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Polypeptides traverse the mitochondrial envelope in an extended state

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Most mitochondrial proteins are synthesized as precursors in the cytosol and imported through contact sites between outer and inner mitochondrial membranes. The molecular mechanism of membrane translocation of precursor proteins is largely unclear. For this report, various hybrid proteins between portions of the precursor of cytochrome b_2 and the entire dihydrofolate reductase (DHFR) were accumulated in mitochondrial contact sites. We unexpectedly found that about 50 amino acid residues of the polypeptide chain in transit were sufficient to span both membranes. This suggests a linear translocation of the polypeptide chain and presents evidence for a high degree of unfolding of polypeptides traversing the mitochondrial membranes.

Mitochondria; Contact site; Protein translocation; Protein unfolding

1. INTRODUCTION

Mitochondria are cell organelles surrounded by two membranes. Most mitochondrial proteins are synthesized in the cytosol and are post-translationally translocated across the mitochondrial membranes [1-4]. The mitochondrial outer membrane proteins MOM19 and MOM72, which are exposed on the mitochondrial surface, function as import receptors for the precursor proteins. Further transfer of the precursors into and across the mitochondrial membranes occurs predominantly at sites of close proximity of outer and inner membranes (contact sites). First, the precursors are inserted into the outer membrane at a common membrane insertion site, termed the general insertion protein (GIP). The entrance of precursors into the inner membrane then occurs in a step requiring the membrane potential ($\Delta\psi$) across the inner membrane. The proteolytic removal of the amino-terminal signal sequence (presequence) by the processing peptidase in the mitochondrial matrix can take place during or after translocation of the polypeptide chain through contact sites.

Little is known about the actual process of membrane translocation of mitochondrial precursor proteins, as is the case with precursor proteins destined for other cell organelles. On their import pathway into mitochondria precursor proteins could be trapped as membrane spanning intermediates that were not fully folded and probably embedded in a proteinaceous environment [5-9]. It remained unclear, however, whether these precursors were translocated in a linear fashion or if folded domains had to cross the membranes. To address this question we have determined the number of amino acid residues in the membrane spanning part of a polypeptide in transit. It turned out that about 50 amino acid residues were sufficient to span both mitochondrial membranes, supporting a view in that mitochondrial precursor proteins cross the membranes in a linear and quite extended state.

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Abbreviations: DHFR, dihydrofolate reductase; BSA, bovine serum albumin; $b_2(1-x)_{+y}$-DHFR, hybrid protein between x amino-terminal amino acid residues of the precursor of cytochrome b_2 and entire DHFR connected by y linker amino acids; p-, i-, m-, precursor-, intermediate-, and mature-sized forms of a protein, respectively; Mops, 3-(*N*-morpholino)propanesulfonic acid; MTX, methotrexate

2. MATERIALS AND METHODS

Published procedures were followed for growth of *Saccharomyces cerevisiae* and *Neurospora crassa* and isolation of mitochondria [10]; construction of hybrid proteins and other DNA manipulations [11,12]; synthesis of precursor proteins in rabbit reticulocyte lysates in the presence of [^{35}S]methionine; import of precursor proteins into isolated mitochondria and treatment with protease [13,14]; sodium dodecyl sulfate polyacrylamide gel electrophoresis; fluorography; quantitation of the fluorographs by laser densitometry [10,14,15].

The import assays contained 10% reticulocyte lysate, 2 mM NADH, 2 mM $MgCl_2$, 8 mM $(NH_4)_2SO_4$, isolated mitochondria (25 μg of mitochondrial protein) and BSA buffer (250 mM sucrose, 3% (w/v) BSA, 80 mM KCl, 10 mM Mops, adjusted to pH 7.2 with KOH) in a final volume of 55 μl. Where indicated, 1 μM MTX or 1 μM valinomycin were added; corresponding samples were supplied with an equivalent amount of solvent. Samples were preincubated with MTX for 5 min at 0°C. Import reactions were carried out for 5 min at 25°C.

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3. RESULTS AND DISCUSSION

To characterize polypeptide chains in transit through the mitochondrial membranes, we studied the mitochondrial import of hybrid proteins between amino-terminal portions of the precursor of cytochrome b_2 [16] and entire dihydrofolate reductase (DHFR) [17] (Fig. 1). After synthesis in rabbit reticulocyte lysates in vitro, the b_2-DHFR hybrid proteins were imported into isolated mitochondria from *Saccharomyces cerevisiae* and proteolytically processed by processing peptidase. Binding of the specific ligand methotrexate (MTX) to DHFR blocked its translocation into mitochondria [18] and thus allowed the accumulation of the hybrid protein $b_2(1\text{–}167)_{+2}$-DHFR in mitochondrial contact sites with the cytochrome b_2-part spanning the mitochondrial membranes and the folded DHFR exposed on the cytosolic side [12]. We preincubated the various hybrid proteins with methotrexate and tested their import into mitochondria. Hybrid proteins with the cytochrome b_2-part as short as 76 amino acid residues (plus 6 linker amino acid residues) could be specifically processed by intact mitochondria (Fig. 1). This suggested that in the presence of methotrexate translocation intermediates in contact sites were accumulated.

This was analyzed in detail with the hybrid protein $b_2(1\text{–}76)_{+6}$-DHFR as shown in Fig. 2. The hybrid protein was processed by mitochondria in the presence and in the absence of methotrexate (Fig. 2, reactions 1 and 2). Processing of $b_2(1\text{–}76)_{+6}$-DHFR by intact mitochondria in the presence of methotrexate was specific according to the following criteria. It was dependent on the membrane potential across the inner membrane since it was blocked by dissipation of the membrane potential by the potassium ionophore valinomycin (Fig. 2, reaction 3). The processing was inhibited by the addition of metal chelators (*o*-phenanthroline and EDTA) that block the metal-dependent processing peptidase [19] in the mitochon-

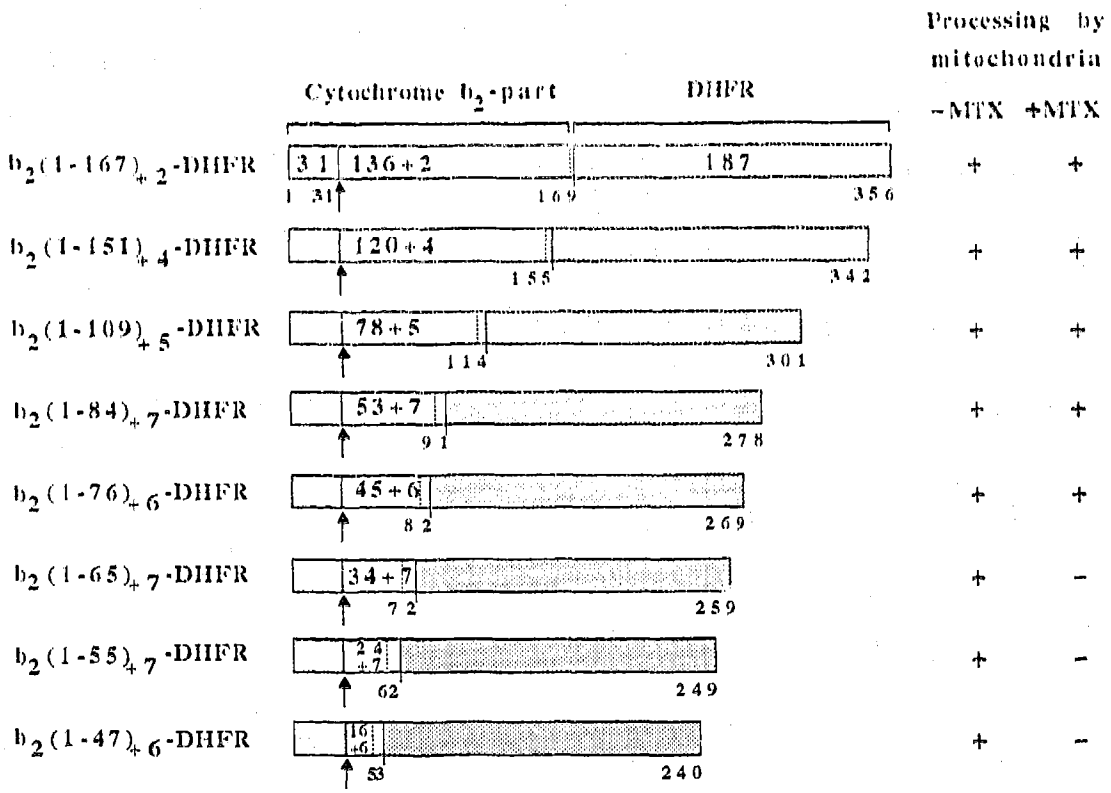

Fig. 1. Import of cytochrome b_2-DHFR hybrid proteins into mitochondria. Hybrid proteins between amino-terminal portions of the precursor of yeast cytochrome b_2 [16] and entire mouse DHFR [17] were constructed as described [12]. The following linker amino acid residues were inserted between the cytochrome b_2 part and DHFR. $b_2(1\text{–}167)_{+2}$-DHFR: Gly, Ile; $b_2(1\text{–}151)_{+4}$-DHFR: Gly, Ser, Gly, Ile; $b_2(1\text{–}109)_{+5}$-DHFR: His, Arg, Ser, Gly, Ile; $b_2(1\text{–}84)_{+7}$-DHFR: Gly, Ile, His, Arg, Ser, Gly, Ile; $b_2(1\text{–}76)_{+6}$-DHFR: Ile, His, Arg, Ser, Gly, Ile; $b_2(1\text{–}65)_{+7}$-DHFR: Arg, Ile, His, Arg, Ser, Gly, Ile; $b_2(1\text{–}55)_{+7}$-DHFR: Arg, Ile, His, Arg, Ser, Gly, Ile; $b_2(1\text{–}47)_{+6}$-DHFR: Ile, His, Arg, Ser, Gly, Ile. The hybrid proteins were synthesized in reticulocyte lysates by coupled transcription/translation and labeled with [^{35}S]methionine. Incubation of the reticulocyte lysate with isolated yeast mitochondria in the presence of a membrane potential was performed as described in section 2. In the absence of methotrexate (−MTX), all hybrid proteins were specifically processed by mitochondria and transported to a location not accessible to externally added proteinase K (40 μg/ml). In the presence of 1 μM MTX, all hybrid proteins remained accessible to proteinase K. The arrow indicates the site of cleavage by mitochondrial processing peptidase to the intermediate-sized form.

drial matrix (Fig. 2, reaction 4). Processing of cytochrome b_2 by processing peptidase occurs between amino acid residues 31 and 32 of the presequence, yielding the intermediate-sized form [12]. The shift in the apparent molecular weight of $b_2(1\text{-}76)_{+6}$-DHFR upon incubation with mitochondria is in good agreement with this processing site. (Processing to the mature-sized protein occurs between amino acid residues 80 and 81 [16,20] and thus cannot take place with $b_2(1\text{-}76)_{+6}$-DHFR.) In the absence of methotrexate, the processed protein was fully imported and thus protected against externally added proteinase K (Fig. 2, reaction 1). In the presence of methotrexate, however, the processed protein was accessible to externally added proteinase K (Fig. 2, reaction 2) under conditions where the outer membrane barrier of mitochondria remained intact [10,12,15,21]. The susceptibility of the protein to proteolytic cleavage both on the matrix side and on the cytosolic side demonstrates that it was accumulated in contact sites [5,6,12,22]. As expected due to the high endogenous protease resistance of DHFR complexed with methotrexate, the treatment with proteinase K released the complete DHFR domain into the supernatant (Fig. 2, reaction 2). Moreover, crystallographic data reveal that the amino-terminal portion of DHFR is located inside the molecule and directly involved in the binding of ligand with its residues 8, 9, 10 and 31 [23,24]. This supports the conclusion that the DHFR domain of the hybrid protein containing methotrexate has not entered the membranes. Thus, 51 amino acid residues are sufficient to span both mitochondrial membranes at contact sites and to allow access for two soluble proteases (proteinase K; matrix processing peptidase). To verify this finding with another organism, we performed the experiments also with mitochondria from *Neurospora crassa* and obtained similar results (data not shown).

Is the $b_2(1\text{-}76)_{+6}$-DHFR that was accumulated in contact sites on the correct import pathway? In Fig. 3A

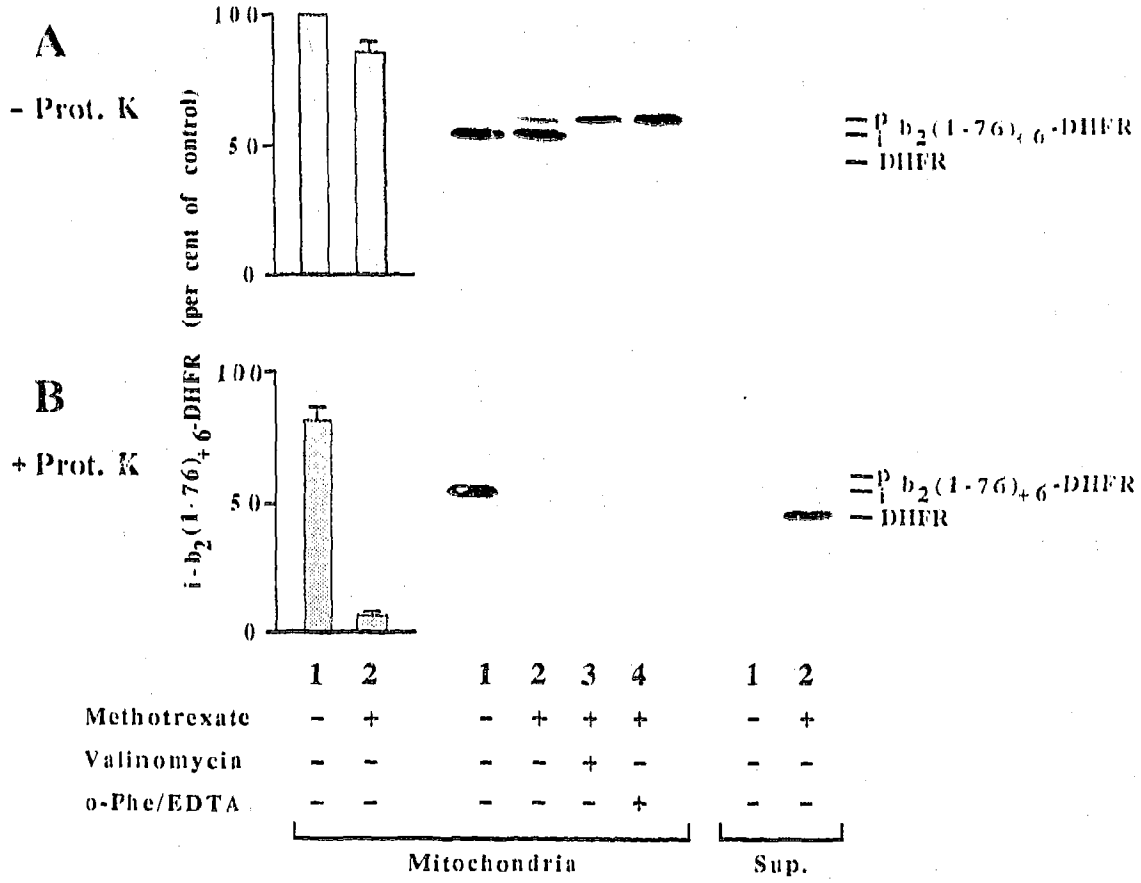

Fig. 2. Accumulation of cytochrome $b_2(1\text{-}76)_{+6}$-DHFR in mitochondrial contact sites. $b_2(1\text{-}76)_{+6}$-DHFR was transported into isolated yeast mitochondria (reaction 1) as described in the legend to Fig. 1. Reactions 2–4 contained 1 μM MTX and in addition 1 μM valinomycin (reaction 3) or 0.4 mM 1,10 phenanthroline/12 mM EDTA (reaction 4). After incubation for 5 min at 25°C, the mitochondria were isolated and washed in buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Mops, pH 7.2, 1 μM MTX, 1 μM valinomycin, and 0.4 mM 1,10-phenanthroline. The samples were divided into halves and one half was treated with proteinase K (+Prot.K). The reisolated mitochondria and the supernatants (Sup.) were analyzed by SDS-PAGE and fluorography. Results quantified by laser densitometry are given as the means of five experiments ± SE. By analysis of the marker proteins cytochrome *c*, cytochrome b_2, and $F_1\beta$ it was confirmed that the mitochondrial outer and inner membranes remained intact at the various conditions (+/− MTX; +/− Prot. K), similarly to controls reported previously [10,12,15,21]. p, precursor protein; i, intermediate sized protein.

we show that the processed protein was stably inserted into the mitochondrial membranes since it could not be released by treatment of the mitochondria at various salt concentrations, as was similarly found with other precursor proteins arrested in contact sites [6,22]. We previously showed that only the translocation across the inner membrane of an amino-terminal part of the precursor depends on the membrane potential whereas translocation of the remainder of the precursor can take place in the absence of $\Delta\psi$ [5,12]. For the experiment shown in Fig. 3B, $b_2(1\text{–}76)_{+6}$-DHFR was first accumulated in contact sites. Then valinomycin was added to dissipate the mitochondrial membrane potential. The mitochondria were reisolated, resuspended in methotrexate-free medium and incubated at 25°C. Fig. 3B demonstrates that the completion of import of $b_2(1\text{–}76)_{+6}$-DHFR indeed occurred in the absence of $\Delta\psi$. This confirms that the processed $b_2(1\text{–}76)_{+6}$-DHFR in contact sites was beyond the $\Delta\psi$-dependent step, i.e. remained inserted into the inner membrane, and was on the correct import pathway.

In summary, we have shown that about 50 amino acid residues of a polypeptide chain in transit are sufficient to span both mitochondrial membranes and to allow access to two soluble proteases. This surprising short length of the membrane spanning polypeptide demonstrates that the penetration of precursor proteins through membranes does not have to occur by a transfer of domains but rather involves translocation of the chain in a linear fashion.

Our results allow a preliminary calculation of the possible conformations of precursor proteins in transit. Fifty amino acid residues correspond to about 7.5 nm in length in α-helical conformation [25], 17.5 nm in β-structure, and 18.1 nm in a fully extended conformation [26]. As the exact structural arrangement of the mitochondrial membranes at contact sites is unknown, we will take in account various estimations for the distance spanned by the ca 50 amino acid residues. The distance across two protein-rich membranes is 15–18 nm [27], in agreement with the thickness of mitochondrial contact sites (18–20 nm) assessed by electron microscopic studies [12]. On the assumption that the precursor protein has to traverse at least the thickness of two adjacent simple unit membranes, the distance would be 12–14 nm [28,29]. Assuming that the two mitochondrial membranes would form a so-called non-bilayer structure (H_{II} phases) [30,31], the thickness would be about 14 nm. Yet, it has to be emphasized that in electron micrographs of mitochondrial contact sites a non-dense structure is always visible between the two membranes. In any of these cases, the polypeptide chain must be in a quite extended conformation in order to span the two membranes.

Considering the complexity of translocation in an at least two-step reaction that involves insertion into the outer membrane at the GIP-site [13,14] and the $\Delta\psi$-dependent translocation through the inner membrane [5,32], it appears very unlikely that the translocation at contact sites would occur through a simple and short channel that would be much shorter than the distances calculated above. Our results therefore provide evidence for an extensive unfolding of polypeptides in transit. As a hypothesis we propose that precursor proteins may be unfolded to such a degree that the

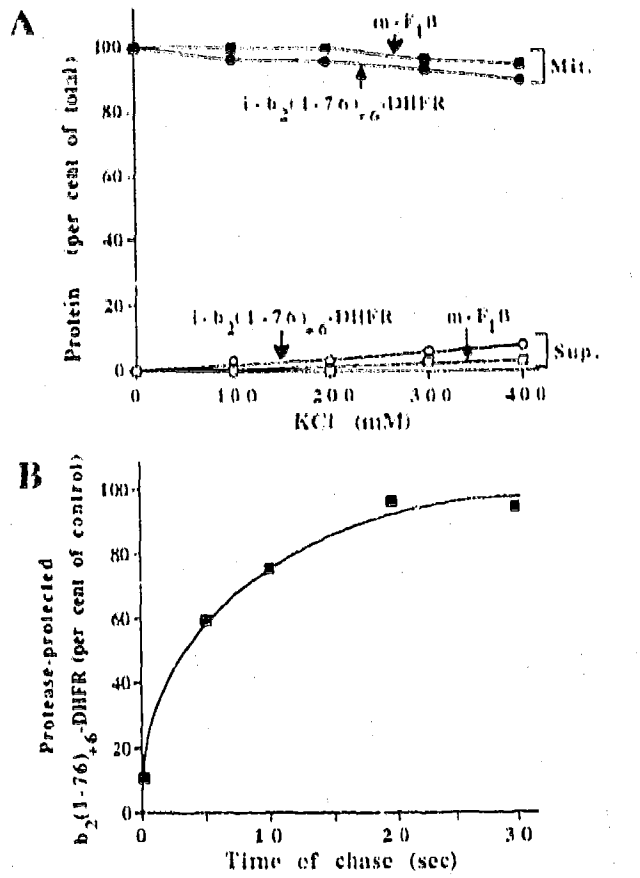

Fig. 3. Cytochrome $b_2(1\text{–}76)_{+6}$-DHFR arrested in contact sites is on the correct import pathway. (A) The translocation intermediate is not released from mitochondria at higher salt concentrations. $b_2(1\text{–}76)_{+6}$-DHFR was accumulated in mitochondrial contact sites in the presence of MTX as described in the legends to Figs 1 and 2. The mitochondria were isolated and incubated in 250 mM sucrose, 1 μM MTX, 10 mM Mops, pH 7.2, and 1 μM valinomycin at the indicated KCl-concentrations for 15 min at 0°C. The mitochondria were reisolated, and mitochondria (Mit.) and supernatants (Sup.) were analyzed for intermediate-sized protein (i-$b_2[1\text{–}76]_{+6}$DHFR) by SDS-PAGE, fluorography and densitometry. As control, F_1-ATPase subunit β imported into the matrix (m-$F_1\beta$) was used. (B) Chase of the translocation intermediate by completion of import in the absence of methotrexate. $b_2(1\text{–}76)_{+6}$-DHFR was accumulated in contact sites in the presence of MTX as described above. Then 1 μM valinomycin was added. The mitochondria were reisolated, washed once in BSA-buffer (250 mM sucrose, 3% BSA, 80 mM KCl, 10 mM Mops, pH 7.2, 1 μM valinomycin) and incubated in this buffer for the indicated times at 25°C. Then treatment with proteinase K was performed. In parallel samples, import was performed in the absence of MTX ('control') or MTX remained present in all incubations ('no chase').

backbone of the polypeptide chain becomes exposed while being translocated through proteinaceous sites in mitochondrial contact zones.

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