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A conserved α helix of Bcs1, a mitochondrial AAA chaperone, is required for the Respiratory Complex III maturation

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ABSTRACT

Bcs1 is a transmembrane chaperone in the mitochondrial inner membrane, and is required for the mitochondrial Respiratory Chain Complex III assembly. It has been shown that the highly-conserved C-terminal region of Bcs1 including the AAA ATPase domain in the matrix side is essential for the chaperone function. Here we describe the importance of the N-terminal short segment located in the intermembrane space in the Bcs1 function. Among the N-terminal 44 amino acid residues of yeast Bcs1, the first 37 residues are dispensable whereas a hydrophobic amino acid in the residue 38 is essential for integration of Rieske Iron-sulfur Protein into the premature Complex III from the mitochondrial matrix. Substitution of the residue 38 by a hydrophilic amino acid residue affects conformation of Bcs1 and interactions with other proteins. The evolutionarily-conserved short α helix of Bcs1 in the intermembrane space is an essential element for the chaperone function.

1. Introduction

The respiratory chain, mainly constituted from five large protein complexes in the mitochondrial inner membrane, is vital for production of ATP, the major energy source required for most cellular processes. Respiratory Chain Complex III, also called as the cytochrome bc_1 complex, actively pumps protons across the inner membrane from the matrix to the intermembrane space upon electron transfer from Complexes I and II using ubiquinol to Complex IV via cytochrome *c*. Complex III is composed of three catalytic subunits, cytochrome *b*, cytochrome c_1, and Rieske Iron-sulfur Protein, and several structural subunits. Cytochrome *b* is encoded by mitochondrial DNA and synthesized in the mitochondrial matrix whereas all other subunits are encoded by nuclear DNA, synthesized in the cytosol, and imported into mitochondria. The assembly pathway of the Complex III subunits is well characterized in the yeast *Saccharomyces cerevisiae* [1]. Briefly, cytochrome *b* forms a core complex with Qcr7 and Qcr8. Cytochrome c_1, Cor1, Cor2, Qcr6, and Qcr9 are then assembled into the core complex to form the premature complex. Qcr10 and Rip1, the yeast homolog of Rieske Iron-sulfur Protein, are finally assembled to form the mature complex. At last, a homo-dimer of Complex III forms a supercomplex with Complex IV. Since Rip1 is essential for electron transfer within Complex III, the premature complex lacking Rip1 is non-functional.

It has been reported that molecular chaperones including Bca1, TTC19, Mzm1, and Bcs1 are involved in the Complex III assembly process [1]. Bca1, which is only found in fungi with no known orthologs in higher eukaryotes, may control formation and/or stability of the premature complex [2]. The Bca1 function can be bypassed and/or redundant with other chaperones, since requirement of Bca1 for yeast growth on non-fermentable carbon sources is limited [2]. TTC19 has been identified from patients suffering from progressive encephalopathy associated with profound Complex III deficiency [3]. Abnormal accumulation of an assemble intermediate composed of Cor1 and Cor2 in the mutant muscle suggests involvement of TTC19 in an early stage of the Complex III assembly process. Mzm1 was identified in yeast and shown to interact with Rip1 in the mitochondrial matrix and protects Rip1 from aggregation and degradation prior to integration into the mitochondrial inner membrane [4,5]. A human homolog of Mzm1, LYRM7/MZM1L, has recently been identified [6].

Bcs1 is the first identified chaperone required for the Complex III assembly in yeast two decades ago [7], and then a human homolog of Bcs1, BCS1L, was identified as a causative gene from patients suffering from fatal human diseases, the GRACILE (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death) syndrome and the Complex III deficiency, and from

Abbreviations: AAA, ATPases associated with diverse cellular activities; BN-PAGE, blue-native polyacrylamide gel electrophoresis; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate).

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the Björnstad syndrome, an autosomal recessive disorder with sensorineural hearing loss and pili torti [8–11]. Loss of Bcs1 activity completely diminished the Complex III activity, indicating that Bcs1 is essential for the Complex III assembly [7]. In particular, it has been shown that Bcs1 is required for the assembly of Rip1 into the premature Complex III. Rip1 is synthesized in the cytosol and imported into the mitochondrial matrix using the general translocators in the outer and inner membranes, the TOM and TIM23 complexes, respectively. After assembly of the iron-sulfur cluster in Rip1, Bcs1 mediates export of Rip1 across the inner membrane and the integration into the premature Complex III [12].

Bcs1 has a single transmembrane domain near the N-terminus spanning the mitochondrial inner membrane. The bulk of Bcs1 is located in the matrix, which is roughly divided into three regions: a mitochondria-targeting signal just following the transmembrane region, a AAA (ATPases associated with diverse cellular activities) domain in the C-terminal end, and the Bcs1-specific highly-conserved middle domain of unknown function [13]. Family proteins harboring a AAA domain, by using energy generated from hydrolysis of ATP, play important roles in many cellular processes including quality control of intracellular proteins, genome stability, and cell proliferation [14]. Although precise mechanisms have not been described, the ATPase activity of the Bcs1 AAA domain has been shown to be required for the Rip1 assembly [15]. In addition, a large number of respiratory-deficient mutations in yeast Bcs1 [16] and disease-related mutations in human BCSlL [17] have been isolated all over the matrix domains, indicating that the highly-conserved C-terminal region is the functional domain of Bcs1. On the other hand, the N-terminal region located in the intermembrane space is significantly variable among species. For instance, the yeast *S. cerevisiae* Bcs1 harbors a 44-amino acid N-terminal region whereas that of human BCS1L is composed of only eight amino acid residues (Fig. 1A).

In the present study, we analyzed the significance of the N-terminal intermembrane space region of yeast Bcs1 on the Complex III assembly. We found that an α helix containing 38th–40th amino acid residues of Bcs1, especially the hydrophobic nature of Leu38, was crucial for the assembly of Rip1 into the Complex III. We also showed that the intermembrane space region was not involved in the Bcs1 complex stability itself but affected interaction with yet-to-be-identified proteins.

2. Materials and methods

2.1. Yeast strains and plasmids

All yeast strains used in this study were derived from the haploid strain W303-1A (*MAT***a**, *ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100*) [18]. The *BCS1* gene on the chromosome was disrupted by replacement with the *KanMX6* module [19], yielding a Δ*bcs1* strain. A single copy plasmid expressing Bcs1 was constructed by amplification of the *BCS1* open reading frame flanking with 480-bp and 180-bp of 5′ and 3′ untranslated regions, respectively, and insertion of the amplified fragment into pRS316. Oligonucleotide-directed mutagenesis was employed to make plasmids expressing mutant Bcs1. The Δ*bcs1* strain was transformed with pRS316 and pRS316-*BCS1*, and grown at 30 °C in a minimal medium composed of 0.67% Difco Yeast Nitrogen Base w/o Amino Acids (BD) and CSM -Ura (ForMedium, UK) supplemented with 2% glucose or 2% lactate.

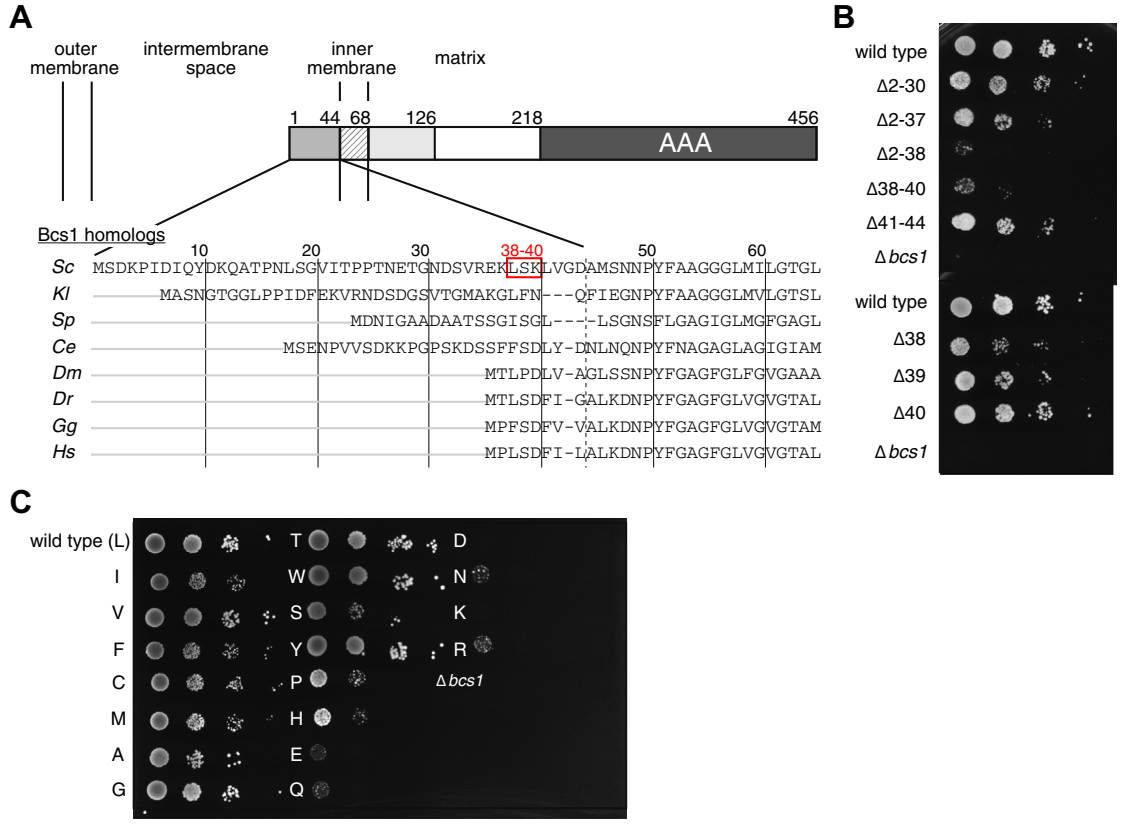

Fig. 1. (A) A schematic representation of yeast Bcs1 and an alignment of the N-terminal region of Bcs1 homologs. *Sc*, *Saccharomyces cerevisiae*; *Kl*, *Kluyveromyces lactis*; *Sp*, *Schizosaccharomyces pombe*; *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Hs*, *Homo sapiens*. (B) Growth of *bcs1* mutants in a non-fermentable carbon source. The Δ*bcs1* strains transformed with pRS316 (Δ*bcs1*) and pRS316-BCS1 were grown in a glucose medium at 30 °C. 0.5 OD_{600} units of the cells were diluted in 10-fold increments, and 3 μl of each dilution (starting with the 1:100 dilution) was spotted onto the minimal medium containing 2% lactate, and incubated at 30 °C for 2–4 days. (C) Growth of *bcs1* strains containing a mutation in the residue 38 in the lactate medium.

2.2. Antibodies

The C-terminal segment of Rip1 (residues 31–215), tagged with hexahistidine at the N-terminus, was expressed as an inclusion body in *Escherichia coli*, purified using Ni–NTA agarose (Qiagen), and used to immunize rabbits for obtaining anti-Rip1 antibody (Medical & Biological Laboratories Co., Ltd.). Anti-Bcs1 antibody was gifted from Dr. Rosemary Stuart (Marquette University, USA) and antibodies raised against Tom40, cytochrome c_1, cytochrome b_2, and Tim44 were gifted from Dr. Toshiya Endo (Nagoya University, Japan).

2.3. Isolation of mitochondria

Cells were grown in the minimal medium with 2% glucose at 30 °C until an OD_{600} reached 1–1.5. Mitochondria were isolated as described previously [20]. Protein concentrations of each isolated mitochondria were determined from the read of OD_{280}.

2.4. Blue native PAGE (BN-PAGE)

Mitochondria were solubilized by incubation in 1% digitonin, 30 mM HEPES-KOH, pH 7.4, 50 mM CH_3COOK, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. The samples were centrifuged at 15,000*g* for 30 min at 4 °C. The supernatants were subjected to BN-PAGE using Novex Native PAGE Bis-Tris gels (Life Technologies) as described previously [21]. High Molecular Weight Calibration kit for native electrophoresis (GE Healthcare) was used to estimate molecular weights.

3. Results

3.1. Requirement of the N-terminal region of Bcs1 exposed to the intermembrane space

Although Bcs1 is highly conserved in eukaryotes, the N-terminal region located in the intermembrane space has apparently shortened during evolution. Bcs1 homologs in higher eukaryotes such as human, birds, and fishes have a shorter N-terminal region than those in worms and fungi (Fig. 1A). This prompted us to analyze the importance of the long N-terminal region of yeast Bcs1. The yeast *S. cerevisiae* would be the most suitable model organism for functional analyses relating to the mitochondrial respiratory chain since the yeast can grow without respiration. Yeast cells produce energy enough to survive by fermentation, and the respiratory activity is required only when they grow in non-fermentable carbon sources. The Δ*bcs1* strain constructed by deletion of the whole open reading frame of the *BCS1* gene in the genome could grow in a medium supplemented with a fermentable carbon source such as glucose but not in that supplemented with a non-fermentable carbon source such as lactate (Fig. 1B, Supplementary Fig. S1A), which is consistent with previous reports showing that Bcs1 is required for the Complex III formation [15,16]. Single-copy plasmids expressing wild type Bcs1 or mutant proteins with deletions of the N-terminal 30 or 37 amino acid residues (Δ2-30 or Δ2-37) or the amino acid segment comprising residues 41–44 (Δ41-44) could complement the growth defect of the Δ*bcs1* strain. In contrast, deletions of the N-terminal 38 residues (Δ2-38) or from residues 38 to 40 (Δ38-40) led to severe growth defects in the lactate medium (Fig. 1B). Further dissections in the amino acid segment 38–40 showed that deletion of Leu38 caused most severe growth defect (Fig. 1B), suggesting Leu38 is crucially important for the function of Bcs1.

To further investigate the importance of Leu38 of Bcs1, we constructed plasmids expressing Bcs1 mutants with a replacement of Leu38 with other 19 amino acid residues. Substitutions at the residue 38 to hydrophobic and less polar amino acid residues except for the proline residue had no effect on the Bcs1 function whereas those to polar and charged amino acid residues caused severe defects (Fig. 1C and Table 1). Especially, substitutions to aspartate and lysine residues led to complete loss of the Bcs1 function. The extent of growth defect correlated with hydrophilicity of replacing amino acids as defined by the hydrophobicity scale (Table 1) [22], suggesting that the hydrophobic nature of the residue 38 plays a critical role.

Computational prediction of secondary structures of proteins [23] revealed that all Bcs1 homologs tested form an α helix around the critical 38th amino acid residue in close vicinity to the inner membrane (Fig. 2A, Supplementary Fig. S2A). To examine whether the α helix is a functional unit, we introduced two proline residues in tandem at the residues 40–41 as a helix breaker. Cells expressing the Bcs1 mutant with mutations K40P and L41P did not grow in the lactate medium (Fig. 2B), suggesting that the α helix in close vicinity to the membrane is a critical component for the function of Bcs1. It should be noted that all Bcs1 mutant cells could grow in a minimal medium containing glucose as a fermentable carbon source (Supplementary Figs. S1 and S2).

3.2. Deficient Complex III assembly in bcs1 mutants

In the absence of Bcs1, Rip1 fails to assemble into the premature Complex III but instead is located in the matrix [12]. We then tested whether the respiratory growth defect observed in the *bcs1* mutant cells is accompanied by deficient Complex III assembly. Mitochondria were isolated from cells expressing wild type Bcs1 and mutant Bcs1(L38A, L38D, or L38N), and solubilized with digitonin. The solubilized proteins were analyzed by BN-PAGE followed by immunoblotting. In mitochondria harboring wild type Bcs1 and Bcs1(L38A), 660-kDa, 750-kDa, and 800-kDa complexes, apparently corresponding to homodimeric Complex III alone and with one and two copies of Complex IV, respectively, were detected by both anti-cytochrome c_1 and anti-Rip1 antibodies (Fig. 3A). In contrast, a 570-kDa complex containing cytochrome c_1 was predominantly observed in mitochondria containing respiratory-deficient Bcs1 mutants (L38D and L38N) as in Δ*bcs1*

Table 1
Substitution of the residue 38.

Amino acid	Hydrophobicity[a]	Growth[b]
Ile	4.5	+++
Val	4.2	++
Leu (WT)	3.8	+++
Phe	2.8	++
Cys	2.5	++
Met	1.9	++
Ala	1.8	+++
Gly	−0.4	+++
Thr	−0.7	+++
Ser	−0.8	++
Trp	−0.9	+++
Tyr	−1.3	+++
Pro	−1.6	+
His	−3.2	+
Glu	−3.5	+/−
Gln	−3.5	+/−
Asp	−3.5	−
Asn	−3.5	+/−
Lys	−3.9	−
Arg	−4.5	+/−

[a] Hydropathy index [22].
[b] Growth, in the lactate medium, of yeast cells expressing a Bcs1 mutant harboring a substitution of the residue 38.

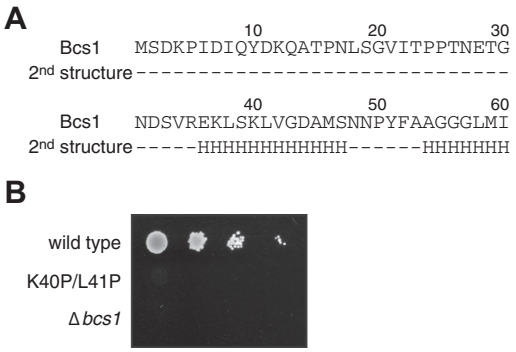

Fig. 2. (A) Predicted secondary structure of the N-terminal region of Bcs1. H, α helix; -, random coil. (B) Growth of *bcs1* mutants in the lactate medium as described in Fig. 1B.

mitochondria (Fig. 3A). No high molecular weight complexes were observed using anti-Rip1 antibody in Bcs1(L38D) and Bcs1(L38N)-expressing mitochondria although low but significant amount of Rip1 was present in the mitochondria (Fig. 3A), suggesting that the Rip1 fails to assemble into the 570-kDa premature Complex III. The 440-kDa Tom40 complex located in the outer membrane were unaffected by the substitutions of Leu38 of Bcs1 (Fig. 3A).

To analyze localization of Rip1 in the respiratory deficient *bcs1* mutants, we employed a protease digestion assay with limited disruption of the outer membrane. Low concentration of digitonin opened the outer membrane but not the inner membrane, allowing proteinase K to digest proteins in the intermembrane space like cytochrome b_2 but not those in the matrix such as Tim44 (Fig. 3B). Matrix-localized proteins were only digested when mitochondria were treated with Triton X-100 (Fig. 3B). In wild type and Bcs1p(L38A)-containing mitochondria, Rip1 was resistant to the protease treatment even in the presence of Triton X-100 (Fig. 3B) probably because Rip1 is tightly embedded in the mature Complex III [12]. In respiratory-deficient Bcs1 mutants (L38D, L38N, and Δ*bcs1*), Rip1 was digested in the presence of Triton X-100 but not of the low concentration of digitonin, suggesting that the Rip1 molecules were located inside the inner membrane.

3.3. Conformational changes of the N-terminal region of Rip1 upon mutations in Leu38

BN-PAGE of digitonin-solubilized mitochondria showed no significant differences in complexes containing Bcs1 upon substitution of Leu38 (Fig. 3A). The protease treatment in the presence of the low concentration of digitonin generated a degradation product of Bcs1 with slightly faster mobility on SDS–PAGE gels (Fig. 3B). The difference of the mobility between full-length and the degradation product of Bcs1 corresponds to ~13 amino acid residues. The respiratory-deficient Bcs1 mutants (L38D and L38N), but not the respiratory-competent Bcs1 mutant (L38A), gave an additional degradation product, whose mobility was almost the same with that of a Bcs1 mutant without first 40 amino

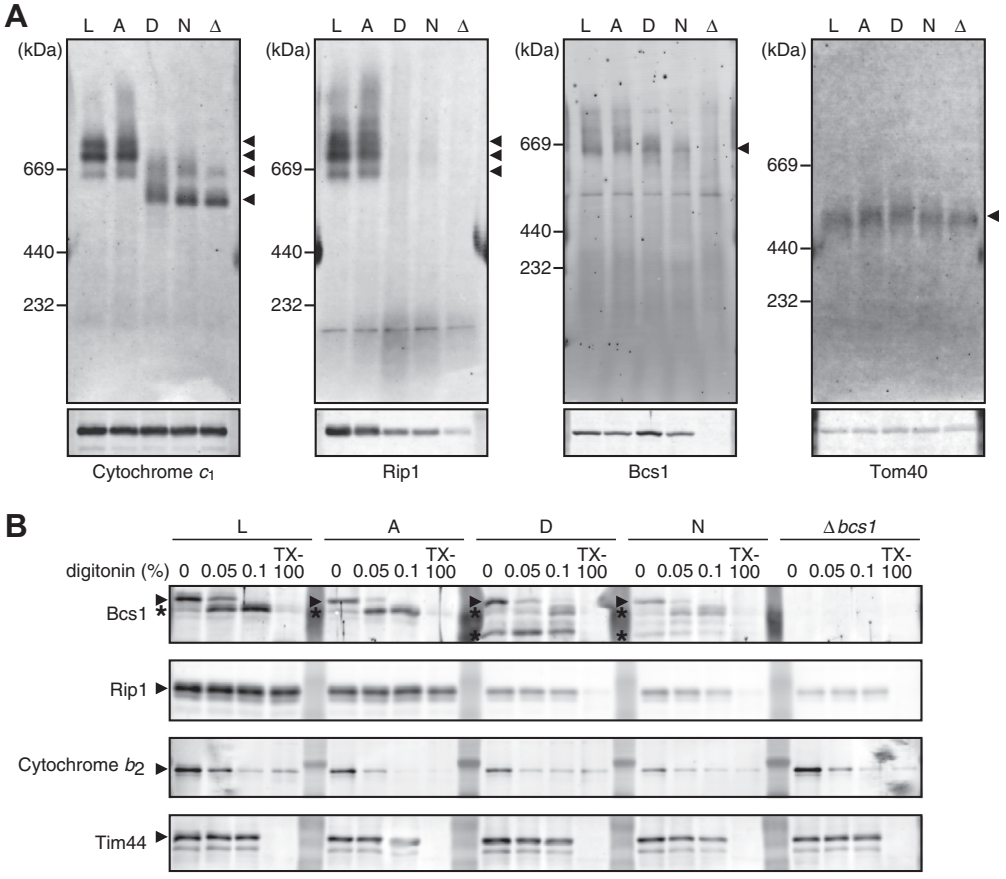

Fig. 3. (A) BN-PAGE of mitochondria solubilized with digitonin. Mitochondria isolated from the Δ*bcs1* cells with the empty vector and cells expressing wild type Bcs1 and Bcs1 mutants harboring substitution of Leu38 with alanine (A), aspartate (D), and asparagine (N) were solubilized with 1% digitonin. The solubilized proteins were subjected to BN-PAGE (Upper panels) and SDS–PAGE (Lower panels), followed by immunodetection with anti-Cytochrome c_1, anti-Rip1, anti-Bcs1, and anti-Tom40 antibodies. (B) Proteinase K digestion of mitochondria. Isolated mitochondria were treated without and with 0.05% and 0.1% digitonin and 1% Triton X-100 on ice for 10 min. The mitochondria were further incubated with 100 μg/ml proteinase K for 5 min at 15 °C. After addition of 20 mM phenylmethylsulfonyl fluoride, all proteins were subjected to SDS–PAGE, followed by immunodecoration with antibodies against Bcs1, Rip1, cytochrome b_2, and Tim44. Arrowheads, full-length proteins; asterisks, major degradation products.

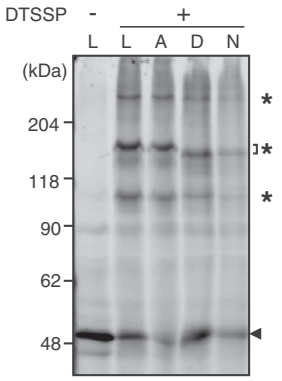

Fig. 4. Crosslink of Bcs1 in the intermembrane space. Mitochondria isolated from cells expressing wild type Bcs1 and Bcs1 with a mutation in Leu38 were treated with 3 mM DTSSP in 10 mM MOPS-KOH, pH 7.2, 250 mM sucrose, and 1 mM EDTA for 30 min at 25 °C. After addition of 10 mM Tris–HCl, pH 8.5 to stop the reaction, the mitochondria were reisolated by centrifugation, and subjected to SDS–PAGE followed by immunodetection with anti-Bcs1 antibody. Arrowheads, unmodified Bcs1; asterisks, major crosslinked products.

acid residues (Fig. 3B, data not shown). These results suggest that the respiratory-deficient mutations lead to conformational changes of the N-terminal region of Bcs1.

To further probe the structural characteristics of the N-terminal region of Bcs1, we performed crosslinking experiments. When mitochondria were treated with a water-soluble membrane-impermeable homo-bifunctional crosslinking reagent, DTSSP (3,3′-dithiobis[sulfosuccinimidylpropionate]), for amine groups, three crosslinked products of wild type Bcs1 were mainly yielded (Fig. 4). Since DTSSP can possibly reach the intermembrane space by traversing the mitochondrial outer membrane through aqueous pores of porin but not inside the inner membrane, the reagent bridged the N-terminal region of Bcs1 and another protein. Treatment of mitochondria harboring Bcs1(L38D) and Bcs1(L38N) generated similar crosslinked products with those of wild type Bcs1 and Bcs1(L38A) except that the mobility of the middle size crosslinked product were slightly but significantly different, suggesting that the respiratory-deficient mutations affect the interaction profile of the N-terminal region of Bcs1.

4. Discussion

Bcs1 is a highly conserved AAA chaperone in eukaryotes since overall identity and homology between yeast Bcs1 and human BCS1L are 50% and 84%, respectively. Nonetheless, the N-terminal region located in the mitochondrial intermembrane space is less conserved (Fig. 1A). We found in the present study that most of the intermembrane space region of yeast Bcs1 is dispensable but at least the last 7 amino acid residues are essential for the respiratory growth. The N-terminal essential short stretch is also conserved in the vertebrate Bcs1 homologs, which have the shortest intermembrane space region among eukaryotes (Fig. 1A). Expression of human BCS1L in a yeast Δ*bcs1* strain restored the respiratory deficiency [11,24], also suggesting that the short N-terminal stretch located in the intermembrane space is enough for the Bcs1 function.

The essential intermembrane space region of Bcs1 homologs is predicted to form an α helix (Supplementary Fig. S2A), which is an essential feature since introducing tandem proline residues resulted in loss of the Bcs1 function (Fig. 2). Deletion of Leu38-Ser39-Lys40 led to severe defect but not complete loss on respiration, suggesting that the nature of the region rather than the exact sequence would be important (Fig. 1B). The size of the side chain is clearly not important since replacements of Leu38 with bulkier amino acids, such as Trp and Tyr, and smaller amino acids, such as Gly and Ala, were acceptable (Fig. 1C). Rather, presence of hydrophobic amino acids in the region would be important. Deletion of the hydrophilic Ser39 and Lys40 residues also affected respiratory growth, suggesting that amphipathic α helix may be a critical feature of the region.

Typical AAA domains function as a homo-hexamer, which is prerequisite for ATP hydrolysis. Thus, it is conceivable that the AAA domain in Bcs1 located in the matrix also forms a hexamer. Although molecular mass of monomer Bcs1 is 51 kDa, digitonin-solubilized wild type Bcs1 migrated as a 660 kDa complex on BN-PAGE (Fig. 3A) [16,25], suggesting that Bcs1 may form a super complex constituted from Bcs1 homo-hexamer and other unknown factors. Treatment of mitochondria with DTSSP generated predominantly three crosslinked products of Bcs1 (Fig. 4). Considering that the apparent molecular mass was nearly double that of monomer Bcs1, the crosslinked product with the fastest mobility looks a homodimer of Bcs1. However, it is unlikely that the other two crosslinked products were homooligomeric crosslinked products of Bcs1 since they had the same and higher intensity than the homodimeric product that should be most efficiently observed. This implies the presence of unknown partner proteins, whose interaction with Bcs1 is affected, but not eliminated, by a mutation of Leu38. Since Rip1 must traverse the inner membrane from the matrix, the supercomplex of Bcs1 may include a translocation channel in the inner membrane. Identification of the Bcs1 partner proteins will reveal the composition of the complex and the mechanism of the translocation of Rip1.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.084.

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