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Protein Folding Causes an Arrest of Preprotein Translocation into Mitochondria In Vivo

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Abstract. With vital yeast cells, a hybrid protein consisting of the amino-terminal third of the precursor to cytochrome b_2 and of the entire dihydrofolate reductase was arrested on the import pathway into mitochondria. Accumulation of the protein in the mitochondrial membranes was achieved by inducing a stable tertiary structure of the dihydrofolate reductase domain. Thereby, three salient features of mitochondrial protein uptake in vivo were demonstrated: its posttranslational character; the requirement for unfolding of precursors; and import through translocation contact sites. The permanent occupation of translocation sites by the fusion protein inhibited the import of other precursors; it did, however, not lead to leakage of mitochondrial ions, implying the existence of a channel that is sealed around the membrane spanning polypeptide segment.

PROTEIN translocation across biological membranes is a key step in the biogenesis of cell organelles (Wickner and Lodish, 1985). The basic problem of how cytosolically synthesized precursor proteins traverse organelle membranes was studied predominantly in cell-free systems. These systems consisted of isolated organelles, such as microsomes (ER), mitochondria, chloroplasts or peroxisomes, and precursor proteins that were synthesized in vitro or expressed in *Escherichia coli* and subsequently purified. With regard to mitochondria, the following steps of protein import have been established (for reviews see Attardi and Schatz, 1988; Hartl and Neupert, 1990; Pfanner and Neupert, 1990). Nuclear-encoded precursor proteins are synthesized on cytosolic polysomes and targeted to specific receptors on the outer mitochondrial membrane. A loosely folded ("unfolded") conformation of a precursor protein is a prerequisite for its translocation across the mitochondrial membranes (Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Chen and Douglas, 1987*a*). Most precursors are translocated through contact sites between outer and inner mitochondrial membranes (translocation contact sites). The membrane potential $\Delta\psi$ across the inner membrane is required for the initial entrance of a precursor into this membrane, while the completion of translocation does not depend on $\Delta\psi$. During or after membrane translocation, the amino-terminal targeting sequence (presequence) is proteolytically cleaved off by the processing peptidase in the mitochondrial matrix.

Though posttranslational translocation of unfolded precursor proteins through mitochondrial contact sites is well established in the isolated system, little is known about protein transport into mitochondria in intact cells. In early studies performed at low temperature, cytoplasmic pools of mitochondrial precursor proteins were found in *Neurospora crassa* cells, representing the first demonstration that at least a fraction of protein translocation occurred posttranslationally (Hallermayer and Neupert, 1976; Hallermayer et al., 1977). With yeast, uncoupling of mitochondria led to the accumulation of precursor proteins that could be posttranslationally imported upon reestablishing a membrane potential (Reid and Schatz, 1982). It remained open, however, if the import occurred posttranslationally under physiological conditions (i.e., normal temperature and energized mitochondria) and, if so, which fraction of import would occur posttranslationally. The current data thus do not rule out that a large fraction of import may occur cotranslationally under physiological conditions. In fact, Fujiki and Verner (1991) recently reported that cotranslational import in a homologous yeast in vitro system was much more efficient than posttranslational protein import. Intimately linked to the question of cotranslational versus posttranslational transport is the problem of how significant the in vitro studies on folding and unfolding of precursor proteins are for protein import in vivo. Does folding and unfolding of complete polypeptides or protein domains take place in vivo or is this problem irrelevant due to a predominantly cotranslational import mechanism? A question of equal importance is the relevance of mitochondrial translocation contact sites for protein import in intact cells.

For this report, we addressed these questions by expressing a hybrid protein between an amino-terminal portion of the mitochondrial precursor protein cytochrome b_2 and the cytosolic enzyme dihydrofolate reductase (DHFR)[1] in intact yeast cells. Binding of the specific ligand methotrexate

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; hsp, heat shock protein.

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to the DHFR part had previously been found to interfere with mitochondrial protein uptake in vitro (Eilers and Schatz, 1986) and to allow the accumulation of a precursor protein in contact sites of isolated mitochondria (Rassow et al., 1989). We found that addition of a methotrexate derivative to yeast cells induced a stable tertiary structure in the hybrid protein and led to its arrest in mitochondrial translocation contact sites in vivo, demonstrating the importance of protein unfolding and posttranslational transport through contact sites. The maintenance of a membrane potential in the mitochondria in which a large number of contact sites were occupied by spanning intermediates, suggests the existence of specific components of the translocation apparatus that are responsible for sealing the inner membrane around the various amino acid residues of the polypeptide in transit.

Materials and Methods

Growth of Yeast Cells and Expression of the Fusion Protein b_2-DHFR

The cDNA for the fusion gene b_2-DHFR was cloned behind the *GAL1*-promoter in the yeast *E. coli* shuttle vector YEp52 (Broach et al., 1983). Yeast cells were transformed as described (Ito et al., 1983). The transformants were grown in media containing 1% yeast extract, 2% peptone, and 2% lactate (pH 5.5). *Saccharomyces cerevisiae* strains DMMA-15 and WT-OTC (Cheng et al., 1987) were used for transformation. The transformants were grown to an OD_{600} of 2, diluted 10-fold with fresh medium and grown for one additional hour. Expression of b_2-DHFR was then induced by addition of 3% galactose. In addition, parallel cultures received aminopterine (0.2 mM) and sulfanilamide (30 mM). Sulfanilamide was added to reduce the intracellular levels of dihydrofolate that might compete with aminopterine. In control experiments, sulfanilamide was shown not to affect the expression and import of mitochondrial precursor proteins. Cells were allowed to grow in the presence of galactose and in the presence or absence of aminopterine and sulfanilamide for up to 4 h. Then cells were harvested, converted to spheroplasts with Zymolyase-20T (Seikagaku Kogyo Co., Tokyo, Japan), and mitochondria were isolated as described (Hartl et al., 1987*a*). Aminopterine (0.2 mM) was added during the isolation of mitochondria from yeast cells that had been grown in the presence of aminopterine. For protease digestion, mitochondria were resuspended to a protein concentration of 1 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, adjusted to pH 7.2 with KOH) and incubated for 20 min with 5 μg/ml proteinase K at 4°C. Where indicated, protease treatment was performed in the presence of 0.5% Triton X-100. Protease digestion was stopped by addition of PMSF to 1 mM. The mitochondria were isolated by centrifugation for 20 min at 12,000 *g* at 4°C. Protein analysis was carried out by SDS-PAGE (Laemmli, 1970), subsequent transfer to nitrocellulose, and immunoblotting with antibodies directed against DHFR (Hartl et al., 1986).

Pulse-Chase Experiments

Yeast cells transformed with b_2-DHFR cDNA were grown in minimal medium (2% lactate, 8% Difco yeast nitrogen base, pH 5.5) to an OD_{600} of 1. Then they were diluted 10-fold with fresh minimal medium and allowed to grow for an additional 2 h at 30°C. From each culture 10 OD_{600} units of cells were collected by centrifugation, resuspended in 1 ml minimal medium, and incubated at 30°C in a shaking waterbath in the presence of 3% galactose and in the presence or absence of 0.2 mM aminopterine and 30 mM sulfanilamide. Where indicated, CCCP was added to a final concentration of 20 μM. After 30 min, [^{35}S]methionine (1,000 Ci/mmol) was added to a final concentration of 0.2 mCi/ml. 5 min later, cycloheximide and unlabeled methionine were added to final concentrations of 0.1 mg/ml and 2 mM, respectively. The incubation was continued and samples (2 OD_{600} units each) were withdrawn after the indicated times of chase. The cells were broken and the proteins were extracted by addition of NaOH and β-mercaptoethanol to final concentrations of 0.94 and 0.45 M, respectively. The proteins were precipitated with TCA, resuspended in 2% SDS, and incubated for 3 min at 95°C. Specific proteins were identified by immunoprecipitation and analyzed by subsequent SDS-PAGE and fluorography. Fluorographs were quantified by densitometry (Hartl et al., 1986).

To analyze protease protection of mitochondrial precursor proteins in pulse-chase experiments, transport was blocked by addition of 20 μM CCCP after the indicated periods of chase with cycloheximide and unlabeled methionine. The yeast cells were converted to spheroplasts by treatment with Zymolyase. The plasma membrane was partly lysed with digitonin (0.1% final concentration), under conditions where the mitochondrial membranes remained intact. The perforated spheroplasts were treated for 10 min with 5 μg/ml of proteinase K. Protease digestion was stopped by addition of 1 mM PMSF. The perforated spheroplasts were broken and proteins were extracted as described for intact yeast cells. Specific proteins were identified by immunoprecipitation and analyzed by SDS-PAGE and fluorography.

Assessment of a Mitochondrial Membrane Potential

The presence of a mitochondrial membrane potential was measured with the potential-sensitive dye (3,3')-dipropylthiadicarbocyanine iodide (Disc3(5)) obtained from Molecular Probes Inc. (Junction City, OR) (Sims et al., 1974). Excitation was at 620 nm and emission at 670 nm. Fluorescence changes were measured at room temperature after addition of mitochondria (final concentration, 15 μg protein/ml) to the dye (final concentration, 2 μM) in 10 mM $MgCl_2$, 80 mM KCl, 0.5 mM EDTA, 20 mM Kp_i, pH 7.4, 0.6 M sorbitol, 1.5 mM BSA. In control experiments, fluorescence changes were measured after subsequent addition of 1 μM valinomycin.

Miscellaneous

Published procedures were used for DNA manipulations (Kleene et al., 1987; Sambrook et al., 1989), protein determination (Bradford, 1976), SDS-PAGE (Laemmli, 1970), immunoprecipitation (Schleyer et al., 1982), fluorography and quantitation of fluorographs by densitometry, TCA precipitation of proteins, Western blotting, generation of antisera against cytosolic mouse DHFR, protease treatment, and reisolation of mitochondria (Hartl et al., 1986).

Results

Accumulation of the Hybrid Protein b_2-DHFR in Mitochondrial Translocation Contact Sites in Intact Yeast Cells

The precursor to the intermembrane space protein cytochrome b_2 is processed twice during import into mitochondria (Guiard et al., 1975; Reid et al., 1982; Guiard, 1985; Hartl et al., 1987*a*,*b*; Rassow et al., 1989; M. Arretz, U. Wienhues, and W. Neupert, unpublished results). An amino-terminal sequence of 31 amino acid residues (containing the matrix targeting signal) is cleaved off by the matrix processing peptidase, yielding the intermediate-sized form. A second proteolytic cleavage between amino acid residues 80 and 81 takes place on the intermembrane space side of the inner membrane, leading to the mature-sized protein. The hybrid protein b_2-DHFR, consisting of the 167 amino-terminal residues of the precursor to cytochrome b_2 and the entire mouse DHFR (Rassow et al., 1989), was found to be efficiently imported into yeast mitochondria and similarly processed in two steps to the intermediate-sized (i) and the mature-sized (m) form (Rassow et al., 1990).

To investigate the import of b_2-DHFR in vivo, intact yeast cells were transformed with the fusion gene b_2-DHFR under control of the galactose-inducible *GAL1*-promoter. The cells were grown at 30°C in the presence or absence of the inducing sugar galactose. Then the mitochondria were isolated and analyzed by SDS-PAGE, followed by immunoblotting with antiserum directed against mouse DHFR. Fig. 1 *a* shows that, in the presence of galactose, b_2-DHFR was expressed and processed to the mature-sized form. The mature-sized b_2-DHFR was fully imported into mitochon-

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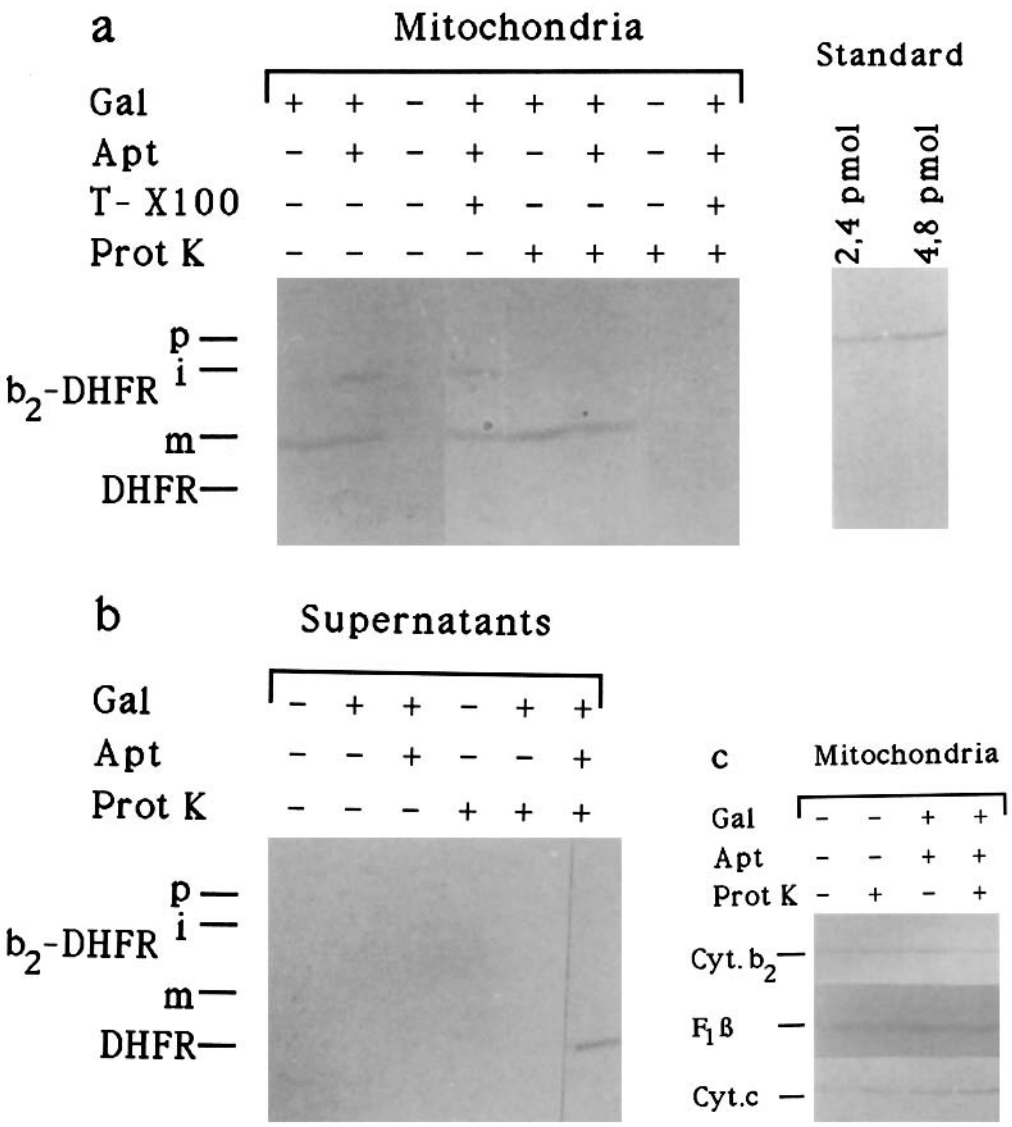

Figure 1. In vivo accumulation of b_2-DHFR spanning mitochondrial outer and inner membranes. The expression of b_2-DHFR in yeast was induced with galactose for 2 h at 30°C in the presence or absence of aminopterine. Then mitochondria were isolated. The mitochondria (0.2 mg of mitochondrial protein in 0.4 ml) were incubated at 0°C in the absence or presence of proteinase K. The samples were separated into mitochondria and supernatants by centrifugation, analyzed by SDS-PAGE, and transferred to nitrocellulose and immunoblotting with antibodies to mouse DHFR. (*a*) Mitochondria. (*b*) Supernatants after reisolation of mitochondria. As a control, one aliquot of mitochondria was lysed with 0.5% Triton-X 100, then incubated at 0°C in the presence or absence of proteinase K and processed as the other samples. Purified p-b_2-DHFR overexpressed in *E. coli* (Rassow et al., 1989) was used as a standard. P, precursor; i, intermediate-sized form; m, mature-sized form; Gal, galactose; Apt, aminopterine; TX-100, Triton-X 100; Prot K, proteinase K. (*c*) The integrity of reisolated mitochondria was controlled by determining the content of marker proteins by immunoblotting: holocytochrome c (*Cyt. c*), cytochrome b_2 (*Cyt. b_2*) (both intermembrane space), and the β-subunit of the F_1-ATPase ($F_1\beta$) (matrix side of the inner membrane).

dria as it was protected against proteinase K added to the isolated mitochondria unless the mitochondrial membranes were disrupted by detergent. In parallel samples, the yeast cells expressing b_2-DHFR received aminopterine, a folate analogue known to stabilize the tertiary structure of DHFR. Thereby a considerable amount of intermediate-sized b_2-DHFR was accumulated with the mitochondria (Fig. 1 *a*). This intermediate-sized form was accessible to proteinase K added to the intact mitochondria, indicating that part of the molecule was located outside of the outer mitochondrial membrane. The intactness of the mitochondrial membranes under the various conditions was controlled by analyzing their content of marker proteins for the intermembrane space (cytochrome b_2 and cytochrome c) and the matrix ($F_1\beta$) that are known to be released or degraded upon damage of membranes (Hartl et al., 1986, 1987*a*; Nicholson et al., 1988). Fig. 1 *c* shows that neither the presence of aminopterine nor the treatment with proteinase K decreased the mitochondrial content of the marker proteins.

Since the processing to i-b_2-DHFR is performed by the processing peptidase in the matrix, the intermediate is accessible to proteases both on the cytosolic side and on the matrix side of the membranes and thereby fulfills the criteria for a translocation intermediate spanning both mitochondrial membranes at contact sites (Schleyer and Neupert, 1985; Schwaiger et al., 1987). As expected due to its high endogenous protease resistance, the folded DHFR domain was not digested by proteinase K but released into the supernatant (Fig. 1 *b*). This confirms that the b_2-part of i-b_2-DHFR arrested in translocation contact sites was spanning the mitochondrial membranes, while the folded DHFR was exposed on the cytosolic side.

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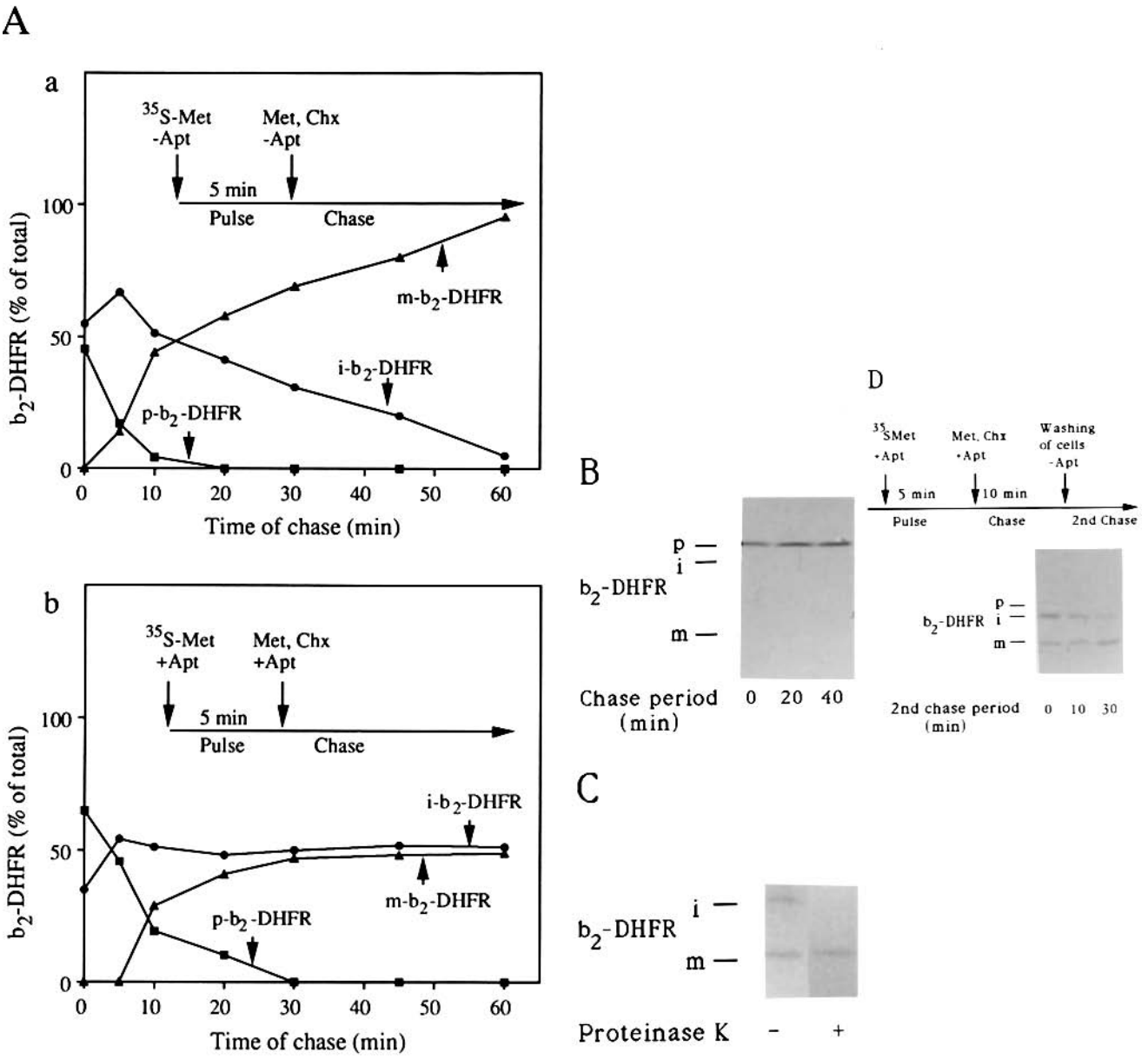

Figure 2. Characterization of membrane spanning b_2-DHFR. (*A*) Membrane-spanning b_2-DHFR arrested in vivo is on the correct import pathway. The expression of b_2-DHFR was induced with galactose for 20 min in the absence (*a*) or presence (*b*) of aminopterine (*Apt*). Then [^{35}S]methionine (*^{35}S-Met*) was added (*pulse*). After 5 min, unlabeled methionine (*Met*) and cycloheximide (*Chx*) were added in the absence (*a*) or presence (*b*) of aminopterine for the time indicated (chase). The cells were lysed and analyzed for the presence of b_2-DHFR by immunoprecipitation, SDS-PAGE, and fluorography. Bands corresponding to precursor-, intermediate-, and mature-sized b_2-DHFR (*p-, i-, m-b$_2$-DHFR*) were quantified by laser densitometry. (*B*) Inhibition of formation of spanning intermediates by uncoupling of mitochondria. The expression of b_2-DHFR was induced in the absence of aminopterine as described in *A*. Then 20 μM CCCP were added to the yeast culture and cells were labeled for 5 min with [^{35}S]methionine. A chase was then performed for 20 or 40 min with unlabeled methionine. b_2-DHFR was immunoprecipitated and analyzed by SDS-PAGE and fluorography. (*C*) Topology of intermediate arrested in the presence of aminopterine. Cells were treated as in *A* in the presence of aminopterine and chased for 40 min. Then they were treated with Zymolyase and digitonin. The perforated spheroplasts were then incubated with or without proteinase K. After lysis of spheroplasts with Triton-X 100, b_2-DHFR was immunoprecipitated and analyzed by SDS-PAGE and fluorography. (*D*) The spanning intermediates are on the correct import pathway. Cells were treated as in *A* in the presence of aminopterine, chased for 10 min, and collected by centrifugation. They were washed and resuspended in fresh medium without galactose, aminopterine, and sulfanilamide, and then chased for additional 10 or 30 min (2nd chase). The cells were then lysed and analyzed for b_2-DHFR by immunoprecipitation, SDS-PAGE, and fluorography.

The amount of translocation intermediates of b_2-DHFR arrested in contact sites was ~20 pmol per mg mitochondrial protein, corresponding to ~400–1,600 molecules per mitochondrion (Fig. 1 *a*). Since isolated mitochondria contain 40–70 pmol translocation contact sites per milligram protein (Vestweber and Schatz, 1988; Rassow et al., 1989), about 30–50% of the import sites determined in vitro were occupied by i-b_2-DHFR.

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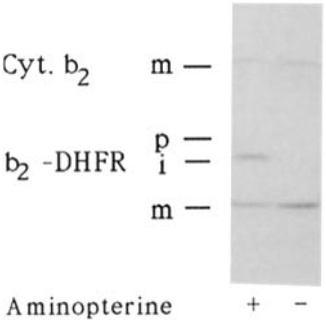

Figure 3. Effect of aminopterine on the import of b_2-DHFR and cytochrome b_2. Cells were grown as described in Fig. 2 *A*, pulse labeled with [^{35}S]methionine for 5 min in the presence or absence of aminopterine, and chased for 40 min with unlabeled methionine and cycloheximide and then cytochrome b_2 or b_2-DHFR were analyzed by immunoprecipitation, SDS-PAGE, and fluorography. p, precursor; i, intermediate-sized form; m, mature-sized form.

Cytochrome b_2-DHFR Arrested in Translocation Contact Sites In Vivo Is on the Correct Import Pathway

To further characterize the import pathway of b_2-DHFR in intact cells, pulse-labeling experiments were performed. Yeast cells were labeled with [^{35}S]methionine for 5 min at 30°C (pulse). Then cycloheximide and an excess of unlabeled methionine were added and the cells incubated for the indicated periods at 30°C (chase) (Fig. 2 *A*). After lysis of the cells, subsequent immunoprecipitation with anti-DHFR antiserum, and SDS-PAGE, labeled b_2-DHFR was visualized on fluorographs. In the absence of aminopterine, b_2-DHFR was found to be efficiently processed to the mature-sized form during the chase period (Fig. 2 *A*, *a*). In the presence of aminopterine, a considerable amount of the intermediate-sized form accumulated and the disappearance of the precursor form and generation of the mature-sized form were slowed down (Fig. 2 *A*, *b*). The formation of i-b_2-DHFR was blocked by addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Fig. 2 *B*), indicating that an energized mitochondrial inner membrane was required. To confirm that the i-b_2-DHFR accumulated in a pulse-chase experiment was arrested in translocation contact sites, yeast cells were converted to spheroplasts by treatment with Zymolyase and the plasma membrane was partly lysed with digitonin (under conditions where the mitochondrial membranes remained intact; H. F. Steger, W. Neupert, and N. Pfanner, unpublished results). Treatment with proteinase K showed that i-b_2-DHFR, but not m-b_2-DHFR, was exposed on the mitochondrial surface, i.e., trapped in translocation contact sites (Fig. 2 *C*).

Did the intermediate-sized form of b_2-DHFR become arrested as a true translocation intermediate, i.e., could its import be completed upon release of the transport block? Cells were pulse labeled, chased for 10 min in the presence of aminopterine, and then reisolated, washed, and grown for an additional 30 min in medium without aminopterine. The intermediate-sized form of b_2-DHFR, which had been arrested in the presence of aminopterine, was indeed converted to its mature form during this second incubation (Fig. 2 *D*). This m-b_2-DHFR was fully imported into mitochondria, as it was protected against proteinase K added to partly lysed spheroplasts (as shown in Fig. 2 *C*).

In summary, by inducing stable folding of the DHFR domain, b_2-DHFR is accumulated in mitochondrial translocation contact sites in vivo. The reversibility of the transport arrest demonstrates that this b_2-DHFR is on the correct import pathway.

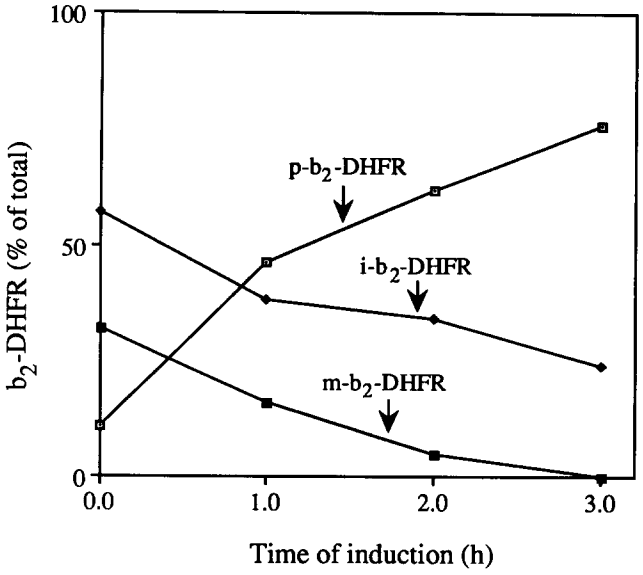

Figure 4. Saturation of mitochondrial translocation contact sites in vivo by prolonged expression of b_2-DHFR in the presence of aminopterine. The expression of b_2-DHFR was induced in the presence of aminopterine for 0, 1, 2, and 3 h. Then cells were labeled with [^{35}S]methionine for 5 min (pulse) and subsequently chased with unlabeled methionine and cycloheximide for 10 min. The cells were lysed and b_2-DHFR was analyzed by immunoprecipitation, SDS-PAGE, fluorography, and laser densitometry. p, precursor; i, intermediate-sized form; m, mature-sized form.

Post-translational Mode of Import of b_2-DHFR and Folding of the DHFR Domain Occur at Physiological Import Rates

To assess the occurrence of posttranslational protein translocation and the relevance of protein folding/unfolding under "physiological" import conditions, we first determined if the amount of b_2-DHFR synthesized and imported into mitochondria was comparable to that of the authentic precursor to cytochrome b_2. The pulse-chase experiment shown in Fig. 3 confirms that the amounts of b_2-DHFR synthesized and imported were similar to those of cytochrome b_2. The presence of aminopterine did not affect the formation of mature cytochrome b_2 (Fig. 3), demonstrating the specificity of aminopterine in inhibiting the import of the DHFR-carrying hybrid protein and excluding unspecific effects of aminopterine on mitochondrial protein uptake. Furthermore, the import kinetics of b_2-DHFR (Fig. 2 *A*, *a*) were comparable to those of cytochrome b_2 (Reid et al., 1982).

About half of the labeled b_2-DHFR accumulated as intermediate in the presence of aminopterine (Fig. 2 *A*, *b*). Thus, the DHFR domain of these intermediates was fully synthesized (that is in a post-translational state) and competent for stable folding before translocation across the membranes. In addition, the majority of the precursor-sized b_2-DHFR present at the beginning of the chase (Fig. 2 *A*) was not associated with mitochondria, but found in the cytosolic fraction (Fig. 1 and data not shown), demonstrating a posttranslational mechanism also for this precursor fraction. It has to

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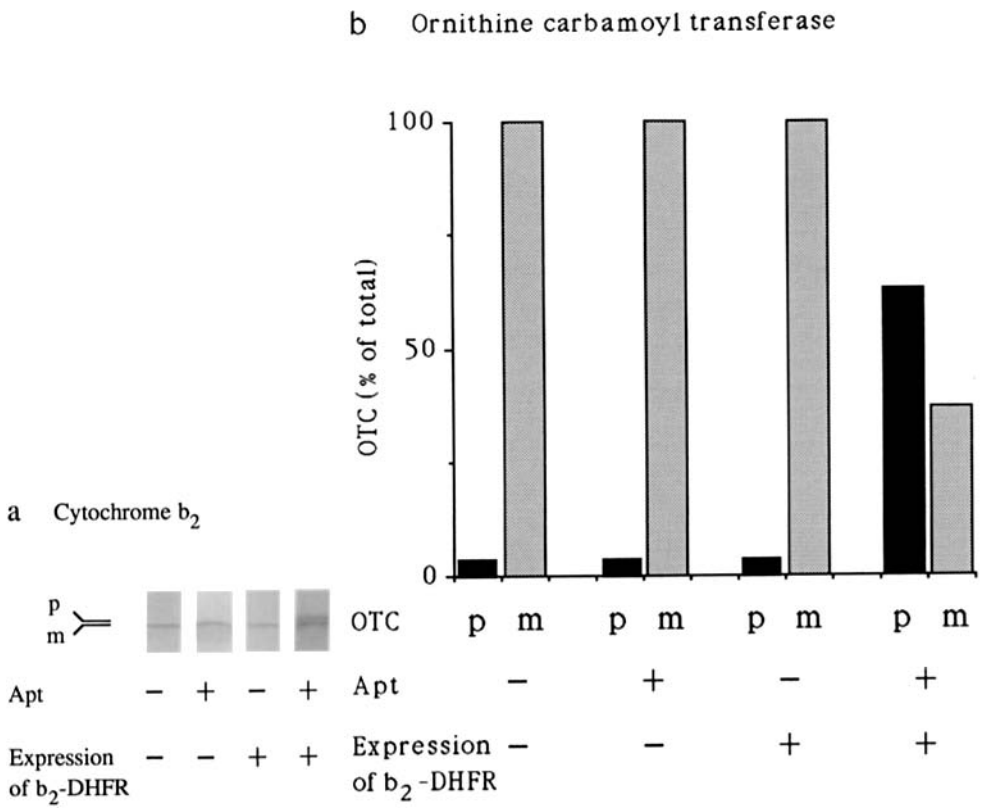

Figure 5. Inhibition of import of cytochrome b_2 and of ornithine carbamoyl transferase (*OTC*) by accumulation of b_2-DHFR in translocation contact sites. The expression of b_2-DHFR was induced for 4 h in the absence or presence of aminopterine in yeast cells transformed with b_2-DHFR and in addition with human ornithine carbamoyl transferase (*OTC*) also under control of the GAL1-promoter (Cheng et al., 1987). The cells were then pulse labeled for 5 min with [^{35}S]methionine and subsequently chased for 40 min after addition of cycloheximide and unlabeled methionine. In addition, the same procedure was performed with yeast cells transformed with human OTC, but not with b_2-DHFR. Cells were then lysed, and cytochrome b_2 or human OTC were analyzed by immunoprecipitation, SDS-PAGE, and fluorography. (*a*) Cytochrome b_2 (*Cyt. b_2*); (*b*) Ornithine carbamoyl transferase (*OTC*); (*p*) precursor; (*m*) mature-sized form.

be emphasized that under the conditions used so far the mitochondrial import sites were not saturated (blocked) by b_2-DHFR (see below), excluding a back-up block as a possible reason for the accumulation of p- and i-b_2-DHFR. In summary, at least 75–80% of import of the b_2-DHFR proteins seems to occur by a post-translational mechanism. With regard to the role of protein folding, a stable tertiary structure can be induced in at least half of the precursors, leading to a block of import. This demonstrates that a transport-competent (loosely folded) structure of precursor proteins is not only required in vitro but also in vivo.

Accumulation of b_2-DHFR in Translocation Contact Sites Leads to Inhibition of the Import of Authentic Precursor Proteins

Under the conditions used in the experiments shown in Figs. 2 and 3, the accumulation of b_2-DHFR in mitochondrial translocation contact sites did not affect the import of other precursor proteins (see Fig. 3, and data not shown), raising the question of whether mitochondrial import sites could be saturated in vivo and if different precursor proteins used the same import sites.

The experiment shown in Fig. 1 *a* indeed indicated that ~30–50% of the import sites determined in vitro were occupied by i-b_2-DHFR when b_2-DHFR was expressed in the presence of aminopterine for a longer time period such as 2 h. We therefore applied similar conditions to the pulse-chase experiments. Unlabeled b_2-DHFR was expressed in yeast cells in the presence of aminopterine for the indicated periods (Fig. 4), followed by a pulse-chase analysis of the processing of b_2-DHFR. The longer the time of induction of unlabeled b_2-DHFR was (that is the more b_2-DHFR was accumulated in mitochondrial translocation contact sites), the more labeled p-b_2-DHFR was detected in the yeast cells and the less mature and intermediate-sized b_2-DHFR were generated. The accumulation of unprocessed precursor depended on the presence of aminopterine (see Fig. 5), indicating that a stable folding of DHFR in a large number of b_2-DHFR molecules led to a saturation of mitochondrial import sites.

In the following the import of authentic cytochrome b_2 and ornithine carbamoyl transferase (OTC) was analyzed in pulse-chase experiments. Neither the presence of aminopterine nor the expression of b_2-DHFR for several hours affected the efficient mitochondrial import of cytochrome b_2 (Fig. 5 *a*) or OTC (Fig. 5 *b*), excluding unspecific inhibition of protein synthesis and import under these conditions. However, when the long-term expression of b_2-DHFR was performed in the presence of aminopterine, the unprocessed precursor forms of cytochrome b_2 and OTC accumulated in the cell. We conclude that a considerable fraction of mitochondrial translocation contact sites was saturated with i-b_2-DHFR and thus the import of other precursor proteins was inhibited.

The possibility existed that the massive accumulation of precursors spanning mitochondrial translocation contact sites dissipated the membrane potential across the inner membrane and thereby protein import was reduced. Therefore, we probed the presence of a membrane potential with the potential-sensitive dye (3,3')-dipropylthiadicarbocyanine iodide (Disc3(5)) (Fig. 6). The change in fluorescence intensity upon interaction of the dye with mitochondria carrying ~20 pmol b_2-DHFR per mg mitochondrial protein in translocation contact sites was comparable to that with control

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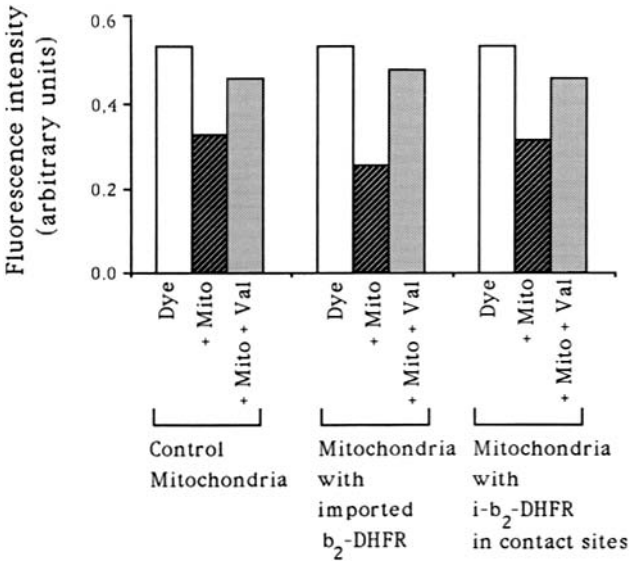

Figure 6. Saturation of mitochondrial translocation contact sites with chemical amounts of membrane-spanning intermediates of b_2-DHFR does not dissipate the mitochondrial membrane potential. The membrane potential was measured fluorometrically with the potential-sensitive dye Disc3(5). Mitochondria were isolated from yeast cells, in which the expression of b_2-DHFR was not induced, or in which the expression of b_2-DHFR was induced for 3 h in the absence or presence of aminopterine. Fluorescence intensity is given in arbitrary units. Where indicated, valinomycin (1 μM) was included to dissipate the membrane potential. Mit, mitochondria; Val, valinomycin.

mitochondria and was sensitive to the K^+-ionophore valinomycin. In agreement with the finding that saturation of translocation contact sites in vitro does not lead to dissipation of the membrane potential (Vestweber and Schatz, 1988; Rassow et al., 1989), it is concluded that accumulation of large amounts of traversing polypeptide chains in mitochondrial translocation contact sites in vivo does not uncouple mitochondria.

Discussion

We report here that the induction of a stable tertiary structure in the carboxyl-terminal domain of a precursor protein leads to arrest of the precursor in mitochondrial translocation contact sites of intact yeast cells. Using this system, several characteristics of protein translocation described in vitro could be analyzed in vivo.

Implications on Folding and Posttranslational Translocation of Precursors

The specific DHFR-ligand aminopterine induced a stable tertiary structure in at least half of the newly synthesized b_2-DHFR molecules. This suggests that the DHFR domains of these precursors were completely synthesized and competent for folding before translocation into the mitochondrial membranes. Upon removing aminopterine, the hybrid proteins were fully imported into mitochondria. This is the first demonstration that folding and unfolding reactions can take place with cytosolically synthesized precursor proteins during the process of transport into cell organelles of intact eucaryotic cells. Maintaining a translocation-competent (loosely folded) conformation of a precursor protein is thus not only important in cell-free systems, but also in vivo. This fits well with the finding of Deshaies et al. (1988) that the deletion of a subset of cytosolic 70-kD heat shock proteins (hsp70s) leads to accumulation of precursor proteins destined for ER or mitochondria in intact yeast cells. By in vitro studies, it was concluded that hsp70s and ATP were required to keep precursor proteins destined for various cell organelles in a translocation-competent conformation (Pfanner et al., 1987*a*; Verner and Schatz, 1987; Chen and Douglas, 1987*b*; Chirico et al., 1988; Murakami et al., 1988; Zimmermann et al., 1988; Pfanner et al., 1990; Waegemann et al., 1990; Murakami and Mori, 1990; Sheffield et al., 1990). The importance of a loosely folded conformation in vivo implies that the function of cytosolic hsp70s in intact yeast cells (Dehaies et al., 1988) is indeed related to conferring transport-competence to precursor proteins. The inefficient posttranslational protein import in a homologous yeast in vitro system observed by Fujiki and Verner (1991) may be due to an inactivation of cytosolic cofactors in their preparation of yeast lysates.

We find that under physiological growth conditions (30°C, non-poisoned mitochondria), at least 75–80% of the b_2-DHFR molecules are imported by a posttranslational mechanism: the import of ~50% of the precursors is sensitive to aminopterine and, in addition, ~30% are found in a cytosolic precursor pool. With the precursors that are sensitive to aminopterine, at least the DHFR domain that comprises more than half of the molecule is translocated posttranslationally. Although it cannot be excluded that the presequences interact with the mitochondria while a carboxyl-terminal part of the precursor is still synthesized, the major portion of the precursors is translocated after the completion of synthesis and thus exhibits the property expected of a posttranslational transport mechanism, i.e., dependence on the conformation of the polypeptide chain. The mechanism of translocation of the remaining ~20% of precursors is unclear. It might occur posttranslationally, yet was not detected in our analysis. Alternatively, this fraction of import may indeed occur cotranslationally, as proposed by Butow and colleagues in view of the association of ribosomes with the mitochondrial outer membranes (Kellems and Butow, 1972; Kellems et al., 1975; Ades and Butow, 1980; Suissa and Schatz, 1982).

We expect that a similar approach can be applied to the analysis of protein translocation into the ER and chloroplasts (and possibly also peroxisomes) in vivo. Thereby the role of hsp70s and the problem of cotranslational vs. posttranslational translocation could be analyzed under conditions that are close to the physiological import situation.

Mitochondrial Translocation Contact Sites

In a series of in vitro studies, contact sites between both mitochondrial membranes were demonstrated to be the major site for import of precursor proteins (Schleyer and Neupert, 1985; Hartl et al., 1986; Schwaiger et al., 1987; Chen and Douglas, 1987*a*; Pfanner et al., 1987*b*; Vestweber and Schatz, 1988; Pon et al., 1989; Rassow et al., 1989). The sites were saturable and different precursors apparently used the same sites: the major unsolved question was if these sites

had the same function in intact cells. Here we show with yeast cells that the b_2-DHFR molecules accumulate in mitochondrial import sites in a two-membrane-spanning fashion. Moreover, mitochondrial import sites could be largely saturated in vivo without affecting the membrane potential of the mitochondria. Thereby the import of other precursor proteins was inhibited, indicating that different precursors use the same translocation contact sites also in intact cells. We propose that translocation contact sites represent the major entry gate of mitochondria. Alternative import pathways are either limited to special precursor proteins, such as apocytochrome c that is directly translocated across the outer membrane (Rietveld and de Kruijff, 1984; Stuart et al., 1990), or are only possible in artificial in vitro situations, e.g., the direct translocation of precursors across the inner membrane in yeast mitochondria with disrupted outer membrane (Ohba and Schatz, 1987; Hwang et al., 1989).

It is remarkable that mitochondria with precursors accumulated in translocation contact sites are not uncoupled. How is the inner membrane sealed around the spanning polypeptide chains to prevent leakage of protons and other ions? It seems difficult to imagine that the lipid molecules of the membrane have the capacity of forming an insulating layer around the various side chains of the polypeptides in transit. We prefer to suggest the existence of specific proteins in a putative translocation channel (Pfanner et al., 1987*b*) that have a function in making close contact to the traversing polypeptide chain and thereby prevent leakage of potential-generating ions.

Finally, it is quite satisfying that several essential mechanisms of mitochondrial protein import uncovered by experiments with isolated mitochondria were confirmed in studies with intact cells. There is no doubt that in vitro analysis bears great experimental advantages due to a direct accessibility to components involved in a certain reaction. However, they also carry the inherent possibility of non-physiological, irrelevant or even artifactual processes. It is thus of importance for the field of protein translocation in general that the validity of major in vitro findings will be assessed in vivo.

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