Published in final edited form as:
FEBS J. 2009 April ; 276(7): 1900–1914. doi:10.1111/j.1742-4658.2009.06916.x.

Evidence that assembly of the yeast cytochrome bc_1 complex involves formation of a large core structure in the inner mitochondrial membrane

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Abstract

The assembly status of the cytochrome bc_1 complex has been analyzed in distinct yeast deletion strains in which genes for one or more of the bc_1 subunits had been deleted. In all the yeast strains tested a bc_1 sub-complex of about 500 kDa was found when the mitochondrial membranes were analyzed by blue native electrophoresis. The subsequent molecular characterization of this sub-complex, carried out in the second dimension by SDS-PAGE and immunodecoration, revealed the presence of the two catalytic subunits cytochrome *b* and cytochrome c_1, associated with the non catalytic subunits core protein 1, core protein 2, Qcr7p and Qcr8p. Altogether these bc_1 subunits build up the core structure of the cytochrome bc_1 complex which is then able to sequentially bind the remaining subunits, such as Qcr6p, Qcr9p, the Rieske iron-sulfur protein and Qcr10p. This bc_1 core structure may represent a true assembly intermediate during the maturation of the bc_1 complex, first because of its wide distribution in distinct yeast deletion strains and second for its characteristics of stability which resemble those of the intact homodimeric bc_1 complex. Differently from this latter, however, the bc_1 core structure is not able to interact with the cytochrome *c* oxidase complex to form respiratory supercomplexes. The characterization of this novel core structure of the bc_1 complex provides a number of new elements for clarification of the molecular events leading to the maturation of the yeast cytochrome bc_1 complex in the inner mitochondrial membrane.

Keywords

cytochrome bc_1 complex; yeast mitochondria; cytochrome bc_1 core structure; cytochrome bc_1 assembly; yeast deletion mutants

Introduction

The cytochrome bc_1 complex, also known as complex III, is a component of the mitochondrial respiratory chain. In the yeast *Saccharomyces cerevisiae* the homodimeric bc_1 complex is located in the inner mitochondrial membrane and each monomer is composed of ten different protein subunits [1–4]. Three of them, cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein (ISP), contain redox prosthetic groups and hence participate in the electron transfer process (catalytic subunits). The remaining seven subunits do not contain any cofactors and their function is largely unknown (non catalytic subunits or

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supernumerary subunits). These latter are represented by the two large core proteins 1 and 2 and by the smaller subunits Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p. Only one bc_1 subunit, cytochrome *b*, is encoded by the mitochondrial DNA and is therefore synthesized inside mitochondria. All the other subunits are nuclear-encoded and imported post-translationally into yeast mitochondria. The cytochrome bc_1 complex has been crystallized from yeast, chicken and bovine mitochondria [5–8]. A high resolution structure of the yeast bc_1 complex with bound cytochrome *c* in reduced form has also been reported [9].

Several investigations have demonstrated that the mitochondrial respiratory complexes are associated with each other when analyzed under non denaturing conditions by blue native electrophoresis (BN-PAGE). This has been found in *Saccharomyces cerevisiae* mitochondria where an association of the cytochrome bc_1 complex with the cytochrome *c* oxidase complex has been clearly shown [10–12], but also in other organisms, such as *Neurospora crassa* [13], mammals [11] and plants [14]. A higher order organization of the respiratory chain complexes was first proposed for bacterial respiratory enzymes [15]. More extensive associations between the respiratory chain complexes, the so-called "respirasomes", have recently been found in mammals, plants and bacteria [16–19]. A further and more complex evolution of this kind of structural organization is represented by the "respiratory string" model [20]. In addition, a surprising interaction between the respiratory supercomplex, made up of the bc_1 and the oxidase complexes, and the TIM23 protein import machinery has recently been demonstrated in yeast mitochondria [21]. Further unexpected developments came with two recent publications, the first showing interaction of the mitochondrial ADP/ATP transporter with the bc_1-oxidase supercomplex and the TIM23 machinery [22], and the second reporting the influence of the ATP synthase complex on the assembly state of the bc_1-oxidase supercomplex and its association with the TIM23 machinery [23].

However, in the midst of this quickly evolving context of macromolecular organization of the mitochondrial proteome comparatively little is known about the assembly pathway leading to the maturation of the cytochrome bc_1 complex in the inner mitochondrial membrane. In fact, the biogenesis of this multi-subunit complex is very complicated if one takes into account the fact that each monomer is made up of ten different subunits and the functional complex assembles in the inner membrane as a symmetrical homodimer. Numerous previous studies on the bc_1 biogenesis have postulated the existence of distinct sub-complexes in yeast mitochondria [12,24–27]. However, it is still uncertain if these sub-complexes represent true bc_1 assembly intermediates, and the sequence in which these putative sub-complexes bind to each other during the assembly of the bc_1 complex is also unknown. Furthermore, as in the case of the biogenesis of other multi-subunit complexes of the mitochondrial respiratory chain, the assistance of specific chaperone proteins is also required. Available data indicate that the accessory factor Bcs1p is involved in the binding of ISP to an immature bc_1 intermediate [28,29] and that Cbp3p and Cbp4p play an essential, but poorly understood role, during bc_1 biogenesis [30–32]. The insertion of the redox prosthetic groups into the apoproteins of the bc_1 complex is yet another aspect which has been investigated only partially [33,34].

In this study we have characterized a bc_1 sub-complex of about 500 kDa which we propose represents a stable and productive intermediate during the assembly of the bc_1 complex in yeast mitochondria. Besides the previously proposed "central core" of the bc_1 complex, made up of cytochrome *b* associated with Qcr7p and Qcr8p [12], we now propose a larger "core structure" of the bc_1 complex which, in addition to the central core subunits, incorporates the two core proteins and cytochrome c_1. Two other small supernumerary subunits, Qcr6p and Qcr9p, may be present or added to this large sub-complex even if they are not essential for its stability. According to this view the subsequent incorporation of ISP

and Qcr10p into the 500 kDa bc_1 sub-complex completes its transition towards the mature homodimeric bc_1 complex which eventually associates with the cytochrome *c* oxidase complex, thereby generating the higher order complexes.

Results

Molecular characterization of a 500 kDa bc_1 sub-complex in the yeast deletion strains lacking Qcr9p, ISP or Bcs1p

The BN-PAGE analysis of a yeast mutant strain in which the gene encoding the Qcr9p subunit had been deleted (ΔQCR9) revealed the presence of a large bc_1 sub-complex of about 500 kDa [12]. A survey of the literature highlighted bc_1 sub-complexes of similar size in other yeast deletion strains, such as the ΔISP and ΔBCS1 strains [10,29]. These large bc_1 sub-complexes were referred to as “dimeric precomplex” or “partial assembly form of the supracomplex”, but their molecular composition has never been investigated [10,29]. In addition, it is still unclear whether they maintain the capability, typical of the mature homodimeric bc_1 complex, of binding the cytochrome *c* oxidase complex in order to form the respiratory supercomplexes [10,12,29,35].

We have therefore analyzed the assembly status of the bc_1 complex in mitochondrial membranes isolated from the two yeast deletion strains ΔISP and ΔBCS1 which were both unable to respire (Table 1). In addition, we analyzed under the same conditions the mutant strain ΔQCR9 which exhibited a reduced growth rate on non-fermentable carbon sources when compared to a yeast wild type strain (Table 1). Fig. 1A shows the BN-PAGE analysis of the mitochondrial membranes isolated from all these deletion strains and from a wild type strain. In the ΔQCR9, ΔISP and ΔBCS1 strains a protein band of approximately 500 kDa was immunodecorated with a polyclonal antiserum directed against the bc_1 core proteins. On the contrary, this bc_1 sub-complex was absent in the wild type strain in which three high molecular mass bands were clearly detected (Fig. 1A). It was previously shown that these three protein bands in the complex from wild type yeast correspond to the bc_1 homodimer (670 kDa), the homodimeric bc_1 plus one copy of the oxidase complex (850 kDa) and the homodimeric bc_1 plus two copies of the oxidase (1000 kDa) [10,12].

Fig. 1B shows the bc_1 subunit composition analysis, carried out in the second dimension by SDS-PAGE and immunodecoration, of the 500 kDa sub-complexes found in the yeast deletion strains. All the bc_1 subunits were present in the ΔISP strain, with the exception of ISP and Qcr10p, the latter having been proposed to be the last subunit incorporated into the bc_1 complex, immediately after ISP [29]. It is interesting to note that the 500 kDa bc_1 sub-complex present in the ΔISP strain also contained the subunit Qcr9p and the chaperone Bcs1p. In the ΔBCS1 strain, on the other hand, ISP and Qcr10p were both missing in the same large sub-complex. These results suggest that Bcs1p, as previously proposed [29], is specifically required for the insertion of ISP into an immature bc_1 complex and that the association of the Qcr9p subunit with the bc_1 complex precedes the binding of ISP. In fact, the direct absence of ISP (ΔISP), or the block of its insertion into the bc_1 complex due to the deletion of the chaperone Bcs1p (ΔBCS1), does not prevent the binding of Qcr9p to the bc_1 complex (Fig. 1B). According to this model, the absence of Qcr9p in the ΔQCR9 yeast strain prevented the binding of both ISP and Qcr10p, even if the bc_1 sub-complex contained the chaperone protein Bcs1p (Fig. 1B). It is also worth noting that in all the sub-complexes of about 500 kDa detected in these yeast deletion strains no association with Cox6bp [36] was found, suggesting that this large bc_1 sub-complex is not able to interact with the cytochrome *c* oxidase. Indeed, the Cox6bp was immunodetected in a different and significantly lower molecular mass region of about 230 kDa (Fig. 1B). The absence of the oxidase complex in the 500 kDa bc_1 sub-complex was further confirmed by the results obtained with another antiserum directed against the yeast Cox1p subunit (Fig. 1B) [37].

The results in Fig. 1B also suggest that Qcr10p may be the last subunit incorporated into the bc_1 complex. In order to test this possibility we analyzed the mitochondrial membranes isolated from the ΔQCR10 strain which, as shown in Table 1, was respiratory competent. In the absence of the Qcr10p subunit, only the two higher molecular mass bands were detected in the BN-PAGE analysis, but not the 670 kDa band corresponding to the homodimeric bc_1 complex (Fig. 2A). This means that in the absence of this supernumerary subunit the formation of the two supercomplexes is still possible. Fig. 2B shows that these two supercomplexes contained all the bc_1 subunits and, as expected, included the cytochrome *c* oxidase complex as demonstrated by the immunoreactivity with an antiserum directed against Cox6bp. However, to exclude the possibility that the disappearance of the homodimeric bc_1 complex observed in this yeast deletion strain could be simply due to a decrease in the endogenous levels of the bc_1 complex we compared, in a parallel experiment, the steady-state protein amount on SDS-PAGE both in wild type and ΔQCR10 strains (Fig. 2C). Such a kind of analysis showed that all the bc_1 subunits were present in comparable amounts in both yeast strains. It is therefore currently unknown the reason causing the disappearance of the bc_1 dimer in the yeast strain in which Qcr10p is missing.

On the basis of all the above reported results we propose that a large bc_1 sub-complex exists in the inner mitochondrial membrane when the bc_1 subunits ISP and Qcr9p, or the chaperone Bcs1p, are missing. This bc_1 sub-complex appears sufficiently stable to resist proteolytic degradation normally occurring inside mitochondria for unassembled protein subunits [38]. The 500 kDa bc_1 sub-complex is made up of cytochrome *b*, cytochrome c_1, the two core proteins, Qcr6p, Qcr7p and Qcr8p. To this stable bc_1 sub-complex the sequential binding of Qcr9p, ISP and Qcr10p occurs.

The 500 kDa bc_1 sub-complex is also present in yeast double deletion strains

We then analyzed the assembly status of the bc_1 complex in a double deletion strain in which the genes encoding ISP and Qcr9p were both deleted (ΔISP/ΔQCR9). This strain, as expected, was respiratory-deficient because of the absence of the catalytic subunit ISP (Table 1). Fig. 3A shows that also in this mutant strain a band of approximately 500 kDa was found when the mitochondrial membranes were analyzed in the first dimension by BN-PAGE. The resolution of this band in the second dimension by SDS-PAGE, followed by immunodecoration with subunit-specific antibodies (Fig. 3B), revealed a structural organization of the bc_1 sub-complex identical to that found in theΔQCR9 strain, i.e. the presence of the two catalytic subunits cytochrome *b* and cytochrome c_1, the two core proteins, Qcr6p, Qcr7p and Qcr8p. This sub-complex, which also contained the chaperone protein Bcs1p, was not able to bind the oxidase complex (Fig. 3B). In fact, the antiserum against the Cox6bp subunit reacted in a molecular mass region of 230 kDa, most probably corresponding to the monomeric form of the cytochrome *c* oxidase complex.

The subsequent analysis of two further double deletion strains, ΔISP/ΔQCR10 andΔQCR9/ΔQCR10 was then performed. The growth phenotype of these yeast deletion strains differed, as shown in Table 1. Indeed, whereas the ΔISP/ΔQCR10 strain was respiratory-deficient, the ΔQCR9/ΔQCR10 strain exhibited a reduced growth rate on non-fermentable carbon sources. Fig. 4A shows that a band of approximately 500 kDa was found in both yeast mutant strains when the mitochondrial membranes were analyzed in the first dimension by BN-PAGE. The subsequent resolution of these bands in the second dimension by SDS-PAGE (Fig. 4B) revealed that in the ΔISP/ΔQCR10 deletion strain all the bc_1 subunits, with the obvious exception of ISP and Qcr10p, were incorporated into the bc_1 sub-complex. This finding corroborates the previous results (Fig. 1B) showing that ISP and Qcr10p represent the last subunits incorporated into the bc_1 complex. On the other hand, the absence of Qcr9p in the ΔQCR9/ΔQCR10 strain prevented the binding of ISP (Fig. 4B). Interestingly, in the absence of Qcr9p the catalytic subunit ISP was still present in the mitochondrial membranes, but it

migrated as a single species in the molecular mass region of 35 kDa (Fig. 4B, right panel). This finding is in agreement with the lack of incorporation of ISP into the 500 kDa bc_1 sub-complex and clearly underlines the importance of Qcr9p for ISP binding. Furthermore, the chaperone Bcs1p was clearly found in the 500 kDa bc_1 sub-complex of both yeast mutant strains (Fig. 4B). On the contrary, these bc_1 sub-complexes were not able to bind the oxidase complex, which migrated in the monomeric form in the molecular mass region of 230 kDa (Fig. 4B).

Altogether, these results reinforce the previous findings (see above) regarding the sequence in which the last subunits are added to the 500 kDa bc_1 sub-complex. Furthermore, the wide distribution of the same bc_1 sub-complex in distinctly different deletion strains supports the hypothesis that it represents a true assembly intermediate during the maturation of the bc_1 complex in the inner mitochondrial membrane.

The subunit Qcr6p is not required for the formation and stabilization of the 500 kDa bc_1 sub-complex

The role played by the Qcr6p subunit during the assembly of the bc_1 complex is particularly enigmatic. In previous studies the Qcr6p subunit was found only in the supercomplex of 1000 kDa in wild type yeast mitochondria, but not in that of 850 kDa or in the dimeric bc_1 complex of 670 kDa [12]. A possible explanation for these results may relate to an easy loss of this small and acidic bc_1 subunit during the electrophoretic analysis carried out by BN-PAGE. However, this possibility now seems unlikely because the Qcr6p subunit was consistently found in all the 500 kDa bc_1 sub-complexes identified in this study by two-dimensional electrophoresis (Fig. 1B,Fig. 3B,Fig. 4B). This finding raises the intriguing possibility that the subunit Qcr6p is specifically required for the stabilization of this large bc_1 sub-complex of approximately 500 kDa. We have thus tested this possibility (see below). In addition, previous data indicated a possible interaction between both the Qcr6p and Qcr9p subunits with the catalytic subunit cytochrome c_1 [24,26]. Interestingly, all these subunits were found in the 500 kDa bc_1 sub-complex characterized in this study.

With this information in mind, we decided to construct a new yeast mutant strain in which both genes encoding Qcr6p and Qcr9p were deleted (ΔQCR6/ΔQCR9). Surprisingly, also in this strain a band of approximately 500 kDa was clearly identified (Fig. 5A). This band, when resolved in the second dimension by SDS-PAGE, showed the presence of cytochrome *b*, cytochrome c_1, the two core proteins and the small subunits Qcr7p and Qcr8p (Fig. 5B). Furthermore, the chaperone protein Bcs1p was also found in this bc_1 sub-complex. The ISP subunit, because of the absence of Qcr9p, was not incorporated into this sub-complex but migrated alone in the molecular mass region of 35 kDa (Fig. 5B). Also the oxidase complex was found in its monomeric form in the 230 kDa molecular mass region (Fig. 5B). The ΔQCR6/ΔQCR9 strain, as shown in Table 1, was respiratory-incompetent.

In order to check whether the absence of Qcr6p prevented the incorporation of the subunit Qcr9p into the 500 kDa bc_1 sub-complex we constructed a further yeast double deletion strain in which the genes encoding ISP and Qcr6p were simultaneously deleted (ΔISP/ΔQCR6). In this mutant strain, which was also respiratory-incompetent like the previous one (Table 1), a bc_1 sub-complex of about 500 kDa was again found (Fig. 6A). This sub-complex, when analyzed in the second dimension by SDS-PAGE and immunodecoration (Fig. 6B), revealed the presence of the small subunit Qcr9p along with the expected cytochrome *b*, cytochrome c_1, the two core proteins, Qcr7p and Qcr8p. Altogether these findings indicate that i) Qcr6p is not required for the formation and stabilization of the 500 kDa bc_1 sub-complex and that ii) Qcr6p is not required for the incorporation of Qcr9p into the bc_1 sub-complex. A further novel finding in theΔISP/ΔQCR6 strain is the appearance of an intermediate form of cytochrome c_1 which migrated in a molecular mass region of about

230 kDa (Fig. 6B). This agrees with previous findings in which it was shown that deletion of QCR6 retards maturation of cytochrome c_1 [39].

The 500 kDa bc_1 sub-complex is stable both in digitonin and in Triton X-100

In order to investigate how stable was the association between the bc_1 subunits in the 500 kDa bc_1 sub-complex, Triton X-100 was used for the solubilization of the mitochondrial membranes, instead of the mild detergent digitonin. Fig. 7A shows the BN-PAGE analysis of the mitochondrial membranes isolated from the wild type or ΔQCR9 yeast strains in the presence of 1% digitonin or 1% Triton X-100. The left panels of Fig. 7A show that the bc_1-oxidase supercomplexes were found in wild type mitochondria only when the mild detergent digitonin was used (lane 1). On the contrary, Triton X-100 caused the disappearance of the two supercomplexes of 1000 and 850 kDa, leaving unaltered only the band of 670 kDa which corresponds to the homodimeric bc_1 complex (lane 2). The same results were obtained when lower concentrations of Triton X-100 were used for the solubilization of the mitochondrial membranes from a wild type yeast strain (data not shown). This finding suggests that the forces stabilizing the association between the bc_1 and the oxidase in the supercomplexes are weaker than those existing among the bc_1 subunits in the homodimeric complex.

Interestingly, the 500 kDa sub-complex was clearly found also when the solubilization was carried out in the presence of Triton X-100, with no detectable difference in comparison to the 500 kDa sub-complex obtained with digitonin solubilization (Fig. 7A, right panels, compare lane 4 with lane 3). We then investigated the stability of the 500 kDa bc_1 sub-complex, solubilized in the presence of digitonin or Triton X-100, at different temperatures. Fig. 7B shows that the stability of this sub-complex was significantly reduced if the solubilization was carried out at 10°C instead of 0°C. At 25°C the Triton X-100-solubilized bc_1 sub-complex completely disappeared, whereas only a tiny amount of the bc_1 sub-complex was detected if the solubilization was carried out with the mild detergent digitonin (Fig. 7B).

We conclude that the forces stabilizing the bc_1 subunits in the 500 kDa sub-complex are sufficiently stable to make it possible the solubilization with Triton X-100. These forces stabilizing the bc_1 subunits in the sub-complex are similar to those present between the subunits in the mature homodimeric bc_1 complex. Furthermore, the association between the subunits is temperature-sensitive, thereby excluding the possible presence of non specific protein aggregates in the 500 kDa bc_1 sub-complex.

Discussion

In this study we have analyzed the molecular composition of a bc_1 sub-complex of about 500 kDa which has been found in several yeast strains in which genes for one or more of the bc_1 subunits had been deleted. Actually, several studies carried out on the biogenesis of the yeast cytochrome bc_1 complex postulated the existence of distinct bc_1 sub-complexes [24–27]. In these studies, however, the interaction between the bc_1 subunits was hypothesized only indirectly by assaying the steady-state levels of the remaining subunits in the mitochondrial membranes of yeast strains in which specific genes encoding bc_1 subunits had been deleted. A significant advance was made by analyzing the mitochondrial membranes from several yeast bc_1 deletion strains under non-denaturing conditions [12]. This kind of analysis showed for the first time a direct physical interaction between distinct bc_1 subunits, thus leading to the proposal of the existence of a common set of bc_1 sub-complexes in numerous yeast deletion strains [12]. The present investigation, on the other hand, gives further insights into the yeast bc_1 biogenesis, describing a 500 kDa sub-complex which most probably represents a *bona fide* intermediate during the assembly of the cytochrome bc_1

complex into the inner mitochondrial membrane. Indeed, the wide distribution of this sub-complex in distinct yeast deletion strains, and its stability, strongly argues against the possibility that it may represent a degradation product or an incorrect assembly intermediate found only in a single mutant strain.

Previous investigations suggested that the central hydrophobic core of the bc_1 complex is represented by the cytochrome *b*/Qcr7p/Qcr8p sub-complex [24–27] (Fig. 8). We propose to refer to this subcomplex as the "membrane core sub-complex." Here we present data indicating that a larger core structure of the bc_1 complex exists which includes cytochrome *b*/Qcr7p/Qcr8p/cytochrome c_1/core protein 1/core protein 2 (Fig. 8). A significant difference between the smaller and the larger sub-complexes is the fact that the first one (cytochrome *b*/Qcr7p/Qcr8p) is very unstable and consequently its identification is extremely difficult, whereas the second (cytochrome *b*/Qcr7p/Qcr8p/cytochrome c_1/core protein 1/core protein 2) is characterized by a much higher stability. It is therefore tempting to speculate that the larger bc_1 core structure acquires a higher stability against proteolytic degradation after the incorporation of the two core proteins.

The minimal, yet stable, composition of the core structure of the yeast bc_1 complex includes the two catalytic subunits, cytochrome *b* and cytochrome c_1, the two core proteins, and the small supernumerary subunits Qcr7p and Qcr8p (Fig. 8). On the one hand, this finding reinforces the previously postulated existence of a nucleating core in the bc_1 assembly pathway, made up of the ternary complex between cytochrome *b* and the two small subunits Qcr7p and Qcr8p [12,24–27]. On the other hand, it does not confirm the previously proposed existence of a sub-complex composed of cytochrome c_1 and the two supernumerary subunits Qcr6p and Qcr9p [24,26,40].

The composition of the 500 kDa bc_1 sub-complex characterized in this study rather lends further support to our recent and unexpected finding of a stable interaction between cytochrome c_1 and each of the two core proteins [12]. As shown in Fig. 8, the large bc_1 core structure is capable of binding the chaperone protein Bcs1p. The binding site of this chaperone, therefore, must reside in the bc_1 subunits composing the core structure, i.e. cytochrome *b* and cytochrome c_1, the two core proteins, Qcr7p and Qcr8p. We can also conclude that Qcr6p and Qcr9p are not required for Bcs1p binding and that the binding of ISP and Qcr10p is subsequent to that of Bcs1p.

Previous studies suggested that the insertion of ISP into the bc_1 complex would replace the bound Bcs1p on the basis of the limited structural similarities between these two proteins which imply a common binding site on the immature bc_1 complex [29]. On the basis of our results this assumption seems unlikely because Bcs1p was also found in the homodimeric bc_1 complex and therefore concomitantly with the ISP [12]. In any case, Bcs1p is primarily required for the incorporation of ISP, even if further functions cannot be excluded. A possibile role of this chaperone in the stabilization of the core structure of the bc_1 complex can be excluded on the basis of the existence of the 500 kDa bc_1 sub-complex also in the ΔBCS1 deletion strain. In addition, the fact that the molecular mass of the bc_1 sub-complex found in this deletion strain is more or less similar to that of the sub-complex found in all the other deletion strains would suggest that the Bcs1p is present as a monomer in the bc_1 core structure. The role of this chaperone protein has also been investigated in humans in which molecular defects of BCS1 were associated with mitochondrial encephalopathy [41]. It was also shown in humans that the accessory factor Bcs1p is involved in ISP binding into the mitochondrial bc_1 complex [41].

On the basis of the present findings, we can now speculate about a possible sequence of binding of the remaining bc_1 subunits to the 500 kDa bc_1 sub-complex. As illustrated in Fig.

8, the bc_1 core structure, associated with the chaperone Bcs1p, binds Qcr6p and/or Qcr9p. Interestingly, our findings showed that there is no mutual interaction between Qcr6p and Qcr9p, at least in the stabilization of the core structure of the bc_1 complex. Such a core structure, in fact, exists and is stable independently of the presence of these two small supernumerary subunits. Furthermore, Qcr6p is not required for the incorporation of Qcr9p into the bc_1 core structure and, vice versa, Qcr9p is not essential for Qcr6p binding. It is also true that when only the Qcr6p subunit is missing, as previously demonstrated, the incorporation of all the other subunits into the bc_1 core structure proceeds normally, thus leading to the formation of the bc_1-oxidase supercomplexes [12]. On the other hand, Qcr9p, as well as Bcs1p, are essential for the subsequent binding of the catalytic subunit ISP to the 500 kDa bc_1 sub-complex. Therefore, the presence of both Qcr9p and Bcs1p is required for the insertion of ISP into the bc_1 sub-complex, but the presence of only one of these two subunits does not substitute for the other. After the addition of ISP the binding of the last subunit, i.e. Qcr10p, finally occurs. These findings are in agreement with previous studies which suggested that ISP and Qcr10p represent the last subunits incorporated into the bc_1 complex [29].

The molecular mass of the bc_1 sub-complex characterized in this study is also a matter of careful consideration. In fact, at least two possibilities may be considered: the first, that the 500 kDa bc_1 sub-complex is already in the dimeric form, i.e. is already containing two copies of each of the bc_1 subunits plus the monomeric form of Bcs1p; the second, that the 500 kDa bc_1 sub-complex contains a single copy of each of the bc_1 subunits which, in this case, may interact with unidentified oligomeric forms of bc_1 assembly factors [12,29,32,41]. Of course, the possibility that other protein components of the respiratory chain, such as subunits of the cytochrome *c* oxidase, or of the TIM23 machinery, or even of the metabolite transporter family, belong to the 500 kDa bc_1 sub-complex cannot be ruled out at the moment. The first hypothesis, i.e. a dimeric bc_1 core structure of approximately 500 kDa, may be compatible with the molecular masses of two copies of the bc_1 subunits found in the core structure. However, it has to be kept in mind that the technique of BN-PAGE does not allow a careful determination of the molecular mass of the oligomers because the electrophoretic migration may be influenced by several factors, such as the variable binding of Coomassie Brilliant Blue to polypeptides, as well as by intrinsic charge of protein complexes [42,43].

If the second hypothesis is correct, i.e. a monomeric form of the bc_1 complex in the 500 kDa band, the following question arises. When does the dimerization of the bc_1 complex occur? An appealing possibility would be that the addition of the ISP to the 500 kDa sub-complex induces the bc_1 dimerization. In this context, it is worth noting that ISP exists as trans-dimer structure, as clearly evidenced in the crystallographic studies [5–8]. The peripheral domain of ISP, which includes the 2Fe-2S cluster, is bound to a bc_1 monomer, whereas its transmembrane helix is directed towards the other monomer [3,8]. Interestingly, immediately after the addition of the ISP to the bc_1 sub-complex, a shift in the molecular mass from approximately 500 to 670 kDa does occur. This change in the molecular mass is too large to be explained by the addition of just two copies of the ISP and two copies of Qcr10p. However, this molecular mass change is too small to explain the dimerization of the bc_1 complex at this stage, i.e. just after the addition of ISP and Qcr10p.

On the other hand, in the transition from the 500 kDa band to the 670 kDa band a structural rearrangement of the bc_1 complex may occur, due to the binding of ISP and Qcr10p, which may lead to the dimerization of the complex. Such a structural rearrangement of the bc_1 complex may also be associated with a concomitant rearrangement of the bound assembly factors. These considerations become even more intriguing if one compares the assembly status of the bc_1 complex in the ΔISP and in theΔQCR10 strain (Fig. 1A and Fig. 2A). In

structural terms, the only (known) difference between these two deletion strains is the absence of ISP in the first strain in comparison to the second. However, a huge difference is seen in the molecular mass of the bc_1 complex in these two deletion strains, thus leading to the hypothesis that the addition of ISP may play a pivotal role in the structural rearrangement of the yeast bc_1 complex which finally leads to the supercomplex formation. These new findings open up several avenues of investigation and illustrate that a significant amount of work is still necessary for a complete understanding of the assembly process of the respiratory complexes in the inner mitochondrial membrane.

Experimental procedures

Materials

Yeast nitrogen base without amino acids, phenylmethylsulfonyl fluoride, digitonin, Triton X-100, glass beads, acrylamide, bis-acrylamide, N,N,N′N′-tetramethylethylenediamine (TEMED), ammonium peroxodisulfate, 6-aminohexanoic acid, di-isopropylfluorophosphate, agar, glucose, molecular weight protein markers for electrophoresis and glycerol were from Sigma (St Louis, MO, USA). Yeast extract and bacto-peptone were purchased from Difco (Detroit, MI, USA). Bis-Tris, ULTROL grade, was from Calbiochem (La Jolla, CA, USA). Coomassie Brilliant Blue G-250 was from Serva (Heidelberg, Germany). Tricine was from USB (Cleveland, OH, USA). Nitrocellulose was from Pall Life Sciences (New York, NY, USA). The ECL Plus Western Blotting detection system was from Amersham Biosciences (Chalfont St Giles, UK). All other reagents were of analytical grade.

Yeast strains and growth media

The genotypes and sources of the *S. cerevisiae* strains are described in Table 2. The ISP deletion strain was prepared by following the procedure of homologous recombination, as previously described [44]. This method requires the creation by PCR of a DNA fragment, in which the coding region for the selectable marker LEU2 is sandwiched by the 5′ and the 3′ flanking sequences of the ISP open-reading frame. Yeast cells were transformed with this construct by treatment with lithium acetate [45] and the transformants were then selected for leucine prototrophy. The double deletion strains were constructed by crossing selected single deletion strains. The resulting diploids were sporulated and tetrads were dissected to obtain the double deletion strains ΔQCR6/ΔQCR9, ΔQCR9/ΔQCR10, ΔISP/ΔQCR6, ΔISP/ΔQCR9 andΔISP/ΔQCR10. The selectable markers exhibited a 2:2 segregation pattern, and some spores were prototrophic for both markers. Haploid spores of ΔQCR6/ΔQCR9,ΔQCR9/ΔQCR10, ΔISP/ΔQCR6, ΔISP/ΔQCR9, ΔISP/ΔQCR10 were then selected for *Leu*$^+$ and *His*$^+$, *His*$^+$ and *Leu*$^+$, *Leu*$^+$ and *Ura*$^+$, *Leu*$^+$ and *His*$^+$ or *Leu*$^+$ and *His*$^+$ prototrophy, respectively. The growth phenotype was determined by incubating the yeast cells at 25°C either on YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) agar and 2% (w/v) glucose] or on YPEG plates [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) agar, 3% (v/v) glycerol and 2% (v/v) ethanol]. For the isolation of mitochondrial membranes, the yeast strains were grown in liquid YPD medium containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose, pH 5.0.

Isolation of mitochondrial membranes

Yeast cells were grown overnight at 25°C in 800 ml of YPD medium to the exponential growth phase (A_{578} of 1–2), harvested at 3200 *g* for 15 min (Avanti J-E centrifuge, JA-14 rotor; Beckman Coulter, Fullerton, CA, USA), washed once with distilled water and then resuspended in 25 ml of MTE buffer (400 mM mannitol, 50 mM Tris/HCl, 2 mM EDTA, pH 7.4). Acid-washed glass beads were added up to a final volume of 30 ml to the mixture kept at 4°C. 1 mM di-isopropylfluorophosphate was then added in order to prevent non specific proteolytic degradation. Afterwards, the cells were broken mechanically with a

vortex mixer at maximum speed for 10 min at 4°C. After the further addition of MTE buffer to a final volume of 50 ml, the mixture was vortexed briefly and then centrifuged at 1000 *g* for 10 min (Avanti J-E centrifuge, JA-14 rotor). The pellet was discarded, while the supernatant was transferred to a fresh tube and re-centrifuged at 18500 *g* for 30 min (5810R centrifuge, F-34-6-3 rotor; Eppendorf, Hamburg, Germany) in order to pellet the mitochondrial membranes. The pellet was then washed with 20–30 ml of MTE buffer and re-isolated by centrifugation as described above. The mitochondrial membranes were finally re-suspended in 1 ml of MTE buffer, divided in aliquots of 50 μl each, and stored at −80°C.

Elettrophoretic techniques

The mitochondrial membranes (75 μg) were lysed in 50 μl of ice-cold solubilization buffer [20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF] containing 1% digitonin (w/v) for 10 min at 0°C. After a clarifying centrifugation at 20000 *g* for 30 min (5810 centrifuge, F-45-30-11 rotor) to remove insoluble material, 2.5 μl of sample buffer (5% Coomassie Brilliant Blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminohexanoic acid) were added to the supernatant. The blue native polyacrilamide gel electrophoresis (BN-PAGE) was then performed as previously reported [46,47]. High molecular mass calibration markers included thyroglobulin (670 kDa), apoferritin (440 kDa), catalase (230 kDa), alcohol dehydrogenase (150 kDa), conalbumin (78 kDa), albumin (66 kDa), and β-lactoglobulin (35 kDa).

In the experiment testing the stability of native complexes, 75 μg of mitochondrial protein were solubilized in 50 μl of ice-cold buffer containing 1% (w/v) digitonin or 1% (w/v) Triton X-100 and incubated for 10 min at different temperatures ranging from 0°C to 25°C. After this treatment, mitochondrial lysates were analyzed by BN-PAGE, as described.

For subunit analysis of native complexes, sample lanes from BN-PAGE were excised from the gel and incubated in a solution containing 60 mM Tris/HCl (pH 6.8) and 0.2% SDS for 20 min at room temperature; each gel strip was then placed horizontally in the gel-pouring apparatus for the second dimension (SDS-PAGE) [48], already containing the separating gel (15% polyacrylamide and 0.1% SDS). The gel slice was subsequently encased in 5% polyacrylamide stacking gel and finally submitted to electrophoresis. The calibration markers used in the SDS-PAGE were albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactoglobulin (14.2 kDa).

Western blotting and ECL detection

After BN-PAGE and two-dimensional BN/SDS-PAGE, the mitochondrial proteins were transferred to nitrocellulose by Western blotting following standard procedures. Immunodetection was performed using polyclonal and monoclonal primary antibodies against the various subunits of the yeast cytochrome bc_1 complex. Another antibody used in this study was that against Bcs1p (a generous gift from R. Stuart, Marquette University, Milwaukee, WI, USA). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG (Chemie, Rockford, IL, USA) or anti-mouse IgG (Amersham Biosciences, Chalfont St Giles, UK). The ECL kit was used for the immunodetection, and the fluorographs were quantified using an Imaging Densitometer GS-700 from Bio-Rad (Hercules, CA, USA).

Other methods

Protein determination was carried out according to published methods [49,50]. Standard procedures were used for the preparation and ligation of DNA fragments, for the transformation of *E. coli* and the isolation of plasmid DNA from bacterial cells [51]. Other yeast genetic methods used were as described in [52].

Acknowledgments

This work was supported by the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) PRIN 2006, and by National Institutes of Health Research Grant GM 20379.

Abbreviations

cyt *b*	cytochrome *b*
cyt c_1	cytochrome c_1
ISP	Rieske iron-sulfur protein
core 1	core protein 1
core 2	core protein 2
Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p	subunits 6, 7, 8, 9 and 10 of the yeast bc_1 complex, respectively
Cox1p and Cox6bp	subunits 1 and 6b of the yeast cytochrome *c* oxidase complex

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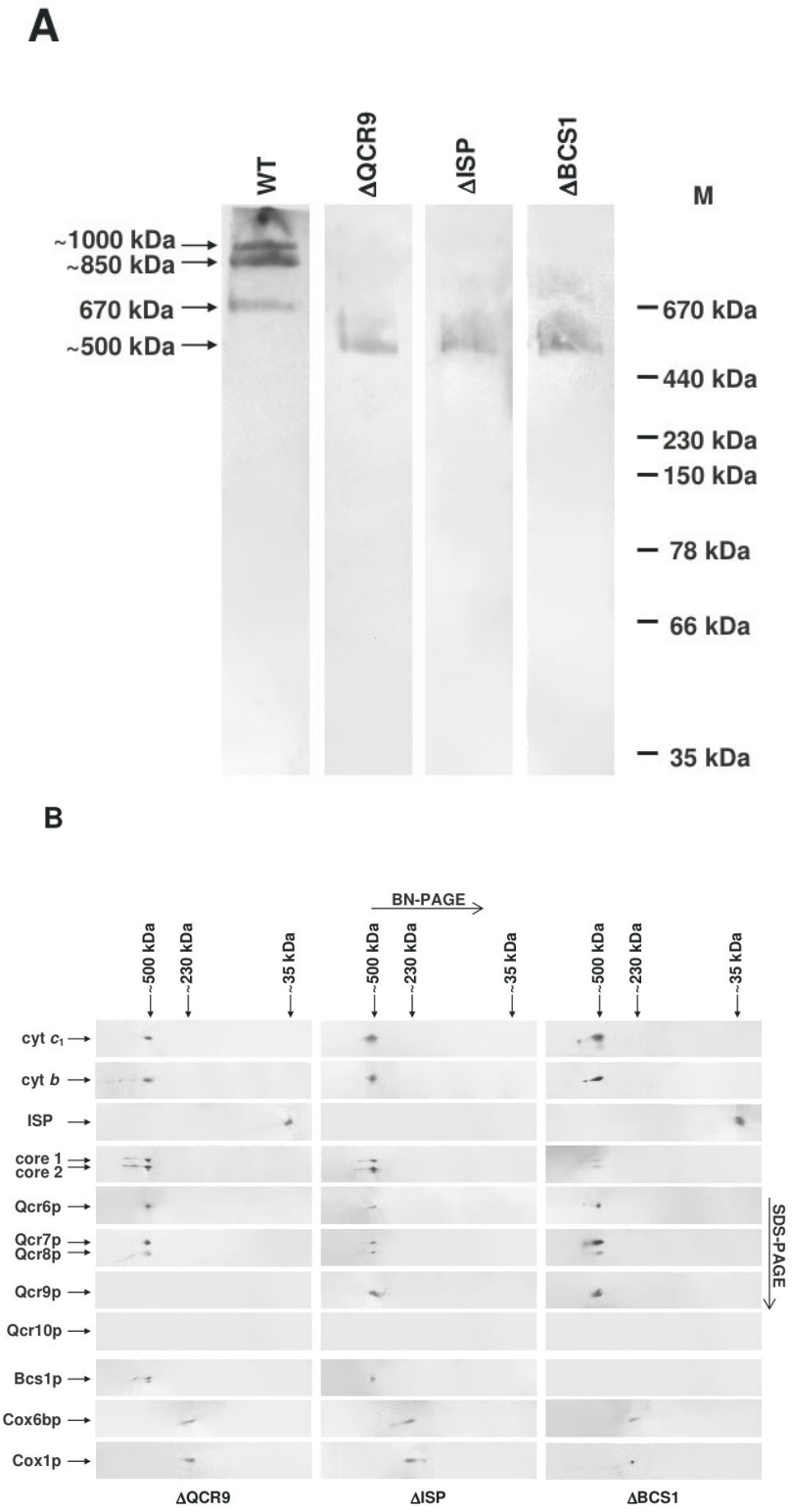

Figure 1.

Characterization of 500 kDa bc_1 sub-complexes in the yeast deletion strains lacking Qcr9p, ISP or Bcs1p. In panel A, mitochondrial membranes from wild type (WT), ΔQCR9, ΔISP

and ΔBCS1 strains were solubilized with 1% digitonin and analyzed by BN-PAGE, as described in Experimental Procedures. The protein complexes were detected by immunoblotting with antisera specific for core protein 1 and core protein 2. The calibration markers are indicated on the right side of the gel blot. In panel B, the mitochondrial membranes from the three yeast deletion strains were analyzed by SDS-PAGE after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blot. Cyt c_1, cytochrome c_1; cyt *b*, cytochrome *b*; ISP, Rieske iron-sulfur protein; core 1, core protein 1; core 2, core protein 2; Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p, subunits 6, 7, 8, 9 and 10 of the yeast bc_1 complex, respectively. Cox1p and Cox6bp, subunits 1 and 6b of the yeast cytochrome *c* oxidase complex, respectively.

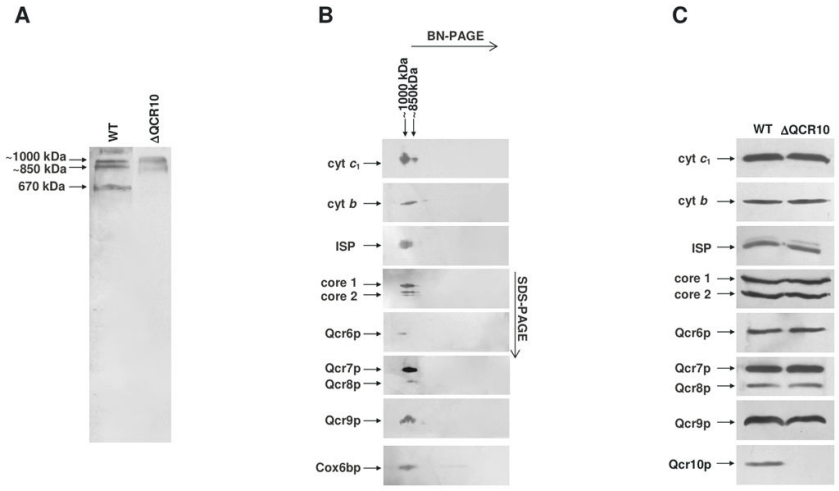

Figure 2.

Resolution of mitochondrial membranes from WT and ΔQCR10 yeast strains by BN-PAGE and SDS-PAGE. In Panel A, mitochondrial membranes were analyzed by BN-PAGE, as described in Fig. 1A. Panel B shows the SDS-PAGE of the subunit 10 deletion strain membranes after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blot. Panel C shows the SDS-PAGE analysis of the mitochondrial membranes from WT and ΔQCR10 yeast strains followed by Western blotting with antibodies to the subunits of the bc_1 complex indicated on the left side of the blots.

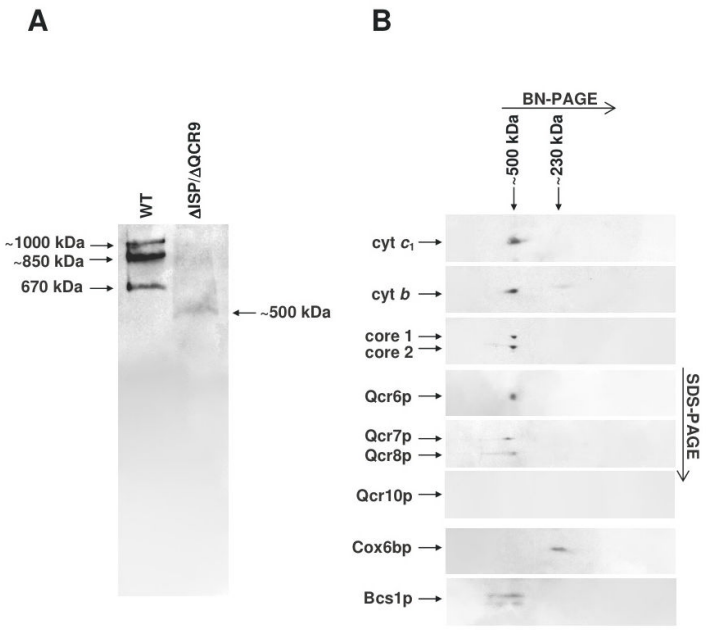

Figure 3.

Resolution of mitochondrial membranes from WT and ΔISP/ΔQCR9 yeast strains by BN-PAGE and SDS-PAGE. In Panel A, mitochondrial membranes were analyzed by BN-PAGE, as described in Fig. 1A. Panel B shows the SDS-PAGE of the ΔISP/ΔQCR9 deletion strain membranes after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blot.

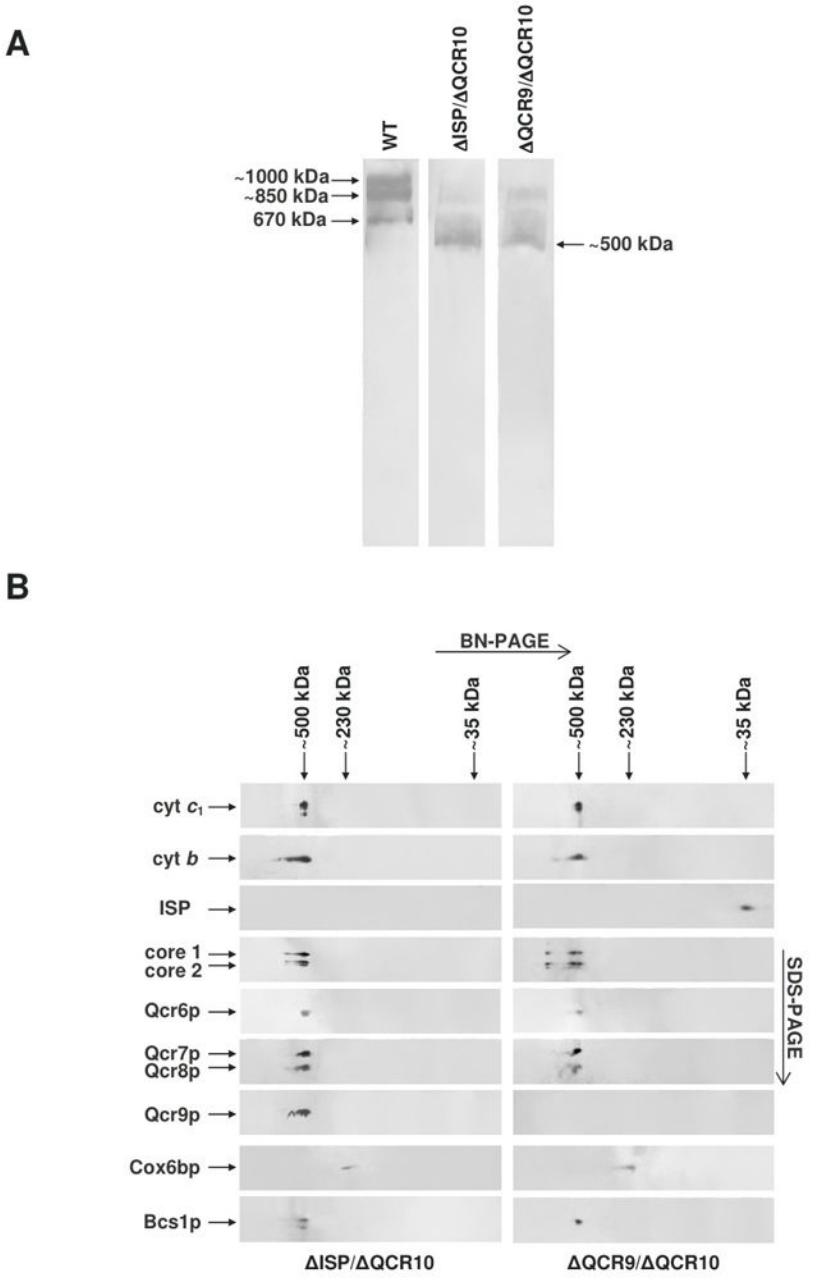

Figure 4.

Resolution of mitochondrial membranes from WT, ΔISP/ΔQCR10 andΔQCR9/ΔQCR10 yeast strains by BN-PAGE and SDS-PAGE. In Panel A, mitochondrial membranes were analyzed by BN-PAGE, as described in Fig. 1A. Panels B show the SDS-PAGE of the ΔISP/ΔQCR10 (left) and the ΔQCR9/ΔQCR10 (right) deletion strain membranes after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blots.

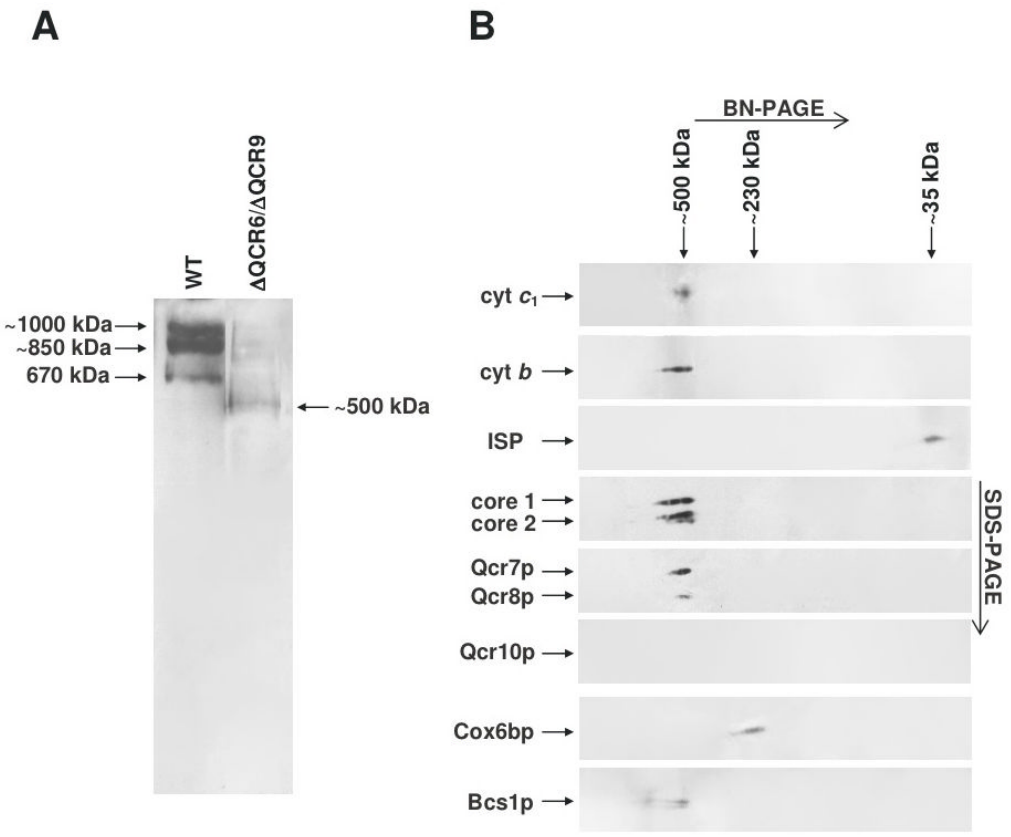

Figure 5.

Resolution of mitochondrial membranes from WT and ΔQCR6/ΔQCR9 yeast strains by BN-PAGE and SDS-PAGE. In Panel A, mitochondrial membranes were analyzed by BN-PAGE, as described in Fig. 1A. Panel B shows the SDS-PAGE of the ΔQCR6/ΔQCR9 deletion strain membranes after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blot.

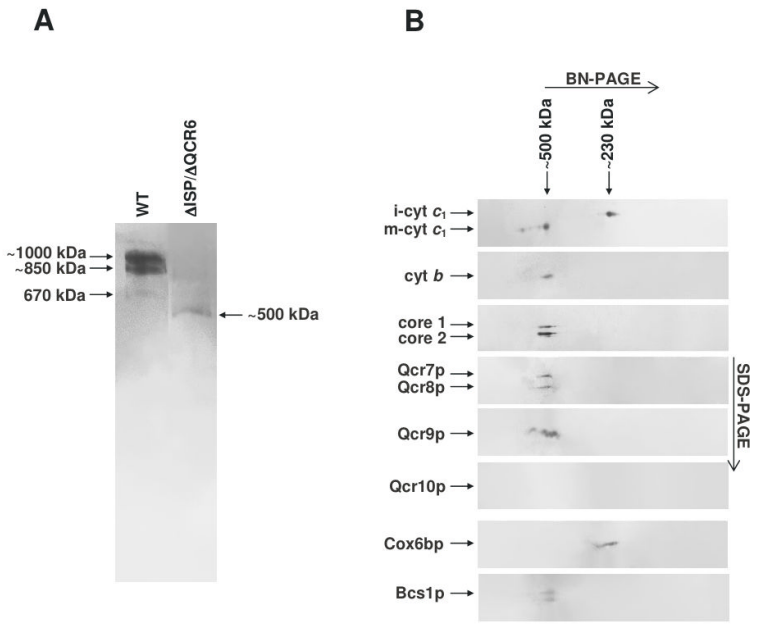

Figure 6.

Resolution of mitochondrial membranes from WT and ΔISP/ΔQCR6 yeast strains by BN-PAGE and SDS-PAGE. In Panel A, mitochondrial membranes were analyzed by BN-PAGE, as described in Fig. 1A. Panel B shows the SDS-PAGE of the ΔISP/ΔQCR6 deletion strain membranes after BN-PAGE in the first dimension. The gel was blotted and probed with antibodies to the proteins indicated on the left side of the gel blot.

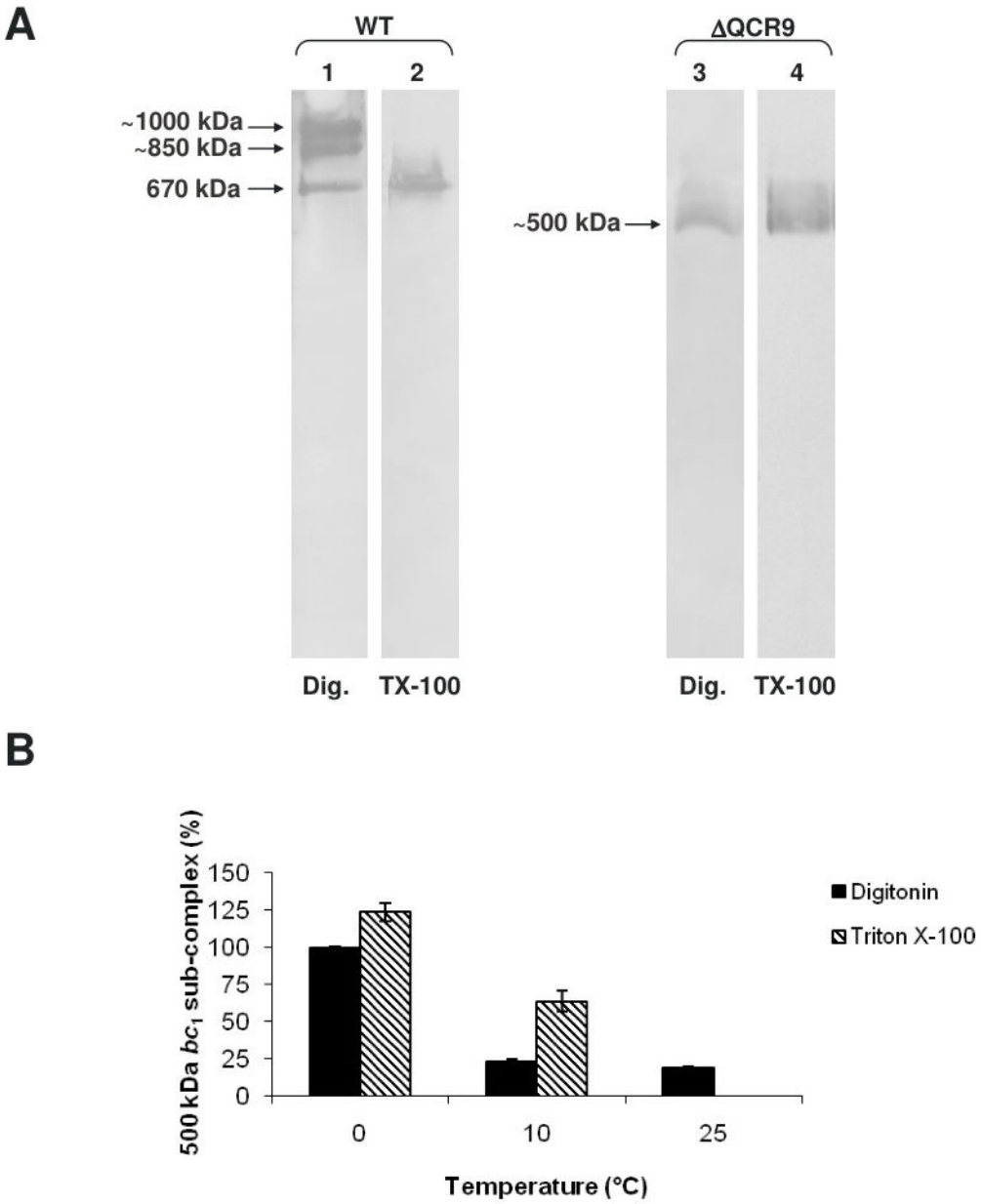

Figure 7.

Stability of the 500 kDa bc_1 sub-complex in different conditions of solubilization. In Panel A, mitochondrial membranes from WT (lanes 1 and 2) and ΔQCR9 (lanes 3 and 4) yeast strains were solubilized with 1% digitonin (lanes 1 and 3) or 1% Triton X-100 (lanes 2 and 4) and protein complexes were analyzed by BN-PAGE, as described in Fig. 1A. In Panel B, mitochondrial membranes from the subunit 9 deletion strain were solubilized with 1% digitonin or 1% Triton X-100 and incubated for 10 min at different temperatures ranging from 0°C to 25°C. After this treatment, mitochondrial lysates were analyzed by BN-PAGE, as described in Fig. 1A. The immunodecorated bc_1 sub-complex of approximately 500 kDa was quantified as described under Experimental Procedures and shown in Panel B; the amount of the 500 kDa bc_1 sub-complex solubilized with 1% digitonin at 0°C was set to 100% (control).

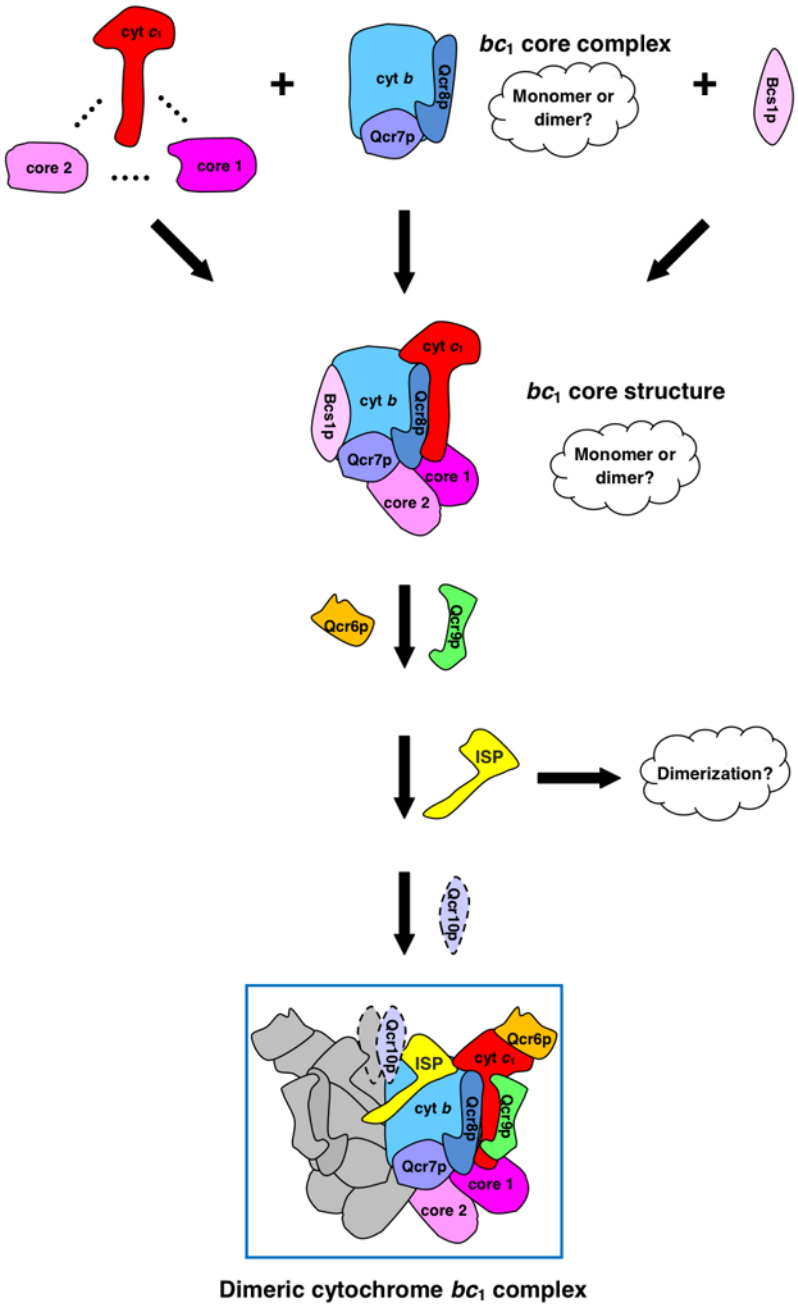

Figure 8.

Schematic model depicting the putative pathway of assembly of the yeast cytochrome bc_1 complex. *De novo* assembly occurs via the association of bc_1 sub-complexes (cytochrome *b*/Qcr7p/Qcr8p and cytochrome c_1/core protein 1/core protein 2) in a large core structure which also includes the chaperone protein Bcs1p. This core structure is then able to sequentially bind the remaining bc_1 subunits in a process which eventually leads to the formation of the homodimeric bc_1 complex in the inner mitochondrial membrane. As Qcr10p is not essential for the dimerization of the bc_1 complex, it is represented with dashed outlines. Indeed, the bc_1 complex apparently can dimerize without the addition of Qcr10p, since the enzyme from the subunit 10 deletion strain and from the wild type strain were

purified by the same chromatography procedure from the mitochondrial membranes of the respective strains [54].

Table 1

Growth phenotype of single and double deletion mutants. All the strains were first grown in liquid YPD medium to the same original density and subsequently plated on solid media containing fermentable (YPD) or non-fermentable carbon sources (YPEG). Normal growth, +; reduced growth rate, (+); no growth, −.

YEAST STRAINS	YPD	YPEG
WT	+	+
ΔQCR9	+	(+)
ΔISP	+	−
ΔBCS1	+	−
ΔQCR10	+	+
ΔISP/ΔQCR9	+	−
ΔISP/ΔQCR10	+	−
ΔQCR9/ΔQCR10	+	(+)
ΔQCR6/ΔQCR9	+	−
ΔISP/ΔQCR6	+	−

Table 2

Saccharomyces cerevisiae strains used in this study.

Strain	Genotype	Reference
WT (W303–1B)	*MATα, ade2–1, his3–11,15, trp1–1, leu2–3,112, ura3–1, can1–100*	Gift from A. Tzagoloff, Columbia University, New York
ΔQCR9	*MATα, leu2–3,112, can1–11, qcr9Δ2::HIS3*	[53]
ΔISP	*MATa, ade2–1, his3–11,15, trp1–1, leu2–3,112, ura3–1, can1–100, rip1Δ::LEU2*	This study
ΔBCS1	*MATa, ade2–1, his3–1,15, leu2–3,112, trp1–1, ura3–1, Δbcs1::HIS3*	Gift from A. Tzagoloff, Columbia University, New York
ΔQCR10	*MATa, ade2–1, his3–1,15, leu2–3,112, ura3–1, can1–100, qcr10Δ2::LEU2*	[54]
ΔISP/ΔQCR9	*MATα, leu2–3,112, his3, rip1Δ::LEU2, qcr9Δ2::HIS3*	This study
ΔISP/ΔQCR10	*MATα, ade2–1, his3–11,15, leu2–3,112, ura3–1, can1–100, rip1Δ::LEU2 qcr10Δ1::HIS3*	This study
ΔQCR9/ΔQCR10	*MATa, ade2–1, leu2–3,112, qcr9Δ2::HIS3, qcr10Δ2::LEU2*	[27]
ΔQCR6/ΔQCR9	*MATα, leu2–3,112, his3, qcr6Δ::LEU2, qcr9Δ2::HIS3*	This study
ΔISP/ΔQCR6	*MATα, ade2–1, his3–11,15, trp1–1, leu2–3,112, ura3–1, can1–100, rip1Δ::LEU2, qcr6Δ::URA3*	This study