The Rieske Iron-Sulfur Protein: Import and Assembly into the Cytochrome $bc_1$ Complex of Yeast Mitochondria

Laura Conte and Vincenzo Zara

Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Via Pro.v.le Lecce-Monteroni, 73100 Lecce, Italy

Correspondence should be addressed to Vincenzo Zara, vincenzo.zara@unisalento.it

Received 31 January 2011; Accepted 12 March 2011

Academic Editor: Takao Yagi

Copyright © 2011 L. Conte and V. Zara. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Rieske iron-sulfur protein, one of the catalytic subunits of the cytochrome $bc_1$ complex, is involved in electron transfer at the level of the inner membrane of yeast mitochondria. The Rieske iron-sulfur protein is encoded by nuclear DNA and, after being synthesized in the cytosol, is imported into mitochondria with the help of a cleavable N-terminal presequence. The imported protein, besides incorporating the 2Fe-2S cluster, also interacts with other catalytic and non-catalytic subunits of the cytochrome $bc_1$ complex, thereby assembling into the mature and functional respiratory complex. In this paper, we summarize the most recent findings on the import and assembly of the Rieske iron-sulfur protein into *Saccharomyces cerevisiae* mitochondria, also discussing a possible role of this protein both in the dimerization of the cytochrome $bc_1$ complex and in the interaction of this homodimer with other complexes of the mitochondrial respiratory chain.

1. Introduction

The Rieske iron-sulfur protein (Rip1p or ISP) is one of the catalytic subunits of the cytochrome $bc_1$ complex (also known as complex III of the mitochondrial respiratory chain). This respiratory enzyme assembles as a mature and functional homodimer in the inner membrane of mitochondria; besides ISP, it incorporates two further catalytic subunits, cytochrome $b$ and cytochrome $c_1$, and seven noncatalytic subunits (core protein 1 and core protein 2, Qcr6p, Qcr7p, Qcr8p, Qcr9p, Qcr10p). These last subunits are also named “supernumerary” because they are absent in some bacterial equivalents of cytochrome $bc_1$ complex [1, 2]. ISP is essential for the activity of this complex and for the respiratory growth of *Saccharomyces cerevisiae* mitochondria [3–5]. The gene R1P1 is conserved across both prokaryotes and eukaryotes [2], and its human homolog is UQCRFS1. A comparison of the amino acid sequence has revealed a high degree of similarity, especially in the C-terminal region of the protein. Functionally, ISP strictly cooperates with cytochrome $b$ and cytochrome $c_1$ in the electron transfer catalyzed by the mitochondrial cytochrome $bc_1$ complex. In doing so, ISP requires the presence of a 2Fe-2S cluster, which is bound to the movable C-terminal domain of the protein located in the mitochondrial intermembrane space. This intermembrane space domain is connected by a short amino acid linker to the hydrophobic N-terminal $\alpha$-helix, which is inserted into the inner mitochondrial membrane. The studies carried out over the last decades on the import and assembly of ISP into mitochondria have revealed a well-defined pathway of interactions of this protein with several $bc_1$ complex subunits. In addition, the topology of ISP in the large homodimeric cytochrome $bc_1$ complex structure is quite characteristic; indeed, while the transmembrane domain of ISP strictly interacts with the $bc_1$ subunits of one monomer, the intermembrane space domain of the same protein strictly interacts with the $bc_1$ subunits of the other monomer. Therefore, the implications of ISP biogenesis and assembly appear intriguing. It is indeed possible that ISP, besides representing an obligatory component for the redox activity of the $bc_1$ complex, may also play a role in building up the mature and functional complex III in the mitochondrial membranes.
2. Structural Characteristics of ISP in the Cytochrome bc₁ Complex

The resolution of the crystal structure of the cytochrome bc₁ complex isolated from beef [6, 7], chicken [8], and yeast [9] mitochondria has revealed a dimeric structural arrangement of this complex. In the crystal structure, the two copies of ISP are intertwined and oriented in a trans manner with respect to each monomer (Figure 1). Each molecule of ISP is localized on the outer surface of the inner mitochondrial membrane and consists of three separate domains: (i) a membrane-spanning α-helix domain at its N-terminus; (ii) a soluble C-terminal extramembranous domain containing the bulk of the protein and the iron-sulfur cluster; (iii) a short segment of 7 to 9 amino acids connecting the extrinsic domain to the membrane-spanning domain [7–9].

The structures of the N-terminal domains of yeast and bovine/chicken Rieske proteins are different, and this difference coincides with a low sequence homology [9]. The amino terminus of yeast ISP is relatively enriched in polar amino acids [3]. There is a stretch of hydrophobic amino acids distal to the N-terminus, beginning at alanine 55; this hydrophobic sequence and the polar amino acids anchor ISP to the bc₁ complex [2]. More in detail, the first ten residues of the N-terminal polypeptide of yeast ISP form one strand of a β-sheet to which cytochrome c₁, Qcr8p, and core protein 1 contribute [9]. This secondary element is also present in the bovine subunit [7]. In the mammalian crystal structure, the transmembrane α-helix of ISP interacts with the transmembrane helices of Qcr9p and Qcr10p [7]. Despite the low sequence homology and because of the similar overall structure of the yeast compared with the bovine and chicken complexes, the authors assumed that the location of the transmembrane helix may be very similar in the complexes [8, 9].

The structure of the soluble extramembranous domain of yeast ISP [9] is highly similar to the homologous domain of the bovine complex [7]. This extrinsic domain (residues 93–215) is a flat spherical module containing three layers of antiparallel β-sheets and forms a functional unit with cytochrome b and cytochrome c₁ of the second monomer. The iron-sulfur cluster is coordinated by Cys159, His161, Cys178, and His181 with Cys164 and Cys180 forming a disulfide bridge, which stabilizes the cluster [9, 10]. Failure to insert the iron-sulfur cluster impairs assembly of ISP into the bc₁ complex, as evidenced by the susceptibility of this catalytic subunit to protease degradation [11]. Eliminating the disulfide bridge did not significantly impair the stability of the cluster but indirectly damaged the ubiquinol oxidation site [12]. The destruction of the Rieske iron-sulfur cluster by hematoporphyrin-promoted photoinactivation resulted in a proton-permeable bc₁ complex [13]. Another study confirmed that the elimination of the iron-sulfur cluster in mutant bc₁ complexes opened up an otherwise closed proton channel within this respiratory complex [14]. Therefore, it was speculated that in the normal catalytic cycle of the bc₁ complex, the iron-sulfur cluster may function as a proton-exiting gate regulating the controlled, vectorial extrusion of protons across the bc₁ complex [14].

One of the striking features of the cytochrome bc₁ complex structures was the finding that the catalytic domain of ISP, which carries the iron-sulfur cluster, is connected to the transmembrane anchor by a flexible linker region, also called tether domain. In fact, despite the changes in the position of the iron-sulfur cluster, the structures of the extrinsic and the membrane-spanning domains remain unchanged in all of the crystal structures, suggesting that the movement of the iron-sulfur cluster results from the rotation of the flexible linker, which allows the catalytic domain to move during electron transfer between two positions, proximal to cytochrome b and to cytochrome c₁. This motion of the catalytic domain of ISP is essential to shuttle reducing equivalents from ubiquinol to the heme group of cytochrome c₁ [7, 8, 15, 16]. The tether domain, a highly conserved region of ISP, shows the sequence TADVLAMA in yeast (residues 85–92). The presence of three highly conserved alanine residues in this region suggests that these small amino acid residues may provide the needed flexibility for the proposed stretching of the “tether” [17]. Mutations that limit the flexibility of the linker region caused a significant decrease in the activity of cytochrome bc₁ complex [18–22]. These results demonstrated that the flexibility of this region is essential for electron transfer activity, consistent with the movement of ISP. Other modifications, such as changes of the length of the linker region, impair the interaction of ubiquinol with cytochrome bc₁ complex [19, 23, 24].

These mutations probably alter the distance between ISP and cytochrome b in such a way that the cleft between these two subunits, which is the ubiquinol oxidase site, does not properly accommodate ubiquinol. Thus, it appears that the tether domain can tune the flexibility, the mobility, and the positioning of ISP to allow the finest movement of this protein between cytochrome b and cytochrome c₁ during catalysis. However, recent structural and biochemical evidence demonstrated that also cytochrome b controls the motion of ISP through its affinity for the ISP extrinsic domain, and this intricate mechanism explains the high fidelity of the electron transfer in the cytochrome bc₁ complex [25–28].

3. Import and Maturation of ISP into Yeast Mitochondria

Like the majority of mitochondrial proteins, ISP is encoded by a nuclear gene, translated on cytosolic ribosomes as a precursor protein, and then imported into mitochondria with the help of an N-terminal presequence [29, 30]. The import of ISP and the two-step processing of the precursor protein to its mature size was first characterized in Neurospora crassa mitochondria [31]. As in the case of import of other mitochondrial proteins, the entry of ISP into the inner mitochondrial membrane required a membrane potential across the lipid bilayer. After import into energized mitochondria, the protein became resistant to externally added proteases because of its integration into the inner mitochondrial membrane. In Saccharomyces cerevisiae, the precursor protein is made up of 215 amino acids and includes an N-terminal presequence of 30 amino
acids. This presequence is cleaved off in two steps inside mitochondria, thus generating a mature ISP of 185 amino acids [32–34]. At first, the matrix processing peptidase (MPP) removes a 22-amino acid peptide from the N-terminal of the ISP precursor protein (p-ISP) producing the intermediate form of this protein (i-ISP). The mitochondrial intermediate peptidase (MIP) then removes an octapeptide from i-ISP to generate the mature length iron-sulfur protein (m-ISP), which contains 185 amino acids. On the contrary, in mammals the presequence is cleaved off in a single step and retained inside the cytochrome \( \text{bc}_1 \) complex [35]. The biological function of the intermediate octapeptide and the reason for the two-steps processing occurring only in distinct ISP precursors belonging to certain organisms, such as \( \text{Saccharomyces cerevisiae} \), are not fully understood. However, it has been demonstrated that the two-step processing of the ISP precursors is not essential for the import of the protein into mitochondria and its assembly into the cytochrome \( \text{bc}_1 \) complex; in fact, mutated forms of ISP that are processed to mature size in a single step are properly and functionally assembled [36].

It was also controversial whether i-ISP was first incorporated into the \( \text{bc}_1 \) complex and then cleaved [37–39] or whether only m-ISP could be assembled into the \( \text{bc}_1 \) complex [33]. Site-directed mutagenesis experiments demonstrated that the second processing step takes place after i-ISP has been assembled into the yeast mitochondrial \( \text{bc}_1 \) complex and that the iron-sulfur cluster is inserted into the apoprotein before MIP cleaves off the second part of the presequence [40]. Interestingly, it was also found that i-ISP was not stably inserted into the complex unless cytochrome \( c_1 \) was processed to its mature size. In fact, the block of cytochrome \( c_1 \) maturation found in a yeast mutant strain lacking Qcr6p also impaired \textit{in situ} maturation of ISP [41]. These findings suggest that the import and assembly of the various subunits into the cytochrome \( \text{bc}_1 \) complex occur at different rates in an ordered manner.

In this context, it is important to highlight that also the assembly of iron-sulfur cluster is a complex process involving multiple highly conserved components [42, 43]. In eukaryotes, mitochondria perform a central role in iron-sulfur cluster maturation because they harbor the so-called ISC (iron-sulfur cluster) assembly machinery. In general, maturation of mitochondrial iron-sulfur proteins, such as ISP, involves two major steps [44, 45]. First, an iron-sulfur cluster intermediate is transiently assembled on a scaffold protein (ISCU in humans or Isu1 or Isu2 in yeast) and, subsequently, transferred to the target apoproteins. The sulfur originates from cysteine via the activity of the cysteine desulfurase Nfs1, which is coordinated with the essential accessory protein Isd11. Although the source of sulfur for iron-sulfur cluster formation is clear, the pathway of iron to Isu1 is less defined; however, frataxin (Yfh1 in yeast), a small acidic mitochondrial protein, has been suggested to serve as the iron donor of the reaction through formation of a complex with Isu1, Nfs1, and Isd11 [46–49]. In the second step, the cluster is released from Isu1, transferred to recipient apoproteins, and assembled into the apoprotein by coordination with specific amino acid residues. These processes need the assistance of specific ISC assembly components: the mitochondrial monothiol glutaredoxin Grx5, the dedicated chaperone of the Hsp70
family termed Ssq1, the DnaJ-like co-chaperone Jac1, and the nucleotide exchange factor Mge1 [50–55]. However, further studies are necessary to characterize the sequence of events of the early steps of de novo iron-sulfur cluster biogenesis.

4. Assembly of ISP into the Cytochrome bc₁ Complex

Due to the complexity of the molecular events involved, a discussion on ISP assembly cannot be made without considering the assembly of the cytochrome bc₁ complex in which ISP inserts and operates [56]. Many experiments have been carried out over the last decades on the molecular mechanisms and steps involved in the assembly of the various protein subunits into the cytochrome bc₁ complex. Since the beginning, it appeared plausible that the assembly of these subunits occurred through distinct steps involving the preliminary formation of small bc₁ subcomplexes followed by their ordered interaction in building up the mature and functional bc₁ complex. These hypotheses were formulated indirectly by assaying the steady-state levels of the remaining subunits in the mitochondrial membranes of yeast strains in which genes for one or more bc₁ subunits had been deleted [57–60]. Different experimental approaches, consisting in the solubilization of the wild-type bc₁ complex with detergents and salts, led to the same conclusions, that is the possible existence of specific bc₁ subcomplexes [57, 61].

On the basis of these investigations, the existence of at least three distinct bc₁ subcomplexes was proposed: the cytochrome b-Qcr7p-Qcr8p subcomplex, the core protein 1-core protein 2 subcomplex, and the cytochrome c₁-Qr6p-Qr9p subcomplex. It is interesting to note that ISP was not included in any of these subcomplexes, thereby leading to the proposal that this catalytic subunit could be one of the last proteins inserted into the growing cytochrome bc₁ complex. Such a conclusion was also reached analyzing the bc₁ subunit composition of a yeast mutant strain in which ISP inserted and operated [56]. Comparable results were obtained some years later with a ΔQCR10 strain, in which the gene encoding ISP had been deleted (ΔISP) [57, 59]. Similar findings were obtained some years later with ΔISP and ΔQCR10 strains, in which the gene encoding the supernumerary subunit Qcr10p had been deleted [62]. In both yeast mutant strains (ΔISP and ΔQCR10), all the bc₁ subunits were found, with the obvious exception of ISP and Qcr10p. These findings suggest that all the other subunits are able to assemble in a protease-resistant form of the bc₁ complex even in the absence of ISP or Qcr10p. It is in fact known that the imported, but not-assembled, bc₁ subunits are susceptible of protein degradation inside yeast mitochondria [58, 63]. The two strains ΔISP and ΔQCR10, however, showed a striking functional difference because the first was respiratory-deficient whereas the second was respiratory-competent. This is due to the different role played by the two proteins in the mature cytochrome bc₁ complex; in fact, whereas ISP is involved in the electron transport activity catalyzed by the bc₁ complex, Qcr10p is a supernumerary subunit apparently devoid of any role in the respiratory activity. Interestingly, some authors suggested that Qcr10p could be added after the processing of i-ISP to m-ISP [40]. In addition, in the crystal structure of the bovine enzyme, the homologous supernumerary subunit interacts with ISP and is peripherally located [7, 8]. Taken together these data are in favour of a possible interaction of ISP and Qcr10p during the last steps of bc₁ complex assembly.

An interaction between ISP and the supernumerary subunit Qcr9p was also suggested. In fact, a structural and functional defect of ISP was found in a mutant yeast strain (ΔQCR9) in which the gene encoding Qcr9p had been deleted [64]. In the ΔQCR9 strain the conformation of ISP was altered in such a way that the apoprotein was not able to insert the iron-sulfur cluster appropriately [64]. In addition, in this yeast mutant strain ISP resulted more sensitive to endogenous proteolytic degradation [60, 64].

It is important to underline that the existence of these bc₁ subcomplexes has been postulated only indirectly on the basis of the analysis of the subunit composition of yeast mutant strains in which single genes or pairs of genes encoding distinct bc₁ subunits had been deleted. More recently, a physical interaction between the bc₁ subunits has been directly demonstrated by a different experimental approach. This latter consisted in the analysis of the subunit composition of the bc₁ complex with a non-denaturing electrophoretic technique, the blue-native polyacrylamide gel electrophoresis (BN-PAGE) [65], followed by immunoblotting with antibodies directed against all the bc₁ protein subunits. This approach led to the identification of a new bc₁ subcomplex of approximately 66 kDa, made up of the two subunits ISP and Qcr9p [66]. This was the first demonstration of a direct physical interaction between ISP and another protein subunit of the cytochrome bc₁ complex. In the past, on the basis of indirect evidence [58, 67, 68], an interaction of Qcr9p with cytochrome c₁, instead of ISP, was proposed. A ternary subcomplex, made up of cytochrome c₁, Qr6p, and Qr9p, was for a long time retained to be an important intermediate during the assembly of the cytochrome bc₁ complex [57–60]. However, the recent approach of BN-PAGE failed to reveal this bc₁ subcomplex. Interestingly, the 66 kDa subcomplex, derived from the association of ISP and Qcr9p, was only found in yeast mutant strains strongly defective in bc₁ complex assembly, such as the ΔCOR1, ΔCOR2, and ΔCYT1 strains [66]. Indeed, in all these strains only some low MW bc₁ subcomplexes, catalytically inactive, were repeatedly found [66]. These findings suggest that ISP can be isolated in a definite subcomplex with Qcr9p only when the incorporation of these two proteins into the nascent bc₁ complex is inhibited. On the other hand, these results do not guarantee that the 66 kDa subcomplex represents a real bc₁ complex assembly intermediate. They only suggest that ISP is able, in certain conditions, to strongly interact with the supernumerary subunit Qcr9p. Furthermore, no data are currently available on the possible presence in this 66 kDa subcomplex of other protein components, such as specific chaperone proteins which may have a role in bc₁ complex maturation.

When comparing these data with those obtained with the crystal structure of the yeast cytochrome bc₁ complex [9] some new implications interestingly emerged. In fact, the transmembrane region of ISP was found in the vicinity of
the corresponding transmembrane region of Qcr9p. More precisely, 14 amino acid residues of the hydrophobic α-helix of ISP were found within 4 Å of distance from 12 residues belonging to the hydrophobic α-helix of Qcr9p. The particularity is that this interaction between ISP and Qcr9p is established in one bc1 monomer, whereas the catalytic function of ISP is exerted within the other bc1 monomer. In fact, it is at the level of the other bc1 monomer that the functional interaction between ISP and cytochrome c1 occurs. These results are therefore in agreement with the transdimeric structure of ISP within the mature homodimeric bc1 complex (Figure 1).

5. Insertion of ISP into a Core Structure of the Cytochrome bc1 Complex

Further information on the ISP biogenesis was obtained by investigating the molecular characteristics of a recently identified bc1 subcomplex of about 500 kDa [69]. This subcomplex was identified in three yeast mutant strains, in which the genes encoding ISP, Qcr9p, or Bcs1p were individually deleted [69]. The minimal composition of this 500 kDa bc1 subcomplex was found in the ΔQCR9 mutant strain and consisted of cytochrome b, cytochrome c1, core protein 1, core protein 2, Qcr6p, Qcr7p, and Qcr8p. Interestingly, this subcomplex also contained Bcs1p, a chaperone involved in cytochrome bc1 assembly [70, 71]. The 500 kDa bc1 subcomplex was proteolytically stable inside yeast mitochondria, was repeatedly found in several yeast mutant strains, and for all these characteristics was therefore named "bc1 core structure." In addition, this bc1 subcomplex was particularly helpful because it allowed a detailed characterization of the last steps of bc1 assembly, including the precise events leading to ISP integration into the respiratory complex. It was indeed discovered that the binding of Qcr9p preceded the binding of ISP and that the binding of this catalytic subunit was required for the integration of Qcr10p into the mature and functional bc1 complex [69]. Furthermore, besides Qcr10p, also the presence of the chaperone protein Bcs1p in the 500 kDa subcomplex was absolutely required for the integration of the catalytic subunit ISP [69]. This finding is in agreement with previous results showing the importance of Bcs1p in the biogenesis of ISP and therefore of the entire bc1 complex. In fact, it has been proposed that the ATP-dependent chaperone Bcs1p maintains the preassembled bc1 complex in a competent state capable of binding ISP and Qcr10p [71]. It is, however, interesting to find that the presence of only one of these two subunits (Qcr9p or Bcs1p) does not substitute for the other [69]. This means that both proteins, the supernumerary subunit Qcr9p and the chaperone protein Bcs1p, are required for the insertion of ISP into the bc1 core structure. It is also possible that different structural and/or functional elements of these proteins are absolutely required for the assembly of ISP into the bc1 complex.

The existence of this stable bc1 subcomplex in various yeast mutant strains strongly supports the hypothesis that it may represent a real intermediate during the process of assembly of the homodimeric cytochrome bc1 complex. However, the possibility that this 500 kDa bc1 subcomplex may instead represent an incorrectly assembled intermediate or a degradation product characteristic of the yeast mutant strains examined cannot be totally excluded. As a consequence, the data on the pathway followed by ISP during its incorporation into the bc1 complex might be incorrect or inconclusive. In order to overcome this possible pitfall, Bcs1p was overexpressed in the yeast mutant strain ΔBCS1 containing the 500 kDa bc1 subcomplex [72], in order to investigate the possibility of a recovery of the mature cytochrome bc1 complex. Interestingly, the overexpressed Bcs1p was able to reconstruct the functional homodimeric cytochrome bc1 complex in a time-dependent fashion [72]. This was not the case when some mutant Bcs1p’s, instead of the wild type Bcs1p, were overexpressed in the ΔBCS1 yeast strain. These mutant forms of Bcs1p were not able to recover the functional bc1 complex, even when the coexpression of ISP was also carried out [72]. These findings therefore validated the previous results concerning the sequence of molecular events involved in the assembly of ISP into the bc1 core structure, which, in its turn, represents a bona fide assembly intermediate of the cytochrome bc1 complex.

6. Is ISP Involved in the Dimerization of the bc1 Complex and/or in the Formation of the Respiratory Chain Supercomplexes?

Although the subunit composition of the bc1 core structure has been carefully analyzed [69], no data are currently available on its aggregation state that could be either monomeric or dimeric. In fact, the molecular mass of 500 kDa could be due to a monomeric state of the immature bc1 complex associated to still unknown assembly factors or, alternatively, to a dimeric state of the immature complex plus a single copy of the chaperone Bcs1p. Previous findings have shown that the sole integration of ISP into the bc1 core structure determines a shift in the molecular mass of this complex from 500 to 670 kDa [69]. This change in the size of the complex is too large to be explained by the inclusion of just ISP and Qcr10p (which, as stated before, is added after ISP binding) into the 500 kDa core structure. On the other hand, the molecular shift observed is too small if a bc1 dimerization occurs at this stage as a consequence of ISP binding. In addition, very little is known on the nature and role played by the bc1 assembly factors in the course of these molecular events. We cannot therefore exclude that ISP integration into the bc1 core structure triggers a general rearrangement of the bc1 complex and of the putative bound assembly factors eventually leading to the dimerization of the functional complex in the inner mitochondrial membrane (Figure 2).

The cytochrome bc1 complex is not only functionally but also structurally associated to other complexes of the mitochondrial respiratory chain. In fact, it has been clearly demonstrated that supercomplexes between cytochrome bc1 and cytochrome c oxidase exist in the inner membrane of yeast and mammalian mitochondria [66, 73, 74]. In particular, two supercomplexes of about 1000 and 850 kDa
have been identified in the yeast mitochondrial membranes solubilized with the non-denaturing detergent digitonin and analyzed by BN-PAGE [66, 73]. It has been proposed that these two supercomplexes correspond to the homodimeric cytochrome bc₁ complex plus one copy (850 kDa) or two copies (1000 kDa) of the monomeric cytochrome c oxidase complex. The role of distinct subunits of the bc₁ or the oxidase complexes in gluing together these respiratory complexes is currently under investigation.

On the one hand, it has been proposed that the formation of the 1000 kDa supercomplex requires the presence of the functionally assembled cytochrome bc₁ and cytochrome c oxidase complexes [73]. In the absence of ISP the formation of this supercomplex was hindered as a consequence of the lack of an assembled cytochrome bc₁ complex. On the other hand, in the same ΔISP yeast mutant strain the authors found some respiratory complexes, smaller than the supercomplex, yet containing protein subunits belonging to both the bc₁ and the c oxidase complexes. This suggests that in the absence of ISP an association between the bc₁ and the oxidase complexes, even if incomplete and non-productive, does occur.

On the contrary, subsequent experiments revealed that in the absence of ISP no binding between the bc₁ core structure and the mature c oxidase complex was possible [69]. In fact, the 500 kDa bc₁ subcomplex found in the ΔISP yeast mutant strain did not contain the oxidase complex which, instead, migrated in its monomeric form in the molecular mass region of about 230 kDa. This suggests that the integration of ISP into the bc₁ complex is an essential prerequisite for the subsequent formation of the supercomplexes (Figure 2). Therefore, these results open up new avenues of investigation on the possible role of ISP in gluing together the respiratory complexes in the higher structures of the supercomplexes. The difficulty of all these studies is due to the necessity of identifying genuine assembly intermediates and not casual protein aggregates that could lead to wrong results and misleading interpretations.

7. Concluding Remarks
ISP has a fundamental role in the electron transport activity catalyzed by the cytochrome bc₁ complex because it represents the primary acceptor of electrons deriving from the oxidation of ubiquinol. The catalytic domain of this protein subsequently cooperates with the corresponding domain of cytochrome c oxidase complex. Many studies have therefore been carried out on the functional properties of ISP along with the molecular characterization of the flexibility of its catalytic domain.

However, also the molecular events involved in the biogenesis of ISP have gained some attention, especially in these last years. The reason for this is due to several factors, first of all to the complicated relationships (structural, spatial, and temporal) existing between the assembly of ISP and that of other protein subunits into the bc₁ complex, which must acquire a homodimeric structure (20 subunits in total) in the inner membrane of Saccharomyces cerevisiae mitochondria. Secondly, ISP is the unique subunit of the bc₁ complex showing a transdimeric topology in the functional complex. This structural property could be in some way responsible for bc₁ complex dimerization or it can be at least related to the process of dimerization. The addition of ISP to the growing bc₁ complex may trigger a complex series of events leading to a general rearrangement of the complex and of the bound assembly factors. As discussed before, some hints already exist about a pivotal role of ISP during bc₁ assembly, even if a significant amount of work is still needed.
necessary in order to better explore this intriguing possibility. Finally, preliminary data indicate that ISP is also required for the formation of the respiratory supercomplexes in the inner mitochondrial membrane. Whether this effect is indirectly due to the lack of a mature $bc_1$ complex (in the absence of ISP) or to a direct interaction of ISP with a putative partner subunit of the oxidase complex is a further matter of future investigations.

8. Abbreviations

ISP and Rip1p, iron-sulfur protein and Rieske iron-sulfur protein, respectively, indicate the same protein subunit belonging to the yeast cytochrome $bc_1$ complex; Qcr6p, Qcr7p, Qcr8p, Qcr9p, and Qcr10p, subunits 6, 7, 8, 9, and 10 of the yeast cytochrome $bc_1$ complex, respectively.

Acknowledgment

The authors thank Bernard L. Trumpower of the Department of Biochemistry at the Dartmouth Medical School (Hanover, NH, USA) for the fruitful discussion and support during the writing of this paper.

References

[1] X. H. Yang and B. L. Trumpower, “Protonmotive Q cycle pathway of electron transfer and energy transduction in the three-subunit ubiquinol-cytochrome c oxidoreductase complex of Paracoccus denitrificans,” Journal of Biological Chemistry, vol. 263, no. 24, pp. 11962–11970, 1988.

[2] B. L. Trumpower, “Cytochrome $bc_1$ complexes of microorganisms,” Microbiological Reviews, vol. 54, no. 2, pp. 101–129, 1990.

[3] J. D. Beckmann, P. O. Ljungdahl, J. L. Lopez, and B. L. Trumpower, “Isolation and characterization of the nuclear gene encoding the Rieske iron-sulfur protein (RIP1) from Saccharomyces cerevisiae,” Journal of Biological Chemistry, vol. 262, no. 18, pp. 8901–8909, 1987.

[4] J. D. Beckmann, P. O. Ljungdahl, and B. L. Trumpower, “Mutational analysis of the mitochondrial Rieske iron-sulfur protein of Saccharomyces cerevisiae. I. Construction of a RIP1 deletion strain and isolation of temperature-sensitive mutants,” Journal of Biological Chemistry, vol. 264, no. 7, pp. 3713–3722, 1989.

[5] P. O. Ljungdahl, J. D. Beckmann, and B. L. Trumpower, “Mutational analysis of the mitochondrial Rieske iron-sulfur protein of Saccharomyces cerevisiae. II. Biochemical characterization of temperature-sensitive RIP1 mutations,” Journal of Biological Chemistry, vol. 264, no. 7, pp. 3723–3731, 1989.

[6] D. Xia, C. A. Yu, H. Kim et al., “Crystal structure of the cytochrome $bc_1$ complex from bovine heart mitochondria,” Science, vol. 277, no. 5322, pp. 60–66, 1997.

[7] S. Iwata, J. W. Lee, K. Okada et al., “Complete structure of the 11-subunit bovine mitochondrial cytochrome $bc_1$ complex,” Science, vol. 281, no. 5373, pp. 64–71, 1998.

[8] Z. Zhang, L. Huang, V. M. Shulmeister et al., “Electron transfer by domain movement in cytochrome $bc_1$,” Nature, vol. 392, no. 6677, pp. 677–684, 1998.

[9] C. Hunte, J. Koepke, C. Lange, T. Rossmanith, and H. Michel, “Structure at 2.3 Å resolution of the cytochrome $bc_1$ complex from the yeast Saccharomyces cerevisiae co-crystallized with an antibody Fv fragment,” Structure, vol. 8, no. 6, pp. 669–684, 2000.

[10] S. Iwata, M. Saynovits, T. A. Link, and H. Michel, “Structure of a water soluble fragment of the ‘Rieske’ iron-sulfur protein of the bovine heart mitochondrial cytochrome $bc_1$ complex determined by MAD phasing at 1.5 Å resolution,” Structure, vol. 4, no. 5, pp. 567–579, 1996.

[11] E. B. Gutierrez-Cirlos, T. Merbitz-Zahradnik, and B. L. Trumpower, “Failure to insert the iron-sulfur cluster into the Rieske iron-sulfur protein impairs both center N and center P of the cytochrome $bc_1$ complex,” Journal of Biological Chemistry, vol. 277, no. 52, pp. 50703–50709, 2002.

[12] T. Merbitz-Zahradnik, K. Zwicker, J. H. Nett, T. A. Link, and B. L. Trumpower, “Elimination of the disulfide bridge in the Rieske iron-sulfur protein allows assembly of the [2Fe-2S] cluster into the Rieske protein but damages the ubiquinol oxidation site in the cytochrome $bc_1$ complex,” Biochemistry, vol. 42, no. 46, pp. 13637–13645, 2003.

[13] T. Miki, L. Yu, and C. A. Yu, “Hematoporphyrin-promoted photoinactivation of mitochondrial ubiquinol-cytochrome $c$ reductase: selective destruction of the histidine ligands of the iron-sulfur cluster and protective effect of ubiquinone,” Biochemistry, vol. 30, no. 1, pp. 230–238, 1991.

[14] B. Gurung, L. Yu, D. Xia, and C. A. Yu, “The iron-sulfur cluster of the Rieske iron-sulfur protein functions as a proton-exiting gate in the cytochrome $bc_1$ complex,” Journal of Biological Chemistry, vol. 280, no. 26, pp. 24895–24902, 2005.

[15] A. R. Crofts and E. A. Berry, “Structure and function of the cytochrome $bc_1$ complex of mitochondria and photosynthetic bacteria,” Current Opinion in Structural Biology, vol. 8, no. 4, pp. 501–509, 1998.

[16] C. A. Yu, D. Xia, H. Kim et al., “Structural basis of functions of the mitochondrial cytochrome $bc_1$ complex,” Biochimica et Biophysica Acta, vol. 1365, no. 1-2, pp. 151–158, 1998.

[17] D. S. Beattie, Y. Wang, and V. H. Obungu, “The role of various domains of the iron-sulfur protein in the assembly and activity of the cytochrome $bc_1$ complex of yeast mitochondria,” Journal of Bioenergetics and Biomembranes, vol. 31, no. 3, pp. 215–224, 1999.

[18] H. Tian, S. White, L. Yu, and C. A. Yu, “Evidence for the head domain movement of the Rieske iron-sulfur protein in electron transfer reaction of the cytochrome $bc_1$ complex,” Journal of Biological Chemistry, vol. 274, no. 11, pp. 7146–7152, 1999.

[19] E. Darrouzet, M. Valkova-Valchanova, and F. Daldal, “Probing the role of the Fe-S subunit hinge region during Q(o) site catalysis in Rhodobacter capsulatus of Biological Chemistry,” vol. 274, no. 11, pp. 7146–7152, 1999.

[20] D. S. Beattie, Y. Wang, and V. H. Obungu, “The role of various domains of the iron-sulfur protein in the assembly and activity of the cytochrome $bc_1$ complex of yeast mitochondria,” Journal of Bioenergetics and Biomembranes, vol. 31, no. 3, pp. 215–224, 1999.

[21] E. Darrouzet, M. Valkova-Valchanova, and F. Daldal, “Probing the role of the Fe-S subunit hinge region during Q(o) site catalysis in Rhodobacter capsulatus of Biological Chemistry,” vol. 274, no. 11, pp. 7146–7152, 1999.

[22] M. Ghosh, Y. Wang, C. E. Ebert, S. Vadlamuri, and D. S. Beattie, “Substituting leucine for alanine-86 in the tether region of the iron-sulfur protein of the cytochrome $bc_1$ complex affects the mobility of the [2Fe2S] domain,” Biochemistry, vol. 40, no. 2, pp. 327–335, 2001.
[23] E. Darrouzet, M. Valkova-Valchanova, C. C. Moser, P. L. Dutton, and F. Daldal, "Uncovering the [2Fe-2S] domain movement in cytochrome bc1 and its implications for energy conversion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 9, pp. 4567–4572, 2000.

[24] J. H. Nett, C. Hunte, and B. L. Trumpower, "Changes to the length of the flexible linker region of the Rieske protein impair the interaction of ubiquinol with the cytochrome bc1 complex," *European Journal of Biochemistry*, vol. 267, no. 18, pp. 5777–5782, 2000.

[25] N. Fisher, I. Bourges, P. Hill, G. Brasseur, and B. Meunier, "Disruption of the interaction between the Rieske iron-sulfur protein and cytochrome b in the yeast bc1 complex owing to a human disease-associated mutation within cytochrome b," *European Journal of Biochemistry*, vol. 271, no. 7, pp. 1292–1298, 2004.

[26] L. Esser, X. Gong, S. Yang, L. Yu, C. A. Yu, and D. Xia, "Surface-modulated motion switch: capture and release of iron-sulfur protein in the cytochrome bc1 complex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 35, pp. 13045–13050, 2006.

[27] D. Xia, L. Esser, L. Yu, and C. A. Yu, "Structural basis for the mechanism of electron bifurcation at the quinol oxidation site of the cytochrome bc1 complex," *Photosynthesis Research*, vol. 92, no. 1, pp. 17–34, 2007.

[28] C. A. Yu, X. Cen, H. W. Ma et al., "Domain conformational switch of the iron-sulfur protein in cytochrome bc1 complex is induced by the electron transfer from cytochrome b5 to b6," *Biochimica et Biophysica Acta*, vol. 1777, no. 7–8, pp. 1038–1043, 2008.

[29] W. Neupert and J. M. Herrmann, "Translocation of proteins into mitochondria," *Annual Review of Biochemistry*, vol. 76, pp. 723–749, 2007.

[30] A. Chacinska, C. M. Koehler, D. Milenkovic, T. Lithgow, and J. H. Nett, "Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p," *The EMBO Journal*, vol. 22, no. 18, pp. 4815–4825, 2003.

[31] M. Yang and B. L. Trumpower, "Deletion of QCR6, the gene encoding subunit six of the mitochondrial cytochrome bc1 complex, blocks maturation of cytochrome c1, and causes temperature-sensitive petite growth in Saccharomyces cerevisiae," *Journal of Biological Chemistry*, vol. 269, no. 2, pp. 1270–1275, 1994.

[32] R. Lill and U. Mühlhöf, "Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases," *Annual Review of Biochemistry*, vol. 77, pp. 669–700, 2008.

[33] R. Lill, "Function and biogenesis of iron-sulfur proteins," *Nature*, vol. 460, no. 7257, pp. 831–838, 2009.

[34] U. Mühlhöf, J. Gerber, N. Richhardt, and R. Lill, "Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p,", *The EMBO Journal*, vol. 22, no. 18, pp. 4815–4825, 2003.

[35] R. Dutkiewicz, J. Marszalek, B. Schilke, E. A. Craig, R. Lill, and U. Mühlhöf, "The Hsp70 chaperone Ssq1p is dispensable for iron-sulfur cluster formation on the scaffold protein Isu1p.," *Journal of Biological Chemistry*, vol. 281, no. 12, pp. 7801–7808, 2006.

[36] J. Gerber, U. Mühlhöf, and R. Lill, "An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1.," *EMBO Reports*, vol. 4, no. 9, pp. 906–911, 2003.

[37] L. A. Graham and B. L. Trumpower, "Mutational analysis of the mitochondrial Rieske iron-sulfur protein of Saccharomyces cerevisiae. III. Import, protease processing, and assembly into the cytochrome bc1 complex of iron-sulfur protein lacking the iron-sulfur cluster," *Journal of Biological Chemistry*, vol. 266, no. 33, pp. 22485–22492, 1991.
[52] R. Dutkiewicz, B. Schilke, H. Knieszner, W. Walter, E. A. Craig, and J. Marszalek, "Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe-S) center biogenesis. Similarities to and differences from its bacterial counterpart," Journal of Biological Chemistry, vol. 278, no. 32, pp. 29719–29727, 2003.

[53] R. Dutkiewicz, B. Schilke, S. Cheng, H. Knieszner, E. A. Craig, and J. Marszalek, "Sequence-specific interaction between mitochondrial Fe-S scaffold protein Isu and Hsp70 Ssq1 is essential for their in vivo function," Journal of Biological Chemistry, vol. 279, no. 28, pp. 29167–29174, 2004.

[54] A. J. Andrew, R. Dutkiewicz, H. Knieszner, E. A. Craig, and J. Marszalek, "Characterization of the interaction between the J-protein Jac1p and the scaffold for Fe-S cluster biogenesis, Isu1p," Journal of Biological Chemistry, vol. 281, no. 21, pp. 14580–14587, 2006.

[55] L. E. Vickery and J. R. Cupp-Vickery, "Molecular chaperones HscA/Ssq1 and HscB/Jac1 and their roles in iron-sulfur protein maturation," Critical Reviews in Biochemistry and Molecular Biology, vol. 42, no. 2, pp. 95–111, 2007.

[56] V. Zara, L. Conte, and B. L. Trumpower, "Biogenesis of the yeast cytochrome bc1 complex," Biochimica et Biophysica Acta, vol. 1793, no. 1, pp. 89–96, 2009.

[57] M. D. Crivellone, M. A. Wu, and A. Tzagoloff, "Assembly of the mitochondrial membrane system. Analysis of structural mutants of the yeast coenzyme QH2-cytochrome c reductase complex," Journal of Biological Chemistry, vol. 263, no. 28, pp. 14323–14333, 1988.

[58] J. A. Berden, P. J. Schoppink, and L. A. Grivell, "Characterization of the interaction between the J-protein Jac1p and the scaffold for Fe-S cluster biogenesis, Isu1p," Journal of Biological Chemistry, vol. 281, no. 21, pp. 14580–14587, 2006.

[59] L. Conte, B. L. Trumpower, and V. Zara, "Bcs1p can rescue the large and productive cytochrome bc1 complex," FEBS Journal, vol. 276, no. 7, pp. 1900–1914, 2009.

[60] F. G. Nobrega, M. P. Nobrega, and A. Tzagoloff, "Bcs1p, a novel gene required for the expression of functional Rieske iron-sulfur protein in Saccharomyces cerevisiae," The EMBO Journal, vol. 11, no. 11, pp. 3821–3829, 1992.

[61] C. M. Cruciat, K. Hell, H. Fölsch, W. Neupert, and R. A. Stuart, "Bcs1p, an AAA-family member, is a chaperone for the assembly of the cytochrome bc1 complex," The EMBO Journal, vol. 18, no. 19, pp. 5226–5233, 1999.

[62] L. Conte, B. L. Trumpower, and V. Zara, "Bcs1p can rescue the large and productive cytochrome bc1 complex assembly intermediate in the inner membrane of yeast mitochondria," Biochimica et Biophysica Acta, vol. 1813, no. 1, pp. 91–101, 2011.

[63] C. M. Cruciat, S. Brunner, F. Baumann, W. Neupert, and R. A. Stuart, "The cytochrome bc1 and cytochrome c oxidase complexes associate to form a single supercomplex in yeast mitochondria," Journal of Biological Chemistry, vol. 275, no. 24, pp. 18093–18098, 2000.

[64] H. Schägger and K. Pfeiffer, "Supercomplexes in the respiratory chains of yeast and mammalian mitochondria," The EMBO Journal, vol. 19, no. 8, pp. 1777–1783, 2000.