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Targeting of cytochrome b_2 into the mitochondrial intermembrane space: specific recognition of the sorting signal

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Cytochrome b_2 contains 2-fold targeting information: an amino-terminal signal for targeting to the mitochondrial matrix, followed by a second cleavable sorting signal that functions in directing the precursor into the mitochondrial intermembrane space. The role of the second sorting sequence was analyzed by replacing one, two or all of the three positively charged amino acid residues which are present at the amino-terminal side of the hydrophobic core by uncharged residues or an acidic residue. With a number of these mutant precursor proteins, processing to the mature form was reduced or completely abolished and at the same time targeting to the matrix space occurred. The accumulation in the matrix depended on a high level of intramitochondrial ATP. At low levels of matrix ATP, the mutant proteins were sorted into the intermembrane space like the wild-type precursors. The results: (i) suggest the existence of one or more matrix components that specifically recognize the second sorting signal and thereby trigger the translocation into the intermembrane space; (ii) indicate that the mutant signals have reduced ability to interact with the recognition component(s) and then embark on the default pathway into the matrix by interacting with mitochondrial hsp70 in conjunction with matrix ATP; (iii) strongly argue against a mechanism by which the hydrophobic segment of the sorting sequence stops translocation in the hydrophobic phase of the inner membrane.

Key words: conservative sorting/cytochrome b_2/mitochondria/protein transport/sorting signal

Introduction

Targeting of proteins to the mitochondrial matrix appears to follow a quite uniform pathway. It requires the presence of a matrix targeting signal at the amino-terminus of the precursor and involves receptors on the mitochondrial surface, as well as transport machineries facilitating the transfer of unfolded polypeptide chains across both outer and inner mitochondrial membranes (Neupert *et al.*, 1990; Pfanner and Neupert, 1990; Pfanner *et al.*, 1992; Baker and Schatz, 1991). In contrast, translocation into other mitochondrial subcompartments, in particular into the intermembrane space (IMS), occurs by less uniform pathways. At least three different mechanisms exist to direct a protein into this compartment. A first pathway has been described for cytochrome c (Stuart and Neupert, 1990). Apocytochrome c is made without a cleavable targeting signal, it can insert into the outer mitochondrial membrane in a presumably spontaneous process and can be trapped in the IMS after the covalent addition of the heme group catalyzed by cytochrome c heme lyase (CCHL). A second pathway has been described for the import of this latter enzyme (Lill *et al.*, 1992). CCHL is also synthesized without a cleavable signal, but uses receptors and the outer membrane translocation machinery. It does not, however, use the inner membrane translocation apparatus. Accordingly, CCHL does not need a membrane potential, nor does it require ATP in the matrix. A third pathway is used by cytochrome b_2 (Guiard, 1985) and by some proteins of the inner membrane which are largely exposed to the IMS, such as cytochrome c_1 (Sadler *et al.*, 1984) and the Rieske Fe/S protein (Harnisch *et al.*, 1985; Beckmann *et al.*, 1987). The precursors of these respiratory chain components contain a matrix targeting sequence at the amino-terminus, followed by a sorting domain which in the first two cases is cleaved off and in the latter case remains presumably as a membrane anchor.

For cytochrome b_2 and cytochrome c_1, two different mechanisms of sorting have been proposed (Hartl and Neupert, 1990). Initially, the sorting sequence was suggested to serve as a 'stop-transfer' domain (Kaput *et al.*, 1982; Hurt and van Loon, 1986; van Loon *et al.*, 1986; van Loon and Schatz, 1987). The hydrophobic segment was proposed to stop the translocation process (triggered by the matrix targeting signal) through an interaction with the hydrophobic membrane phase. Completion of translocation of the polypeptide chain across the outer membrane into the IMS and cleavage by specific proteases on the outer face of the inner membrane (Ohashi *et al.*, 1982; Behrens *et al.*, 1991; Schneider *et al.*, 1991) is then thought to occur. As a possible alternative or additional pathway, it was proposed that cytochrome b_2 could be targeted initially to the matrix and subsequently sorted into the IMS using the sorting sequence as a topogenic signal (Hartl *et al.*, 1987). Evidence in favor of sorting through the matrix was provided by showing that, under appropriate conditions, precursors processed to intermediate size were located in the matrix space, that ATP in the matrix was required for sorting and that the matrix-localized heat shock proteins hsp70 and, under certain conditions, hsp60 were involved (Cheng *et al.*, 1989; Koll *et al.*, 1992). Recently, sorting of cytochrome c_1 and cytochrome b_2 via the matrix space was questioned; however, the argumentation was based on negative evidence (Glick *et al.*, 1992).

A detailed understanding of the mechanisms by which the sorting signals direct proteins into the IMS, and a characterization of the components involved in these reactions, may shed new light on the so far conflicting conclusions. This approach requires a careful analysis of the

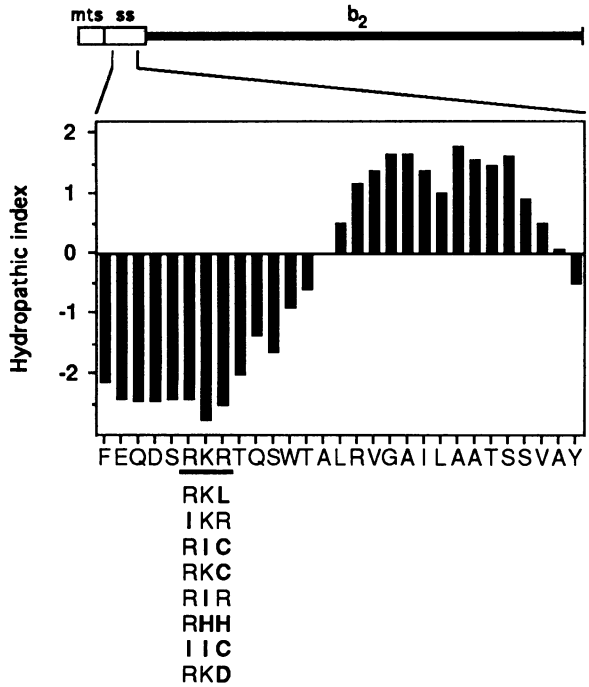

Fig. 1. Mutations introduced into the sorting signal of pre-cytochrome b_2. The two signal sequences of cytochrome b_2 are indicated (mts, mitochondrial targeting signal; ss, intramitochondrial sorting sequence), the mature part of the protein is shown in bold. The hydrophobic indices from amino acid 42 to 70 were calculated according to Kyte and Doolittle (1982). The amino acid exchanges introduced by *in vitro* mutagenesis are listed below the wild-type sequence.

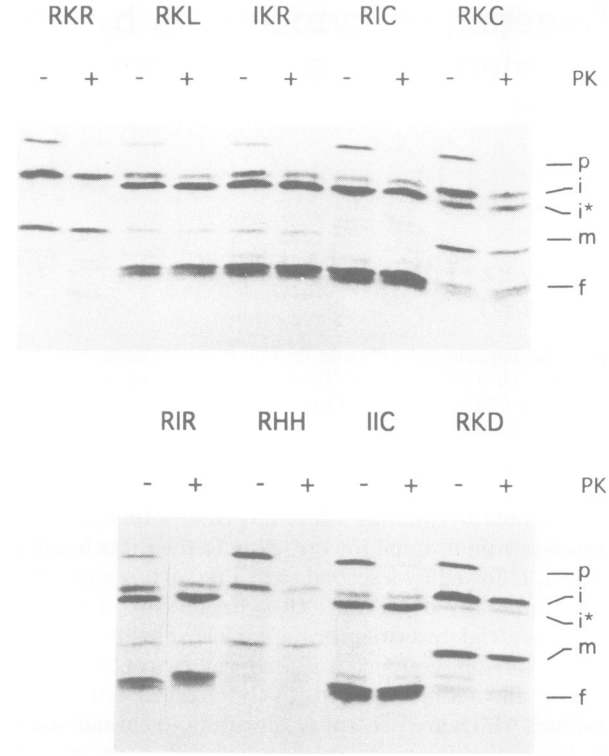

Fig. 2. Import and processing of pb_2(167)DHFR fusion proteins with alterations in the sequence positions 47–49 in comparison to the wild-type (RKR). Import conditions and SDS–PAGE are described in Materials and methods. p, precursor form; i, intermediate form; i*, intermediate* form; m, mature form; f, fragment forms; +PK, samples treated with proteinase K.

sequences believed to play an essential role in targeting. The sorting signals for the IMS appear to contain at least two typical motifs: (i) a core of hydrophobic uncharged amino acid residues preceded by (ii) one or more positive charges. It has been noted before that the sorting signals display considerable similarity to the export signals of bacterial proteins and secreted proteins in eukaryotes (Hartl and Neupert, 1990; von Heijne, 1990).

An analysis on secretion-defective *Escherichia coli* mutants has stressed the importance of the positive charges preceding the hydrophobic core in the secretion process (Puziss *et al.*, 1989). In this report, we are focusing on the positive charges of the sorting domain in the cytochrome b_2 precursor. We show that a number of mutants in which these charged residues were replaced by uncharged ones became mislocated in the mitochondrial matrix at high, but not at low levels of matrix ATP. We conclude that there must be a specific recognition of this part of the sorting signal in the mitochondria and that 'stop-transfer' by interaction of the hydrophobic part of the signal with the hydrophobic membrane phase is not a likely mechanism for sorting.

Results

Specific mutations in the positively charged cluster amino-terminal of the hydophobic sequence of the sorting signal lead to accumulation of cytochrome b_2 in the mitochondrial matrix

The sorting signal of cytochrome b_2 precursor contains a cluster of positive charges at the amino-terminal side of the hydrophobic core (amino acids RKR in positions 47–49) (Guiard, 1985). By *in vitro* mutagenesis, we replaced each or combinations of these three positively charged residues by uncharged or negatively charged ones (Figure 1). Mutations in the RKR motif were analyzed in the context of two different precursor proteins. One was a fusion protein consisting of the first 167 amino acids of cytochrome b_2 followed by the mouse cytosolic dihydrofolate reductase [pb_2(167)DHFR]. The second one was the authentic precytochrome b_2. The mutant proteins were synthesized *in vitro* in reticulocyte lysate and import into isolated mitochondria was analyzed.

Some of the mutant pb_2(167)DHFR precursors were imported and processed to the intermediate (*i*) and mature sized forms (*m*) like the wild-type precursor protein (e.g. RKD and RHH; Figure 2). Other pb_2(167)DHFR mutants showed reduced processing to (*i*) and (*m*) forms (e.g. RKC and RIR), and some mutants (e.g. RIC and IIC) were not processed to the (*m*) form at all. However, when processing to the mature form was totally absent or reduced, other products were observed (Figure 2). In particular, some mutants showed, in addition to an intermediate sized form with the same apparent molecular weight as the intermediate in wild type, a product that was ~1 kDa smaller (*i**) than (*i*) and fragment forms (*f*) that were smaller than the wild-type mature sized cytochrome b_2 DHFR fusion protein.

In order to determine the intramitochondrial location of the various mutant proteins, following import mitochondria were exposed to hypotonic medium. Under these conditions, the outer membrane was selectively opened (as judged by the release of cytochrome b_2), whilst the inner membrane remained to a large extent intact (controlled by the

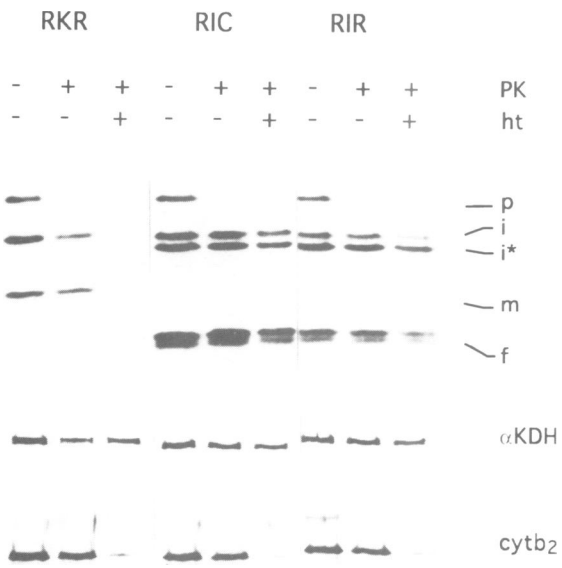

Fig. 3. Intramitochondrial localization of wild-type and mutant pb_2(167)DHFR proteins. Wild-type (RKR) and mutant (RIC and RIR) pb_2(167)DHFR were imported into mitochondria. Import was performed with a total of 50 μg mitochondrial protein in sorbitol buffer (for details see Materials and methods). After import, mitochondria were collected by centrifugation and resuspended in 100 μl fresh import buffer. Two aliquots were diluted into 5 vols of isotonic buffer containing 0.6 M sorbitol, 20 mM Hepes/KOH, 1 mg/ml BSA (pH 7.4). One sample remained untreated (−PK), whereas the second aliquot was treated with 100 μg/ml proteinase K (+PK). The third aliquot was diluted into 5 vols of 20 mM Hepes/KOH, 1 mg/ml BSA, 100 μg/ml proteinase K (hypotonic treatment, + ht). Aliquots of the samples were blotted to nitrocellulose and decorated with antibodies against α-ketoglutarate dehydrogenase (αKDH, matrix marker) and cytochrome b_2 ($cytb_2$, IMS marker) to check for the intactness of the matrix space and the opened outer membrane.

accessibility of α-ketoglutarate dehydrogenase to proteinase K). The intermediate and mature sized forms generated from the wild-type precursor were almost completely degraded under these conditions, demonstrating that they were partially or completely present in the intermembrane space (Figure 3). In contrast, the (*i*) and (*i**) forms, as well as the (*f*) forms of the mutant proteins were proteinase K inaccessible following swelling. Apparently, mutations in the positively charged cluster led to mistargeting to the matrix space. This mistargeting was accompanied by limited proteolytic degradation to the characteristic (*i**) and (*f*) forms. In the case of those mutant precursors which were partly affected, the fraction processed to (*i*) and (*m*) forms was translocated into the IMS.

In order to confirm that the mislocating effect of the mutations was also exhibited by the full-length cytochrome b_2, the effects of the amino acid exchanges were also investigated in the context of the full-length cytochrome b_2. As was the case with the fusion protein, the mutant full-length precytochrome b_2 containing the RIC mutation was processed almost exclusively to the intermediate sized form. Similarly, the mutation RIR in the full-length precytochrome b_2 gave rise to the same intermediary phenotype as was observed in the corresponding DHFR fusion protein. In contrast, wild-type precytochrome b_2 was processed to both intermediate and mature size (Figure 4), and both forms were proteinase K sensitive after opening the IMS by hypotonic swelling (Figure 4). The intermediate sized forms

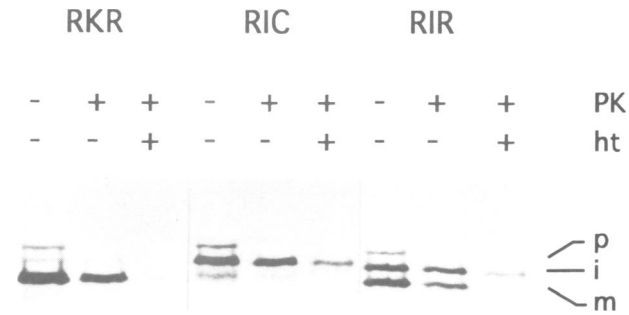

Fig. 4. Import of wild-type (RKR) and mutant (IIC) full-length cytochrome b_2, and their intramitochondrial localization. After import, the samples were processed as described in Figure 3. PK, proteinase K; ht, hypotonic treatment.

of mutants RIC and RIR remained partially proteinase K resistant which is indicative for partial mistargeting to the matrix (~45% of total (*i*) form in the case of RIC).

Degradation of the matrix-localized mutant full-length precytochrome b_2 was not observed, although minor proteolytic events cannot be excluded. Apparently, the proteolytic enzymes which degrade mislocated proteins have certain structural requirements that differ between various precursor proteins.

In summary, these data demonstrate that subtle changes in the positively charged cluster of the sorting signal lead to mistargeting into the matrix of both full-length precytochrome b_2 and the derived fusion pb_2(167)DHFR proteins. We conclude that for correct sorting, a specific recognition of the sorting signal must occur. Since mutants in which the hydrophobic stretch was extended by creating two or three additional hydrophobic residues at the amino-terminal side of the hydrophobic core were localized in the matrix, a stop of the transfer by simple interaction with the hydrophobic membrane is therefore unlikely.

Mutant pb_2(167)DHFR proteins require high ATP levels for translocation into the matrix and reach the intermembrane space when matrix ATP is low

The energy requirements for import of the mutant cytochrome b_2 fusion proteins were analyzed and compared to those of the wild-type protein. Mitochondria were treated with oligomycin to inhibit ATP synthesis by the F_1F_o ATPase. One half of these mitochondria were then supplemented with NADH and an ATP-regenerating system to restore intra- and extramitochondrial ATP ('matrix ATP replenished'). The other half of the mitochondria was first treated with carboxyatractyloside to block the ADP/ATP translocator and then also received NADH and an ATP-regenerating system ('matrix ATP depleted'). The import of wild-type pb_2(167)DHFR into the IMS was not affected by depletion of matrix ATP. This is in contrast to the behavior of most mitochondrial precursor proteins, including authentic cytochrome b_2 and fusion proteins consisting of longer segments of cytochrome b_2 linked to DHFR, which all have a distinct requirement for high levels of matrix ATP and heat shock proteins (Kang *et al.*, 1990; Koll *et al.*, 1992). When mutant RIC of pb_2(167)DHFR was imported into mitochondria with reduced levels of matrix ATP, accumulation of intermediate sized forms in the matrix and the abnormal processing to the (*i**) and (*f*) fragments was

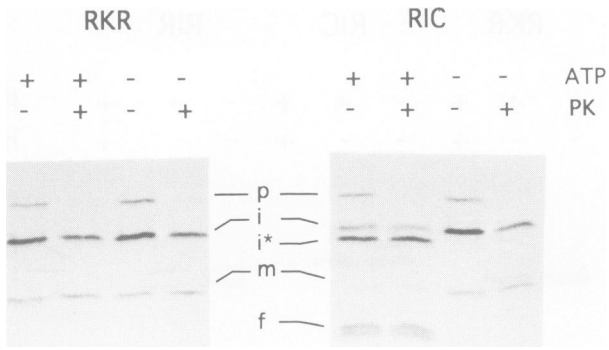

Fig. 5. Import of wild-type (RKR) pb_2(167)DHFR and mutant (RIC) pb_2(167)DHFR into ATP-depleted mitochondria. Mitochondria were incubated with 30 μM oligomycin and 2.5 mM ADP for 3 min at 25°C. The samples were split into two aliquots. One half received an ATP-regenerating system and 5 mM NADH ('+ATP'), while the other aliquot was incubated for 3 min at 25°C with 30 μM carboxyatractyloside before addition of the ATP-regenerating system and NADH ('−ATP'). After a further incubation for 3 min at 25°C, radiolabeled precursors were imported and the samples were processed as described (see Figure 3).

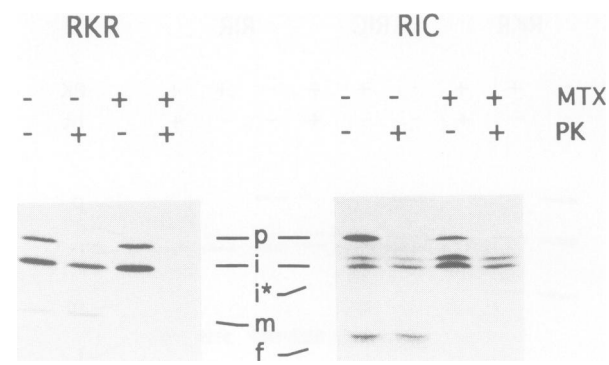

Fig. 6. Import of wild-type (RKR) and mutant (RIC) pb_2(167)DHFR in the presence and absence of methotrexate. Wild-type and mutant radiolabeled precursor proteins were imported for 25 min at 25°C into mitochondria, in the absence (−MTX) and presence of 2 μM methotrexate (+MTX). After import, each sample was split and one half was treated with 100 μg/ml proteinase K (+PK), whereas the other half remained untreated (−PK). The samples were further processed as described in Materials and methods.

practically completely abolished (Figure 5). Rather, (*i*) and (*m*) forms were generated as with the wild-type pb_2(167)DHFR. These (*i*) and (*m*) species formed in ATP-depleted mitochondria were localized in the intermembrane space (data not shown), in other words reduction of matrix ATP led to a suppression of the mutant phenotype. Essentially the same results were obtained when a decrease of the level of matrix ATP was achieved by pretreatment with apyrase (data not shown). It should be emphasized that in all these experiments the ATP concentration outside the matrix was kept at high level.

When the RIC or IIC mutations were introduced into the full-length precytochrome b_2, results obtained were similar to those observed with the fusion proteins. At high matrix ATP levels, the intermediate sized forms of the mutant proteins were found in the matrix and no mature forms were detectable. At low matrix ATP, however, intermediate and mature sized forms of the mutated cytochrome b_2 were observed in the IMS (data not shown). Thus, correct targeting of the full-length mutant cytochrome b_2 to the IMS also depended on the intramitochondrial ATP level.

Arrest of fusion proteins in a transmembrane arrangement by methotrexate leads to a tight membrane apposition of the DHFR domain of the mutant proteins but not of the wild-type fusion protein

pb_2(167)DHFR, when synthesized in reticulocyte lysate, allows folding of the DHFR part which then assumes a protease-resistant conformation and can bind the substrate analogue methotrexate (Eilers and Schatz, 1986; Rassow *et al.*, 1990; Koll *et al.*, 1992). The precursor complexed with methotrexate can then enter the mitochondria only up to the point where the stabilized DHFR domain reaches the outer membrane, the folded domain being unable to traverse the membrane. The DHFR moiety can then be cleaved off by adding protease such as proteinase K or trypsin to the intact mitochondria (Figure 6). The mutant proteins analyzed in this study were all able to form a folded DHFR domain in reticulocyte lysate (results not shown). Upon import, they

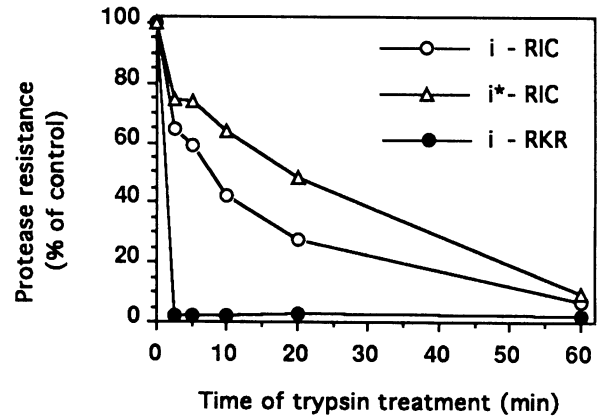

Fig. 7. Prolonged trypsin treatment of the methotrexate stabilized translocation intermediates. Wild-type (RKR) and mutant (RIC) pb_2(167)DHFR proteins were imported for 15 min in the presence of 2 μM methotrexate. Trypsin was added to a final concentration of 20 μg/ml and incubated for the indicated time periods. Trypsin treatment was stopped by addition of soybean trypsin inhibitor and the samples were reisolated by centrifugation and processed on SDS−PAGE. The autoradiograph was quantified by laser densitometry (Ultroscan XL, LKB Pharmacia).

became arrested as spanning intermediates, the (*i**) forms being produced but not the (*f*) forms. Apparently, there is a protease in the matrix that gets access to the amino-terminus of the mutant precursors after the matrix targeting sequence has been cleaved.

Unexpectedly, the DHFR domain in these arrested fusion proteins could not be cleaved off with protease under the conditions used for the wild type (Figure 6). The reason for this could be that either the link between the cytochrome b_2 and the DHFR domain was not accessible to the added protease, or that it became translocated across the outer/inner membrane despite the stabilization by methotrexate. Protease treatment for prolonged periods on ice eventually led to cleavage of the mutant proteins also (Figure 7). Under these conditions, the intermembrane space was not compromised. Thus, with the mutant proteins the methotrexate-stabilized DHFR domain was still outside the outer membrane;

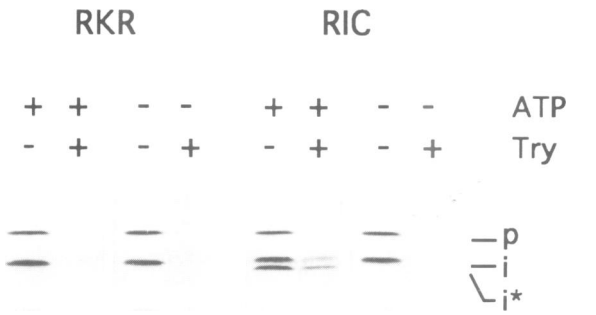

Fig. 8. Protease sensitivity of methotrexate-stabilized translocation intermediates in mitochondria with reduced ATP levels. Mitochondria were incubated in import buffer containing 2 μM methotrexate and matrix ATP was decreased as described in Figure 5. After incubation with oligomycin and ADP, the samples were split into two portions, of which one received carboxyatractyloside and the other remained untreated. An ATP-regenerating system and NADH were added to both samples after a 3 min incubation at 25°C. Radiolabeled precursor was added to the mitochondria and import was performed as described. After import, one-half of the sample was treated with 200 μg/ml trypsin on ice for 15 min, followed by a 5 min incubation with 1 mg/ml soybean trypsin inhibitor (+Try). The samples were collected by centrifugation and analyzed by SDS−PAGE.

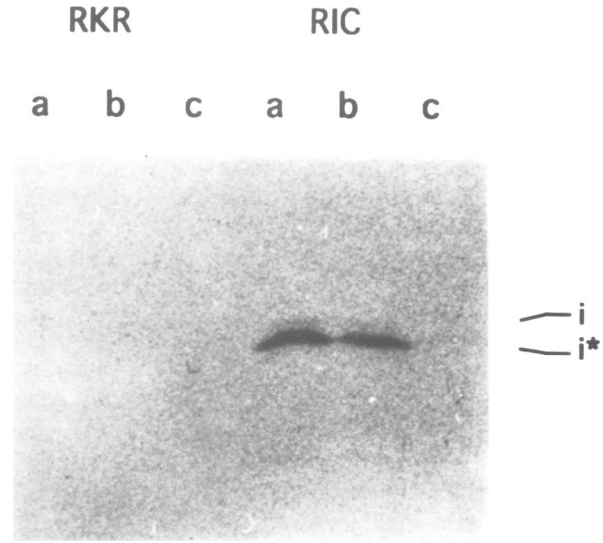

Fig. 9. Co-immunoprecipitation of wild-type (RKR) and mutant (RIC) pb_2(167)DHFR with mt-hsp70. Methotrexate-stabilized translocation intermediates were generated as described in Figure 6 and subjected to co-immunoprecipitation with antiserum against either mt-hsp70 (**lane a**), bacterial DnaK (**lane b**) and/or preimmune serum (**lane c**).

however, it was apparently more tightly apposed to the outer membrane, preventing access of the added protease. One possible explanation for this behavior is that with the mutant proteins mt-hsp70 remains tightly associated with that part of the spanning intermediate that is exposed to the matrix space, thereby blocking a back-sliding of the polypeptide chain in the putative translocation pore. In contrast, the wild-type protein, after reacting with components of the export machinery, should not be fixed in the matrix space or at least in a less tight manner.

Accordingly, a reduction of matrix ATP which had been shown to suppress the mutant phenotype (see Figure 5) should lead to an increased protease susceptibility of the methotrexate-stabilized mutant pb_2(DHFR). This in fact was observed, as shown in Figure 8. In ATP-replenished mitochondria, resistant (*i*) and (*i**) forms were observed with mutant RIC, whereas the intermediate sized form was protease sensitive in mitochondria with reduced ATP levels.

Association of the mutant, but not the wild-type methotrexate-arrested intermediates, with mt-hsp70 could, in fact, be demonstrated by co-immunoprecipitation. Mitochondria with methotrexate-stabilized translocation intermediates accumulated in the presence of ATP were lyzed with detergent and immunoprecipitation with antibodies against mt-hsp70 was performed. This antibody precipitated the mutant (*i*) and (*i**) forms, but not wild-type intermediate (Figure 9). The efficiency of this co-immunoprecipitation was not very high (~5−10% of the total spanning intermediate) which can be explained by the unstable interaction between precursor and mt-hsp70 after lysis of mitochondria. The fact that the (*i*) form was less efficiently precipitated might be accounted for by the eventual conversion of the (*i*) to the (*i**) form, as we have observed in time course experiments (results not shown).

In summary, these experiments reveal a strong difference between wild-type and mutant protein, namely that the former is not or much less tightly associated with mt-hsp70 when spanning the membranes, whereas the latter is bound to hsp70 at this stage.

Discussion

Targeting of cytochrome b_2 into the mitochondrial intermembrane space apparently involves a highly specific recognition step in which the sorting signal is decoded. Mutations in the cluster of the three positively charged amino acid residues preceding the hydrophobic stretch of the sorting signal led to accumulation of the protein in the matrix. Most probably, the element to be recognized includes both the positive charges and the hydrophobic core (Hartl and Neupert, 1990; Beasley and Schatz, 1991). We therefore propose that a protein component exists in the mitochondria that specifically recognizes this sorting signal. It has been noted previously that the mitochondrial sorting sequences bear similarity to the signal sequences of secreted proteins both in bacteria and in eukaryotic cells (Hartl and Neupert, 1990; von Heijne, 1990). In bacteria these sequences are handled by a complex set of proteins which eventually facilitate the translocation across the plasma membrane of bacteria [for a review, see Wickner *et al.* (1991)]. In eukaryotes, very similar signal sequences interact with the signal-recognition particle and subsequently with specific components in the membrane of the endoplasmic reticulum [for a review, see Rapoport (1992)]. In line with the generally accepted theory of the endosymbiontic origin of mitochondria, we propose that a (possibly homologous) signal-recognition protein or particle is present in the matrix or at the inner face of the inner mitochondrial membrane.

The function of such components might be conserved in yet another aspect, namely that they assist in membrane translocation by allowing co-translocational transfer to the matrix space and intermembrane space, as cytoslic SRP allows co-translational translocation into the endoplasmic reticulum. We have recently provided evidence in support of such a simultaneous import and export reaction (Koll *et al.*, 1992). Coupling of translocation across outer and inner membrane and back into the IMS, however, is not necessarily obligatory. Segments of the polypeptide chain

in transit, or even the whole precursor, may be exposed to the matrix space. The latter situation may occur when import is much faster than export (Koll *et al.*, 1992). In that case, mitochondrial chaperones were found to bind to polypeptide chains as they pass through the matrix.

The existence of a mitochondrial SRP-like component would also explain our observation that wild-type and mutant proteins interact differently with mt-hsp70 and have different ATP requirements. Previous work has suggested that the initial interaction of mt-hsp70 with the polypeptide chain as it appears on the inner face of the inner membrane is an important, if not essential, driving force for moving the precursors across both membranes. With the wild-type precursor of cytochrome b_2, we suggest that when the sorting sequence appears on the matrix side the component recognizing the sorting signal binds to it and directs it to the inner membrane. In contrast, in the case of the mutants the putative recognition protein (or a component reacting subsequently in the pathway) would not be able to bind strongly enough to the altered sorting sequence, allowing mt-hsp70 to bind to the precursor. Thus, a competition between mt-hsp70 and the putative export components seems to exist. In the presence of high matrix ATP levels, the equilibrium would be favored in the direction of mt-hsp70 interaction and import would occur into the matrix. In contrast, in the presence of low matrix ATP levels, i.e. when mt-hsp70 cannot function properly, the equilbrium would be shifted towards an interaction with the signal-recognition component and targeting to the IMS would take place.

In ATP-depleted mitochondria, yet another pathway may exist that would lead to the formation of intermediate sized and mature cytochrome b_2. The lack of mt-hsp70 function is known to cause the accumulation of import intermediates spanning both outer and inner membranes, and of intermediates that have completed translocation across the outer membrane, but have only initiated translocation across the inner membrane (Hwang *et al.*, 1991; Rassow and Pfanner, 1991; Jascur *et al.*, 1992). In such a configuration the second cleavage site could become accessible to IMP1 protease catalyzing the cleavage of the sorting signal on the surface of the inner membrane, and both mature and intermediate sized cytochrome b_2 located in the intermembrane space would be generated. It can presently not be decided which of the two pathways is the correct one, or whether both pathways might exist at the same time.

These conclusions are in agreement with the 'conservative sorting' model which proposes that proteins like cytochrome b_2, cytochrome c_1 and the Rieske Fe/S protein first become directed to the matrix (via the general import pathway) and subsequently sorted along pathways that already existed in the prokaryotic ancestors of mitochondria using signals that have also been conserved in their essential features (Neupert and Hartl, 1990). The essential role of the positively charged residues in these sorting signals seems to be functionally conserved, as suggested by a comparison of the data presented here to those on the corresponding motif in bacterial leader sequences. The present model specifies 'conservative sorting' by proposing the existence of protein components that selectively recognize sorting signal sequences in the matrix or at the inner face of the inner membrane.

Recently, 'conservative sorting' of cytochrome b_2 and cytochrome c_1 has been challenged, and the 'stop-transfer model' originally proposed for the sorting of precursors with dual signals has been claimed to be exclusively valid (Glick *et al.*, 1992). The data presented here show that stop-transfer by a hydrophobic domain in a sorting signal cannot exist as a topogenic principle in the sorting of cytochrome b_2. In the mutants analyzed here, the hydrophobic segment has not been altered, rather in some cases it was even extended. This supports our previous hypothesis that in translocation across outer and inner mitochondrial membranes, hydrophobic sequences are not sequestered by default into the lipid phase of the membranes (Hartl *et al.*, 1987; Mahlke *et al.*, 1990).

In a preliminary report, selection of a series of mutations was described in a fusion protein consisting of an amino-terminal segment of cytochrome b_2 and cytochrome oxidase subunit IV (Beasley and Schatz, 1991). The mutant fusion proteins were able to complement a deficiency in cytochrome oxidase subunit IV, suggesting that they could enter the matrix space. Some of the mutants contained amino acid exchanges in the region preceding the hydrophobic core. These observations are in agreement with the results from our analysis of mutant precursor proteins generated *in vitro*.

We want to point out, however, that the results presented here do not exclude a modified concept of sorting by 'stop-transfer'. One could imagine that it is not the hydrophobic segment alone that is required to arrest translocation at the level of the inner membrane, but that additional information, such as positive charges at the amino-terminus of the hydrophobic stretch, is crucial. Such a variation of the 'stop-transfer' mechanism would then infer a highly specific binding protein in the mitochondrial inner membrane or at the outer surface of the inner membrane. The experiments described here do not exclude such a modified 'stop-transfer'. Moreover, the two pathways are *a priori* not mutually exclusive. The present data, however, in our view are best explained in the framework of a pathway that involves sorting through the matrix space. This is supported by a recent report in which a mutational analysis of the targeting signal of cytochrome c_1 was described (Jensen *et al.*, 1992). Mutations in the hydrophobic segment of the sorting signal were observed to cause targeting to the matrix. Analysis of the import of such a mutant form also led to the view that transport through the matrix is the most likely mechanism for the sorting of cytochrome c_1 to the intermembrane space.

Materials and methods

Isolation of mitochondria

Wild-type *Saccharomyces cerevisiae* (strain D 273-10B) was grown on lactate medium according to Daum *et al.* (1982) and harvested at an OD_{578} of 1.5–2. Mitochondria were isolated as described previously (Daum *et al.*, 1982) with the exception that the purified mitochondria were finally resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA and 10 mM MOPS/KOH, pH 7.2) at a protein concentration of 5–10 mg/ml.

Recombinant DNA techniques and mutagenesis

Standard molecular biology techniques were carried out as described by Sambrook *et al.* (1989). Oligonucleotide-directed mutagenesis was performed according to the Muta-Gene™ manual by Bio-Rad. For **in vitro** mutagenesis, DNA coding for cytochrome b_2(167)DHFR (Guiard, 1985) was cloned as an *Eco*RI/*Hin*dIII fragment into the polylinker of M13mp19. Mutants were scored by DNA sequence analysis (Sequenase™ US Biochemicals Corp.). Inserts carrying the desired mutations were recovered by *Eco*RI/*Hin*dIII digestion and ligated into the polylinker of pGEM4 (Promega). Constructs containing the mutations in the full-length cytochrome b_2 were obtained by replacing the DHFR gene with the residual part of the cytochrome b_2 gene on a *Bam*HI–*Hin*dIII fragment.

Synthesis of radiolabeled precursor proteins and import in vitro

Precursor proteins were synthesized in the presence of [^{35}S]methionine by coupled transcription/translation in reticulocyte lysates (Amersham) (Pelham and Jackson, 1976; Krieg and Melton, 1984; Stüber *et al.*, 1984). Post-ribosomal supernatants of reticulocyte lysates were prepared as described previously (Zimmermann and Neupert, 1980).

Import of radiolabeled precursors into mitochondria was performed in 120 μl of freshly prepared import buffer (3% fatty acid-free bovine serum albumin, 220 mM sucrose, 80 mM KCl, 25 mM KP_i, 5 mM MgOAc, 5 mM DTT, 5 mM NADH, 2.5 mM ATP, 1 mM $MnCl_2$, pH 7.2) with 30 μg of mitochondrial protein. After incubation for 3 min at 25°C, radiolabeled precursor was added (2–5% of the total vol) and incubation was continued for 20 min at 25°C. For import in the presence of methotrexate (MTX), the import buffer containing 2 μM MTX was divided into two portions. One part was incubated with 2–4 μl radiolabeled precursor for 10 min on ice, the other part was incubated with the mitochondria for 3 min at 25°C. Then the two portions were combined and import was performed for 20 min at 25°C. The import reactions were stopped by dilution with 400 μl of ice-cold SMKCl (250 mM sucrose, 80 mM KCl, 10 mM MOPS/KOH, pH 7.2). One half of the sample was treated with 30–50 μg/ml proteinase K on ice for 25 min, whereas the other half remained untreated. To stop protease digestion, PMSF was added to a concentration of 1.6 mM. Mitochondria were reisolated by centrifugation and washed once with 400 μl SEM buffer. The pellet was subjected to SDS–PAGE (Laemmli, 1970) and analyzed by fluorography.

ATP depletion of the matrix

Mitochondria were incubated for 3 min on ice in buffer containing 0.6 mg/ml mitochondrial protein in 3% fatty acid-free bovine serum albumin, 220 mM sucrose, 80 mM KCl, 25 mM KP_i, 5 mM MgOAc, 1 mM $MnCl_2$, 30 μM oligomycin (pH 7.2). ADP was added to a final concentration of 2.5 mM and incubation was continued for 3 min at 25°C. Then the suspension was split into two aliquots. One half of the sample received 30 μM carboxyatractyloside and was incubated for 3 min. Both suspensions were then supplemented with 5 mM NADH, 10 mM creatine phosphate and 0.1 mg/ml creatine kinase. After 3 min at 25°C, radiolabeled precursor was added and import was performed as described above.

Intramitochondrial localization of imported proteins

For localization experiments, import was performed for 20 min at 25°C in a total vol of 200 μl import buffer [0.6 M deionized sorbitol, 50 mM Hepes/KOH (pH 7.2), 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2 mM KH_2PO_4, 2.5 mM ATP, 5 mM NADH, 5 mM DTT and 1 mg/ml fatty acid-free bovine serum albumin (BSA)] containing 50 μg of mitochondrial protein and 10 μl of radiolabeled precursor. After import mitochondria were collected by centrifugation for 4 min at 12 000 *g* and resuspended in 100 μl fresh import buffer (Glick *et al.*, 1992). For non-swelling conditions, the suspension was diluted with 5 vols of ice-cold isotonic buffer [0.6 M sorbitol, 20 mM Hepes/KOH, 1 mg/ml BSA (pH 7.4)], while for the generation of mitoplasts the suspension was diluted with 5 vols of ice-cold hypotonic buffer [20 mM Hepes/KOH, 1 mg/ml BSA (pH 7.4)]. Samples were treated with proteinase K (0.1 mg/ml) as indicated. After incubation for 30 min on ice, the protease-treated samples received 1.6 mM PMSF and mitochondria or mitoplasts were collected by centrifugation. To fully inactivate the proteinase K, the samples were treated with trichloroacetic acid as described previously (Glick *et al.*, 1992). Aliquots of the samples were subjected to SDS–PAGE (Laemmli, 1970) and immunoblotting of protein to nitrocellulose was performed.

Immunoblotting

Immunoblotting to nitrocellulose was performed using a semi-dry blotting system in continuous buffer (Kyhse-Andersen, 1984). Antibody labeling and visualization by alkaline phosphatase conjugated anti-antibodies was carried out as described by Blake *et al.* (1984). The washed nitrocellulose filters were air dried and exposed to Kodak XR films.

Antibodies against α-ketoglutarate dehydrogenase were generously provided by B.Glick (Biocenter, University of Basel).

Immunoprecipitation

Radiolabeled precursor protein was imported into 50 μg mitochondria in the presence of MTX as described above. After import, 30 μM oligomycin and 40 U/ml apyrase were added and incubation continued for 5 min at 25°C. The suspension was diluted with 4 vols of SMKCl containing 5 mM ADP and 2 μM MTX. Mitochondria (40 μg) were reisolated by centrifugation and lyzed on ice for 15 min in 600 μl lysis buffer [0.1% Triton X-100, 150 mM NaCl, 10 mM MOPS, 5 mM EDTA, 0.5 mM PMSF (pH 7.4)]. After centrifugation for 10 min at 12 000 *g* supernatant corresponding to 10 μg mitochondrial protein was added to 3 mg Protein A–Sepharose (Pharmacia) coupled to the immunoglobulin fraction from 25 μl antiserum. The suspension was gently shaken for 1 h at 4°C. The Sepharose beads were collected by centrifugation in an Eppendorf centrifuge, and washed twice with lysis buffer and once with 10 mM MOPS (pH 7.4). The immunocomplex was dissociated by incubation in SDS and 2-mercaptoethanol containing electrophoresis sample buffer, and then subjected to SDS–PAGE and fluorography.

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