing of these two genes revealed that R3 carries a mutation in ATP1, and R2, R12, and R18 carry mutations in ATP2.

**Gene Deletion, Site-Directed Mutagenesis, and Epitope Tagging**

The genes were deleted in the WT strain (CW252) by replacing the open reading frames (ORFs) with the *URA3, LEU2, or KanR* markers (see Table S1). The *bcs1-F401I* mutant was constructed by site-directed mutagenesis with the Stratagene QuickChange Kit and inserted at the chromosomal *BCS1* locus. Gct7 as well as the WT and mutant Bcs1 proteins were tagged at their C-termini with c-Myc or hexahistidine epitopes, respectively (Longtine et al., 1998). We verified that the introduction of the tag did not induce a respiratory deficiency. All the constructions were verified by PCR amplification and sequencing.

**Mitochondria Preparation, SDS-PAGE, and BN-PAGE**

Cells were grown overnight at 28°C in galactose medium, and mitochondria were isolated according to Lemaire and Dujardin (2008). Mitochondrial proteins were analyzed on 12% SDS-PAGE. For BN-PAGE, mitochondria were isolated according to Lemaire and Dujardin (2008). Mitochondrial proteins were analyzed on 12% SDS-PAGE. For BN-PAGE, mitochondria were isolated according to Lemaire and Dujardin (2008).

**Coimmunoprecipitation Experiments**

Mitochondria were solubilized in 50 mM Tris HCl (pH 7.4), 100 mM NaCl, 1% digitonin for 30 min at 4°C and centrifuged for 15 min at 100,000 × g. The supernatants were incubated with polyclonal anti-c-Myc antibodies and Alexa Bourand-Plantefol for technical assistance. J.O. was supported by Fondation pour la Recherche Médicale.

**Determination of the Activities of the Respiratory Complexes III and IV**

The activities were measured spectrophotometrically at 550 nm at 25°C on 2.5–10 μg of isolated mitochondria (Lemaire and Dujardin, 2008). The ubiquinol cytochrome c oxidoreductase (complex III) activity was assayed by the rate of reduction of cytochrome c in the presence of saturating amounts of decylubiquinol, and the cytochrome c oxidase (complex IV) activity was assayed by the rate of cytochrome c oxidation. The inhibitors, antimycin for complex III and KCN for complex IV, were used to test the specificity of the signal.

**Membrane Potential, ATP Synthesis, and Hydrolysis Measurements**

Variations of the membrane potential (∆ψ) were evaluated in respiration buffer by measurement of rhodamine 123 (Rh-123) fluorescence quenching with a SAFAS Monofluor spectrofluorometer. Mitochondria were energized using ethanol as a respiratory substrate instead of NADH because fluorescence of the latter overlaps that of Rh-123. To determine ATP synthesis rates, mitochondria (0.3 mg) were placed in a 2 ml thermostatically controlled chamber at 26°C in respiration buffer. The reaction was started by the addition of 4 mM NADH and 1 mM ADP and stopped with 3.5% perchloric acid, 12.5 mM EDTA. Samples were then neutralized to pH 6.5 by addition of 2 M KOH/0.3 M MOPS. The luciferin/luciferase assay (Thermo Labystems) was used to determine ATP concentrations. The specific ATPase activity at pH 8.4 of nonosmotically protected mitochondria (20 μg of proteins) was measured in the presence of saturating amounts of ATP with or without oligomycin (Lemaire and Dujardin, 2008).

**Purification by Ni-NTA Chromatography of Bcs1 Proteins and Measurements of In Vitro ATPase Activity**

A total of 10 mg of mitochondrial proteins was solubilized in 1 ml of buffer A (50 mM NaCl, 15% w/v glycerol, 15 mM imidazole, 50 mM sodium phosphate [pH 7.9]) with 2% digitonin. After 30 min of incubation at 4°C, the extract was clarified by centrifugation at 25,000 × g for 30 min at 4°C. The supernatant was mixed with 0.25 ml Ni-NTA agarose beads (QiAGEN) and washed with buffer A. After an overnight incubation at 4°C, the flow through was collected by centrifugation at 1,000 × g for 1 min at 4°C. Beads were washed with 40 volumes of buffer A by centrifugation at 1,000 × g for 1 min at 4°C, and Bcs1 proteins were eluted with buffer E (50 mM NaCl, 15% w/v glycerol, 250 mM imidazole, 50 mM sodium phosphate [pH 7.9]). The in vitro ATPase activity was measured at 28°C under magnetic stirring in 1 ml of activity buffer (20 units ml−1 of pyruvate kinase, 30 units ml−1 of lactate dehydrogenase, 2 mM phosphoenol pyruvate, 0.2 mM NADH, 5 mM MgCl2, 50 mM HEPES [pH 7.5], and ATP at the concentrations 0, 2.5, 5, 10, or 20 mM). ATP concentrations of 0–5 mM were previously used by Augustin et al. (2009). The molar extinction coefficient of NADH at 340 nm was 6.22 M−1 cm−1, and the path length was 1 cm.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.08.017.

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