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Bcs1p, an AAA-family member, is a chaperone for the assembly of the cytochrome bc_1 complex

Cristina-Maria Cruciat, Kai Hell, Heike Fölsch, Walter Neupert and Rosemary A.Stuart[1,2]

Institut für Physiologische Chemie der Universität München, Goethestrasse 33, 80336 München, Germany

[1]Present address: Department of Biology, Marquette University, Milwaukee, WI 53201, USA

[2]Corresponding author
e-mail: stuartr@marquette.edu

Bcs1p, a mitochondrial protein and member of the conserved AAA protein family, is involved in the biogenesis of the cytochrome bc_1 complex. We demonstrate here that Bcs1p is directly required for the assembly of the Rieske FeS and Qcr10p proteins into the cytochrome bc_1 complex. Bcs1p binds to a precomplex in the assembly pathway of the cytochrome bc_1 complex. Binding of Bcs1p to and release from this assembly intermediate is driven by ATP hydrolysis. We propose that Bcs1p acts as an ATP-dependent chaperone, maintaining the precomplex in a competent state for the subsequent assembly of the Rieske FeS and Qcr10p proteins.

Keywords: AAA family/Bcs1p/chaperones/cytochrome bc_1 complex/mitochondria

Introduction

The assembly of the mitochondrial respiratory chain complexes involves the coming together of both nuclear and mitochondrially encoded protein subunits and the incorporation of a number of prosthetic groups. This is not a trivial process, as each of these complexes has a defined stoichiometry and appears to be assembled in a coordinated fashion. Failure to synthesize one subunit of a complex often results in the rapid proteolytic turnover of the remaining synthesized subunits, unless they have progressed sufficiently in their assembly pathway to form stable assembly intermediates, termed subcomplexes. Over the past few years, the genetic analysis of respiratory-deficient yeasts has provided evidence for a number of assembly co-factors, essential for the formation of these respiratory chain complexes (Tzagoloff and Myers, 1986; Grivell, 1989; Tzagoloff and Dieckmann, 1990; Tzagoloff, 1995). These co-factors are not subunits of the final complexes, but play an essential role in their formation. The roles of some of these proteins have been characterized and range from enzymes involved in the synthesis and/or insertion of prosthetic groups, to those which facilitate the insertion of proteins into the inner membrane, and finally to others which are proposed to act as chaperones coordinating the formation of stable assembly subcomplexes. The molecular details of the modes of operation of these chaperones are unknown to date.

The ubiquinol–cytochrome *c* oxidoreductase, herein referred to as the cytochrome bc_1 complex (complex III), of the respiratory chain is composed of three catalytic subunits: the mitochondrially encoded cytochrome *b*, the nuclearly encoded cytochrome c_1 and Rieske FeS proteins. These proteins, together with a series of other non-catalytic subunits, assemble to form an enzymatically active complex (Trumpower, 1990; Tzagoloff, 1995). In the yeast *Saccharomyces cerevisiae* these non-catalytic subunits are Core1, Core2, Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p (Tzagoloff, 1995). The analysis of the steady-state levels of these subunits in yeast mutants deficient in the synthesis of one of these subunits has suggested that the assembly pathway of this complex occurs in a coordinate fashion, involving the formation of specific assembly intermediates (for a review see Grivell, 1989; Tzagoloff, 1995). According to this model, cytochrome *b* initially forms a subcomplex with Qcr7p and Qcr8p, which subsequently joins with the Core1 and Core2 proteins. Cytochrome c_1, on the other hand, is proposed to form another subcomplex with Qcr6p and Qcr9p. Formation of each of these subcomplexes ensures stability against proteolytic attack for the individual subunits contained within them (Grivell, 1989). The cytochrome *b* and cytochrome c_1 subcomplexes subsequently unite to form a 'cytochrome bc_1 precomplex', prior to the assembly of the Rieske FeS protein and, presumably, the non-essential subunit, Qcr10p. The final assembled cytochrome bc_1 complex can be purified as a dimeric complex from the mitochondrial membranes (Xia *et al*., 1997; Iwata *et al*., 1998; Zhang *et al*., 1998).

A number of genes have been identified encoding proteins that potentially assist in the assembly of the cytochrome bc_1 complex (Wu and Tzagoloff, 1989; Nobrega *et al*., 1992; Crivellone, 1994). So far the biochemical function of none of these proteins is understood. Bcs1p was recently reported to be specifically involved in the assembly of the Rieske FeS protein (Nobrega *et al*., 1992). In the absence of Bcs1p, the steady-state levels of the Rieske FeS are reported to be strongly reduced (Nobrega *et al*., 1992). Whether Bcs1p was required for the submitochondrial sorting of the Rieske FeS, the insertion of the FeS cluster prosthetic group or directly facilitating assembly of this protein into the precytochrome bc_1 complex was not known until now.

Bcs1p is a mitochondrial inner membrane protein and a fringe member of the highly conserved ATPases, the AAA protein family (ATPases associated with different cellular activities) (Confalonieri and Duguet, 1995; Beyer, 1997; Patel and Latterich, 1998). Members of this diverse family are united by their conserved AAA sequence motif, which encompasses an ATP-binding site comprising Walker A and B boxes (Confalonieri and Duguet, 1995).

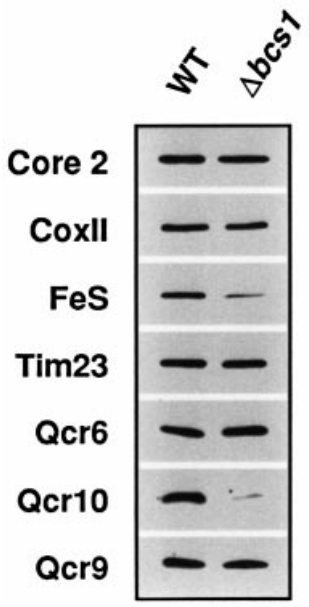

Fig. 1. Bcs1p is required for the stable expression of Rieske FeS protein and Qcr10p. Equivalent amounts of mitochondria isolated from the Δ*bcs1* deletion strain and the corresponding isogenic wild-type strain W303-1A were subjected to SDS–PAGE and analysed by Western blotting for the steady-state levels of components of the cytochrome bc_1 complex and unrelated other marker proteins, as indicated.

AAA-family members are involved in a variety of cellular processes, including vesicle-mediated transport, proteolytic degradation and cell cycle regulation (Latterich *et al*., 1995; Arlt *et al*., 1996; Mayer *et al*., 1996; Rep and Grivell, 1996; Patel and Latterich, 1998; Leonhard *et al*., 1999).

We characterized Bcs1p and show here that it acts as a molecular chaperone facilitating the assembly of the Rieske FeS and Qcr10p proteins into the cytochrome bc_1 complex. Bcs1p binds to the partially formed pre-cytochrome bc_1 complex in an ATP-dependent manner. By doing so, we propose that Bcs1p maintains this complex in a state competent for incorporation of the Rieske FeS and Qcr10p proteins.

Results

Bcs1p is required for stable expression of both Qcr10p and the Rieske FeS protein

Deletion of the BCS1 gene results in respiratory-incompetent mitochondria due to a defective cytochrome bc_1 complex (Nobrega *et al*., 1992). Analysis of the mitochondria isolated from the Δ*bcs1* strain had indicated that the levels of the Rieske FeS protein were reduced in comparison with wild-type mitochondria (Nobrega *et al*., 1992). The levels of subunits Core1, Core2, cytochrome c_1, Qcr7p and Qcr8p, in contrast, remained unchanged. In order to address whether the FeS protein was the only protein affected by the absence of Bcs1p, the steady-state levels of the remaining subunits of the cytochrome bc_1 complex, Qcr6p, Qcr10p and Qcr9p in the Δ*bcs1* strain were analysed here (Figure 1). The presence of Bcs1p was required for the stable expression of Qcr10p, in addition to FeS, as both were strongly reduced in the Δ*bcs1* strain. Deletion of Bcs1p had no appreciable effect on the steady-state levels of Qcr6p and Qcr9p. In addition, the levels of two unrelated proteins, subunit II of the cytochrome oxidase complex (CoxII) and Tim23, a component of the inner-membrane translocase, as well as Core2 (as previously documented by Nobrega *et al*., 1992), were unaffected by the absence of Bcs1p.

We conclude that Bcs1p plays a crucial role in the stable expression of only two subunits of the cytochrome bc_1 complex: the Rieske FeS protein and the small non-catalytic subunit, Qcr10p.

Bcs1p is directly required for the assembly of Rieske FeS into the cytochrome bc_1 complex

The assembly state of the cytochrome bc_1 complex was analysed in the Δ*bcs1* mitochondria (Nobrega *et al*., 1992) and compared with that of the wild type. Isolated mitochondria were subjected to lysis using the mild detergent digitonin. This solubilization procedure proved successful for the isolation of an intact cytochrome bc_1 complex, as was evident from the co-elution upon gel filtration of the Core2, cytochrome c_1, Rieske FeS and Qcr10p signals (Figure 2A, upper two panels). The estimated molecular mass of the eluted complex was ~1000 kDa. Thus the dimeric cytochrome bc_1 complex (~500 kDa) appears to form a supracomplex in the mitochondrial inner membrane which remains intact under the solubilization conditions used. The molecular identity of the complex interacting with the dimeric cytochrome bc_1 complex is currently under investigation.

Gel filtration analysis of solubilized Δ*bcs1* mitochondria indicated that complete assembly of the cytochrome bc_1 complex had not occurred in the absence of Bcs1p (Figure 2A, lower two panels). Core2 and cytochrome c_1 co-eluted from the column two fractions later than in the wild-type analysis, indicating that they were present in a complex that was smaller than the fully assembled cytochrome bc_1 complex; we designate this complex here the cytochrome bc_1 pre-supracomplex or 'precomplex'. This precomplex could also be immunodecorated with antisera specific for cytochrome *b* and Qcr6p subunits (results not shown), but not Qcr10p and Rieske FeS. Rather, Qcr10p and Rieske FeS were recovered in fractions with estimated molecular masses of ~70 and 40 kDa, respectively. The cytochrome bc_1-containing complex which accumulated in the Δ*bcs1* mitochondria had a larger apparent molecular mass than the functional dimeric cytochrome bc_1 complex, suggesting that partial assembly of the supracomplex had occurred. Hence incorporation of Qcr10p and Rieske FeS was not a prerequisite for the formation of such a supracomplex. Low amounts of Core2 and cytochrome c_1 were also recovered in a smaller complex (~100 kDa) in the Δ*bcs1* mitochondria. On the basis of molecular mass analysis this may correspond to the monomeric precytochrome bc_1 subcomplex.

Blue native gel electrophoresis (BN–PAGE) was also used to study the oligomeric state of the cytochrome bc_1 complex. Wild-type and Δ*bcs1* mitochondria were solubilized with digitonin and analysed by BN–PAGE followed by Western blotting (Figure 2B). The wild-type cytochrome bc_1 complex remained intact under this analysis procedure, as cytochrome *b* and Rieske FeS proteins were found to migrate together. Furthermore the estimated molecular mass of the complex indicated that

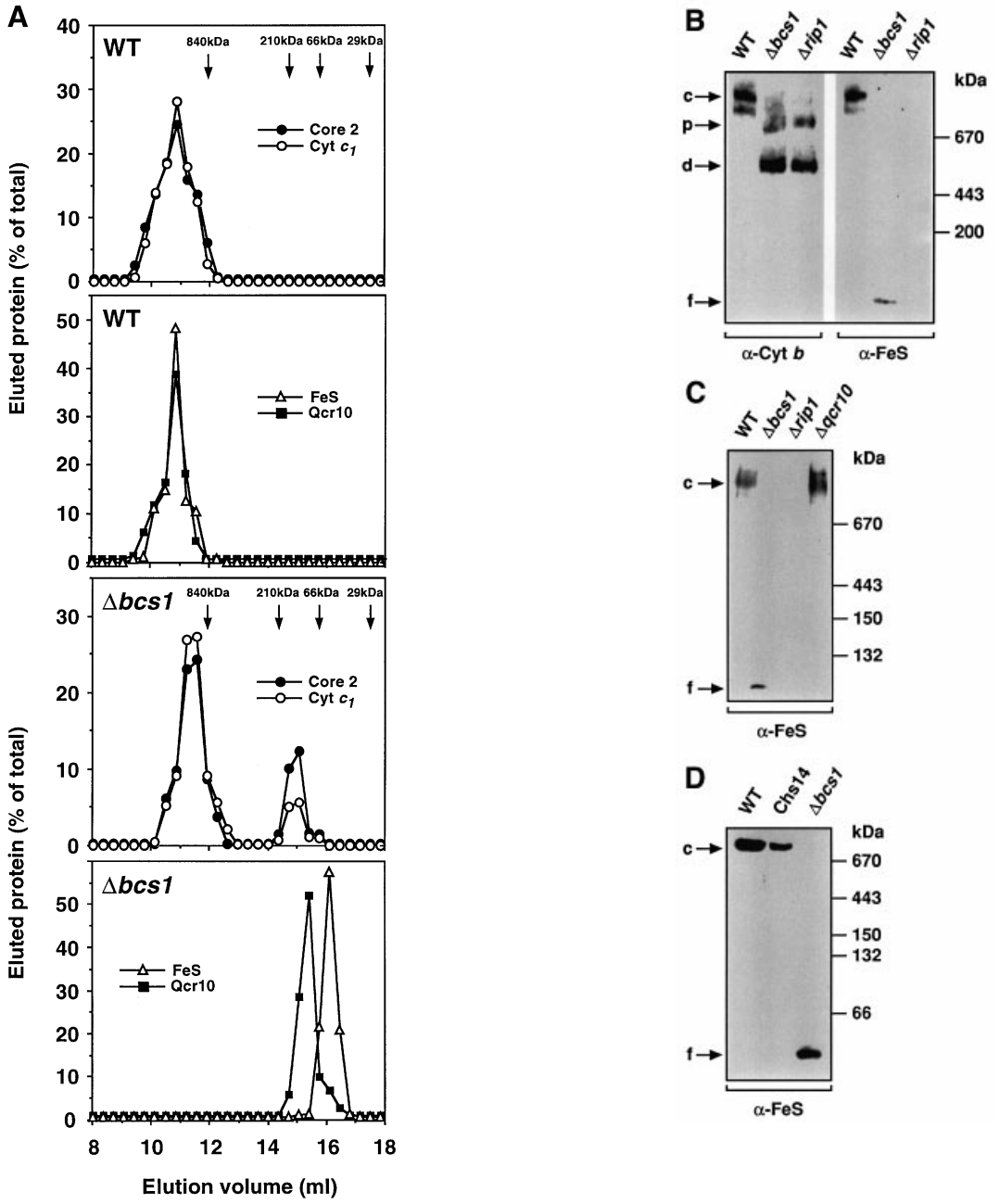

Fig. 2. Bcs1p is required for the assembly of the Rieske FeS and Qcr10p proteins into the cytochrome bc_1 complex. (**A**) Gel filtration analysis of the cytochrome bc_1 complex of wild-type (WT) and Δ*bcs1* strains. The proteins Core2 (●), cytochrome c_1 (○), Rieske FeS (△) and Qcr10p (■) were detected in the eluate fractions by Western blotting and immunodecoration. See Materials and methods for details of calibration standards. (**B**) BN–PAGE analysis of the cytochrome bc_1 complex of wild-type (WT), Δ*bcs1* and Δ*rip1* strains. A 4–8% polyacrylamide gradient gel was used. The protein complexes were detected by Western blotting and immunodecoration with antibodies against cytochrome *b* (α-Cyt *b*) and Rieske FeS (α-FeS). c, cytochrome bc_1 supracomplex; p, a precomplex of the cytochrome bc_1 supracomplex; d, cytochrome bc_1 dimeric precomplex (without Rieske FeS and Qcr10p); f, free, non-assembled monomeric Rieske FeS. Note that a minor amount of a second form of complex 'c' was sometimes observed with slightly faster mobility than the supracomplex; this form may result from partial instability of the supracomplex. (**C**) BN–PAGE analysis of the cytochrome bc_1 complex from wild-type (WT), Δ*bcs1*, Δ*rip1* and Δ*qcr10* strains. A 4–8% polyacrylamide gradient gel was used. Assembled and unassembled Rieske FeS proteins were detected by Western blotting, using antibodies specific for Rieske FeS protein (α-FeS). Abbreviations as in (B). (**D**) BN–PAGE analysis of the cytochrome bc_1 complex from wild-type (WT), and the Chs14 and Δ*bcs1* strains. The assembly state of Rieske FeS was monitored by immunoblotting using antibodies specific for Rieske FeS protein (α-FeS). A 6–13% polyacrylamide gradient gel was used. Calibration standards, see Materials and methods; abbreviations as in (B).

the cytochrome bc_1 complex had been isolated as a supracomplex, as was observed in the gel filtration analysis. In the Δ*bcs1* mitochondria, the Rieske FeS protein was not assembled into the cytochrome bc_1 complex, but was present in the low molecular mass range. The incompletely assembled cytochrome bc_1 complex appears to be less stable under the conditions of BN–PAGE than it is upon gel filtration. Whereas the predominant form isolated by gel filtration analysis was a large complex of ~100 kDa less than the wild-type supracomplex, it was resolved into two distinct complexes by the BN–PAGE, a larger, less abundant form and a second, smaller, predomin-

ant form. From their molecular masses, these complexes correspond to a partially assembled form of the cytochrome bc_1 pre-supracomplex (the minor species) (as observed in the gel filtration) and a smaller form of the cytochrome bc_1 precomplex (lacking Rieske FeS and Qcr10p) (predominant species). Interestingly, the same assembly state of the cytochrome bc_1 complex was observed in mitochondria isolated from a strain, Δ*rip1*, where the RIP1 gene encoding for the Rieske FeS protein had been deleted (Figure 2B).

Analysis of mitochondria isolated from a Δ*qcr10* yeast strain by BN–PAGE indicated the presence of assembled cytochrome bc_1 supracomplex containing the Rieske FeS protein. Thus the presence of Qcr10p is not essential for the assembly of Rieske FeS into the cytochrome bc_1 complex (Figure 2C). Although essential for the assembly of both Rieske FeS and Qcr10p, we conclude that Bcs1p acts primarily at the level of the Rieske FeS protein. The assembly of Rieske FeS thus appears to be a prerequisite for the incorporation of Qcr10p into the cytochrome bc_1 complex. In accordance with this, the levels of Qcr10p are strongly reduced in the Δ*rip1* mitochondria, indicating that Qcr10p requires the presence of the Rieske FeS protein for its stabilization. Furthermore, the Qcr10p present in the Δ*rip1* mitochondria is not assembled into the cytochrome bc_1 precomplex, as indicated by gel filtration analysis (results not shown).

As discussed previously, Bcs1p might be involved in the formation or addition of the FeS cluster into the apo-Rieske FeS protein (Nobrega *et al*., 1992). In order to address whether the incorporation of this prosthetic group is a prerequisite for assembly of this protein, mitochondria were isolated from a yeast strain expressing only a mutant Rieske FeS derivative (Chs14) which was unable to form an FeS cluster (Denke *et al*., 1998). Despite its inability to bind the FeS cluster, as shown by BN–PAGE, the mutated Rieske FeS was correctly assembled into the cytochrome bc_1 supracomplex (Figure 2D). Thus, addition of its prosthetic group, a FeS cluster, is not essential for assembly of Rieske FeS into the cytochrome bc_1 complex.

In conclusion, Bcs1p is directly and specifically involved in mediating the assembly of the Rieske FeS protein into the cytochrome bc_1 complex. In the absence of Bcs1p, the assembly of Qcr10p is prevented in a secondary reaction due to the inhibition of Rieske FeS assembly.

The activity of Bcs1p is compromised by mutations in its ATP-binding domain

We isolated yeast mutants with an impaired Bcs1p function. The first mutant, *bcs1-2*, was identified in a screen for temperature-sensitive mutants in the Bcs1p. This mutant was capable of growing on non-fermentable carbon sources, such as glycerol, at 24°C, but not at the non-permissive temperature of 37°C (Figure 3A). Sequencing the BCS1 gene in the *bcs1-2* strain revealed the exchange of Pro268, which is located in the Walker A box, for a Leu residue. A second mutant, *bcs1(K273A)*, was constructed whereby the conserved Lys273 residue in the Walker A box was exchanged by site-directed mutagenesis for an Ala residue. The *bcs1(K273A)* mutant was able to grow on glucose, but not on glycerol at 24 or 37°C (Figure 3A). Thus, the ability to bind ATP is intimately related to the function of the Bcs1p protein.

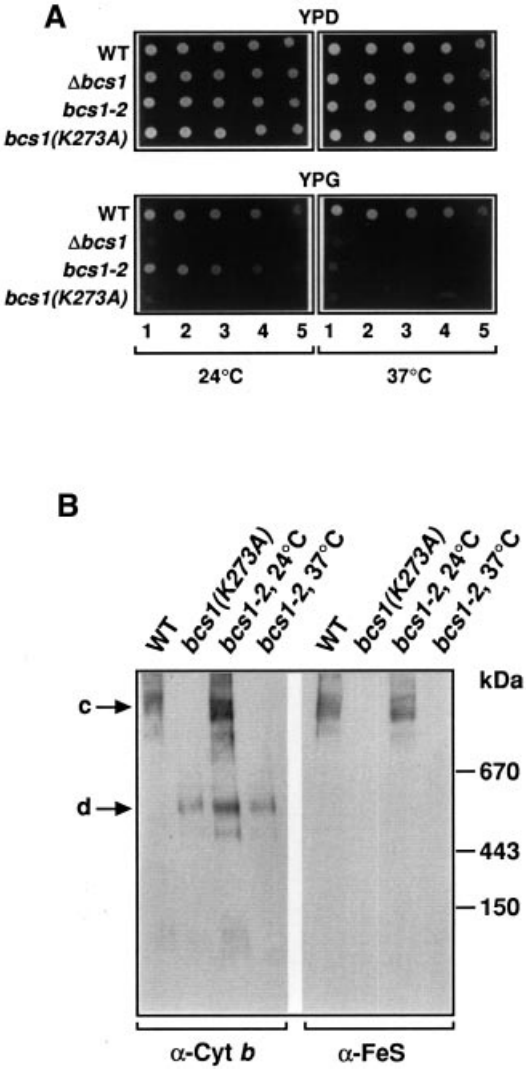

Fig. 3. The ATP-binding domain is required for Bcs1p function. (**A**) Growth of yeast cells with mutations in BCS1. Cells from the deletion strain Δ*bcs1*, *bcs1-2*, *bcs1(K273A)* and the corresponding isogenic wild-type (WT) W303-1A grown in the presence of glucose were resuspended in sterile water at a concentration of 10 OD_{578}/ml. A dilution series was generated by serially diluting this suspension 10-fold each time. Two microlitres of each of the resulting dilutions were spotted onto YPD and YPG plates (spots 1–5) and were incubated for 2 days at 24 or 37°C. (**B**) BN–PAGE of solubilized mitochondria from the Walker A box mutant *bcs1(K273A)*, the temperature-sensitive mutant *bcs1-2* and the corresponding isogenic wild type (WT) W303-1A. Protein complexes were detected by Western blotting, using antibodies against cytochrome *b* (α-Cyt *b*) and Rieske FeS (α-FeS). Calibration standards (see Materials and methods) and abbreviations are as in Figure 2B.

Both of these mutations in the Walker A box affected the assembly process of the cytochrome bc_1 complex (Figure 3B). Partial assembly of the cytochrome bc_1 supracomplex was observed in mitochondria of the *bcs1-2* mutant that had been grown at 24°C. The main fraction of the complex, however, accumulated as a form corresponding to the incompletely assembled cytochrome bc_1 precomplex, which did not contain the Rieske FeS protein. When grown at the non-permissive temperature, only this precomplex was observed in the *bcs1-2* mitochondria. Complete assembly of the supracomplex was not detected, consistent with the lack of respiratory

competence of this mutant at 37°C. Mitochondria isolated from the *bcs1(K273A)* yeast strain also contained only the partially assembled cytochrome bc_1 complex, which accumulated largely in the form of the precomplex.

In summary, the assembly of the complete cytochrome bc_1 complex requires the ATP-dependent activity of the Bcs1p protein.

Bcs1p associates with the cytochrome bc_1 precomplex in an ATP-dependent manner

A number of possibilities exist for the molecular mechanism of Bcs1p-mediated assembly of the Rieske FeS protein into the cytochrome bc_1 complex. One could imagine that Rieske FeS interacts directly with the Bcs1p protein prior to its assembly, to facilitate its sorting to the intermembrane space and/or subsequent assembly into the complex. Alternatively, Bcs1p might interact directly with an assembly intermediate of the cytochrome bc_1 complex, such as the precomplex observed in the Δ*bcs1* and Δ*rip1* mitochondria.

Several different approaches to find an interaction of Bcs1p with unassembled Rieske FeS were met with negative results. Therefore, we tested the ability of the Bcs1p to bind to the cytochrome bc_1 precomplex, which is assembled in the absence of the Rieske FeS protein. Mitochondria isolated from the Δ*rip1* strain were solubilized with digitonin and co-immunoprecipitation using antibodies specific for the Bcs1p was performed (Figure 4A). Several subunits of the cytochrome bc_1 complex, as indicated by the detection of Core1, Core2, cytochrome *b*, cytochrome c_1 and Qcr6p, were co-immunoprecipitated with Bcs1p (Figure 4A). None of the cytochrome bc_1 complex subunits were detected in controls with the preimmune antibodies. Co-immunoprecipitation of Bcs1p with subunits of the cytochrome bc_1 complex was not observed in mitochondria prepared from a yeast strain where the gene for cytochrome c_1 had been disrupted (Δc_1) (Figure 4A). This was true despite the fact that these mitochondria contained similar amounts of cytochrome bc_1 subunits (Core1, Core2 and Qcr6p, in particular) as did the Δ*rip1* mitochondria (Figure 4B). Mitochondria from the Δc_1 strain did not accumulate steady-state levels of the cytochrome bc_1 pre-supracomplex observed in the Δ*rip1* and Δ*bcs1* strains due to the lack of cytochrome c_1, as verified by BN–PAGE (Figure 4C). In conclusion, the interaction of Bcs1p with these subunits of the cytochrome bc_1 complex reflects a functional interaction with a specific assembly intermediate of the cytochrome bc_1 complex formed late in the assembly pathway.

Is the association of Bcs1p with the cytochrome bc_1 precomplex influenced by the presence of ATP? Mitochondria isolated from the Δ*rip1* strain were lysed with digitonin either in the presence or absence of added ATP. Subunits of the cytochrome bc_1 complex such as Core1, Core2, cytochrome *b*, cytochrome c_1 and Qcr6p were found in association with Bcs1p, in the absence but not in the presence of ATP (Figure 5A). Thus the addition of ATP apparently causes the release of Bcs1p from the cytochrome bc_1 precomplex. This release event is dependent on the hydrolysis of the ATP, as the addition of non-hydrolysable analogues, ATPγS and in particular AMP–PNP, stabilized the association of Bcs1p with subunits of the cytochrome bc_1 precomplex (Figure 5B).

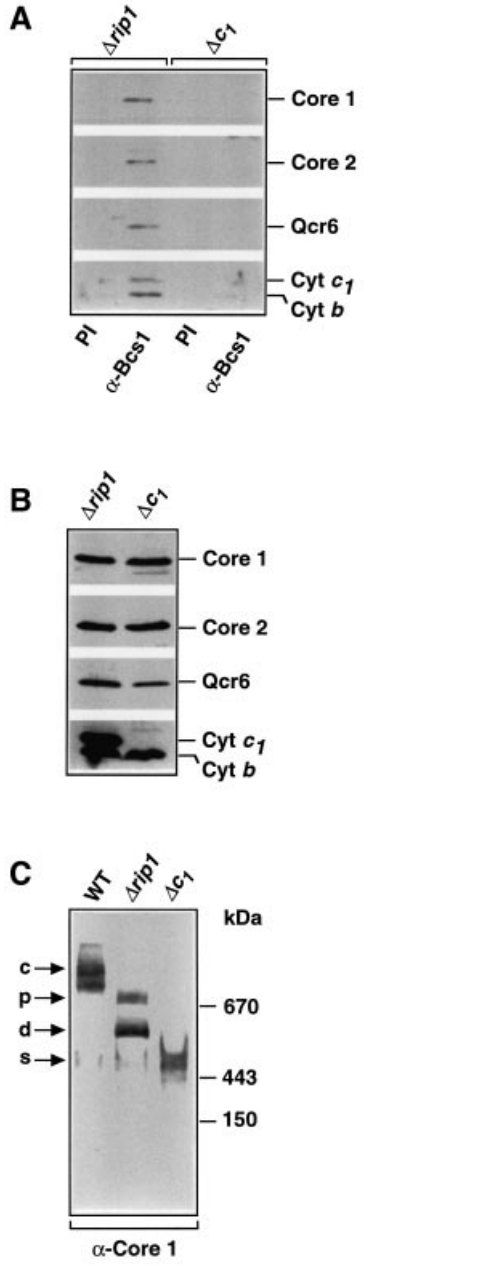

Fig. 4. Bcs1p interacts with the cytochrome bc_1 precomplex. (**A**) Co-immunoprecipitation of components of the cytochrome bc_1 complex with antibodies specific for Bcs1p. Isolated mitochondria from the deletion strains Δ*rip1* and Δc_1 were solubilized with digitonin. After a clarifying spin, the supernatant was divided and incubated either with anti-Bcs1p IgG (α-BCS1) or the respective preimmune IgG (PI), covalently bound to protein A–Sepharose. Immunoprecipitates were analysed by SDS–PAGE and Western blotting. Western blots were decorated with antibodies against the proteins Core1, Core2, cytochrome *b* (Cyt *b*), cytochrome c_1 (Cyt c_1), and Qcr6p. (**B**) Equivalent amounts of mitochondria isolated from Δ*rip1* and Δc_1 strains were analysed by SDS–PAGE and Western blotting. Immunodecoration of blots was performed as in (A). (**C**) BN–PAGE of solubilized mitochondria from the Δ*rip1* and Δc_1 deletion and wild-type (WT) strains. The assembly forms of the cytochrome bc_1 complex were detected by Western blotting and immunodecoration with antibodies against Core1 (α-Core1). c, cytochrome bc_1 supracomplex; p, precomplex of the cytochrome bc_1 supracomplex; d, cytochrome bc_1 dimeric precomplex (without Rieske FeS and Qcr10p); s, subcomplex containing Core1 (an assembly intermediate).

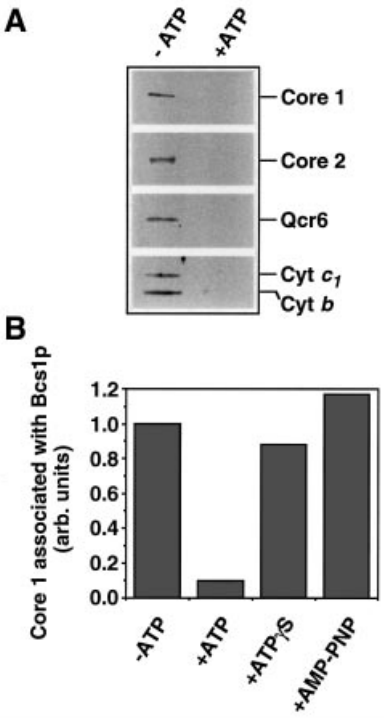

Fig. 5. Release of Bcs1p from cytochrome bc_1 precomplex is dependent on ATP hydrolysis. (**A**) Co-immunoprecipitation of components of the cytochrome bc_1 complex with antibodies specific for Bcs1p in the presence and absence of ATP. Mitochondria from the Δ*rip1* deletion strain were lysed with digitonin in the presence or absence of added ATP. Co-immunoprecipitation with Bcs1p using anti-Bcs1p IgG was performed, and samples were further analysed, as described in Figure 4A. (**B**) Solubilization of mitochondria was performed in the absence or presence of ATP (2 mM), ATPγS (2 mM) or AMP–PNP (2 mM). Co-immunoprecipitation with anti-Bcs1p IgG was performed as in Figure 4A.

In summary, Bcs1p physically associates with the cytochrome bc_1 complex during a late step in its assembly process. Release of Bcs1p, prior to the incorporation of the Rieske FeS and Qcr10p proteins, is driven by ATP hydrolysis.

Discussion

Bcs1p, as we show here, is required for the assembly of the Rieske FeS and the Qcr10p proteins into the cytochrome bc_1 complex. Neither the addition of the prosthetic group to the apo-FeS protein nor the prior assembly of Qcr10p protein is a prerequisite for the assembly of the Rieske FeS protein into the cytochrome bc_1 complex. Our current evidence suggests that Bcs1p is directly involved in the assembly of the Rieske FeS protein into the cytochrome bc_1 complex. Qcr10p most likely assembles after the Rieske FeS protein. Thus absence of the assembled Qcr10p in the Δ*bcs1* mitochondria appears to be an indirect effect due to the lack of prior assembled Rieske FeS.

How does Bcs1p facilitate the assembly of Rieske FeS into the cytochrome bc_1 complex? Despite an extensive series of experiments, no evidence could be obtained to suggest a direct interaction between these two proteins (results not shown). Bcs1p does not appear to guide or chaperone intermediate assembly states of the Rieske FeS

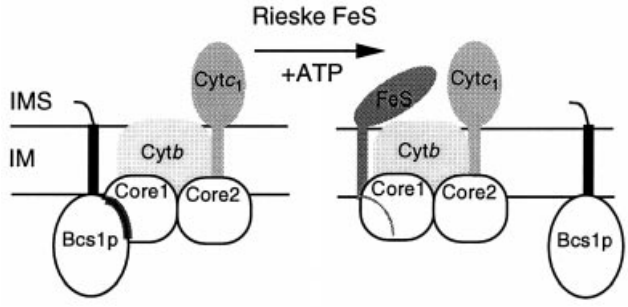

Fig. 6. Model of ATP-dependent interaction of Bcs1p with the cytochrome bc_1 pre-supracomplex. See text for details. The shaded area in Bcs1p indicates that Bcs1p and Rieske FeS may bind to a common binding site in the cytochrome bc_1 precomplex. For simplification of drawing, we have omitted other subunits from the cytochrome bc_1 pre-supracomplex. IM, inner membrane; IMS, intermembrane space; FeS, Rieske FeS protein; Cyt*b*, cytochrome *b*; Cytc_1, cytochrome c_1.

protein. Rather, Bcs1p interacts in an ATP-dependent manner with the cytochrome bc_1 precomplex; it appears to maintain it in a state competent for the subsequent assembly of the Rieske FeS (Figure 6). We propose that Bcs1p acts by binding to an assembly intermediate to prevent adverse folding/subunit interactions of this pre-complex. In this respect, the membrane-bound Bcs1p is a chaperone in its own right, like, for example, Hsp70 of the endoplasmic reticulum, BiP, which chaperones the assembly of antibodies by transiently binding to immuno-globulin heavy chains (Bole *et al*., 1986). Bcs1p at the cytochrome bc_1 precomplex may occupy a site sub-sequently taken by the Rieske FeS, suggesting that both proteins could share limited structural similarities. Interes-tingly, N-terminal matrix-exposed regions of Rieske FeS (amino acid residues 21–53 of the mature protein) display significant sequence similarity to non-contiguous regions of the C-terminal hydrophilic domain of Bcs1p (amino acid residues 92–100 and 344–363). Whether this sequence similarity (and possibly a common tertiary structure) reflects the basis for a common site of interaction of Bcs1p and Rieske FeS with the cytochrome bc_1 precomplex remains a matter of speculation. The crystal structure of the bovine cytochrome bc_1 complex indicates that the N-terminal region of Rieske FeS interacts with the Core1 protein in the assembled complex (Xia *et al*., 1997; Iwata *et al*., 1998; Zhang *et al*., 1998).

Binding of Bcs1p to and release from the cytochrome bc_1 precomplex appears to be a dynamic process regulated by ATP hydrolysis. Bcs1p forms a large oligomeric complex in the mitochondrial inner membrane (our unpublished results). Whether the Bcs1p binds as a monomer or oligomer to the cytochrome bc_1 precomplex is not clear. No evidence for co-migration of the Bcs1p complex with the cytochrome bc_1 precomplex in the Δ*rip1* strain under BN–PAGE, for example, was obtained. This may mean either that only a small percentage of these complexes under equilibrium conditions form a supracomplex between the Bcs1p and cytochrome bc_1 precomplex, or that the monomeric form of the Bcs1p binds to the precytochrome bc_1 complex and addition of this protein to such a large complex does not result in a noticeable increase in the size of the complex. Furthermore, the interaction of Bcs1p and the cytochrome bc_1 precomplex

which is observed by co-immunoprecipitation may not be stable under the conditions of BN–PAGE analysis, which is true for other mitochondrial complexes (our unpublished results).

How is the Rieske FeS protein sorted to the cytochrome bc_1 precomplex? It has been proposed that Rieske FeS is initially imported to the mitochondrial matrix, from where it is inserted into the inner membrane (Hartl *et al*., 1986; van Loon and Schatz, 1987). An alternative explanation for the sorting of FeS could involve a role of Bcs1p and the cytochrome bc_1 precomplex during its import into the mitochondria. The complex of Bcs1p and precytochrome bc_1 may recognize and bind the incoming Rieske FeS protein at the level of import over the inner membrane. Interaction of N-terminal presequence of Rieske FeS with the Core1 and/or Core2 proteins, relatives of the MPP α and β subunits, could support such a reaction. Accumulation of non-assembled Rieske FeS in the mitochondrial matrix (results not shown) occurring in the absence of the Bcs1p is consistent with both pathways.

Members of the AAA protein family are involved in protein assembly and dissociation events (Latterich *et al*., 1995; Arlt *et al*., 1996; Mayer *et al*., 1996; Patel and Latterich, 1998; Rabouille *et al*., 1998; Leonhard *et al*., 1999). Our data here demonstrate that the AAA-family member, Bcs1p, acts as a true ATP-dependent chaperone with the highly specialized function in the assembly of an oligomeric complex of which it is not a final constituent.

Materials and methods

Yeast strains

All yeast strains used in this study were constructed in the same genetic background of W303-1A, *ade2*, *his3*, *trp1*, *leu2* and *ura3* were as follows: wild-type yeast (W303-1A); the Δ*bcs1* strain (W303-1A, BCS1::HIS3) (Nobrega *et al*., 1992); the *bcs1-2* and *bcs1(K273A)* strains (see below) which were constructed in the background of the Δ*bcs1* strain; the Chs14 strain, carrying the S183C mutation in the RIP1 gene encoding Rieske FeS protein, expressed from centromeric plasmid in the W303-1A RIP::LEU2 strain (Denke *et al*., 1998); and the Δ*qcr10* strain (W303-1A, QCR10::LEU2) (Brandt *et al*., 1994). The Δ*rip1* (W303-1A RIP::HIS3) and Δc_1 (W303-1A CYT1::HIS3) strains were constructed by replacing the entire open reading frames encoding the Rieske FeS (*RIP1* gene) or cytochrome c_1 with the *HIS3* gene, as described previously (Wach *et al*., 1994; Arnold *et al*., 1998).

All strains with the exception of the *bcs1-2* strain were grown at 30°C on YPGal (2% peptone, 1% yeast extract, 2% galactose) supplemented with 0.5% lactate. The temperature-sensitive mutant *bcs1-2* was grown at 24°C and shifted overnight to 37°C in YPD (2% peptone, 1% yeast extract, 2% glucose) supplemented with 0.5% lactate, when indicated. All cells were harvested at an OD_{578} of ~1–1.5. Mitochondria were isolated as described previously (Herrmann *et al*., 1994), with the exception of the *bcs1-2* mutant, for which the zymolyase treatment was performed at either 24 or 37°C (depending on the growth temperature).

Construction of the temperature-sensitive bcs1-2 mutant

For construction of the temperature-sensitive mutant of the *BCS1* gene product, bcs1-2, the *BCS1* open reading frame together with its promoter and terminator region was amplified as a *Bam*HI–*Hin*dIII fragment from the plasmid DNA pG2/T1 (Nobrega *et al*., 1992) by PCR and subcloned into the centromeric vector pRS315 (Sikorski and Hieter, 1989), yielding pRS315-BCS1. Hydroxylamine mutagenesis of pRS315-BCS1 plasmid DNA was performed according to the method of Rose *et al*. (1990) with the exception that a 3-fold concentration of hydroxylamine was used. The mutagenized DNA was transformed into the Δ*bcs1* strain, and leucine/histidine-independent transformants were selected. Temperature-sensitive mutants (ts mutants) were selected by screening for conditional growth by replica plating on YPD (glucose, 2%) and YPG (glycerol, 2%) at 24 and 37°C. The plasmid DNA was isolated from the ts transformants and transformed into *Escherichia coli* for amplification. The re-isolated plasmid was then retransformed into the Δ*bcs1* strain to confirm that the ts phenotype was plasmid linked.

Construction of the Walker Box A-mutant bcs1(K273A)

A single point mutation in the BCS1 gene which resulted in an exchange of Lys273 located in the Walker Box A for an Ala residue *bcs1(K273A)* was introduced according to the protocol of the ExSite™ PCR-Based-Site-Directed Mutagenesis kit (Stratagene). The resulting modified BCS1 ORF was cloned together with the BCS1 promotor and terminator regions in the multicopy vector pRS315 containing the LEU2 marker. The resulting plasmid DNA pRS315-BCS1(K273A) was transformed into the Δ*bcs1* strain, and leucine/histidine-independent transformants were selected.

Gel filtration analysis

Isolated mitochondria (1 mg protein) were lysed in 200 µl digitonin buffer [1% (w/v) digitonin, 150 mM K-acetate, 30 mM HEPES–KOH pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml α_2-macroglobulin, 1 µg/ml aprotinin and 1 µg/ml leupeptin] for 30 min on ice. Following a clarifying spin (30 min, 226 000 *g*, TLA45 rotor, Beckman TL-100 ultracentrifuge), the supernatant was applied to a Superose 6 gel-filtration column (25 ml column volume, Pharmacia) equilibrated with the same digitonin buffer. Fractions (0.35 ml) were collected, precipitated by adding trichloroacetic acid to a final concentration of 12.5% (w/v) and analysed by SDS–PAGE and Western blotting. The proteins Bcs1p, cytochrome c_1, Core2, Rieske FeS and Qcr10p were detected in the eluate fractions by immunoblotting. The calibration standards used in the gel filtration and BN–PAGE (see below) analysis (as indicated) are as follows: Hsp60 (840 kDa), bovine thyroglobulin (670 kDa), horse spleen apoferritin (443 kDa), cytochrome b_2 (210 kDa), potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin dimer (132 kDa), bovine serum albumin monomer (66 kDa) and bovine erythrocyte carbonic anhydrase (29 kDa).

Blue native gel electrophoresis

Isolated mitochondria (200 µg) were lysed in 40 µl digitonin buffer containing 10% glycerol, as described above, with the exception that 50 mM KOAc was used. Following a clarifying spin, 4 µl of sample buffer [5% (w/v) Serva Blue G in 500 mM aminocaproic acid] were added to the supernatant. The supernatant was analysed next by BN–PAGE (Schägger and von Jagow, 1991) using either 4–8% or 6–13% polyacrylamide gradient gels, as indicated, followed by Western blotting. The protein complexes were detected by immunoblotting.

Co-immunoprecipitations

For co-immunoprecipitations of cytochrome bc_1 complex subunits with antibodies against the C-terminus of the Bcs1p protein, the Bcs1p antibodies and the respective preimmune IgG (140 µl of serum) were covalently bound to protein A–Sepharose (50 µl wet volume, Pharmacia Biotech, Inc.) with the cross-linker DMP (dimethyl-pimelimidate) (Harlow and Lanes, 1989). Isolated mitochondria (700 µg protein) were lysed in 700 µl digitonin buffer and subjected to a clarifying spin. The detergent extract was divided and incubated under gentle shaking for 2 h at 4°C either with the anti-Bcs1p IgG or the respective preimmune IgG, coupled to protein A–Sepharose. Co-immunoprecipitates were washed three times for 5 min at 4°C with digitonin buffer and analysed by SDS–PAGE and immunoblotting. For co-immunoprecipitations performed in the presence of ATP, or analogues, mitochondria were lysed with digitonin buffer containing 2 mM MgOAc and 2 mM ATP, or 2 mM ATPγS, or 2 mM AMP–PNP (as indicated). The washing steps of the immunoprecipitates were also performed in the presence of Mg–ATP or the respective ATP analogues.

Miscellaneous

Protein determination and SDS–PAGE were performed according to the published methods of Bradford (1976) and Laemmli (1970), respectively. The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system according to the supplier's instructions (Amersham).

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