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The sorting signal of cytochrome b_2 promotes early divergence from the general mitochondrial import pathway and restricts the unfoldase activity of matrix Hsp70

Frank Gärtner, Ulf Bömer, Bernard Guiard[1] and Nikolaus Pfanner[2]

Biochemisches Institut, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany and [1]Centre de Génétique Moleculaire CNRS, Université Pierre et Marie Curie, F-91190 Gif-sur-Yvette, France

[2]Corresponding author

Cytochrome b_2 is imported into mitochondria and sorted to the intermembrane space by a bipartite N-terminal presequence, which is a matrix targeting sequence followed by an intermembrane space sorting signal. The N-terminus of the mature protein forms a folded heme binding domain that depends on the unfoldase function of matrix (mt) Hsp70 for import. We report that the distance between the presequence and the heme binding domain is critical for the ability of mt-Hsp70 to promote import of the domain. Hybrid proteins with 40 or more amino acids between the presequence and the heme binding domain are arrested in the import machinery. The translocation arrest can be overcome by unfolding of the preprotein or by inactivation of the intermembrane space sorting signal. Moreover, the sorting signal prevents backsliding of the precursor polypeptide in the import site in the initial import step, when the signal has not made contact with the matrix. The results indicate that the sorting signal interacts with component(s) of the inner membrane/intermembrane space during the initial import step and promotes an early divergence of b_2 preproteins from the general matrix import pathway, precluding an unfolding role for mt-Hsp70 in the translocation of most of the mature portions of a preprotein. We propose a sorting model of cytochrome b_2 which explains the apparently divergent previous results by a unifying hypothesis.

Keywords: cytochrome b_2/intermembrane space/mitochondria/protein sorting/*Saccharomyces cerevisiae*

Introduction

The 70 kDa heat shock protein in the mitochondrial matrix (mt-Hsp70) is essential for the import of nuclear-encoded preproteins into the matrix. mt-Hsp70 binds to the precursor polypeptide chain emerging on the matrix side of the inner membrane and promotes its complete import in an ATP-dependent reaction. By using temperature-sensitive mutants of *Saccharomyces cerevisiae* mt-Hsp70 (Ssc1p) and by inhibiting the function of mt-Hsp70 at low ATP levels, two roles have been ascribed to Hsp70 in the membrane translocation of preproteins (Gambill *et al.*, 1993; Glick *et al.*, 1993; Voos *et al.*, 1993; Stuart *et al.*, 1994; Wachter *et al.*, 1994). (i) The translocase function is assumed to be mediated by a Brownian ratchet mechanism, i.e. soluble Hsp70 traps imported preprotein segments in the matrix and prevents their backsliding through the import channel (Ungermann *et al.*, 1994; Pfanner and Meijer, 1995). (ii) The unfoldase or pulling function of mt-Hsp70 is needed for those preproteins that carry folded domains in the cytosol. Binding of mt-Hsp70 to precursor segments in the matrix supports unfolding of domains on the cytosolic side. This involves a dynamic interaction of mt-Hsp70 with Mim44 (also termed Isp45), an essential 44 kDa protein of the mitochondrial inner membrane import machinery (Maarse *et al.*, 1992; Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). It is thought that Mim44-bound Hsp70 changes its conformation upon binding an incoming polypeptide and thereby exerts a pulling force on the preprotein, which facilitates unfolding of outside domains (Pfanner *et al.*, 1994; Glick, 1995; Pfanner and Meijer, 1995). Preproteins destined for the matrix typically require both Hsp70 functions. When a preprotein is in a loosely folded conformation or requires only low energy input for unfolding it depends only on the translocase function for complete import into the matrix (Voos *et al.*, 1993).

Preproteins destined for the inner membrane or intermembrane space follow distinct routes with a differential Hsp70 dependence. Two preproteins have received special attention. (i) The Fe/S protein of the bc_1 complex is first completely imported into the matrix in a mt-Hsp70-dependent reaction and then exported to the intermembrane space side of the inner membrane. This pathway was termed conservative sorting, as export follows the mechanisms established in the prokaryotic ancestors of mitochondria (Hartl *et al.*, 1986; Hartl and Neupert, 1990; Kang *et al.*, 1990). (ii) Cytochrome b_2, which finally ends up in the intermembrane space, carries a bipartite 80 residue presequence (Reid *et al.*, 1982; Guiard, 1985). The first portion of the presequence (31 residues) is positively charged, functions as a typical matrix targeting sequence and is removed by the matrix processing peptidase. The second portion of the presequence includes a hydrophobic segment that, together with preceding positively charged residues, mediates sorting of the preprotein to the intermembrane space (Beasley *et al.*, 1993; Schwarz *et al.*, 1993). Removal of the second portion of the presequence is performed by the inner membrane peptidase 1 on the intermembrane space side of the inner membrane (Pratje and Guiard, 1986; Schneider *et al.*, 1991; Nunnari *et al.*, 1993). Two conflicting models are currently proposed for the sorting pathway of cytochrome b_2. Sorting by a conservative pathway such that each part of the precursor polypeptide passes through the matrix (Hartl *et al.*, 1987; Koll *et al.*, 1992; Gruhler *et al.*, 1995) or by a stop transfer mechanism (Blobel, 1980; Kaput *et al.*, 1982; Hurt and van Loon, 1986) which involves arrest of the sorting

sequence in the inner membrane. According to the stop transfer mechanism the mature part of this intermembrane space protein does not even enter the inner membrane (Glick *et al.*, 1992, 1993; Rospert *et al.*, 1994).

The import of cytochrome b_2 revealed an unusual dependence on matrix Hsp70. A hybrid protein with the entire presequence of cytochrome b_2 was sorted to the intermembrane space without the need for mt-Hsp70 (Glick *et al.*, 1992, 1993; Voos *et al.*, 1993; Stuart *et al.*, 1994), however, the authentic cytochrome b_2 precursor required functional mt-Hsp70 for import. It turned out that the tightly folded heme binding domain (HB), located in the first 99 amino acid residues of mature cytochrome b_2, rendered import dependent on the unfoldase function of mt-Hsp70 (Voos *et al.*, 1993; Glick *et al.*, 1993; Stuart *et al.*, 1994). It was concluded that a hypothetical mitochondrial sorting machinery (MSM) bound to the sorting sequence and thereby substituted for the translocase function of mt-Hsp70 (Beasley *et al.*, 1993; Schwarz *et al.*, 1993; Voos *et al.*, 1993; Gärtner *et al.*, 1995).

Attempts were made to explain the differential role of mt-Hsp70 in b_2 sorting, in view of each of the conflicting hypotheses. The mt-Hsp70 independence of sorting of some b_2 preproteins was seen as support for the stop transfer hypothesis (Glick *et al.*, 1992, 1993). The requirement for mt-Hsp70 for unfolding of the mature protein may be seen to favor the conservative sorting hypothesis, yet a model was proposed that allows explanation by a stop transfer mechanism. mt-Hsp70 should bind to the presequence and thereby exert an inward-directed force that is transferred to the HB, which is just outside the outer membrane. The pulling force imposed on the folded domain would facilitate its unfolding during translocation across the outer membrane (Glick *et al.*, 1993). On the other hand, in the conservative sorting hypothesis the ability of the b_2 presequence to promote mt-Hsp70-independent import of a preprotein (as long as unfolding is not rate limiting) was explained by a 'short-cut' of the typical sorting pathway. While the preprotein is typically completely imported into the matrix and then exported across the inner membrane (Hartl *et al.*, 1987; Koll *et al.*, 1992; Gruhler *et al.*, 1995), in special cases the export reaction may be initiated when only part of the preprotein is in the matrix. This may lead to a situation where only loops of the preprotein are in the matrix and the export reaction drives the import reaction, as long as unfolding is not rate limiting (however, every part of the preprotein must still pass through the matrix space) (Koll *et al.*, 1992; Gruhler *et al.*, 1995; Ono *et al.*, 1995).

For this report we designed an assay to distinguish between the predictions about the role of mt-Hsp70 made by the two models for the sorting of precytochrome b_2. We moved the HB towards the C-terminal end, such that according to the stop transfer model it should be impossible for mt-Hsp70 to generate a pulling force on the domain. In the conservative sorting model each part of the preprotein has to be transported through the matrix and therefore mt-Hsp70 should be able to facilitate unfolding of preprotein domains located further away from the presequence. We found that the presence of the intermembrane space sorting signal restricts the unfoldase activity of mt-Hsp70 to domains in proximity to the presequence, whereas folded domains which are located more C-terminal can only be unfolded after deletion of the sorting signal. Moreover, the sorting signal seems to be recognized by components of the inner membrane/intermembrane space under conditions where previous contact of the sorting signal with the matrix space is excluded. We propose a hypothesis that explains these and the previous apparently divergent results by a new unifying hypothesis of mitochondrial protein sorting which incorporates aspects of both the stop transfer hypothesis and the conservative sorting hypothesis.

Results

A b_2–DHFR hybrid protein with the HB at the C-terminus is arrested in the mitochondrial import site

Hybrid proteins containing the non-covalent HB of cytochrome b_2 and mouse dihydrofolate reductase (DHFR) are particularly useful for the study of the role of mt-Hsp70 in promoting import of polypeptides into mitochondria, since three distinct types of folded structures can be presented to mitochondria (Glick *et al.*, 1993; Voos *et al.*, 1993). In the presence of heme (present in reticulocyte lysate), HB requires functional mt-Hsp70 for unfolding. When mt-Hsp70 is defective, the HB domain is unable to cross the outer membrane and remains on the cytosolic side. DHFR requires only low energy input for unfolding (Pace, 1990) and in the context of b_2 hybrid proteins can be unfolded during translocation in the absence of functional mt-Hsp70 (Voos *et al.*, 1993). Addition of the specific ligand methotrexate stabilizes the folded structure of DHFR, such that it cannot be unfolded by mitochondria when mt-Hsp70 is fully functional (Eilers and Schatz, 1986; Rassow *et al.*, 1989; Voos *et al.*, 1993). mt-Hsp70 is able to generate an inward-directed force on a polypeptide in transit, such that methotrexate-stabilized DHFR is tightly adpressed to the outer membrane and cannot be removed by protease added to isolated mitochondria (Schwarz *et al.*, 1993).

We constructed a hybrid protein containing the entire presequence of cytochrome b_2 (plus four amino acids of mature cytochrome b_2), followed by DHFR and HB (Figure 1A). pb_2–DHFR–HB was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with isolated energized *S.cerevisiae* wild-type mitochondria. The mitochondria were re-isolated and analyzed by SDS–PAGE and digital autoradiography (storage phosphorimaging system). The hybrid protein was efficiently processed to the intermediate (i) form and very slowly to the mature (m) form (Figure 1B, upper panel, lanes 1–4). However, all forms of b_2–DHFR–HB were accessible to protease added to the isolated mitochondria, demonstrating that they were not fully imported into the mitochondria (Figure 1B, lower panel, lanes 1–4). Instead, fragments of b_2–DHFR–HB were observed (Figure 1B, lower panel, lanes 1–4). The two major fragments have apparent molecular masses of ~16 and 13 kDa. In the absence of a membrane potential ($\Delta\psi$) across the mitochondrial inner membrane neither processing of pb_2–DHFR–HB nor formation of the fragments took place (Figure 1B, lane 5). The length, membrane potential ($\Delta\psi$) dependence and kinetics of appearance of the two major fragments suggest that they

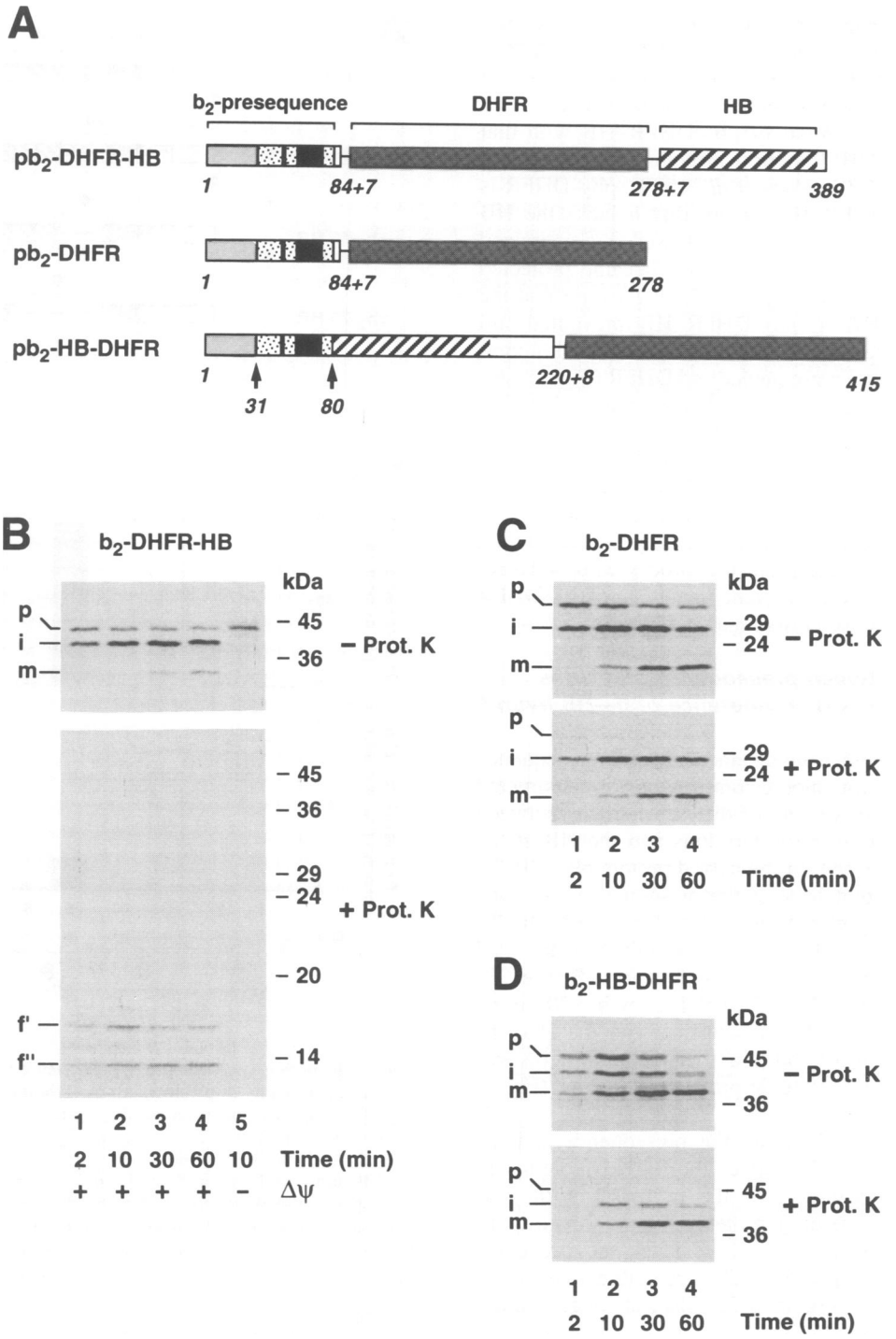

Fig. 1. A cytochrome b_2–DHFR hybrid protein with the HB at the C-terminus is arrested in mitochondrial import sites. (**A**) Hybrid proteins employed. pb_2–DHFR–HB contains the first 84 amino acid residues of the cytochrome b_2 precursor, a seven residue linker, the entire mouse dihydrofolate reductase (DHFR), a seven residue linker and cytochrome b_2 amino acid residues 81–184, with the HB (HB, residues 81–179). The bipartite b_2 presequence (positions 1–80) is composed of the N-terminal matrix targeting sequence, cleaved by the matrix processing peptidase at position 31, and residues 32–80 (stippled), which include the intermembrane space sorting signal (shown in black) and are removed by the inner membrane peptidase 1. Construct pb_2–DHFR lacks the HB (and the linker preceding it). In pb_2–HB–DHFR the first 220 amino acid residues of the authentic cytochrome b_2 precursor, including the complete HB (positions 81–179), are fused to DHFR (with an eight residue linker) (Koll *et al.*, 1992). The constructs pb_2–DHFR–HB (**B**), pb_2–DHFR (**C**) and pb_2–HB–DHFR (**D**) were incubated with isolated wild-type *S.cerevisiae* mitochondria (50 μg protein in a total volume of 100 μl) at 25°C for the times indicated in Materials and methods. In (B), lane 5 the membrane potential had been dissipated prior to import by including 1 μM valinomycin and 20 μM oligomycin. Samples were divided and one half treated with proteinase K (50 μg/ml final concentration). After re-isolation and separation by SDS–PAGE the import reactions were analyzed using a storage phosphorimaging system. p, i and m, precursor, intermediate and mature forms of the preprotein respectively. f' and f'', proteolytic fragments derived from b_2–DHFR–HB.

represent the imported N-terminal portions of the precursor and intermediate forms of b_2–DHFR–HB (~65 residues of the mature part plus the 80 residue presequence or 49 residues of the second portion of the presequence).

We compared the import of pb_2–DHFR–HB with that of two other pb_2–DHFR hybrid proteins (Rassow *et al.*, 1990; Koll *et al.*, 1992; Voos *et al.*, 1993). pb_2–DHFR is identical to pb_2–DHFR–HB except that it lacks the HB (Figure 1A). pb_2–DHFR was processed by energized mitochondria and the processed forms became protected against external protease (Figure 1C), suggesting that the presence of the HB in pb_2–DHFR–HB prevented full import of the protein. pb_2–HB–DHFR contains the complete HB between the presequence and DHFR (Figure 1A). pb_2–HB–DHFR was also processed to the intermediate and mature forms and the processed forms became protease-protected (Figure 1D), raising the possibility that the lack of complete import of pb_2–DHFR–HB might be due to the location of the HB within the hybrid protein. A quantification by digital autoradiography indicated that the formation of protease-protected, processed b_2–DHFR–HB was ≥40-fold less efficient than that of b_2–DHFR and b_2–HB–DHFR (see also Figure 4B).

The distance between presequence and HB is critical for the import competence of b_2–HB hybrid proteins

In order to test whether the distance between presequence and HB is crucial for import competence we constructed hybrid proteins with spacers of different lengths between the b_2 presequence at the N-terminus and the HB at the C-terminus (Figure 2A). In the hybrid protein pb_2–HB the HB follows immediately after the presequence, as is the case in authentic cytochrome b_2 and pb_2–HB–DHFR. pb_2–HB was processed by energized mitochondria and transported to a protease-protected location (Figure 2B, columns 1). With pb_2–20–HB, which contains 20 amino acid residues between the presequence and the HB, the major fraction of mitochondria-associated preproteins was also transported to a protease-protected location (Figure 2B, columns 3).

When the distance between the presequence and the HB was extended to 40 residues the hybrid protein was still efficiently processed, but the processed form was largely accessible to external protease (Figure 2B, columns 5). When the distance was 70 residues the processed hybrid protein was quantitatively accessible to external protease (Figure 2B, columns 7). Also, at longer import times no protease protection of b_2–70–HB was observed (not shown). We conclude that the distance between the presequence and the HB is of critical importance to whether a pb_2–HB hybrid protein is transported to a protease-protected location or not.

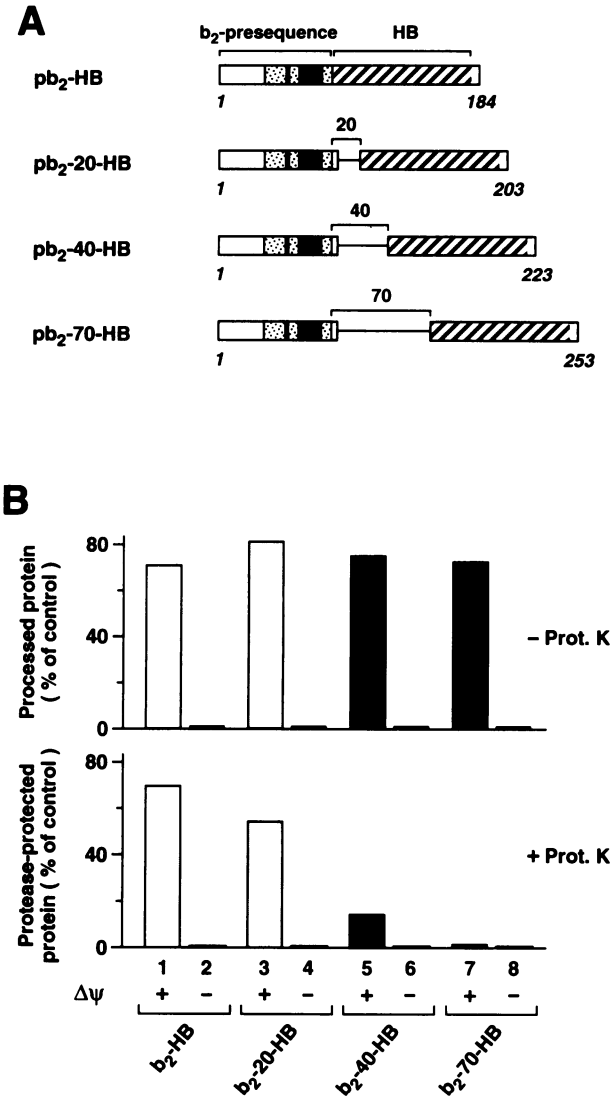

Fig. 2. The distance between the b_2 presequence and the HB is critical for the import ability of preproteins. (**A**) Hybrid proteins. The protein pb_2–HB comprises amino acid residues 1–184 of the authentic cytochrome b_2 precursor. In this case the HB (positions 81–179) immediately follows the presequence (positions 1–80). In pb_2–20–HB the HB starts at relative position 20 from the cleavage site at residue 80. For pb_2–40–HB and pb_2–70–HB this distance has been extended to 40 and 70 residues respectively. (**B**) The ^{35}S-labeled precursor proteins were imported into isolated wild-type mitochondria for 40 min at 25°C in a standard assay, described in Materials and methods. In every second reaction (lanes 2, 4, 6 and 8) the membrane potential had been dissipated prior to incubation. Proteinase K treatment (50 μg/ml final concentration) of one half of each sample was performed for 15 min at 4°C. Then the mitochondria were re-isolated by centrifugation, separated by SDS–PAGE and exposed to storage phosphor screens. The total amount of labeled protein associated with non-protease-treated mitochondria was set to 100% (control).

Unfolding of the preprotein allows complete import of a b_2–HB hybrid protein with a long spacer into the intermembrane space

pb_2–70–HB was denatured by pre-incubation in 8 M urea and then added to energized mitochondria. The efficiency of specific processing (Figure 3A, column 2) was comparable with that of direct import from reticulocyte lysate (Figure 3A, column 1). However, a large fraction of the urea-treated hybrid protein was transported to a protease-protected location (Figure 3A, column 5). In the absence of a $\Delta\psi$ across the inner membrane no protease protection of b_2–70–HB was observed (Figure 3A, column 6), excluding the possibility that protease protection was caused by aggregation of the preprotein.

Mature b_2–70–HB was not protease protected after opening of the intermembrane space by swelling of the mitochondria (formation of mitoplasts; Figure 3B, column 2),

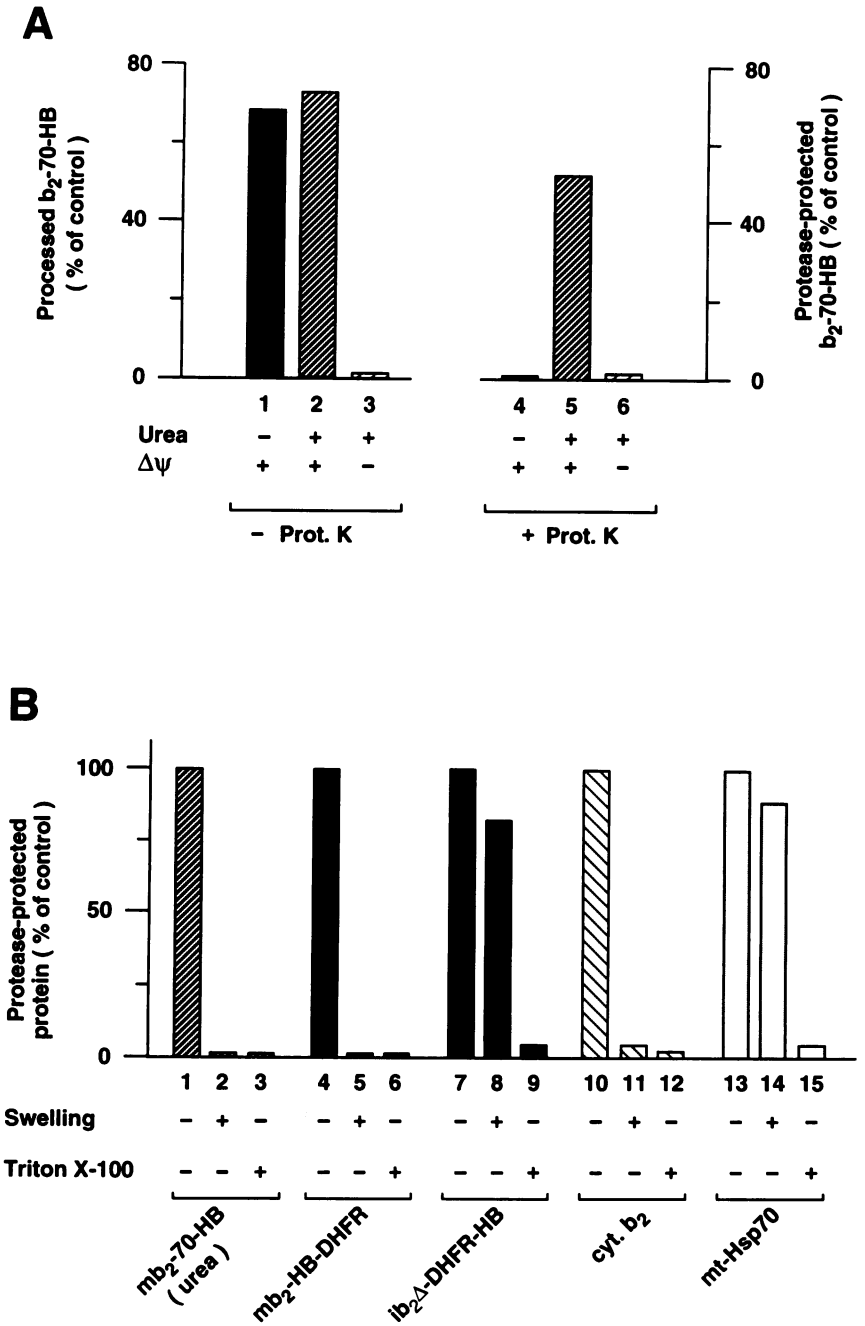

Fig. 3. Unfolding allows complete import of a preprotein with a long spacer between the presequence and the HB. (**A**) Import of b_2–70–HB. pb_2–70–HB was imported into wild-type mitochondria for 5 min at 25°C as described in Materials and methods (lanes 1 and 4, import out of reticulocyte lysate; lanes 2, 3, 5 and 6, urea-denatured preprotein). In reactions 3 and 6 the membrane potential had been dissipated prior to addition of precursor. Where indicated the samples were treated with proteinase K (50 μg/ml) for 15 min at 4°C. After re-isolation the mitochondria were analyzed by SDS–PAGE and digital autoradiography. The total amount of b_2–70–HB associated with non-protease-treated mitochondria was set to 100% (control). (**B**) Sub-mitochondrial localization of imported preproteins. The radiolabeled precursor proteins were imported into wild-type mitochondria at 25°C. Different import times (5 min for urea-denatured b_2–70–HB, 45 min for b_2–HB–DHFR and 15 min for b_2Δ–DHFR–HB) were chosen in order to obtain appropriate fractions of mature or intermediate proteins. The samples were divided into three portions and the mitochondria were re-isolated. After washing with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH, pH 7.2) one portion was resuspended in $SEMK_{80}$ (SEM plus 80 mM KCl) and treated with proteinase K (50 μg/ml). The second aliquot was converted to mitoplasts by hypotonic swelling and the third was lysed by Triton X-100 [0.2% (w/v) Triton X-100, 150 mM NaCl, 10 mM MOPS–KOH, pH 7.2], both in the presence of proteinase K (50 μg/ml). After re-isolation of the mitochondria/mitoplasts or TCA precipitation (third portion) the proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. Imported proteins were analyzed by autoradiography and marker proteins (cytochrome b_2 and mt-Hsp70) by immunodecoration. The total amount of protease-resistant protein in intact mitochondria was set to 100% for each protein. i and m, intermediate and mature forms of the preprotein.

indicating that the urea-denatured protein was correctly transported to the mitochondrial intermembrane space, as is the case with b_2–HB–DHFR imported directly from reticulocyte lysate (Figure 3B, column 5; Koll *et al.*, 1992). The fractionation was controlled by marker proteins: endogenous cytochrome b_2 was not protease protected in

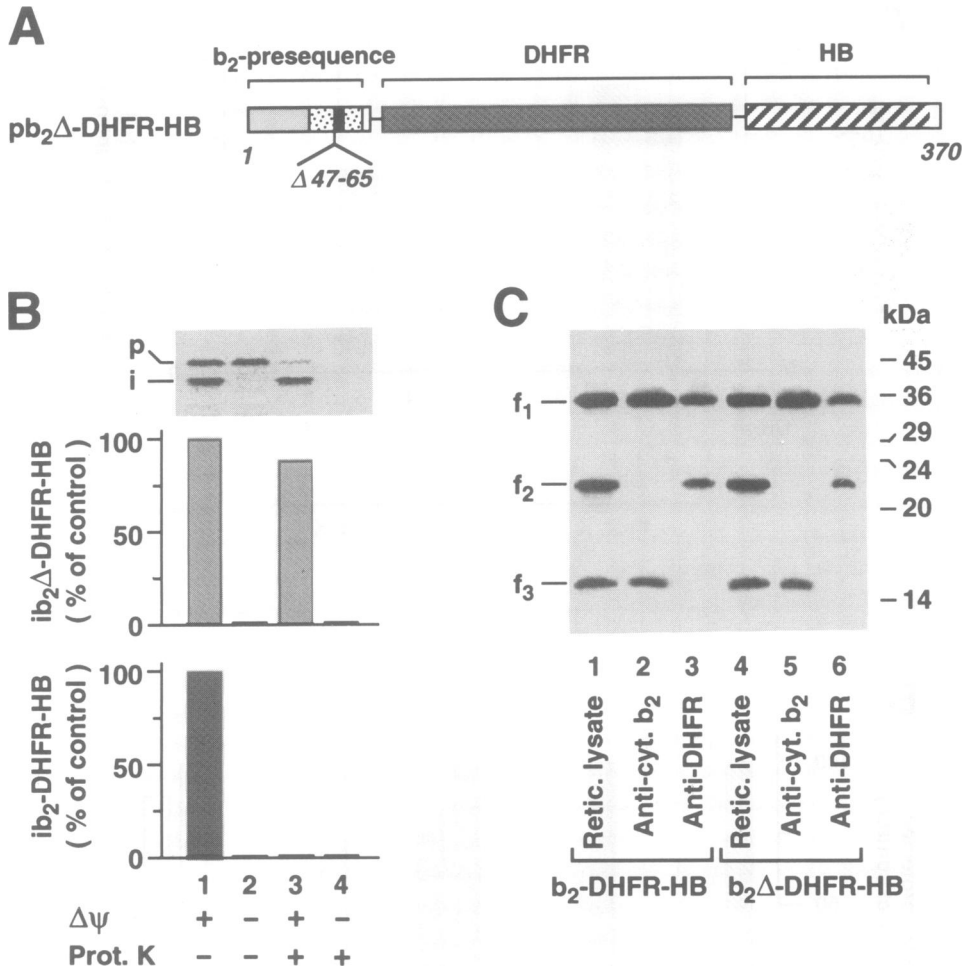

Fig. 4. Inactivation of the intermembrane space sorting signal restores the import competence of b_2–DHFR–HB. (**A**) Construct employed. The hybrid protein $pb_2\Delta$–DHFR–HB represents a derivative of pb_2–DHFR–HB (Figure 1A) lacking amino acid residues 47–65 within the second portion of the presequence. Thereby the intermembrane space sorting signal is inactivated. (**B**) Standard import reactions (see Materials and methods) with $pb_2\Delta$–DHFR–HB and pb_2–DHFR–HB respectively were carried out for 10 min at 25°C employing yeast wild-type mitochondria. In lanes 2 and 4 no membrane potential ($\Delta\psi$) was present during the incubation. Lanes 3 and 4 show the protease-protected protein after addition of 50 μg/ml proteinase K for 15 min. The amount of intermediate protein formed in the presence of $\Delta\psi$ was set to 100% (control). p and i, precursor and intermediate forms of the preprotein. (**C**) ^{35}S-Labeled precursor proteins of pb_2–DHFR–HB and $pb_2\Delta$–DHFR–HB (9 μl rabbit reticulocyte lysate) respectively were treated with 50 μg/ml proteinase K for 15 min on ice in a final volume of 12 μl. The reactions were stopped by addition of phenylmethylsulfonyl fluoride and divided into three portions. Two of the portions were incubated with antibodies against cytochrome b_2 (lanes 2 and 5) and DHFR (lanes 3 and 6) respectively, pre-bound to protein A–Sepharose, for 30 min at 4°C, as described in Materials and methods. The immunoprecipitated material was analyzed on an SDS gel together with the third portion (lanes 1 and 4), showing all three proteolytic fragments (f_1, f_2 and f_3) of the two preproteins.

mitoplasts (Figure 3B, column 11), whereas matrix Hsp70 was protease protected in mitoplasts and became accessible only after opening of the inner membrane by detergent (Figure 3B, columns 14 and 15). We conclude that unfolding of b_2–70–HB permits correct translocation into the intermembrane space.

A b_2–HB hybrid protein with a long spacer can be imported after deletion of the intermembrane space sorting signal

The second portion of the presequence of cytochrome b_2 contains a cluster of positively charged residues (positions 47–49) and a segment with a predicted hydrophobic character (residues 55–71; Guiard, 1985). A selection for mutations which inactivate the intermembrane space sorting signal yielded a high preference for point mutations in the positive cluster or the hydrophobic segment (Beasley *et al.*, 1993). A deletion of 19 residues (positions 47–65), including the positive cluster and part of the hydrophobic segment, was shown to completely inactivate the intermembrane space sorting signal, leading to protein import into the matrix (Koll *et al.*, 1992; Voos *et al.*, 1993). We inactivated the sorting signal of b_2–DHFR–HB by deletion of residues 47–65 (Figure 4A). The resulting hybrid protein $b_2\Delta$–DHFR–HB was transported to a protease-protected location (Figure 4B, lane 3), in contrast to b_2–DHFR–HB. This indicates that a large distance between the presequence and the HB does not impair import of the HB when the intermembrane space sorting signal is inactivated. On inactivation of the sorting signal $b_2\Delta$–DHFR–HB was only processed by the matrix processing peptidase to the intermediate form. $ib_2\Delta$–DHFR–HB was protected against external protease in mitoplasts (Figure 3B, column 8) and was accessible after opening of the

matrix (Figure 3B, column 9), in agreement with transport of $b_2\Delta$–DHFR–HB into the matrix.

DHFR and the HB form protease-resistant folded domains in preproteins synthesized *in vitro* (Glick *et al.*, 1993; Voos *et al.*, 1993). In order to exclude the possibility that inactivation of the sorting signal led to unfolding of mature portions of the hybrid protein we compared the fragmentation of b_2–DHFR–HB and $b_2\Delta$–DHFR–HB in reticulocyte lysates after addition of proteinase K. Both hybrid proteins yielded a comparable fragmentation into three major fragments (Figure 4C, lanes 1 and 4). By immunoprecipitation with antisera specific for DHFR or the mature part of cytochrome b_2 the large fragment f_1 was found to contain both DHFR and a mature part of cytochrome b_2, the middle fragment f_2 consisted of DHFR and the small fragment f_3 reacted with anti-cytochrome b_2 serum (Figure 4C, lanes 2, 3, 5 and 6). The size and specific immunorecognition of fragments f_1–f_3 thus suggest that they contain DHFR–HB, DHFR and HB respectively. This excludes the possibility that deletion of 19 residues from the presequence led to unfolding of the HB in the hybrid protein and suggests that the mature portions of b_2–DHFR–HB and $b_2\Delta$–DHFR–HB are in comparable folded states.

Import of the hybrid proteins depends on functional mt-Hsp70

We asked whether the hybrid proteins b_2–DHFR–HB and $b_2\Delta$–DHFR–HB depended on the function of mt-Hsp70. The *S.cerevisiae* mutant *ssc1-3* carries a temperature-sensitive defect in mt-Hsp70. The mutant Hsp70 is defective in interaction with incoming polypeptide chains. Import steps requiring functional mt-Hsp70 are thus strongly impaired in *ssc1-3* mitochondria (Gambill *et al.*, 1993; Voos *et al.*, 1993).

Mitochondria were isolated from *ssc1-3* cells and the corresponding wild-type cells and pre-incubated at 37°C to induce the mutant phenotype (Gambill *et al.*, 1993). Processing of $b_2\Delta$–DHFR–HB and transport to a protease-protected location were inhibited in *ssc1-3* mitochondria (Figure 5A, lanes 2 and 4), indicating a dependence on mt-Hsp70.

In contrast, the efficiency of processing of b_2–DHFR–HB by *ssc1-3* mitochondria was close to that of wild-type mitochondria (Figure 5B, upper panel, lanes 1 and 2). The first portion of the presequence could thus be transported into the matrix independently of functional mt-Hsp70. A similar behavior has been found for other b_2 hybrid proteins with an intact intermembrane space sorting signal, e.g. for b_2–HB–DHFR (Voos *et al.*, 1993). Interaction of the sorting signal with the MSM is assumed to substitute for the translocase function of mt-Hsp70 (Voos *et al.*, 1993; Glick *et al.*, 1993; Stuart *et al.*, 1994). Preproteins like b_2–HB–DHFR then require the unfoldase function of mt-Hsp70 for unfolding of the HB and thus depend on functional mt-Hsp70 for complete import (i.e. transport to a protease-protected location; Voos *et al.*, 1993). As b_2–DHFR–HB cannot be fully imported by wild-type mitochondria, it is obvious that no complete import was observed with *ssc1-3* mitochondria (Figure 5B, lower panel, lane 2).

The results reported so far do not provide evidence for an interaction of mt-Hsp70 with b_2–DHFR–HB. Two possibilities were conceivable. (i) b_2–DHFR–HB is unable to functionally interact with mt-Hsp70, therefore the HB cannot be unfolded and the preprotein is not completely imported. (ii) b_2–DHFR–HB can functionally interact with mt-Hsp70, but the sorting signal arrests translocation in the inner membrane and thus an inward-directed pulling force of mt-Hsp70 is limited to mature domains close to the presequence, i.e. domains that are close to the mitochondrial outer membrane before the sorting signal is arrested in the inner membrane. We therefore asked if mt-Hsp70 showed an influence on a domain closer to the presequence than the HB. The DHFR segment is close to the presequence and appeared to be a suitable candidate. Methotrexate strongly stabilizes the folded state of DHFR and has been shown to prevent its import into mitochondria (Eilers and Schatz, 1986; Rassow *et al.*, 1989). When the precursor polypeptide interacts with mt-Hsp70 the pulling force of Hsp70 can lead to a tight membrane apposition of the stable DHFR domain at the outer membrane, such that the DHFR cannot be cleaved off by external protease (Schwarz *et al.*, 1993). b_2–DHFR–HB was pre-incubated with methotrexate and then added to energized wild-type and *ssc1-3* mitochondria. This did not affect processing by wild-type mitochondria (Figure 5B, upper panel, lane 3), but reduced processing by *ssc1-3* mitochondria by ~70% (Figure 5B, upper panel, lane 4), suggesting that mt-Hsp70 facilitates import of the matrix targeting sequence when transfer of the DHFR domain is blocked. When the mitochondria were treated with proteinase K after the import reaction protease-resistant ib_2–DHFR–HB was detected in the re-isolated wild-type mitochondria (Figure 5B, lower panel, lane 3). In addition, a smaller protease-resistant form, f, of the same size as ib_2–DHFR was detectable (Figure 5B, lower panel, lane 3). Both the intermediate and f forms were recognized by antibodies directed against DHFR (not shown), whereas only the intermediate form, and not the f form, was recognized by antibodies directed against mature cytochrome b_2 (Figure 5B, lane 6), indicating that fragment f lacked the HB. Formation of the protease-resistant intermediate and f forms did not occur when the membrane potential across the inner membrane was dissipated during incubation of preprotein with mitochondria (Figure 5B, lower panel, lane 5), demonstrating that the protease-resistant forms were only generated from those preproteins which had undergone $\Delta\psi$-dependent translocation of part of their polypeptide chain. In *ssc1-3* mitochondria no formation of protease-resistant ib_2–DHFR–HB or fragment f was observed (Figure 5B, lower panel, lane 4). Thus functional mt-Hsp70 is needed to transport the methotrexate-stabilized DHFR domain of b_2–DHFR–HB close enough to the outer membrane for efficient processing of the presequence to take place and for the DHFR domain not to be removed by external protease. This excludes possibility (i) and demonstrates that mt-Hsp70 can promote inward movement of the N-terminal portion of b_2–DHFR–HB.

When $b_2\Delta$–DHFR–HB was pre-incubated with methotrexate processing to the intermediate form was inhibited (Figure 5C, lane 2). Previous studies have shown that a precursor polypeptide which spans the two mitochondrial membranes as a linear chain must possess at least ~50 amino acid residues between the matrix processing

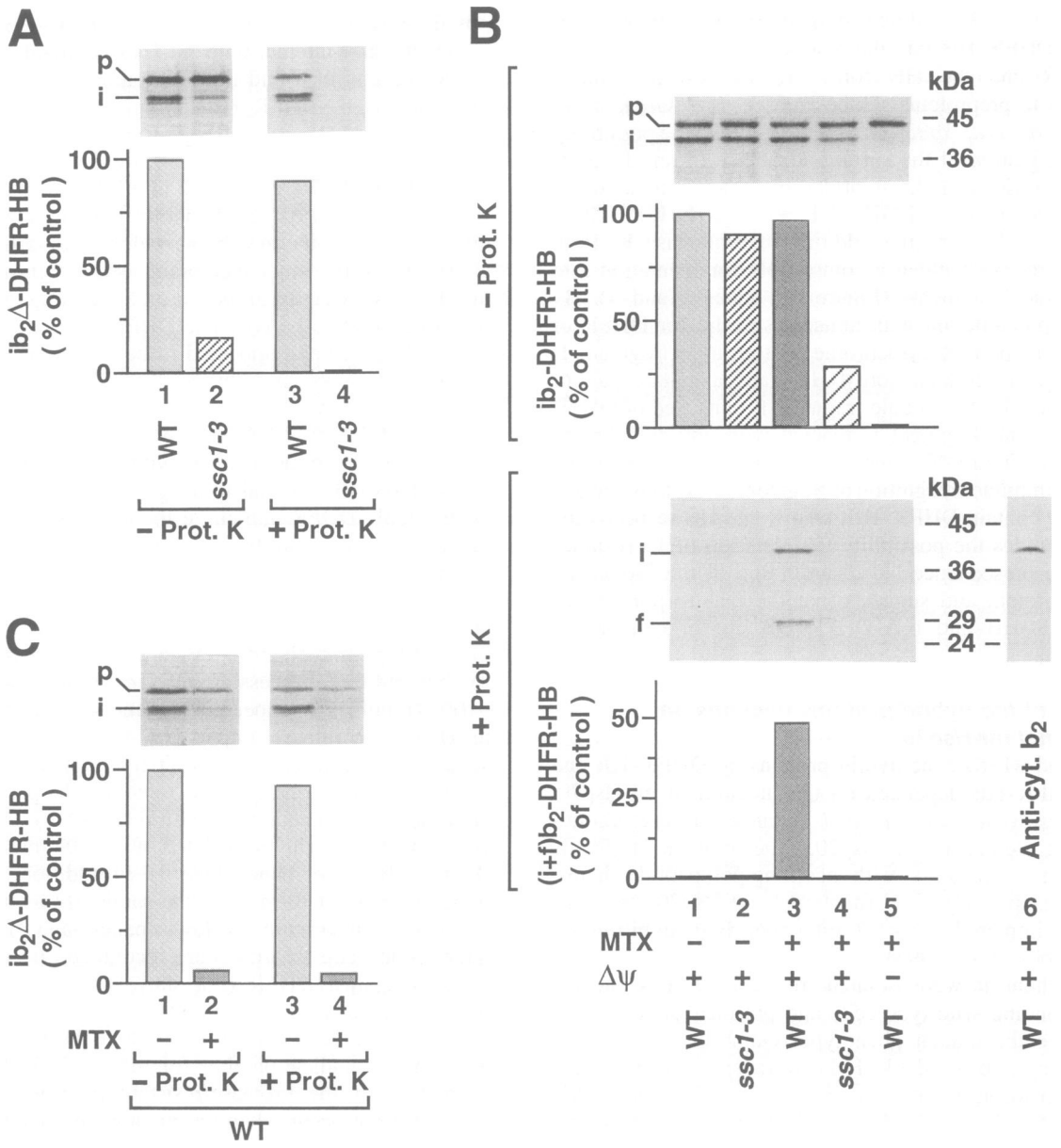

Fig. 5. The b_2 hybrid proteins require functional matrix Hsp70. (**A**) The precursor of b_2Δ–DHFR–HB (5 μl reticulocyte lysate) was incubated with yeast mitochondria (50 μg protein) from wild-type (WT; lanes 1 and 3) and the temperature-sensitive mt-Hsp70 mutant *ssc1-3* (lanes 2 and 4), which had been pre-incubated for 15 min at 37°C prior to import in order to stably induce their phenotype (Gambill *et al.*, 1993). The reactions took place for 10 min at 25°C and were stopped by adding 1 μM valinomycin (see Materials and methods). One half of the samples was treated with 50 μg/ml proteinase K. The amount of intermediate protein generated by wild-type mitochondria was set to 100% (control). (**B**) The experiment was performed as described above with the following modifications. The precursor of b_2–DHFR–HB was employed, where indicated the reticulocyte lysate had been pre-incubated with 1 μM methotrexate (MTX) for 10 min on ice prior to addition of the mitochondria and in lane 5 the incubation took place in the absence of a membrane potential (Δψ). The amount of intermediate protein generated in wild-type mitochondria in the absence of MTX and protease was set to 100% (control). For quantification of the samples treated with protease the amount of fragment f was corrected for the lower content of labeled amino acids compared with the intermediate form. For sample 6 the experiment was performed as described for sample 3 (using twice the amount of material), followed by treatment with proteinase K, lysis of the mitochondria with Triton X-100 and immunoprecipitation with antibodies directed against cytochrome b_2. (**C**) In the import reactions pb_2Δ–DHFR–HB was incubated with wild-type mitochondria for 10 min at 25°C in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1 μM methotrexate. The experiment was continued as described in (A) and (B). p and i, precursor and intermediate forms of the preprotein; f, proteolytic fragment of MTX-arrested b_2–DHFR–HB.

peptidase (MPP) cleavage site and methotrexate-stabilized DHFR to allow processing by MPP (Rassow *et al.*, 1990; Ungermann *et al.*, 1994). DHFR starts at position 92 of b_2–DHFR–HB, giving a 60 residue spacer to the cleavage site of MPP in b_2–DHFR–HB and only a 41 residue spacer in b_2Δ–DHFR–HB. This explains the inhibition of processing of methotrexate-treated b_2Δ–DHFR–HB and excludes the possibility that the intermembrane space sorting signal (located within residues numbers 47–71 of the b_2 presequence, i.e. only 45–21 residues in front of DHFR) of methotrexate-treated b_2–DHFR–HB is located in the matrix space. When the latter preprotein is arrested in energized mitochondria, calculations according to Rassow *et al.* (1990) and Ungermann *et al.* (1994) indicate a location of the sorting signal in the inner membrane/ intermembrane space.

The intermembrane space sorting signal prevents backsliding of accumulated preprotein in the mitochondrial import site

When the function of mt-Hsp70 is compromised by depletion of ATP, preproteins accumulated as membrane-spanning intermediates can slide back in the import channel (Cyr *et al.*, 1993; Schwarz *et al.*, 1993; Ungermann *et al.*, 1994). Schwarz *et al.* (1993) demonstrated that methotrexate-stabilized DHFR in a matrix-targeted preprotein was so tightly apposed to the outer membrane that its removal by external protease was impaired. When matrix ATP was depleted after accumulation of the hybrid protein the polypeptide slid back in the import channel, such that accessibility to protease increased. Such an example is shown in Figure 6A, with the hybrid protein b_2(167)Δ–DHFR, which contains the 167 N-terminal amino acid residues of cytochrome b_2 with the deletion of residues 47–65 described above. b_2(167)Δ–DHFR is imported into the matrix of energized mitochondria. After pre-incubation with methotrexate the hybrid protein is arrested in a two membrane-spanning topology. The matrix targeting sequence is cleaved off, while the stabilized DHFR is still on the outside. The DHFR is so tightly apposed to the outer membrane that added proteinase K does not remove it and a considerable fraction of ib_2(167)Δ–DHFR is resistant to protease treatment (Figure 6A, lower panel, column 5). After depletion of matrix ATP the protease accessibility of ib_2(167)Δ–DHFR is increased >3-fold (Figure 6A, lower panel, column 6).

We asked whether a hybrid protein that was arrested by the intermembrane space sorting signal similarly slid back in the import channel after depletion of matrix ATP. b_2–DHFR–HB was pre-incubated with methotrexate and then accumulated in mitochondria in the presence of ATP, yielding the protease-resistant forms described above (Figure 6A, lower panel, column 2). After depletion of matrix ATP, however, the protease resistance of the accumulated hybrid protein did not decrease (Figure 6A, lower panel, column 4). Therefore, the behavior of b_2–DHFR–HB is different from that of b_2(167)Δ–DHFR.

How is methotrexate-bound b_2–DHFR–HB kept in the import channel? We tested the stability of association of the accumulated protease-resistant hybrid protein with the mitochondrial membranes by treatment with sodium carbonate, pH 11.5 (Fujiki *et al.*, 1982). Proteins which are embedded in the lipid phase of the membranes are not extracted by this treatment (e.g. ADP/ATP carrier; Figure 6B, column 5), whereas soluble proteins (e.g. mt-Hsp70; Figure 6B, column 10) and proteins which are associated with membranes via protein–protein interactions, such as Mim44 (Figure 6B, column 8) and membrane-spanning intermediates of preproteins (Pfanner *et al.*, 1987), are extracted. The protease-protected forms of b_2–DHFR–HB were completely extracted from the membranes in the presence and in the absence of ATP (Figure 6B, columns 2 and 4), indicating that they were held in the membranes by protein–protein interactions.

In summary, these results suggest that the sorting signal in b_2–DHFR–HB interacts with proteinaceous components of the inner membrane/intermembrane space that, similarly to mt-Hsp70, impair backsliding of the preprotein in the import channel.

Discussion

We have analyzed the mitochondrial import characteristics of preproteins containing distinct parts of the precursor of cytochrome b_2 and suggest a unifying sorting hypothesis for this preprotein.

The ability of mitochondria to import preproteins which contain the entire presequence of cytochrome b_2 and the folded non-covalent HB in the mature part depends on the position of the domain in the preprotein. When the HB is 40 or more residues away from the bipartite presequence complete import of the preproteins is strongly inhibited. These preproteins are efficiently processed to the intermediate forms by the matrix processing peptidase, yet remain in the mitochondrial membranes and are accessible to external protease (Figure 7B). Complete import of these preproteins can be achieved by the following means. (i) Unfolding of the preprotein prior to import, leading to correct import into the intermembrane space. (ii) Movement of the HB closer to the bipartite presequence. Unfolding of the HB therefore requires functional matrix Hsp70, which binds to the precursor polypeptide (Glick *et al.*, 1993; Voos *et al.*, 1993; Stuart *et al.*, 1994) and apparently exerts a pulling force (Glick, 1995; Pfanner and Meijer, 1995). (iii) Inactivation of the intermembrane space sorting signal. The preprotein is then completely imported into the mitochondrial matrix in a mt-Hsp70-dependent manner, regardless of where in the polypeptide the HB is located (Figure 7C). Inactivation of the sorting signal does not influence the folding state of *in vitro*-synthesized preproteins, as assessed by the protease resistance of preprotein domains. This suggests that in the case of matrix-targeted preproteins mt-Hsp70 continuously exerts a pulling force on the polypeptide chain in transit, permitting unfolding of domains at any position in the preprotein. In contrast, the presence of the intermembrane space sorting signal of cytochrome b_2 restricts the unfoldase activity of mt-Hsp70 to domains that are in close proximity to the presequence.

Our studies demonstrate the following two mechanisms in sorting of cytochrome b_2. (i) The sorting signal is recognized during the initial import stage by proteinaceous components of the inner membrane/intermembrane space, as evidenced by the prevention of backsliding in the import channel despite impairment of mt-Hsp70 function by depletion of matrix ATP. A preprotein lacking the sorting signal slides back as soon as the function of mt-Hsp70 is compromised. It is important to note that recognition by inner membrane/intermembrane space components occurs before the sorting signal is able to reach the matrix space (denoted in the term 'initial import stage'). This was demonstrated by the short length of the preprotein segment that was able to enter the mitochondria, in agreement with the determinations of Neupert and colleagues (Rassow *et al.*, 1990; Ungermann *et al.*, 1994). The initial recognition in the inner membrane provides direct evidence for a prediction made by the stop transfer hypothesis. (ii) The sorting signal promotes an early divergence of the preprotein from the general mt-Hsp70-driven import pathway into the matrix: mt-Hsp70 cannot promote unfolding of domains that are 40 residues or more away from the presequence; domains with distances of 20 residues or less from the presequence are unfolded.

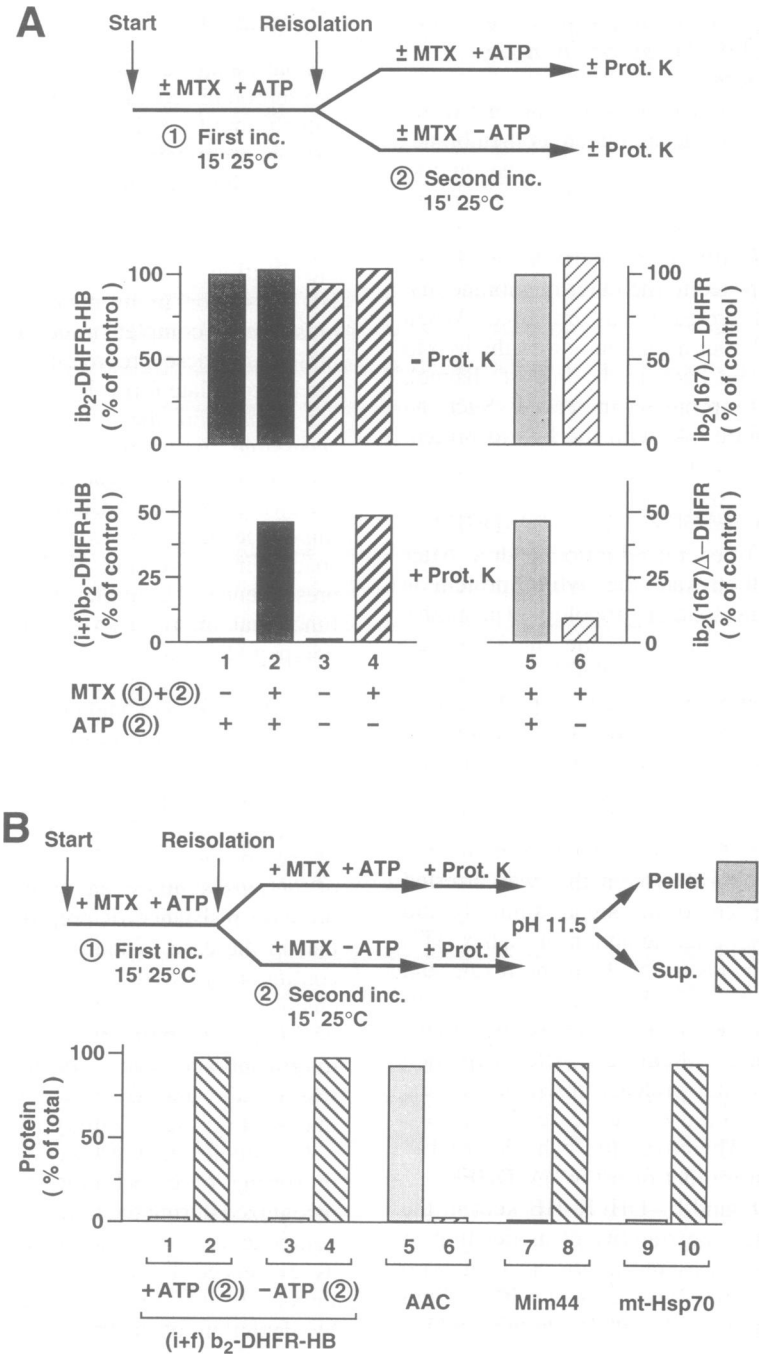

Fig. 6. The intermembrane space sorting signal impairs backsliding of the precursor polypeptide in the mitochondrial import site. (**A**) In a first step the precursor proteins pb_2–DHFR–HB and $pb_2(167)\Delta$–DHFR were incubated with wild-type yeast mitochondria (100 µg protein) for 15 min at 25°C in the presence of 2 mM ATP, as described in Materials and methods. Where indicated the preproteins were pre-incubated with methotrexate (MTX) and MTX (1 µM) was included during both incubations in order to stabilize the DHFR domain (samples 2, 4, 5 and 6). After re-isolation the mitochondria were resuspended in import buffer and divided in half. One portion again received 2 mM ATP (lanes 1, 2 and 5), whereas the other received 20 U/ml apyrase and oligomycin. After incubation for 15 min one half of each sample was treated with 50 µg/ml proteinase K for 15 min at 4°C. The mitochondria were re-isolated, separated by SDS–PAGE and autoradiograms were analyzed for the processed forms of the preproteins. Quantification was as described in the legend to Figure 5. i, intermediate form of the preprotein; f, proteolytic fragment of MTX-arrested b_2–DHFR–HB (see Figure 5B). (**B**) Accumulated pb_2–DHFR–HB is extractable at alkaline pH. Mitochondria were incubated with pb_2–DHFR–HB in the presence of MTX as described above. After 15 min at 25°C the import mixture was divided in half and the mitochondria were re-isolated. One portion was resuspended in import buffer supplemented with ATP as before, the other one in buffer containing apyrase and oligomycin. After a second incubation for 15 min both samples were treated with proteinase K and the mitochondria re-isolated. The mitochondria were resuspended in 1 ml freshly prepared 0.1 M Na_2CO_3, pH 11.5, and kept on ice for 30 min. Centrifugation at 266 000 *g* for 1 h yielded a pellet and a supernatant (Sup.) fraction (TCA precipitated). The samples were analyzed by SDS–PAGE and Western transfer to nitrocellulose was performed. Imported proteins were analyzed by autoradiography and marker proteins [ADP/ATP carrier (AAC), Mim44 and mt-Hsp70] were determined by immunodecoration. The total amount of protease-protected protein (pellet plus supernatant) was set to 100%.

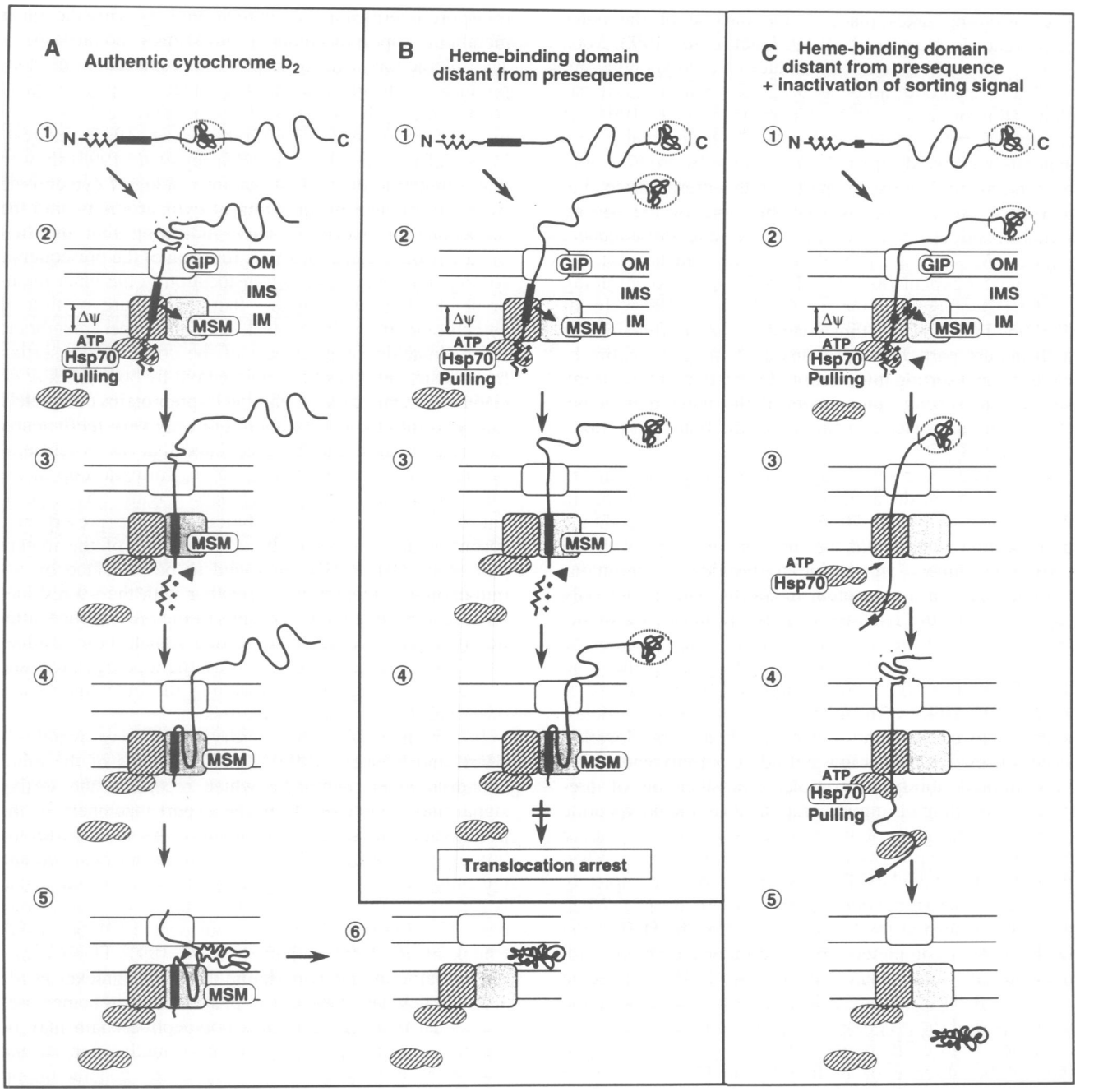

Fig. 7. Hypothetical model of mitochondrial sorting of cytochrome b_2 and derived hybrid proteins. (**A**) Unifying sorting hypothesis for import of cytochrome b_2. The N-terminal part of the presequence is imported via the general matrix targeting pathway driven by the membrane potential $\Delta\psi$ and mt-Hsp70 (stage 2). The sorting signal in the second part of the presequence (denoted by a box) is recognized by the mitochondrial sorting machinery (MSM) as soon as it enters the inner membrane and thereby the preprotein is taken out of the general mt-Hsp70-driven import pathway (stages 2 and 3). Then the N-terminus of the mature part inserts with a loop into the inner membrane sorting machinery (in a hydrophilic proteinaceous environment) (stage 4). Proteolytic removal of the second part of the presequence on the outer side of the inner membrane and completion of translocation across the outer membrane lead to full import of the protein into the intermembrane space (stages 5 and 6) (for further details see Discussion). A folded domain in the preprotein (HB, indicated by a dashed circle, stage 1), which requires the unfoldase activity of mt-Hsp70, can be imported when it is located close to the presequence, since the domain is then unfolded at the outer membrane in the initial mt-Hsp70-dependent import step (stage 2). (**B**) When the folded HB is located more towards the C-terminus (stage 1) it cannot be unfolded when the sorting signal directs the preprotein out of the mt-Hsp70-dependent general import pathway (stages 2 and 3), leading to translocation arrest (stage 4). (**C**) After inactivation of the sorting signal mt-Hsp70 molecules can exert a pulling force on all parts of the preprotein (stages 2–4), permitting unfolding of a HB located far away from the presequence (stage 4). C, C-terminus of the preprotein; GIP, general insertion pore in the outer membrane; IM, inner membrane; IMS, intermembrane space; N, N-terminus of the preprotein; OM, outer membrane. Quoins mark proteolytic processing.

The unfolding takes place at the outside of the outer membrane (Rassow *et al.*, 1989; Glick *et al.*, 1993; Voos *et al.*, 1993) and at least 50 residues of a polypeptide are needed to span both mitochondrial membranes as a linear chain (Rassow *et al.*, 1990; Ungermann *et al.*, 1994). It can therefore be calculated that Hsp70 in the matrix can generate an inward-directed force on the b_2 proteins only by binding to N-terminal parts of the presequence, i.e. parts that precede the hydrophobic core of the sorting signal (residues 55–71 of the 80 residue presequence; Figure 7A, stage 2), providing strong evidence for the stop transfer explanation of mt-Hsp70-supported unfolding of the HB of cytochrome b_2 (Glick *et al.*, 1993). In all likelihood mt-Hps70 is thus unable to interact functionally with mature parts of preproteins containing the entire b_2 targeting and sorting information. Interaction of the sorting signal with sorting components of the inner membrane/intermembrane space substitutes for the translocase function of mt-Hsp70.

Our studies bear some implications for the mechanism of preprotein translocation across the outer membrane. In the absence of mt-Hsp70 function the outer membrane translocation system and the inner membrane-associated sorting machinery are unable to provide a continuous pulling force on a preprotein in transit. This is not only apparent from the inability to promote unfolding of the HB, but also from the only partial outer membrane translocation of segments between the presequence and the HB (Figure 1B). This extends the studies of Mayer *et al.* (1993, 1995) with isolated outer membrane vesicles, which showed that the outer membrane translocation system itself has only a low unfolding activity and thus a very limited ability to complete translocation of preproteins. Binding of import components to the polypeptide chain and refolding of translocated domains may drive the translocation process across the outer membrane. When the HB is absent the DHFR can be unfolded and completely translocated across the outer membrane (Figure 1C). When the HB is placed at the C-terminal end of the DHFR, the DHFR cannot completely cross the outer membrane and, interestingly, more than half of the DHFR molecule remains on the cytosolic side (only ~65 residues of the mature part of the preprotein are protected against external protease) (Figure 7B, stage 4). It is tempting to speculate that in the absence of pulling by mt-Hsp70 precursor polypeptides slide back and forth in the import channel (Cyr *et al.*, 1993; Ungermann *et al.*, 1994) until segments of sufficient length are in the intermembrane space to allow (partial) folding. The energy derived from step-wise folding of preprotein portions may thus contribute to a trapping of the domain in the intermembrane space, leading to a step-wise (discontinuous) translocation process in the absence of mt-Hsp70 action.

Previous discussions on the sorting pathway of cytochrome b_2 were based on two mutually exclusive hypotheses (Glick *et al.*, 1992, 1993; Koll *et al.*, 1992; Gruhler *et al.*, 1995). It is difficult to understand how the numerous detailed studies on mitochondrial sorting of one and the same preprotein led to such different conclusions. We reasoned that the explanation for the apparently divergent results is that different aspects of the same sorting mechanism were analyzed. Neupert and colleagues (Gruhler *et al.*, 1995) reported co-precipitation of a mature transport intermediate of cytochrome b_2 with the inner membrane import machinery (with Mim44 and mt-Hsp70). The interaction with mt-Hsp70 was reported to be non-productive and to occur mainly under non-physiological conditions, such as elevated temperature and use of a mutant form of mt-Hsp70 (Gruhler *et al.*, 1995). Interestingly, Schatz and colleagues (Beasley *et al.*, 1993) showed that a mutation of the first mature residue of cytochrome b_2 led to efficient mistargeting of cytochrome b_2 into the mitochondrial matrix *in vivo*, indicating that the first mature residue can abolish the function of the presequence sorting signal in arresting translocation in the inner membrane. Both results suggest that the mature portion of cytochrome b_2 is in contact with the inner membrane sorting machinery, an aspect of the conservative sorting hypothesis, yet it has been pointed out by both Glick *et al.* (1992) and Ono *et al.* (1995) that b_2 preproteins completely imported into the matrix are not or only very inefficiently exported. It may thus be speculated that the N-terminus of the mature part of cytochrome b_2 forms a loop in the inner membrane sorting machinery (Figure 7A, stage 4) and thereby influences the function of the presequence sorting signal. Interestingly, ~65 residues of the mature part of b_2–DHFR–HB are found to be protected by the mitochondrial membranes. Together with the ~9 residues that are located at the C-terminus of the presequence, after the hydrophobic core of the sorting signal, these residues are of just sufficient length to form the topology proposed in stage 4 of Figure 7A (spanning the outer membrane once and the inner membrane twice).

In agreement with the conservative sorting hypothesis (Hartl and Neupert, 1990), the components of the mitochondrial inner membrane which recognize the sorting signal may be derived from the export machinery of the prokaryotic ancestor of mitochondria. As shown with the homologous transport machineries of *Escherichia coli* and the endoplasmic reticulum, the hydrophobic sorting signal is arrested in the membrane and at the same time mature parts of the preprotein can be translocated (Blobel, 1980; Chuck *et al.*, 1990; Schiebel *et al.*, 1991; Dobberstein, 1994; Gafvelin and von Heijne, 1994; Jungnickel *et al.*, 1994; Schekman, 1994). It is possible that in some cases more than two segments of a polypeptide chain may be present at a transport site. In mitochondria the sorting machinery is in spatial proximity to the general import machinery and thus recognizes and traps the sorting signal during the initial import stage in the inner membrane, preventing translocation of the sorting signal into the matrix (a short-cut between import and sorting in the inner membrane, as predicted by the stop transfer hypothesis; Figure 7A, stages 2 and 3). Since the prokaryotic machinery permits forward and backward movement of limited segments of a preprotein (Schiebel *et al.*, 1991), the derived MSM may be able to insert a loop of the mature part of the preprotein from the intermembrane space side.

We propose the following unifying sorting hypothesis for cytochrome b_2 (Figure 7A). Specific recognition of the sorting signal during initial import into the inner membrane/intermembrane space (stage 2) promotes early divergence of b_2 import from the general matrix import pathway (stage 3) and then directs a loop of the mature part of the protein into the sorting machinery associated

with the inner membrane (stage 4). The looping into the inner membrane may provide some driving energy to promote translocation of the remainder of the polypeptide across the outer membrane (stage 5) (although, as outlined above, it clearly does not achieve the import pulling force of mt-Hsp70). Eventually, proteolytic removal of the second portion of the presequence and re-transport of the loop into the intermembrane space complete the import of cytochrome b_2. Some modifications of this model are conceivable that can be addressed in future studies. (i) The loop (stage 4) may be relatively short and mainly located on the outer surface of the inner membrane. (ii) Under special conditions, e.g. high temperature (Gruhler *et al.*, 1995), the loop may be extended and reach into the matrix to contact mt-Hsp70. (iii) After initial recognition in the inner membrane (stage 3) the sorting signal may emerge on the matrix side and immediately re-insert into the inner membrane, thereby leading to loop formation (stage 4) by a conservative sorting-type mechanism. In this case, however, the loop emerging on the matrix side must not productively interact with mt-Hsp70.

The mitochondrial inner membrane seems to use at least two mechanisms to sort preproteins to the intermembrane space or the inner membrane. (i) The pathway discussed here for cytochrome b_2 may be used by other preproteins with bipartite presequences, such as cytochrome c_1 (Hurt and van Loon, 1986; van Loon *et al.*, 1986; Glick *et al.*, 1992; Wachter *et al.*, 1992) and cytochrome c peroxidase (Kaput *et al.*, 1982). It will be interesting to see if polytopic integral inner membrane proteins such as the ADP/ATP carrier, which are assumed to be sorted by a non-conservative mechanism (Mahlke *et al.*, 1990; Wachter *et al.*, 1992), use a similar mechanism to insert their membrane loops into the inner membrane. (ii) The Fe/S protein of the bc_1 complex and the F_0-ATPase subunit 9, which is nuclear-encoded in *Neurospora crassa* and higher eukaryotes, are sorted by a conservative mechanism, including complete import into the the matrix space and export to the inner membrane (Hartl *et al.*, 1986; van Loon and Schatz, 1987; Mahlke *et al.*, 1990; Rojo *et al.*, 1995).

Materials and methods

Construction of cytochrome b_2 hybrid proteins

pb_2–DHFR–HB. The DNA fragment encoding the chimeric protein b_2–DHFR–HB was engineered in three steps using PCR with Pfu DNA polymerase (Stratagene). First, $pb_2(84)$–DHFR (Rassow *et al.*, 1990) was used as the template. The *Eco*RI site was conserved in the 5′-end, the stop codon (TAA) of the DHFR gene was removed and an *Acc*65I site was added in the 3′-end. The primers used for amplification were: CGM 4957, GCCCGAATTCCCAAACAAAAGTAGTCAATG; CGM 4956, CCGCGGTACCCCGTCTTTCTTCTCGTAGACTTC. The resulting *Eco*RI–*Acc*65I DNA fragment was cloned into the pGEM4 vector to give the pGEM785 construct. Second, $pb_2(220)$–DHFR (pb_2–HB–DHFR) (Koll *et al.*, 1992) was used as the template. The DNA fragment encoding the HB of cytochrome b_2 (residues 81–184) was obtained. A *Xba*I site was introduced in the 5′-end, a stop codon (TAA) after the residue glutamic acid 184 was created and a *Hin*dIII site was added in the 3′-end. The primers used for amplification were: CGM 4955, GCCCTCTAGAGCCGAAACTGGATATGAATAAAC; CGM 4954, CCGGAAGCTTATTCCTTAGTTTCACCAGGAGC. The resulting *Acc*65I–*Hin*dIII fragment was cloned into the pGEM4 vector to give the pGEM786 construct. Third, the *Acc*65I–*Hin*dIII fragment from plasmid pGEM786 was introduced between the *Acc*65I and *Hin*dIII sites of pGEM785 to give the pGEM791 (pb_2–DHFR–HB) construct. In this case seven extra residues (Gly-Val-Pro-Gly-Asp-Pro-Leu) were introduced between the DHFR domain and the HB.

$pb_2\Delta$–DHFR–HB. The DNA fragment *Msc*I–*Hin*dIII encoding the C-terminus of the presequence of cytochrome b_2 fused to the DHFR–HB(81–184) domains was substituted for the *Msc*I–*Hin*dIII DNA fragment of the $b_2(1–167)_{\Delta 19}$–DHFR construct (Koll *et al.*, 1992) to give the pGEM797 ($pb_2\Delta$–DHFR–HB) construct. A deletion of residues 47–65 was thereby introduced into the cytochrome b_2 presequence.

Cytochrome b_2 hybrid proteins with 70 amino acid residues or less between the presequence and the HB were obtained from the vector pGEM791 containing pb_2–DHFR–HB by PCR amplification of the entire plasmid except for different portions of the (spacer) sequence between the b_2 presequence and the HB domain. Primer-mediated introduction of corresponding cleavage sites allowed recircularization of the plasmids with the spacers of different lengths. These spacers comprised residues 81–84 of the cytochrome b_2 precursor, the two seven residue linkers preceding and following DHFR (see Figure 1A), one leucine resulting from ligation of the introduced *Bln*I sites and different portions of the DHFR sequence (N-terminal part) necessary to let the HB start 20, 40 and 70 residues respectively after position 80 of the cytochrome b_2 presequence (b_2–20–HB, b_2–40–HB and b_2–70–HB). PCRs were performed employing the Expand™ Long Template PCR System (Boehringer-Mannheim). Restriction digestion, gel purification and ligation of the PCR products followed published procedures (Sambrook *et al.*, 1989). *Escherichia coli* strain DH1 was used for transformation with the ligation product by electroporation (Bio-Rad Gene Pulser). The construct b_2–HB, representing the first 184 residues of cytochrome b_2, was derived from a pGEM4Z vector encoding authentic precytochrome b_2 (Guiard, 1985). We introduced a stop codon and a *Hin*dIII site with the backward primer and another *Hin*dIII site with the forward primer and amplified the complete plasmid except the sequence corresponding to residues 185–591 of cytochrome b_2. Recircularization steps and transformation were as described above.

Import of preproteins into isolated yeast mitochondria

We used the *S.cerevisiae* strains S150 2B (MATa, *leu2-3,112*, *his3-Δ1*, *trp1-289*, *ura3-52*; 'wild-type'), PK83 [MATα, *ade2-101*, *lys2*, *ura3-52*, *leu2-3,112*, *Δtrp1*, *ssc1-3(LEU2)*; '*ssc1-3*'] and PK82 (MATα, *his4-713*, *lys2*, *ura3-52*, *leu2-3,112*, *Δtrp1*; 'wild-type') (Gambill *et al.*, 1993). The cells were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol) and mitochondria were prepared according to published procedures (Daum *et al.*, 1982; Hartl *et al.*, 1987; Kang *et al.*, 1990; Gambill *et al.*, 1993). Radiolabeled precursor proteins were obtained by *in vitro* transcription of the above constructs with SP6 polymerase followed by synthesis in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and [^{35}S]cysteine as described (Söllner *et al.*, 1991). For import of urea-denatured preprotein we used the procedure described by Glick *et al.* (1993). The translation mixture was centrifuged for 30 min at 266 000 *g* and the resulting pellet was solubilized in 8 M urea, 30 mM MOPS–KOH, pH 7.2, 50 mM dithiothreitol. In a standard import assay isolated yeast mitochondria (50–100 μg protein), energized by addition of 2 mM ATP and 2 mM NADH, were incubated with preprotein (5 μl reticulocyte lysate or urea solution) in bovine serum albumin (BSA)-containing buffer (100 μl final volume) at 25°C (Söllner *et al.*, 1991). The reactions were stopped by including 1 μM valinomycin and placing them on ice. Samples with a dissipated membrane potential $\Delta\psi$ received 1 μM valinomycin and 20 μM oligomycin prior to incubation. For generation of mitoplasts by hypotonic swelling re-isolated mitochondria were resuspended in S_{25}EM buffer (25 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH, pH 7.2) to a final concentration of 250 μg protein/ml and left on ice for 20 min. Swelling was stopped by adding the same volume of S_{475}EM buffer (475 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH, pH 7.2). Proteinase K treatment, re-isolation of the mitochondria and separation by SDS–PAGE have been described previously (Söllner *et al.*, 1991). Autoradiograms were obtained and quantified employing a storage phosphorimaging system (Molecular Dynamics Inc.). Standard procedures were used for Western blotting, TCA precipitation and treatment with sodium carbonate (Fujiki *et al.*, 1982; Pfanner *et al.*, 1987).

Immunoprecipitation of radiolabeled polypeptides

Specific antibodies (10 μl serum) were pre-bound to protein A–Sepharose (7 μl PAS; Pharmacia Biotech Inc.) for 1 h in 30 μl buffer containing Triton X-100 (TB; 1% Triton X-100, 10 mM Tris–HCl, pH 7.5, 300 mM NaCl) and incubated with the polypeptide-containing sample (^{35}S-labeled translation product) for another 30 min at 4°C with gentle shaking. After washing three times with 1 ml TB the PAS–antibody-associated material was dissolved in sample buffer at 95°C, separated by SDS–PAGE and analyzed by autoradiography.

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