Folding of Fumarase during Mitochondrial Import Determines its Dual Targeting in Yeast*

Received for publication, March 6, 2003, and in revised form, August 21, 2003
Published, JBC Papers in Press, September 5, 2003, DOI 10.1074/jbc.M302344200

Ehud Sass‡, Sharon Karniely‡, and Ophry Pines§
From the Department of Molecular Biology, Hebrew University Medical School, Jerusalem 91120, Israel

We have previously proposed that a single translation product of the FUM1 gene encoding fumarase is distributed between the cytosol and mitochondria of Saccharomyces cerevisiae and that all fumarase translation products are targeted and processed in mitochondria before distribution. Thus, fumarase processed in mitochondria returns to the cytosol. In the current work, we (i) generated mutations throughout the coding sequence which resulted in fumarases with altered conformations that are targeted to mitochondria but have lost their ability to be distributed; (ii) showed by mass spectrometry that mature cytosolic and mitochondrial fumarase isoenzymes are identical; and (iii) showed that hsp70 chaperones in the cytosol (Ssa) and mitochondria (Ssc1) can affect fumarase distribution. The results are discussed in light of our model of targeting and distribution, which suggests that rapid folding of fumarase into an import-incompetent state provides the driving force for retrograde movement of the processed protein back to the cytosol through the translocation pore.

There are only a limited number of examples in which a single translation product has been shown to be distributed between two subcellular locations (1–6). The molecular mechanisms underlying these situations have not been fully elucidated. Dual targeting of cytochrome P450B1 by two targeting signals to the endoplasmic reticulum and mitochondria is controlled by phosphorylation of the protein, which activates its mitochondrial targeting signal and functionally inhibits its ER targeting signal (3). The NADH-cytochrome b5 reductase (Mcr1p) is sorted to the outer mitochondrial membrane or the mitochondrial intermembrane space in yeast because of what seems to be an incomplete translocation through the outer membrane. More recently, Bandlow and co-workers (5) have suggested that competition between spontaneous protein folding and mitochondrial targeting determines the distribution of the yeast major adenylate kinase (Aky2). Their model proposes that Aky2 is imported into mitochondria before commencement of significant tertiary structure formation, yet once folded in the cytosol, it is in an enzymatically active state and not competent for import. In plant cells, there are cases in which a protein is targeted by means of a single targeting sequence to both mitochondria and chloroplasts. Such signal sequences are called “ambiguous” even though different regions of the targeting sequence may be recognized by the different organelles (7). Cytosolic and mitochondrial fumarase isoenzymes are encoded by the same gene (FUM1) in Saccharomyces cerevisiae (8). We have previously shown that these proteins follow a unique mechanism of subcellular localization and distribution. There is only one translation product of FUM1 that is targeted to mitochondria by an N-terminal presequence, which is then removed by the mitochondrial matrix peptidase (MPP) (1). The working model proposes that a subset of the processed fumarase molecules is fully imported into the matrix, whereas the majority (~70%) is partially translocated, so that their amino termini become accessible to MPP. These latter molecules are released back into the cytosol as soluble enzyme by retrograde movement through the translocation pore (9). Another unique feature of fumarase is that both in vivo and in vitro, its translocation into the mitochondrial matrix occurs only while it is being translated (1, 9).

We have previously shown that although a mitochondrial targeting sequence is required for interaction of the protein with mitochondria, the specific fumarase targeting signal and the immediate sequence downstream are not crucial for the dual distribution phenomenon. In this study, we have isolated fumarase mutants in which dual targeting is abolished because of perturbation of its conformation. We show that the two isoenzymes are identical and that their distribution can be affected by hsp70 molecular chaperones. These results support the notion that fumarase distribution is determined by its folding in the cell.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The S. cerevisiae strains used were DMM1–15A (lea2, ura3, ade2, his5) (1), YPH499 (ura3, lys2, ade2, trp1, his3, leu2) (10), E290 (his4, trp1) (11), MW141 (lea2, ury3, lys2, trp1, his3, ssa1: H11011, ssa2::LEU2, ssa1::URA3, pGA1-SSA1 (12), W36-1A (lea2, his3, trp1, ury3, ade2, can1), and PR81 (a-ade2, lys2, ury3, leu2, trp1, ssc1::LEU2) (13). Strains harboring the appropriate plasmids were grown overnight at 30 °C in synthetic depleted medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% glucose or galactose (w/v) supplemented with the appropriate amino acids (50 μg/ml). Plasmids pFT2 (Fum-WT) and pFSE24 (ASF) are described elsewhere (1, 14). Plasmids encoding six histidine-tagged versions of fumarase and derivatives are described elsewhere (14). For pFUMA255, pFT2 was cut with BglII (and filled in) and HpaI and ligated. For, pFUMA59, the NsiI of pFT2 was filled in and changed to BamHI by subcloning into the Bluescript (+/-) Smal site. The resulting plasmid was cleaved with BamHI and ligated. For pFUMA32, pFT2 was cut with BamHI and AcoI was filled in and ligated. For pFUMA35, an Smal site was introduced into pFT2 with the oligonucleotide 5′-TCACCCGGGGGTACCTACTTGCCCA-3′, and the result-
Folding of Fumarase Determines its Distribution

The fumarase amino terminus contains the mitochondrial-targeting sequence and the deletion of the signal peptide results as expected in exclusive cytosolic localization (1, 8, 23). Because fumarase targeting to mitochondria occurs only while it is being translated, we initially assumed that distribution information would be located at the amino terminus of the protein. Nevertheless, as reported previously, the exchange of the fumarase signal peptide for that of cytochrome b2 or deletion of the sequence immediately downstream to the cleavage site (through amino acid 37) did not abolish the dual distribution phenomenon (14). Here, in a rather brutal approach, a fumarase gene was created and expressed harboring a 255 amino acid deletion from a total of 488 amino acids (Fig. 1A, Δ96–350). Cultures of S. cerevisiae were grown on galactose as the carbon source to induce the expression of fumarase derivatives under the GAL10 promoter. Subcellular fractionation of these cultures revealed that Δ255 exhibited a complete alteration of distribution; the vast majority was targeted to mitochondria, whereas for the wild type, most of the fumarase (~70%) is in the cytosol. The exclusive targeting and localization of mHis60 to mitochondria demonstrates the integrity of the organelles in these experiments (Fig. 1A). In addition, each of our subcellular fractionation experiments were carefully controlled by assaying for the activity of mitochondrial (citrate synthase or isocitrate dehydrogenase) and cytosolic (glucose 6-phosphate dehydrogenase) enzymes. We then created shorter deletions within fumarase based on the structure of the protein. The fumarase subunit, based on the crystal structure (24), has three domains (amino acids 24–134, amino acids 161–419, and amino acids 420–488). The middle domain forms a 5-helix bundle, which in the full tetramer is part of a 20-helix bundle. The active site of fumarase is predicted to be situated between interfaces of the amino-terminal, carboxyl terminal, and middle domains. Here we show that deletions within each one of these domains abolished dual targeting, with essentially all of the fumarase being targeted to mitochondria (Fig. 1A, Δ33–350, Δ255–350, and Δ421–488). All of these fumarase derivatives, including the 255-amino acid deletion, were efficiently processed in yeast cells in a membrane potential-dependent manner (Fig. 1B). This was shown by labeling yeast cultures expressing the various fumarases with [35S]methionine either in the presence or absence of the proton ionophore CCCP and was followed by preparation of cell extracts, immunoprecipitation, PAGE, and autoradiography. Upon blocking import of these proteins into mitochondria, the precursor forms were detected (Fig. 1B, compare CCCP with +). Thus, these derivatives, like the wild

---

**RESULTS**

**Subcellular Localization of Mutant Fumarases**—The fumarase amino terminus contains the mitochondrial-targeting sequence and the deletion of the signal peptide results as expected in exclusive cytosolic localization (1, 8, 23). Because fumarase targeting to mitochondria occurs only while it is being translated, we initially assumed that distribution information would be located at the amino terminus of the protein. Nevertheless, as reported previously, the exchange of the fumarase signal peptide for that of cytochrome b2 or deletion of the sequence immediately downstream to the cleavage site (through amino acid 37) did not abolish the dual distribution phenomenon (14). Here, in a rather brutal approach, a fumarase gene was created and expressed harboring a 255 amino acid deletion from a total of 488 amino acids (Fig. 1A, Δ96–350). Cultures of S. cerevisiae were grown on galactose as the carbon source to induce the expression of fumarase derivatives under the GAL10 promoter. Subcellular fractionation of these cultures revealed that Δ255 exhibited a complete alteration of distribution; the vast majority was targeted to mitochondria, whereas for the wild type, most of the fumarase (~70%) is in the cytosol. The exclusive targeting and localization of mHis60 to mitochondria demonstrates the integrity of the organelles in these experiments (Fig. 1A). In addition, each of our subcellular fractionation experiments were carefully controlled by assaying for the activity of mitochondrial (citrate synthase or isocitrate dehydrogenase) and cytosolic (glucose 6-phosphate dehydrogenase) enzymes. We then created shorter deletions within fumarase based on the structure of the protein. The fumarase subunit, based on the crystal structure (24), has three domains (amino acids 24–134, amino acids 161–419, and amino acids 420–488). The middle domain forms a 5-helix bundle, which in the full tetramer is part of a 20-helix bundle. The active site of fumarase is predicted to be situated between interfaces of the amino-terminal, carboxyl terminal, and middle domains. Here we show that deletions within each one of these domains abolished dual targeting, with essentially all of the fumarase being targeted to mitochondria (Fig. 1A, Δ33–350, Δ255–350, and Δ421–488). All of these fumarase derivatives, including the 255-amino acid deletion, were efficiently processed in yeast cells in a membrane potential-dependent manner (Fig. 1B). This was shown by labeling yeast cultures expressing the various fumarases with [35S]methionine either in the presence or absence of the proton ionophore CCCP and was followed by preparation of cell extracts, immunoprecipitation, PAGE, and autoradiography. Upon blocking import of these proteins into mitochondria, the precursor forms were detected (Fig. 1B, compare CCCP with +). Thus, these derivatives, like the wild
type, have retained their ability to be targeted and processed in mitochondria but, in contrast, have lost the ability to move back into the cytosol by retrograde movement.

To examine the effect of shorter deletions, two sequences of fumarase were chosen, one that seemed more conserved in eukaryotes than in prokaryotes and another that is part of the active site of the enzyme. The first was acquired by comparing the fumarase sequence from eukaryotes and prokaryotes using the ClustalX program. Fumarase was distributed between the cytosol and mitochondria of all eukaryotes examined (26). The notion was that if a sequence dedicated to distribution exists in fumarase, then it may be conserved in eukaryotes but not in bacteria, for which distribution is meaningless. One of a number of such candidate sequences is located between amino acids 102–119. Deletion of this sequence caused exclusive targeting of fumarase to mitochondria (Fig. 1A, Δ102–119). This was also true for substitutions within this sequence, and, as shown in Fig. 1, substitutions of five amino acids (M5-SALVA) of yeast fumarase for amino acids frequently found at corresponding positions in bacterial fumarases still abolished dual targeting. The second sequence examined was via a deletion of a 16 amino acid sequence of fumarase that has been implicated as being required for mitochondrial targeting (Fig. 1A, Δ335–350).

The various deletion and mutant fumarases described above seem to be targeted to mitochondria and processed, yet whether they are fully imported or remain adhered to the surface of the organelle remained to be determined. Fumarase expressed in yeast in vivo is very resistant to proteases because of its very compact folding, and therefore, protease sensitivity was not our first choice for such localization studies. For this reason, we employed cross-linking with biotin in which the linking agent was not membrane permeable. Only proteins that are inside the mitochondrial membranes are protected from this labeling, whereas treatment with detergent dissolves the membranes and exposes all the protein to the linker. Isolated mitochondria from cultures expressing the various fumarase derivatives were treated with Biotin-X-NHS in the presence or absence of Triton. Quencher (NH₄Cl) was added, and extracts of the mitochondria were prepared, immunoprecipitated with fumarase antiserum, run on SDS-PAGE, electro-blotted, and detected with horseradish peroxidase-streptavidin. As shown in Fig. 2, mHsp70, a matrix resident protein, was labeled weakly in the absence of Triton, whereas fumarase lacking a signal peptide (small amounts of which nonspecifically adhere to mitochondria, not shown) presents identical labeling in the presence or absence of Triton. Based on this analysis (Fig. 2), one can conclude that wild-type fumarase, ΔE, ΔB, and ΔCter are translocated across the membrane into mitochondria, because their signal is significantly weaker in the absence of Triton than in its presence (Fig. 2, top panels, compare + and −). An exception, which we do not understand at present, is Δ59, for which there is a very similar signal with and without Triton. This protein had not leaked out of the mitochondria, according to subcellular fractionation controls (see above), but rather remained adhered externally to mitochondria (after being completely processed by MPP) because it was fully biotinylated (Fig. 1B). The amount of fumarase in treated and untreated samples was determined by regular Western blot analysis with mHsp70 and fumarase antiserum (Fig. 1B, bottom panels).

Activity, Resistance to Proteolysis, and Tetramerization of Fumarase—Importantly, none of the deletions or substitutions exhibited enzymatic activity when expressed in yeast. Table I presents the activities found in the cell extracts of yeast strains expressing the various fumarases under the GAL10 promoter. Whereas extracts from cells expressing the wild-type protein or the protein lacking a signal peptide exhibit high fumarase specific activity, extracts from all the mutants exhibit a low specific activity, which is similar to the background activity provided by the chromosomally encoded enzyme. The reason we did not use chromosomal knockouts in most experiments was to avoid complications of petite phenotypes. Nevertheless, the ability to complement the fum1 petite phenotype of a
knockout strain is a means of examining fumarase activity in vivo (Table I). In this regard, the ability to complement the petite phenotype is not dependent on mitochondrial targeting because fumarase lacking a signal sequence (ΔSP), which is not imported into mitochondria, is capable of complementation of a fumarase knockout strain. None of the mutants in the current study were capable of complementing the FUM1 deletion, indicating that they were devoid of activity in the cells. Even the M5-SALVA exhibited no enzymatic activity or capability of complementation, even though its alteration did not lie within the active site of fumarase. One can assume that the inactivity of M5-SALVA was the result of a change in conformation.

Another indication for changes in the conformation in the various mutant fumarases described here comes from examination of protease sensitivity of fumarase derivatives in cell extracts. Changes in conformation that affect the compactness of the protein are expected to increase sensitivity to cleavage by proteases. Two experiments in which cell extracts were treated with increasing concentrations of PK (Fig. 3) were subsequently analyzed by Western blotting. Our deletion mutants (e.g. Δ32, Δ59, ΔB, and ΔCter) were very sensitive to PK. On the other hand, M5-SALVA and ΔE show only a slight increase in sensitivity to PK. Thus, even minor effects detected in the final conformation of the protein may indicate an alteration in fumarase folding during import which, in turn, causes mis-distribution.

The quaternary structure of fumarase and derivative mutants was examined by testing their ability to form tetramers, which is the structure of the active enzyme. Extracts of yeast cultures expressing the different fumarases were subjected to Blue native PAGE and then were blotted and detected with anti-fumarase antiserum. Wild-type fumarase is partially dis-assembled in 1% CHAPS, thus allowing detection of both the monomer and tetramer in a single sample. As shown in Fig. 4, WT, ΔE, ΔB, and ΔCter readily form tetramers, whereas for ΔB there is a smear of higher and lower bands, indicating a problem in normal assembly of this mutant fumarase into tetramers. Interestingly, the deletion in ΔB occurs within the middle domain, whereas the other deletions occur within the amino and carboxyl-terminal domains. In fact, the crystal structure of fumarase (24) indicates that the middle domain forms a five-helix bundle which, in the full protein, together with the middle domains of the other three subunits, forms a bundle of 20 α-helices, which, in turn, probably hold the tetramer together. With respect to subcellular distribution, formation of the tetramer does not ensure that dual targeting will occur.

**Mass Spectrometry of Fumarase Isoenzymes**—It has been shown previously that fumarase mitochondrial and cytosolic isoenzymes migrate identically on SDS-PAGE (1) and on two-dimensional gel electrophoresis (27). In addition, we have recently shown that the amino and carboxyl termini of the two isoenzymes are identical by employing mass spectrometry of BNPS-skatole (3-bromo-3-methyl-2-(O-nitrophenyl) sulfonyl)indolenine)-generated fumarase peptides (14). Although these data support the notion that fumarase isoenzymes are identical, there still remains a possibility of undetected differences in modification between the two isoenzymes.

To characterize fumarase, product genes encoding histidinyl-tagged fumarase derivatives were previously constructed. Fum-6h was essentially identical to the nontagged version in its specific activity, processing by MPP, tetramerization, and distribution in the cell (27). Fum-6h was purified by affinity chromatography from yeast subcellular fractions and subjected to tryptic digestion. The resulting peptides were analyzed by MALDI-TOF mass spectrometry. As shown in Table II, identical peptides were detected in these fractions, which correspond to 50% of the total fumarase amino acid sequence. Because accounting for every amino acid in fumarase sequences of the two isoenzymes by employing mass spectrometry of peptides is questionable, the mass of the full protein was determined. Fum-6h purified from total extracts (including both isoenzymes) was solid-phase extracted and nanosprayed into the QTOF MS system. Deconvolution was carried out by the Maxent1 program (see “Experimental Procedures”) and resulted in a single peak corresponding to a mass of 51,063 daltons with a half-height width of 7 daltons (Fig. 5, lower panel). As a control, purified FumΔSP-6h was subjected to the same analysis. This mutant fumarase, which lacks the signal peptide and is localized to the cytosol, has been shown previously to contain an additional acetylated methionine at its N terminus (14). As for Fum-6h, the MS analysis of FumΔSP-6h resulted in a single peak corresponding to a mass of 51,237 daltons with a half-height width of 8 daltons (Fig. 5, top panel). The difference in mass corresponding to the two peaks is 174 daltons, which is the calculated mass of acetylated methionine. The fact that Fum-6h (including both isoenzymes) appears as single peak in MS analysis and its mass is within 2 daltons of the calculated mass (according to sequence) indicates that the cells from which this protein was purified contain a single population of unmodified fumarase molecules.

**The Effect of hsp70 Molecular Chaperones on Distribution**—hsp70 molecular chaperones are crucial factors for the import of proteins into mitochondria. Cytosolic hsp70s of the SSA family (of which there are four: SSA1–SSA4), when depleted, cause the accumulation of mitochondrial precursor proteins in the cytosol (12). These molecular chaperones keep precursor proteins in an unfolded import-competent conformation, which is required for passage of the proteins through the translocases in the mitochondrial membranes. Yeast strains harboring plasmid pGAL1-SSA1 that encodes SSA1 under the control of the GAL1 promoter were used (12). The distribution of fumarase encoded by the endogenous chromosomal gene was examined under conditions of induction (growth on galactose) and repression (growth on glucose) of SSA1. An approximate two-fold increase in fumarase distribution to mitochondria upon induction of SSA1 was found (Fig. 6, compare third and fourth bars). In contrast, the distribution of fumarase in the control wild-type strain which does not contain the pGAL1-SSA1 plasmid (but rather an empty pGAL1 vector) is essentially the same.
The mitochondrial hsp70/Ssc1 is not only a molecular chaperone, but is also a component associated through Tim44 with overexpression (not shown). Overexpression of Ssc1 and citrate synthase does not change because of SSA1 expression (growth on galactose), an even higher percentage of fumarase activity was found in the yeast strain MW141, which contains mutations in the genes encoding hsp70 molecular chaperones, SSA1, SSA2, SSA3, SSA4. This strain also contains the plasmid pGAL1-SSA1. Under conditions of SSA1 repression (growth on glucose), a much lower percentage of total fumarase activity was found in the yeast strain MW141 than with the wild-type type strain (Fig. 6; about 10% compared). The ability of the respective plasmid to complement the petite phenotype of a fum1 knockout yeast strain. The exclusive mitochondrial targeting of proteins such as acetyl-CoA synthetase and citrate synthase does not change because of SSA1 expression (growth on galactose), and SSA1 and SSA4. This strain also contains the plasmid pGAL1-SSA1. Under conditions of SSA1 repression (growth on glucose), a much lower percentage of total fumarase activity was found in the yeast strain MW141 than with the wild-type type strain (Fig. 6; about 10% compared). The ability of the respective plasmid to complement the petite phenotype of a fum1 knockout yeast strain.

_regardless of whether the cells were grown on glucose or galactose (Fig. 6, compare first and second bars).

A more pronounced effect on fumarase distribution was observed in the yeast strain MW141, which contains mutations in the genes encoding hsp70 molecular chaperones, SSA1, SSA2, and SSA4. This strain also contains the plasmid pGAL1-SSA1. Under conditions of SSA1 repression (growth on glucose), a much lower percentage of total fumarase activity was found in mitochondria with the MW141 strain than with the wild-type E290 strain (Fig. 6; about 10% versus 20%). This is probably because of the depletion of SSA1 (hsp70) molecular chaperones, which have been shown to be required for mitochondrial targeting, from the yeast cytosol. Under conditions of SSA1 induction (growth on galactose), an even higher percentage of fumarase is distributed to mitochondria than in the wild-type strain. The exclusive mitochondrial targeting of proteins such as acetyl-CoA synthetase and citrate synthase does not change because of SSA1 overexpression (not shown).

The mitochondrial hsp70/Ssc1 is not only a molecular chaperone but is also a component associated through Tim44 with the Tim23 translocase of the inner membrane (28). This function of Ssc1 is vital for protein import into mitochondria and, therefore, is required for yeast viability. To examine the import of fumarase in mthsp70-compromised cells, cultures of the wild-type and the ssc1– mutant were labeled with [35S]methionine, and cell extracts were prepared and immunoprecipitated with fumarase antiserum. As shown in Fig. 6A, the ssc1 mutant is blocked for import at the restrictive temperature as detected by non-processing of the fumarase precursor (compare ssc1– at 37 and 25 °C). In contrast, fumarase is processed efficiently at both temperatures in the wild-type strain (compare WT at 37 and 25 °C). The positions of processed and unprocessed fumarase can be observed in the absence and presence of CCCP (compare WT at 30 °C –/+) regardless of whether the cells were grown on glucose or galactose (Fig. 6, compare first and second bars).

A more pronounced effect on fumarase distribution was observed in the yeast strain MW141, which contains mutations in the genes encoding hsp70 molecular chaperones, SSA1, SSA2, and SSA4. This strain also contains the plasmid pGAL1-SSA1. Under conditions of SSA1 repression (growth on glucose), a much lower percentage of total fumarase activity was found in mitochondria with the MW141 strain than with the wild-type E290 strain (Fig. 6; about 10% versus 20%). This is probably because of the depletion of SSA1 (hsp70) molecular chaperones, which have been shown to be required for mitochondrial targeting, from the yeast cytosol. Under conditions of SSA1 induction (growth on galactose), an even higher percentage of fumarase is distributed to mitochondria than in the wild-type strain. The exclusive mitochondrial targeting of proteins such as acetyl-CoA synthetase and citrate synthase does not change because of SSA1 overexpression (not shown).

The mitochondrial hsp70/Ssc1 is not only a molecular chaperone but is also a component associated through Tim44 with
The objective was to examine yeast strains in which Ssc1 was not fully blocked but in which its activity was reduced, thereby possibly causing slower import. Although ssc1–2 mutants are capable of fully processing fumarase and other mitochondrial precursors at the permissive temperature, effects of the ssc1–2 mutation were clearly detected at this permissive temperature in growth and other characteristics. Based on the assumption that the ssc1–2 mutant is partially impaired at the permissive temperature, we examined the distribution of fumarase in the cell at the permissive temperature. Cultures of the wild type and ssc1–2 were labeled with [35S]methionine and subjected to subcellular fractionation, and aliquots were immunoprecipitated and analyzed by PAGE and autoradiography. Although all fumarase molecules are targeted to and processed in mitochondria, less fumarase is localized to mitochondria in the ssc1–2 mutant when compared with the wild type (Fig. 7B, compare the two top panels; Fig. 7C, compare the two left pairs of bars). The distribution of aconitase and Hsp60, which are fully imported, was not affected under these conditions, and they therefore served as mitochondrial matrix markers (Fig. 7, B and C). We assumed that the ssc1–2 mutation slows down import, thereby providing the portion of fumarase outside the organelle more time to fold, thereby reducing the amount that will eventually be fully imported.

**DISCUSSION**

We have shown previously that all the fumarase molecules in the cell are synthesized as precursors and are targeted to and processed in mitochondria prior to distribution between the cytosol and mitochondria (1, 14). In addition, previous data are consistent with the partial insertion of fumarase amino termini through the mitochondrial membranes, followed by retrograde movement of the majority of the protein back through the translocation pore into the cytosol (9), whereas the minority of the molecules are fully imported into mitochondria. There are a number of questions regarding this phenomenon. Where does the distribution-information reside within the fumarase protein sequence? What is the driving force for retrograde movement? And, do cellular factors play a role in distribution? Although we do not have complete answers to these questions, our data provide a very probable scenario for the events of translocation, retrograde movement, and distribution. For the first question, we have shown (14) that swapping of the fumarase signal peptide for that of another protein and mutations in the sequence immediately downstream do not
Folding of Fumarase Determines its Distribution

Folding of Fumarase Determines its Distribution

45115

Fig. 7. Processing and distribution of fumarase in ssc1-2 strains. A, fumarase processing was blocked by inhibiting import into mitochondria. Yeast cells induced in galactose medium and expressing the indicated fumarase derivatives were labeled with [35S]methionine for 10 min at the indicated temperatures, either in the absence (−) or presence (+) of 20 μM CCCP. Total cell extracts were prepared, immunoprecipitated with fumarase antiserum, and analyzed on SDS-PAGE. Dual arrows show positions of the precursor (upper) and mature (lower) fumarases. B, induced cultures of ssc1-2 and wild-type strains expressing the indicated fumarase derivatives were labeled with [35S]methionine for 15 min at 24 °C and then subjected to subcellular fractionation. Aliquots were immunoprecipitated with fumarase antiserum and analyzed on SDS-PAGE, followed by autoradiography. Immunoprecipitation with yeast aconitase and Hsp60 antiserum provided the pattern of mitochondrial markers (bottom panel). C, results from B. White bars, cytosolic fraction; black bars, mitochondrial fractions.

We have argued in favor of the folding of fumarase as the driving force for retrograde movement. Yet, for this to be the sole driving force is, in our opinion, very questionable. It has been shown in vitro that, whether one follows the “Brownian ratchet” (“trapping”) or the “power stroke” (25) (“pulling”) model (22), the import process can unfold very tightly folded proteins such as titin (22). Hence, for protein folding to block import, there must be assistance, as for example in the case of dihydrofolate reductase, in which methotrexate stabilizes the tertiary structure. Another way to achieve this in vitro is by having too short of a polypeptide from the folded domain to the MMP cleavage site, so that the Tim and mHsp70 do not have enough peptide to trap or pull (22, 31, 32). Thus, we believe that additional sorting-relevant information should be found toward the amino terminus of the protein. As pointed out above, there does not seem to be an autonomous independent sequence with dual targeting information because deletions and mutations throughout fumarase abolish distribution. Nevertheless, this does not rule out the possibility that there may be other shorter (i.e. folding-dependent) linear signals with secondary targeting information. A good candidate for such a sequence is the “eukaryotic-like sequence,” and in fact, the M5-SALVA mutations have the weakest effect, among the mutations, on fumarase conformation, according to proteinase K sensitivity. Yet, this mutant fumarase which harbors substitutions for five amino acids frequently found at corresponding positions in bacterial fumarases is fully mis-distributed to mitochondria. Thus, this sequence may be important for fumarase distribution by slowing down or temporarily blocking import, thereby allowing more of the protein to fold outside the organelle. Another possibility is that such a sequence may provide binding sites for factors that may enhance fumarase folding and stabilize folded domains. In this regard, hsp70/SSA is a general factor that can shift fumarase distribution; however, a specific factor, if one exists, could be much more potent. These questions will be the objectives of our future studies.

It is very interesting to point out that protein folding probably plays a major role in the distribution of other single translation products. It has been suggested (5) that Aky2 is imported into mitochondria before acquiring a folded structure, yet once folded, it remains in the cytosol in an import-incompetent conformation. Dual targeting of cytochrome P4502B1 by two targeting signals to the endoplasmic reticulum and mitochondria is controlled by phosphorylation of the protein, which activates its mitochondrial targeting signal and functionally inhibits its ER targeting signal (3). In this case, however, phosphorylation occurs on a serine residue 128 amino acids from the amino terminus, whereas the mitochondrial and ER signals are within the first 40 residues. The effect of phospho-
rylation is explained by changes in conformation making one or the other of the signals accessible. Thus, folding of proteins in transit to and through membranes may allow determination of their final destination at different stages; blocking interaction with the membrane (Aky2), choice of the membrane (P4502B1), and retrieval from the membrane (Fum1).

Acknowledgments—We thank Walter Neupert for his collaboration and support throughout this study, Elizabeth Craig for strains and plasmids, Ariel Gaaton (Hebrew University, Jerusalem) for the mass spectrometry, Yudit Karp for her assistance, and Eitan Bibi and Doron Rapaport for critical reading of the manuscript.

REFERENCES
1. Stein, I., Peleg, Y., Even-Ram, S., and Pines, O. (1994) Mol. Cell. Biol. 14, 4770–4778
2. Hauke, V., Ocana, C. S., Honlinger, A., Tekatidis, K., Pfanner, N., and Schatz, G. (1997) Mol. Cell. Biol. 17, 4024–4032
3. Anandatheerthavarada, H. K., Biswas, G., Mullick, J., Sepuri, N. B., Otvos, L., Pain, D., and Avadhani, N. G. (1999) EMBO J. 18, 5494–5504
4. Addya, S., Anandatheerthavarada, H. K., Biswas, G., Bhagwat, S. V., Mullick, J., and Avadhani, N. G. (1997) J. Cell Biol. 139, 589–599
5. Strobel, G., Zollner, A., Angermann, M., and Bandlow, W. (2002) Mol. Biol. Cell 13, 1439–1448
6. Bhagwat, S. V., Biswas, G., Anandatheerthavarada, H. K., Addya, S., Pandak, W., and Avadhani, N. G. (1999) J. Biol. Chem. 274, 24014–24022
7. Peeters, N., and Small, I. (2001) Biochim. Biophys. Acta 1541, 54–63
8. Wu, M., and Traaglof, A. (1987) J. Biol. Chem. 262, 12275–12282
9. Knox, C., Sass, E., Neupert, W., and Pines, O. (1998) J. Biol. Chem. 273, 25587–25593
10. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
11. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) The Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., and Schekman, R. (1988) Nature 332, 800–805
13. Gambill, B. D., Voss, W., Kang, P. J., Miao, B., Langer, T., Craig, E. A., and Pfanner, N. (1993) J. Cell Biol. 123, 109–117
14. Sass, E., Blachinsky, E., Karniely, S., and Pines, O. (2001) J. Biol. Chem. 276, 46111–46117
15. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
16. Kanarek, L., and Hill, R. (1964) J. Biol. Chem. 239, 465–474
17. Worthington, C. C. E., ed (1988) Worthington Manual, pp. 159–161
18. Weizman, L., and Hill, R. (1964) J. Biol. Chem. 239, 465–474
19. Wilm, M., and Mann, M. (1996) Anal. Chem. 68, 1–8
20. Okamoto, K., Brinker, A., Paschen, S. A., Maerfi, I., Hayer-Hartl, M., Neupert, W., and Brunner, M. (2002) EMBO J. 21, 3659–3671
21. Peleg, Y., Rokem, J. S., Goldberg, I., and Pines, O. (1999) Appl. Environ. Microbiol. 56, 2777–2783
22. Compasso, K., Brinker, A., Paschen, S. A., Maerfi, I., Hayer-Hartl, M., Neupert, W., and Brunner, M. (2002) EMBO J. 21, 3659–3671
23. Peleg, Y., Rokem, J. S., Goldberg, I., and Pines, O. (1999) Appl. Environ. Microbiol. 56, 2777–2783
24. Weaver, T., Lee, M., Zaitsev, V., Zaitseva, I., Duke, E., Lindley, P., McSweeney, S., Svensson, A., Kerushenko, J., Kerushenko, I., Gladilin, K., and Banaszak, L. (1998) J. Mol. Biol. 280, 431–442
25. Lin, J., Martin, F., Guiard, B., Pfanner, N., and Voss, W. (2001) EMBO J. 20, 941–950
26. Akiba, T., Hiraoka, K., and Tuboi, S. (1984) J. Biochem. (Tokyo) 96, 189–195
27. Blachinsky, E. (2001) Molecular Biology M.Sc. thesis, Hebrew University, Jerusalem
28. Voss, W., and Rottgers, K. (2002) Biochim. Biophys. Acta 1592, 51–62
29. Otte, H., Gruhler, A., Stuart, R. A., Guiard, B., Schwarz, E., and Neupert, W. (1995) J. Biol. Chem. 270, 16922–16928
30. van Leen, A. P., and Schatz, G. (1987) EMBO J. 6, 2441–2448
31. Ungermann, C., Neupert, W., and Cyr, D. M. (1994) Science 266, 1250–1253
32. Schneider, H., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994) Nature 271, 768–774
