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Internal targeting signal of the BCS1 protein: a novel mechanism of import into mitochondria

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The BCS1 protein is anchored in the mitochondrial inner membrane via a single transmembrane domain and has an N_{out}–C_{in} topology. Unlike the majority of nuclear encoded mitochondrial preproteins, the BCS1 protein does not contain an N-terminal targeting sequence. A positively charged segment of amino acids which is located immediately C-terminal to the transmembrane domain acts as an internal targeting signal. In order to function, we postulate that this sequence co-operates with the transmembrane domain to form a tight hairpin loop structure. This loop is translocated across the inner membrane via the MIM/mt-Hsp70 machinery in a membrane potential-dependent manner. This novel mechanism of import and sorting of the BCS1 protein is proposed to represent a more general mechanism used by a number of inner membrane proteins.

Keywords: BCS1/inner membrane proteins/membrane insertion/mitochondrial targeting signals/*Saccharomyces cerevisiae*

Introduction

Most nuclear encoded mitochondrial precursor proteins bear N-terminal cleavable presequences which serve to target them to mitochondria. These presequences are positively charged with a notable absence of negatively charged residues, and have the potential to form amphipathic α-helices (von Heijne, 1986; for a review, see Brunner *et al.*, 1994). Such presequences not only target the precursor to mitochondria (Emr *et al.*, 1986), but are also necessary to facilitate early steps in protein translocation across the inner membrane in a ΔΨ-dependent manner (Schleyer *et al.*, 1982). Upon import into the matrix, N-terminal sequences are proteolytically removed by the mitochondrial processing peptidase (MPP) (Böhni *et al.*, 1983; Schmidt *et al.*, 1984). However, a number of precursor proteins, in particular inner membrane proteins, do not contain N-terminal targeting signals and therefore must be targeted to mitochondria by means of internal signals. Relatively little is known about the nature and mechanism of operation of such internal signals. Do such precursors use the same import machinery as those targeted by N-terminal sequences? How do these precursors traverse the outer and inner membrane protein import channels? How do such internal mitochondrial targeting signals operate in conjunction with those signals determining submitochondrial localization, to ensure both the specific delivery of these proteins to the mitochondria and the attainment of the correct topology within the mitochondria?

In order to address these questions, we analyzed the import pathway of the BCS1 protein. The BCS1 protein was recently identified following the complementation of a yeast respiratory deficient mutant, displaying a deficiency in the Rieske FeS protein of the cytochrome bc_1 complex (Nobrega *et al.*, 1992). The precise role of this protein in the functional assembly of the FeS protein is so far not clear. The primary structure of BCS1 has an N-terminal region that does not bear any resemblance to a mitochondrial targeting signal, rather it contains more negative than positive charges. Subcellular fractionation had indicated the BCS1 protein to be a mitochondrial integral membrane protein (Nobrega *et al.*, 1992).

The BCS1 protein, as shown here, is anchored to the inner membrane by a single transmembrane domain (residues 45–68). A short N-terminal tail is exposed to the intermembrane space, whilst the bulk of the protein is in the matrix (an N_{out}–C_{in} orientation). This membrane anchor is followed by a short positively charged segment (residues 69–83). We demonstrate that this charged region functions as an internal mitochondrial targeting signal and facilitates the import of the BCS1 protein across the inner membrane. In order to do so, this region seems to traverse the inner membrane import machinery as a hairpin loop structure. The internal targeting information co-operates with the single transmembrane domain to sort this protein to the N_{out}–C_{in} topology. Our current data suggest a sorting pathway in which the hydrophobic transmembrane domain of the BCS1 protein appears to become initially translocated into the matrix. In the matrix, it operates as a membrane insertion signal to facilitate the export of the N-terminal tail (~44 residues) across the inner membrane into the intermembrane space.

Results

Import and topology of the BCS1 protein

Radiolabeled BCS1 precursor (apparent molecular mass of 50 kDa) was efficiently imported into mitochondria in the presence, but not in the absence, of a membrane potential (Figure 1A, lanes 2 and 6). No proteolytic processing by MPP was observed. Following import, mitochondria were subfractionated by hypotonic swelling which disrupts the integrity of the outer membrane whilst leaving the inner membrane intact. This hypotonic swelling procedure in the presence of low amounts of proteinase K resulted in the generation of smaller fragments of the imported BCS1 protein (Figure 1A, lane 3), which were reduced down to two fragments when higher amounts of

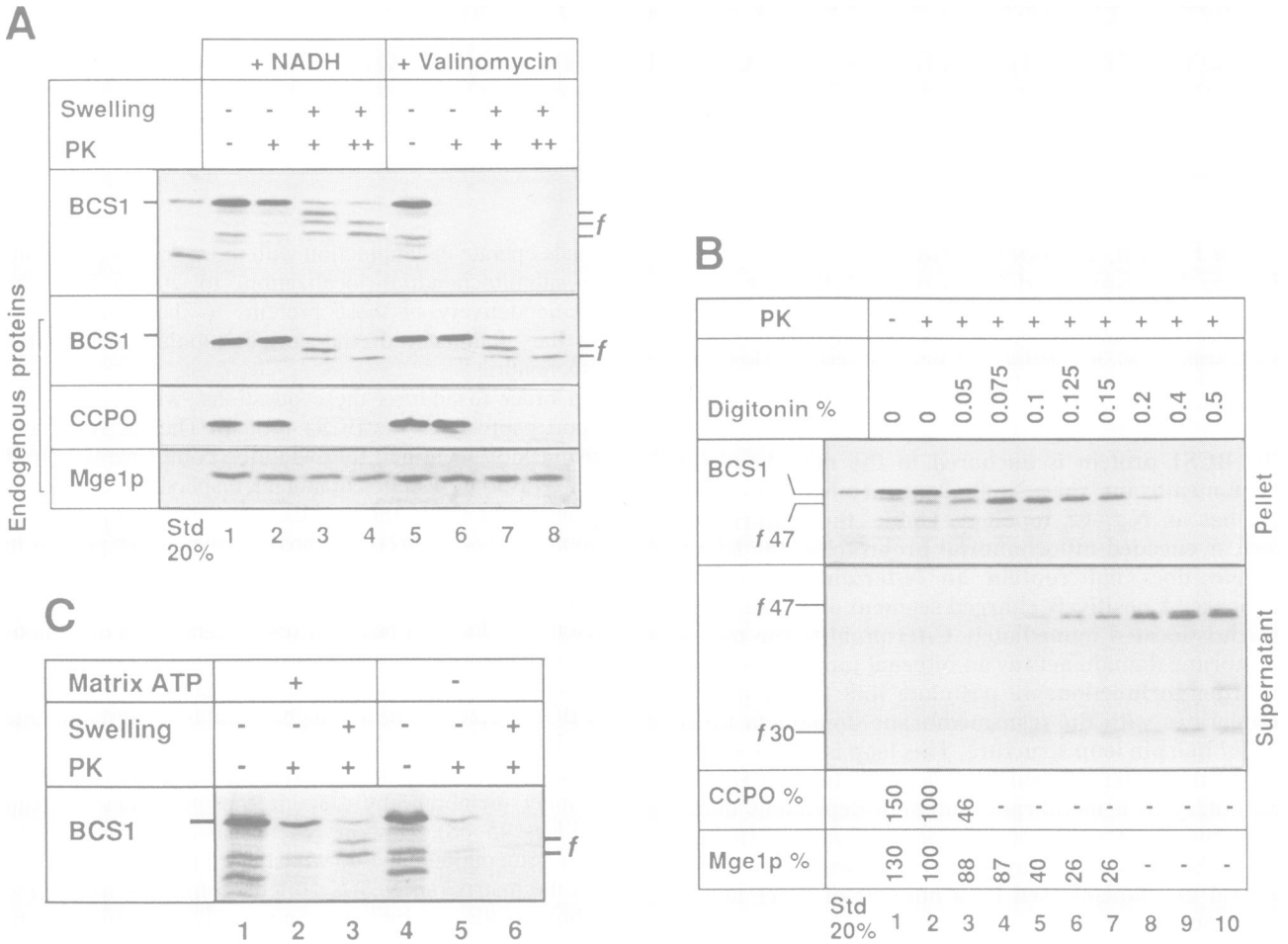

Fig. 1. Import of BCS1 into mitochondria is ΔΨ- and matrix ATP-dependent, and results in an N_{out}–C_{in} topology in the inner mitochondrial membrane. (**A**) and (**B**) ΔΨ-dependent import of BCS1 precursor leads to correct topology. (A) Radiolabeled BCS1 was imported into mitochondria for 5 min at 25°C either in the presence (lanes 1–4, + NADH) or absence (lanes 5–8, + valinomycin) of a membrane potential. After import, mitochondria were reisolated, either mock treated (lanes 1 and 5) or proteinase K (PK) treated with either 20 μg/ml (+) or 200 μg/ml (++) under non-swelling (lanes 2 and 6) or swelling conditions (lanes 3, 4, 7 and 8). All samples were analyzed by SDS–PAGE and blotted onto nitrocellulose. Immunoblotting of the endogenous BCS1 and of the marker proteins CCPO and Mge1p was performed. *f*, fragments generated by proteinase K treatment of mitoplasts. (B) After import of radiolabeled BCS1 into mitochondria for 15 min at 25°C, the sample was trypsin treated, mitochondria were reisolated by centrifugation and resuspended in SEM–KCl buffer, and treated with increasing amounts of digitonin in the presence of proteinase K. Samples were centrifuged, the pellet was lysed in SDS-sample buffer and the supernatant was TCA precipitated. Pellets and supernatants were analyzed by SDS–PAGE and blotted onto nitrocellulose. Immunoblotting of the endogenous marker proteins CCPO and Mge1p was performed and quantified by laser densitometry. The signal of lane 2 (0% digitonin, + PK) was set to 100%. The 47 and 30 kDa fragments generated upon proteinase K treatment are indicated by *f* 47 and *f* 30, respectively. (**C**) Import of BCS1 into mitochondria is dependent on matrix ATP. Radiolabeled BCS1 was imported into mock treated (+ matrix ATP) (lanes 1–3) or matrix ATP-depleted (– matrix ATP) mitochondria (lanes 4–6) in the presence of external ATP for 5 min at 25°C. Mitochondria were reisolated and subjected to proteinase K treatment either under non-swelling (lanes 2 and 5) or swelling (lanes 3 and 6) conditions. Samples were analyzed by SDS–PAGE and blotted onto nitrocellulose. Immunoblotting of the marker proteins CCPO (intermembrane space) and Mge1p (matrix) was performed, swelling was >95% efficient, whilst the integrity of the inner membrane was not perturbed (data not shown). The mobility of the fragments generated upon proteinase K treatment of mitoplasts is indicated by *f*. (A–C) Std 20%, 20% of the amount of radiolabeled precursor added to each reaction.

proteinase K were used (Figure 1A, lane 4). The endogenous BCS1 species also gave rise to the same characteristic fragments, as demonstrated by immunoblotting (Figure 1A, endog. BCS1).

In addition, digitonin fractionation in the presence of proteinase K was used to analyze the topology of the BCS1 protein following import (Figure 1B). Increasing amounts of digitonin led to the sequential opening of the outer and inner membranes. Upon opening of the outer membrane, the imported species was degraded to a slightly smaller, 47 kDa fragment that was recovered with the membrane pellet (Figure 1B). This C-terminal fragment was largely resistant to proteinase K even when the matrix was opened; it was released into the supernatant at higher digitonin concentrations. A similar behavior was observed for the endogenous BCS1 protein (result not shown).

The protein sequence of BCS1 indicated the presence of a single transmembrane domain (residues 45–68) (Nobrega *et al.*, 1992). Together with the data presented here, we conclude that a small domain of the protein (N-terminal ~44 amino acid residues) protrudes into the intermembrane space, whilst the bulk of the protein (C-terminal ~380 residues) resides in the matrix. Upon import into isolated mitochondria, the BCS1 protein becomes correctly sorted across the inner membrane.

In ATP-depleted mitochondria, the efficiency of import

of the BCS1 protein was strongly reduced as compared with matrix ATP-containing mitochondria (Figure 1C, lanes 2 and 5) and the residual imported species was not assembled, but was located in the intermembrane space (Figure 1C, lane 6). A similar observation was made when the BCS1 protein was imported at 37°C into mitochondria from the mutant *ssc1-3* harboring a temperature-sensitive mt-Hsp70 (results not shown). Thus, the import of BCS1 and attainment of its correct topology across the inner membrane requires ATP-dependent mt-Hsp70 action; in the absence of mt-Hsp70 action, translocation of the BCS1 protein across the outer membrane can occur, albeit to a lesser degree than when it is coupled with translocation across the inner membrane.

The BCS1 protein is targeted to mitochondria by an internal matrix targeting signal

The positively charged sequence comprising residues 69–83, immediately following the transmembrane segment of BCS1, has the potential to form an amphipathic α-helix (Figure 2A). In order to investigate whether this internal sequence contains targeting information, we created a series of truncation and fusion BCS1 proteins depicted in Figure 2B.

The first set of constructs, BCS1(Δ40), BCS1(Δ65) and BCS1(Δ82), contain deletions of the N-terminal 40, 65 or 82 amino acid residues, respectively. Deletion of the N-terminal 40 or 65 amino acids did not adversely affect the efficiency or rate of import into isolated mitochondria (Figure 3A). In contrast, BCS1(Δ82) was not imported into mitochondria at all (Figure 3A). Thus, information crucial for mitochondrial protein targeting is contained in the amino acid segment comprising residues 65–82.

In addition to being efficiently imported, BCS1(Δ40) became correctly sorted across the inner membrane and accessible to added proteases in mitoplasts (Figure 3A). The slightly smaller product present in the reticulocyte lysate in addition to BCS1(Δ40) was also imported [Figure 3A, BCS1(Δ40), Std.]. This product most likely originated from translational initiation at methionine 46 at the beginning of the transmembrane domain; as this smaller species does not contain an N-terminal tail, it could not be tested whether it was correctly sorted or not.

In the case of BCS1(Δ65), a second, smaller product was also observed (Figure 3A). This product was generated upon import into mitochondria. When imported in the presence of EDTA/*o*-phenanthroline (EDTA/*o*-phe) to inhibit MPP activity, the BCS1(Δ65) precursor accumulated in the mitochondria as non-processed species (Figure 3B), like preSu9(1–69)–DHFR (where DHFR is mouse cytosolic dihydrofolate reductase) which was co-imported as a control. When MPP was reactivated by the addition of Mg^{2+}/Mn^{2+}, efficient processing of BCS1(Δ65) was observed.

In conclusion, the BCS1 protein is targeted to mitochondria by way of an internal mitochondrial signal located right at the C-terminal side of the transmembrane domain. Furthermore, this targeting signal contains an MPP processing site which is not cleaved when present in the context of an internal targeting sequence. It is, however, efficiently processed when the N-terminus is deleted, hence when the protein is imported into the matrix in a linear fashion, i.e. in a similar manner to presequence-targeted preproteins.

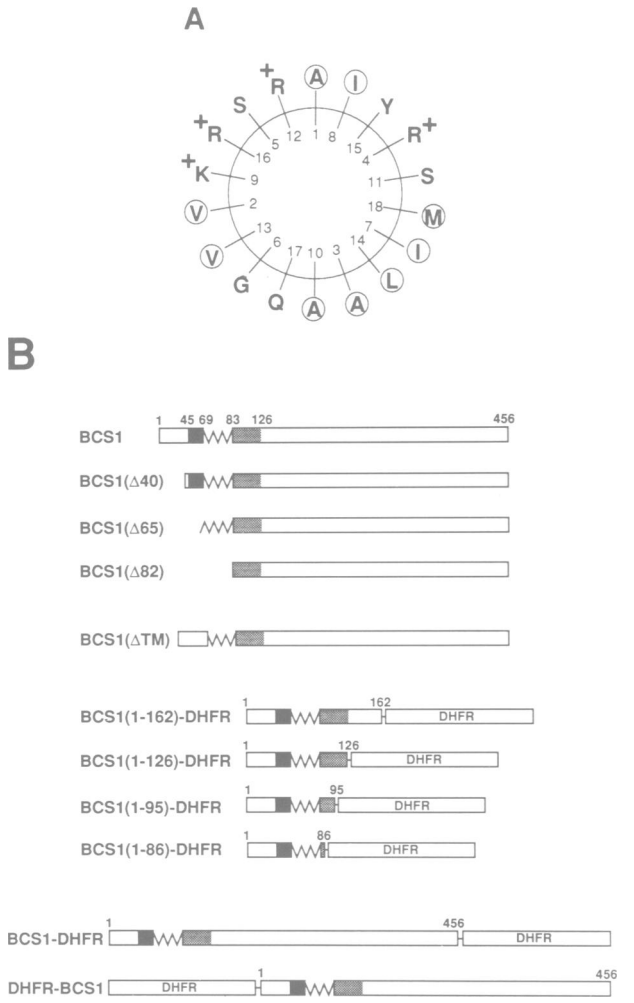

Fig. 2. Structure of the BCS1 protein and derived constructs. (**A**) Mitochondrial targeting sequence. α-Helical plot of amino acid residues 66–83, + denotes positively charged amino acids and apolar residues are circled. (**B**) BCS1 truncation and fusion proteins. Black areas denote the single transmembrane domain (amino acids 45–68), zigzag lines denote the targeting sequence (amino acids 69–83), whilst the shaded areas depict the auxiliary import region (amino acids 95–126). DHFR, mouse cytosolic dihydrofolate reductase.

BCS1 import requires sequences at N- and C-terminal sides of targeting signal

In order to be targeted and imported into mitochondria via an internal targeting signal, the BCS1 precursor most likely forms a loop structure in the region of the polypeptide chain where the targeting signal is present. The loop structure may be stabilized by an interaction of the apolar face of the amphipathic α-helix with the hydrophobic transmembrane domain (see Figure 2A). A BCS1 derivative in which the transmembrane domain was deleted BCS1(ΔTM) (see Figure 2B) was not imported into isolated mitochondria. It remained completely accessible to exogenously added protease (Figure 3C). These findings demonstrate that the transmembrane domain is required in addition to the positively charged segment.

Import of a series of BCS1–DHFR fusion proteins was

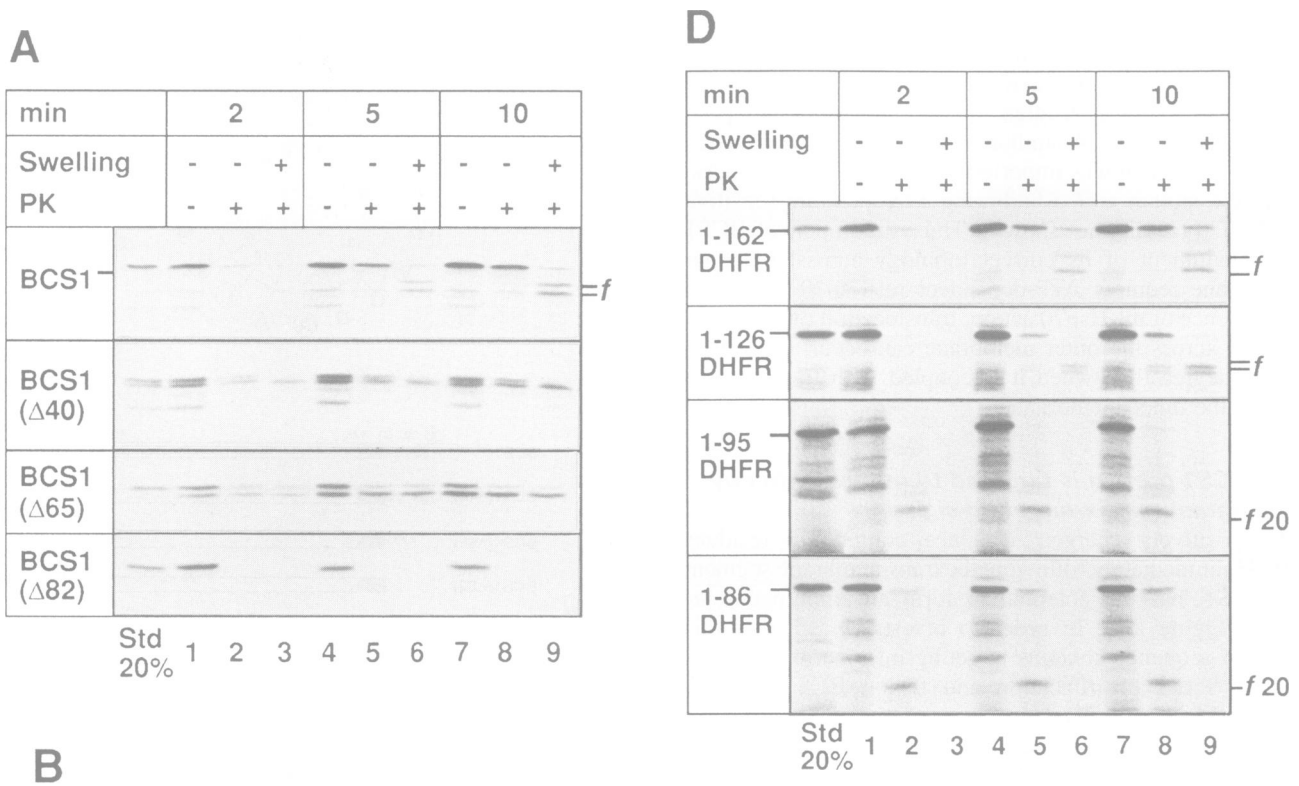

Fig. 3. Import of deletion and fusion proteins derived from BCS1. (**A**) Import kinetics of BCS1 and N-terminal BCS1 deletion proteins. Radiolabeled proteins were imported into mitochondria for 2, 5 or 10 min, as indicated, mitochondria were reisolated, either mock treated (lanes 1, 4 and 7) or subjected to proteinase K treatment, under non-swelling (lanes 2, 5 and 8) or swelling (lanes 3, 6 and 9) conditions, as described in Materials and methods. Samples were analyzed by SDS–PAGE and blotted onto nitrocellulose. Immunoblotting of the marker proteins CCPO and Mge1p was performed, swelling was >95% efficient (data not shown). *f* denotes fragments of BCS1 generated after proteinase K treatment of mitoplasts. (**B**) Reversible inhibition of processing of BCS1(Δ65) by the addition of EDTA/*o*-phe. Radiolabeled BCS1(Δ65) and pSu9(1–69)–DHFR were co-imported into mitochondria for 30 min at 12°C in the presence of $MnCl_2/MgCl_2$ (lanes 1 and 2) or EDTA/*o*-phe (lanes 3–5) as described in Materials and methods (1. Inc.). The samples were divided and trypsin treated as indicated. All samples were centrifuged and the mitochondrial pellet was either lysed directly in SDS-sample buffer (lanes 1, 2 and 3) or resuspended in fresh import buffer in the presence of EDTA/*o*-phe (lane 4) and was maintained on ice or chased in the presence of $MnCl_2/MgCl_2$ for 10 min at 25°C (lane 5) (2. Inc.). Mitochondria were then reisolated and lysed in SDS-sample buffer. All samples were analyzed by SDS–PAGE and the resulting fluorograph is presented. p, precursor; i, intermediate; m, mature forms of BCS1(Δ65) and Su9(1–69)–DHFR. (**C**) The presence of the transmembrane domain is essential for import of BCS1 protein. BCS1(ΔTM) lacks amino acid residues 44–65 encompassing the transmembrane domain of BCS1. Import of radiolabeled BCS1(ΔTM) into mitochondria followed by protease treatment and hypotonic swelling was performed as described in (A). (**D**) Import kinetics of BCS1–DHFR fusion proteins. Import of radiolabeled BCS1(1–162)–DHFR, BCS1(1–126)–DHFR, BCS1(1–95)–DHFR and BCS1(1–86)–DHFR into mitochondria followed by protease treatment and hypotonic swelling was performed as described in (A). *f* denotes fragments of fusion proteins generated after proteinase K treatment of mitoplasts; *f* 20 denotes fragments of the two shorter fusion constructs generated after proteinase K treatment of mitochondria. (A–D) Std 20%, 20% of the amount of radiolabeled precursor added to each reaction.

analyzed that consisted of N-terminal regions of BCS1 fused to DHFR, namely, BCS1(1–162)–DHFR, BCS1(1–126)–DHFR, BCS1(1–95)–DHFR and BCS1(1–86)–DHFR (see Figure 2B). Both BCS1(1–162)–DHFR and BCS1(1–126)–DHFR were imported into mitochondria with an efficiency and rate similar to that of authentic

BCS1 protein (Figure 3D). These fusion proteins became correctly sorted, both giving rise to the characteristic fragments protected in mitoplasts. In contrast, the import of the two shorter fusions, BCS1(1–95)–DHFR and BCS1(1–86)–DHFR, occurred with much lower efficiency (Figure 3D). In both cases, protease treatment of mitochondria resulted in the generation of an ~20 kDa fragment protected in the mitochondria. Neither this fragment nor the imported full-length species were sorted across the inner membrane as they were completely accessible to added protease when the outer membrane was disrupted. This 20 kDa fragment encompasses the BCS1 domain (~8–9 kDa) plus approximately half of the DHFR domain, demonstrating that the DHFR domain is in the process of undergoing translocation. Notably, accumulation of this intermediate required the presence of a membrane potential (results not shown).

In summary, the internal positively charged amino acid segment (69–83), though essential, is not sufficient for directing BCS1 to the correct mitochondrial sublocation. In addition, the transmembrane domain is also necessary together with elements C-terminal to the putative α-helical signal (in the region of residues 95–126).

BCS1 uses the MIM machinery for translocation across the inner membrane

To analyze whether the putative loop structure would use the general inner membrane translocation machinery (the MIM complex; Maarse *et al.*, 1992; Scherer *et al.*, 1992; Blom *et al.*, 1993; Schneider *et al.*, 1994; Berthold *et al.*, 1995), the import of BCS1(1–162)–DHFR into mitoplasts was investigated. Like authentic BCS1 protein, BCS1(1–162)–DHFR was translocated into mitoplasts and became correctly sorted across the inner membrane (results not shown).

In order to investigate the environment of the translocating chain, radiolabeled BCS1(1–162)–DHFR was imported into mitoplasts in the presence of methotrexate (MTX), thereby arrested as a translocation intermediate, and cross-linking was performed. A number of distinct cross-linked products were observed [Figure 4, total + disuccinimidyl suberate (DSS)] that were not seen when import was performed in the absence of MTX (data not shown). The majority of the cross-linking adducts could be immunoprecipitated with known components of the MIM machinery (Figure 4). Thus, the MTX-arrested BCS1(1–162)–DHFR was in the vicinity of MIM17, MIM23, MIM44 and mt-Hsp70. A number of other unidentified cross-linked products were observed (Figure 4, lane 2, indicated by arrowheads). These adducts would reflect cross-linking to components of ~55, 10 and 2–3 kDa. The two larger species could represent new putative components of the MIM machinery, namely a 55 kDa protein (Blom *et al.*, 1995) and either the 10 kDa protein observed by Blom *et al.* (1995) or MIM14 (Berthold *et al.*, 1995).

Sorting mechanism of the BCS1 protein

How does the membrane anchor become inserted into the inner membrane? In an attempt to discriminate between various possible sorting pathways, we analyzed the import and sorting of two further DHFR fusion proteins, DHFR–BCS1 and BCS1–DHFR, containing DHFR placed at either the N- or the C-terminus of the full-length BCS1 protein (see Figure 2B).

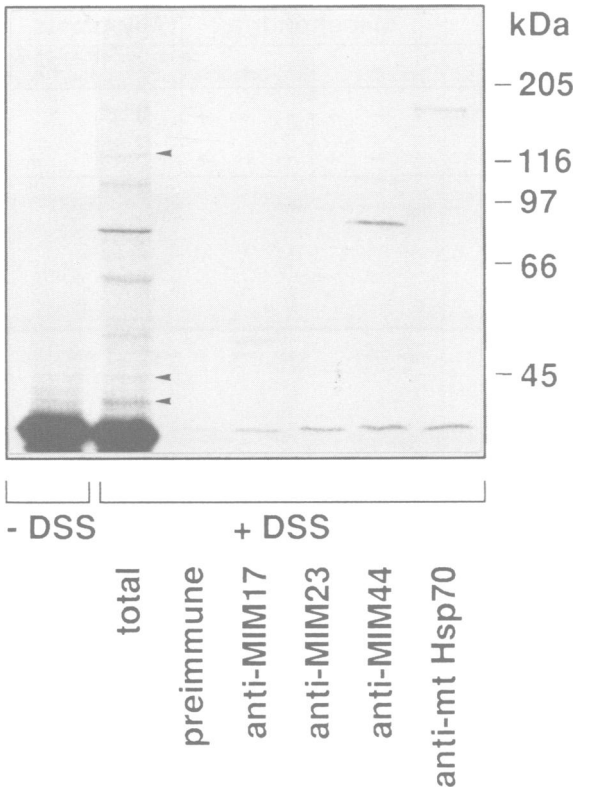

Fig. 4. Cross-linking analysis of BCS1(1–162)–DHFR arrested with MTX in mitoplasts. BCS1(1–162)–DHFR was imported into mitoplasts in the presence of MTX, as described in Materials and methods. Cross-linking with disuccinimidyl suberate (+ DSS) or mock treatment (– DSS) was performed and then mitoplasts were reisolated and lysed as described in Materials and methods. Solubilized cross-linked products were immunoprecipitated with either preimmune serum, or affinity-purified antibodies against MIM17, MIM23 and antiserum against MIM44 or mt-Hsp70, as indicated. The immunoprecipitates were solubilized in SDS-sample buffer and analyzed by SDS–PAGE and fluorography.

Upon import into mitochondria, DHFR–BCS1 was accumulated in the matrix. The N-terminus was not sorted to the intermembrane space (Figure 5, lane 3). In the presence of MTX, the DHFR moiety, as expected, hindered the import across the outer membrane. A similar result was obtained with mitoplasts, where the DHFR–BCS1 protein accumulated completely in the matrix in the absence of MTX (Figure 5, lane 8), whereas in the presence of MTX, import into the matrix was blocked (Figure 5, lanes 6 and 10). Thus, passage across the inner membrane of the N-terminal tail appears to be correlated with that of C-terminal regions.

Placement of the DHFR moiety at the C-terminus of the BCS1 protein (BCS1–DHFR), on the other hand, did not hamper the import and sorting of the protein (Figure 5, lower panel). The N_{out}–C_{in} topology across the membrane was efficiently attained in both mitochondria and mitoplasts (Figure 5, lanes 3 and 8). In the presence of MTX, BCS1–DHFR was found to be protease resistant with mitochondria (Figure 5, lane 5). If, however, protease treatment was performed under matrix ATP-depletion conditions (i.e. after treatment with apyrase and oligomycin), the DHFR domain became accessible to added

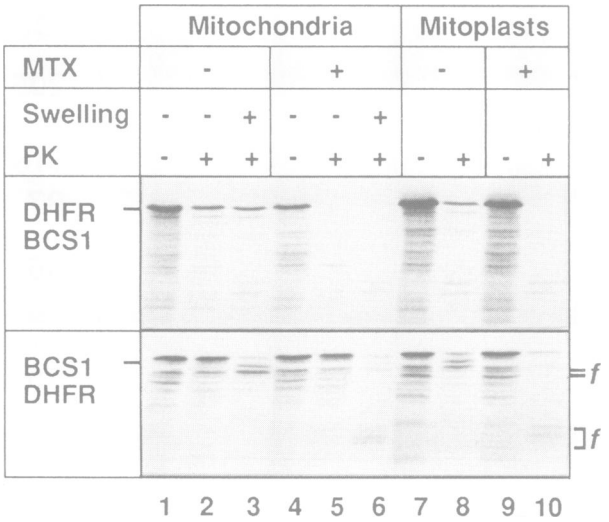

Fig. 5. Import of DHFR–BCS1 and BCS1–DHFR into mitochondria and mitoplasts. Radiolabeled BCS1–DHFR and DHFR–BCS1 were imported into mitochondria (lanes 1–6) or into mitoplasts (lanes 7–10) in the absence (lanes 1–3, 7 and 8) or presence of MTX and NADPH (lanes 4–6, 9 and 10). After import for 15 min at 25°C, mitochondria were reisolated and subjected to swelling, mitoplasts were diluted and proteinase K treated as indicated. All samples were analyzed by SDS–PAGE and blotted onto nitrocellulose. Immunoblotting of the marker proteins CCPO and Mge1p was performed, and indicated that the swelling was >95% efficient. *f*, fragments of fusion proteins generated by PK treatment of swollen mitochondria.

protease (results not shown). The folded DHFR domain was apparently held tightly opposed to the outer membrane by mt-Hsp70/ATP, so that it could not be cleaved off by the protease (Schneider *et al.*, 1994; Ungermann *et al.*, 1994). Protease-protected fragments generated after the opening of the outer membrane were similar to those seen with the authentic BCS1 protein (Figure 5, lane 6). Apparently, translocation proceeded until the DHFR moiety prevented further import at the level of the outer membrane. The generation of similar protease fragments was observed following arrest of BCS1–DHFR in mitoplasts, suggesting correct import and sorting of the BCS1 moiety (Figure 5, lane 10). These fragments were slightly larger than those formed after import into mitochondria (cf. Figure 5, lanes 6 and 10); apparently, more of the BCS1 domain was translocated into the mitoplasts as compared with mitochondria, due to the absence of the barrier of the outer membrane.

In summary, correct sorting of the N-terminus was hampered when the N-terminal tail was extended to include the DHFR moiety. In addition, prevention of translocation of the N-terminal tail resulted in a complete block of import, thus demonstrating close coupling of import of the C-terminus to that of the N-terminus. Our results also show that the N-terminus can be imported and sorted to the intermembrane space in the absence of complete import of the C-terminus. Sorting of the BCS1 protein can occur concomitantly with import across the MIM machinery, indicating that the sites of import and membrane insertion are in close proximity to each other.

Discussion

The location of the BCS1 protein in the inner membrane of mitochondria and its topology, as determined here, are consistent with its proposed function in the assembly of the cytochrome bc_1 complex. A hydrophobic segment (amino acid residues 45–68) anchors the BCS1 protein in the inner membrane. The N-terminal tail of ~44 amino acid residues protrudes into the intermembrane space. The major part of the BCS1 protein is present as a tightly folded protease-resistant domain in the mitochondrial matrix. This latter domain shows similarity to CDC48 and NSF/SEC18 (Nobrega *et al.*, 1992).

Upon import into isolated mitochondria, the precursor form of the BCS1 protein attains this N_{out}–C_{in} topology. It is targeted into mitochondria by means of an internal targeting signal located immediately after the single transmembrane domain. This positively charged segment (amino acids 69–83) has a predicted amphipathic α-helical structure and contains the necessary information to direct the protein into the mitochondria. Both N- and C-terminal neighboring regions co-operate with the positively charged segment to ensure not only efficient mitochondrial targeting, but also mt-Hsp70-dependent translocation across the inner membrane.

We propose that during translocation across the inner membrane, a tight loop structure is formed in which the transmembrane domain contacts the hydrophobic side of the amphipathic α-helix. Abundant Gly residues, known α-helix breakers, are present at the end of the transmembrane domain. Deletion of the transmembrane domain resulted in an overall inhibition of import into mitochondria. Although this loop structure contains an MPP cleavage site, it does not become processed. The co-operation of the internal targeting signal with the transmembrane segment may provide a higher degree of specificity for mitochondrial targeting and sorting; other non-mitochondrial proteins that contain a stretch of amino acids with a potential to form a positively charged amphipathic helix would not be missorted into the mitochondria in the absence of such a neighboring transmembrane domain.

Translocation across the inner membrane requires an additional segment C-terminal of the targeting sequence contained in the region of residues 95–126. In its absence, mitochondrial targeting and initiation of transport across the outer membrane occurred; however, translocation across the inner membrane was only partial. On the basis of these observations, we are entertaining the following scenario. A hairpin loop structure inserts through MIM machineries, whereby it becomes stabilized on the trans side of the inner membrane. Completion of translocation would then involve mt-Hsp70 binding to the auxiliary segment C-terminal to the loop (Figure 6). This segment may function, for example, by providing high-affinity binding sites for mt-Hsp70. Analysis of the sequence in this region reveals a number of Trp and Phe residues, known preferential binding sites for the mt-Hsp70 homolog, Bip (Fourie *et al.*, 1994). Completion of translocation of the C-terminus would then occur in the same manner as described for N-terminal targeted proteins, involving the ATP-dependent action of MIM44 and mt-Hsp70 (Schneider *et al.*, 1994; Stuart *et al.*, 1994a; Ungermann *et al.*, 1994).

The fate of the N-terminal tail during this process is particularly interesting. Our working model includes two possible variations [Figure 6, (a) and (b)]. Upon insertion

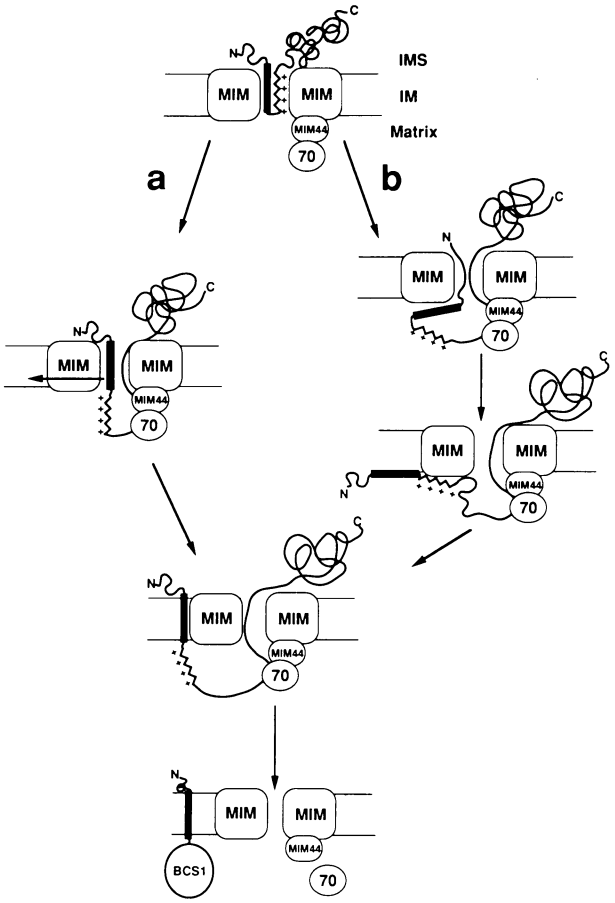

Fig. 6. Model of import and sorting of the BCS1 protein. See the text for details. IM, inner membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane import machinery; 70, mt-Hsp70; the transmembrane domain (amino acids 45–68) is indicated by a black box; the internal targeting sequence (amino acid residues 69–83) is represented by a zigzag line with positive charges.

of the hairpin loop into the MIM machinery, further translocation could involve passage of only the C-terminal domain into the matrix. The hydrophobic transmembrane domain could partition into the lipid bilayer. Consequently, the N-terminal domain would reside in the intermembrane space at all times [Figure 6, (a)]. Alternatively, the hairpin loop could insert into the inner membrane, and further import would involve the complete translocation of both N- and C-termini across the membrane. The N-terminus would then undergo a transfer from the matrix side, resulting in the export of the N-terminal tail into the intermembrane space [Figure 6, (b)].

The following observations provide support for model (b), which involves export of the N-terminal tail from the matrix. First, extension of the N-terminus by the addition of DHFR (DHFR–BCS1 protein) caused accumulation in the matrix and inhibition of sorting. According to model (a), this would imply that the DHFR domain interferes with the sorting of the N-terminus of BCS1, although it is normally retained in the intermembrane space at all times. Rather, these data are consistent with the alternative model (b), as extension of the N-terminal tail by DHFR (~220 amino acids) would most likely render it too long to be exported. Export of N-terminal tails in *Escherichia coli* was shown to be sensitive to length, whereby export of tails longer than ~40 amino acids required the presence of an additional export leader sequence (Cao and Dalbey, 1994). Second, when the DHFR moiety of the DHFR–BCS1 protein was blocked with MTX, translocation of both N- and C-termini was inhibited. This observation is inconsistent with model (a) as prevention of translocation of the N-terminus should not influence the import of C-terminal parts. Again, this would be in agreement with a pathway where complete translocation of the loop into the matrix is necessary to expose mt-Hsp70 binding sites on the single polypeptide chain and to ensure translocation of the C-terminus. Third, insertion of membrane anchors accompanied by export of N-terminal tails from the mitochondrial matrix is an established reaction (Herrmann *et al.*, 1995; Rojo *et al.*, 1995). This export process resembles prokaryotic export events with respect to its energetic requirements (Herrmann *et al.*, 1995; Rojo *et al.*, 1995) and apparent adherence to the positive-inside rule (von Heijne, 1989; Boyd and Beckwith, 1990; Cao and Dalbey, 1994) (E.E.Rojo and R.A.Stuart, unpublished results). Indeed, the preponderance of negative charges in the N-terminal tail is consistent with such an export process.

Our observations, taken together, favor a sorting process whereby the N-terminus undergoes export from the matrix across the inner membrane. However, further experimental evidence is required to confirm such a mechanism.

Finally, is this mitochondrial targeting pathway described here unique to the BCS1 protein? Similar hydrophobic elements immediately followed by positively charged segments with predicted amphipathic α-helical structures, and which are known from topological analysis to be present in the matrix of the finally sorted protein, can be observed in other inner membrane proteins. We would predict that this novel mechanism of import and sorting described here for the BCS1 protein could therefore represent a more general mechanism used by a number of inner membrane proteins.

Materials and methods

Cloning of BCS1 fusion and deletion proteins

Both the full-length BCS1 as well as the N-terminal BCS1 deletion proteins were cloned by polymerase chain reaction (PCR) using the original BCS1 clone pG2/T1 (Nobrega *et al.*, 1992) as template. The N-terminal primers (containing *Eco*RI restriction site for subsequent cloning) used for the PCR were as follows: 5′-GAGAGAATTCATGTCGGATAAGCCGATT-3′ for the full-length protein, and 5′-CCCGAATTCGGATCCATGCTAGTTGGCGATGCTATGTCA-3′, 5′-CCCGAATTCGGATCCATGGCTGTAGCTAGATCCGGTATA-3′ and 5′-CCCGAATTCGGATCCATGATTGTCGACTTAGAGATTCAG-3′ for the BCS1(Δ40), BCS1(Δ65) and BCS1(Δ82) deletion proteins, where the first 40, 65 or 82 amino acids of the BCS1 protein, respectively, have been deleted. In the case of the BCS1(Δ40) and BCS1(Δ65) proteins, a new start ATG was introduced. In each case, the same C-terminal primer (with *Hin*dIII restriction site for subsequent cloning) was used: 5′-GAGAAAGCTTGTTATGTAGTAAGGCG-3′. All PCR products were then cloned using *Eco*RI/*Hin*dIII in the *in vitro* transcription vector pGEM4 under the control of the SP6 RNA polymerase.

The BCS1(1–126)–DHFR and BCS1(1–95)–DHFR constructs were also cloned by PCR using the N-terminal primer as described above and the following C-terminal primers: 5′-CCCCGGATCCTGATTTGTCCTTCGACTGAA-3′ for the BCS1(1–126)–DHFR and 5′-CCCCGGATCCATTGTCATGTTGTATGT-3′ for the BCS1(1–95)–DHFR. Both PCR products were cloned using *Eco*RI/*Bam*HI in front of DHFR, thereby introducing two extra amino acids (Gly-Ser) into the joining region. The BCS1(1–162)–DHFR was cloned by ligating an *Eco*RI/

*Eco*RV BCS1 fragment (codons 1–162) in-frame with a DHFR insert. By doing so, two extra amino acids, Arg-Ser, were introduced in the joining region. The BCS1(1–86)–DHFR was cloned by ligating an *Eco*RI/*Sal*I fragment (codons 1–86) in-frame with DHFR. In this case, four extra amino acids residues were introduced into the final protein (Leu-Gln-His-Arg).

Deletion of the membrane anchor, BCS1(ΔTM), was achieved by amplifying the first 43 amino acids of BCS1 by PCR using the N-terminal primer as above for the full-length clone and the following C-terminal primer: 5′-CCCCGGATCCGCCAACTAGTTTTGAAAG-3′. The PCR product was cloned as an *Eco*RI/*Bam*HI fragment in front of the BCS1(Δ65) construct. By doing so, two extra amino acids (Gly-Ser) were introduced into the joining region.

The full-length BCS1–DHFR constructs (BCS1–DHFR and DHFR–BCS1) were cloned as follows: the BCS1 domain was initially cloned by PCR amplification of the BCS1 gene using the following N-terminal primer, 5′-CCCCGAATTCGGATCCATGTCGGATAAGCCGATT-3′, and using a C-terminal primer where the stop codon had been replaced by a *Bgl*II site as follows: 5′-CCCCAAGCTTAGATCTGAAAATATG-ATTAGCGTT-3′. The resulting product was cloned as a *Bam*HI/*Bgl*II fragment into pQE13 (Qiagen Inc.), cleaved either with *Bgl*II in the case of the N-terminal DHFR fusion protein or cleaved with *Bam*HI for the C-terminal DHFR fusion protein. Both fusion constructs were then cloned as *Bam*HI/*Hin*dIII fragments from the pQE13 vector into pGEM4 and could be transcribed with SP6 RNA polymerase.

Antibody production

The *Sal*I/*Hin*dIII fragment of the BCS1 gene (corresponds to the complete C-terminal matrix-localized domain of BCS1) was amplified by PCR using the following N-terminal primer, 5′-GATTGTCGACTTAGA-GATT-3′, and the same C-terminal primer used for the N-terminal truncated BCS1 proteins described above. This PCR product was cloned in the bacterial expression vector pQE9 (Qiagen Inc.), thus providing an N-terminal His_6-tag. After production in *E.coli*, the protein was purified under denaturing conditions by Ni-NTA chromatography (Hochuli *et al.*, 1988). Purified His_6-tagged BCS1 fragment was used for the generation of antibodies in rabbits.

Isolation of mitochondria and protein import

Standard procedures were used for cell growth (*Saccharomyces cerevisiae* wild type, D273-10B) and isolation of mitochondria (Herrmann *et al.*, 1994). BCS1 and BCS1 truncation and DHFR fusion proteins were synthesized in rabbit reticulocyte lysate (Promega Corp., USA) in the presence of [^{35}S]methionine, as previously described (Pelham and Jackson, 1976).

Import into mitochondria. Unless otherwise indicated, import was performed as described before in the following buffer: 3% (w/v) bovine serum albumin (BSA), 50 mM HEPES (pH 7.2), 0.5 M sorbitol, 80 mM KCl, 10 mM MgOAc, 2 mM K phosphate, 2.5 mM EDTA, 2.5 mM $MnCl_2$ (buffer A). Import mixtures usually contained 4 mM NADH, 2 mM ATP, 0.5 mg mitochondrial protein/ml and 1% (v/v) reticulocyte lysate (Stuart *et al.*, 1994b). Import was performed at 25°C for the times indicated. Following import, samples were treated with proteinase K under non-swelling or swelling conditions (see below).

Import into mitoplasts. Mitochondria were initially converted into mitoplasts by hypotonic swelling by diluting 10-fold in 20 mM HEPES (pH 7.2) and incubation on ice for 30 min. Resulting mitoplasts were reisolated by centrifugation, resuspended in buffer A (0.5 mg/ml) and import was performed as described above for mitochondria. Proteinase K treatment (200 μg/ml) was performed directly without reisolating the mitoplasts and following a 10-fold dilution of the sample with SH buffer [0.6 M sorbitol, 20 mM HEPES (pH 7.2)].

Submitochondrial localization of imported precursor proteins

Conversion to mitoplasts. Hypotonic swelling of mitochondria was performed to determine the presence of the N-terminal tail in the intermembrane space as follows. After import, mitochondria were reisolated by centrifugation (Sigma, rotor 12153, 5 min at 16 300 *g*, 2°C), resuspended in buffer A at a concentration of 50 μg/100 μl and then diluted 10-fold in 20 mM HEPES (pH 7.2) in the presence of 200 μg/ml proteinase K, unless otherwise indicated. Control mitochondria (non-swelling conditions) were diluted to the same extent in SH buffer and also subjected to proteinase K treatment, when indicated. Samples were kept on ice for 30 min and phenylmethylsulfonyl fluoride (PMSF) (2 mM) was added. Mitoplasts/mitochondria were reisolated and washed once with SEM–KCl/PMSF buffer [250 mM sucrose, 10 mM MOPS (pH 7.2), 1 mM EDTA, 100 mM KCl, 0.3 mM PMSF] and then lysed directly in SDS-sample buffer. Samples were analyzed by SDS–PAGE and immunoblotting onto nitrocellulose. The efficiency of swelling was assessed following immunodecoration of the blot with antisera against endogenous cytochrome *c* peroxidase (CCPO) (soluble intermembrane space protein) and Mge1p (matrix located protein).

Digitonin treatment. Following import, mitochondria were treated with trypsin (45 μg/ml) for 15 min on ice and then reisolated by centrifugation (Sigma, rotor 12153, 5 min at 16 300 *g*, 2°C). Mitochondria were resuspended in SEM–KCl buffer (10 mg/ml) in the presence of trypsin inhibitor and treated with increasing concentrations of digitonin in the presence of proteinase K (20 μg/ml) as described previously (Glick *et al.*, 1992; Segui-Real *et al.*, 1993). Following the addition of 2 mM PMSF, samples were subjected to centrifugation (Sigma, rotor 12153, 10 min at 16 300 *g*, 2°C) and the resulting pellets were lysed in SDS-sample buffer; solubilized proteins in the supernatants were trichloroacetic acid (TCA) precipitated. All samples were analysed by SDS–PAGE and immunoblotting onto nitrocellulose.

Inhibition of MPP cleavage by EDTA and o-phenanthroline

The import of BCS1(Δ65) and preSu9(1–69)–DHFR was performed for 30 min at 12°C in the following buffer: 3% (w/v) BSA, 50 mM HEPES (pH 7.2), 0.5 M sorbitol, 80 mM KCl, 2 mM K phosphate, 10 mM EDTA, 2 mM *o*-phe (buffer B), or in buffer A for import control. Samples were trypsin treated (20 μg/ml) for 15 min on ice. Mitochondria were reisolated by centrifugation (Sigma, rotor 12153, 5 min 16 300 *g*, 2°C) and resuspended in fresh import buffer containing either 10 mM EDTA/2 mM *o*-phe (buffer B) or 10 mM Mg^{2+}/2.5 mM Mn^{2+} (buffer A) and incubated for a further 10 min at 25°C. Mitochondria were reisolated, washed once with SEM–KCl and then directly lysed in SDS-sample buffer and analyzed by SDS–PAGE.

MTX arrest of BCS1–DHFR fusion proteins

In order to block complete import of the various BCS1–DHFR derivatives with MTX/NADPH, translation of the radiolabled proteins was performed at 25°C in the presence of 1 μM MTX (or in the absence of MTX for the import controls). Import was performed for 15 min at 25°C in mitochondria or mitoplasts as described above, with the exception that 1 μM MTX and 1 mM NADPH were added to the samples as indicated.

Cross-linking analysis

BCS1(1–162)–DHFR was arrested as an MTX translocation intermediate across the inner membrane in mitoplasts. DSS (200 μM) was added and samples were incubated for a further 30 min at 25°C. The cross-linker was quenched by the addition of Tris–HCl (pH 8.0) to a final concentration of 100 mM. Mitochondria were then reisolated and lysed in SDS-containing buffer. Immunoprecipitations of cross-linked adducts were performed as previously described (Berthold *et al.*, 1995).

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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