Import into Mitochondria, Folding and Retrograde Movement of Fumarase in Yeast

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A single translation product of the FUM1 gene encoding fumarase is distributed between the cytosol and mitochondria of Saccharomyces cerevisiae. All fumarase translation products are targeted and processed in mitochondria before distribution. Here we show that targeting of fumarase is coupled to translation and initially involves insertion of the protein across the mitochondrial membranes and processing by the matrix protease. Rapid folding of fumarase may determine its requirement for coupling of its translocation with translation and unique route of distribution. The amino termini of most fumarase molecules are translocated across the mitochondrial membranes and processed. Unlike the in vivo situation where these molecules are released into the cytosol, in vitro they remain externally attached to the mitochondria, thereby positioned for release from the organelle. Our model suggests that fumarase displays a unique mechanism of targeting and distribution, which occurs cotranslationally and involves folding and retrograde movement of the processed protein back through the translocation pore.

Cytoplasmic and mitochondrial fumarase isoenzymes are encoded by the same gene (FUM1) in Saccharomyces cerevisiae (1). We have shown previously that these proteins follow a unique mechanism of subcellular localization and distribution in vivo. First, there is only one translation product of FUM1, and it is targeted to mitochondria by a characteristic NH₂-terminal peptide presequence, which is subsequently removed by the matrix protease. Second, it appears that a subset of the processed fumarase molecules are fully imported into the matrix, whereas the majority (70–80%) are released back into the cytosol as soluble active enzyme by an unknown mechanism (2).

The question as to whether in vivo initiation of protein import into mitochondria must occur during (cotranslational) or can, with equal efficiency, occur after completion of protein synthesis (posttranslational) has been addressed previously. It has been proposed that in vivo the normal mode of import of such proteins is co- rather than posttranslational, as suggested by the observations that ribosomes synthesizing mitochondrial proteins were found associated with mitochondria, only minute amounts of some precursors of mitochondrial proteins were detected in yeast cells in vivo, and inhibition of translation inhibits import of mitochondrial proteins (3–7). On the other hand, in vivo accumulated precursors were observed to be chased into mitochondria. Thus, practically all naturally occurring proteins that have been studied could be imported posttranslationally in vivo, suggesting that translation and import are not necessarily coupled. In addition, in vitro, virtually all mitochondrial precursor proteins so far investigated are, under appropriate conditions, successfully imported posttranslationally. In the unique case of fumarase, translocation into the mitochondrial matrix in vivo appears to be strictly cotranslational (2). When fumarase precursors are accumulated in the cytosol by treating cells with CCCP1 (carbonyl cyanide m-chlorophenylhydrazone) treatment, restoration of the membrane potential did not bring about import and the formation of mature fumarase (2).

A so far unresolved question is how fumarase ends up in the cytosol, given that processing of all FUM1 products takes place in mitochondria in vivo (2). Either full translocation of fumarase takes place followed by processing and export back into the cytosol, or these molecules are partially translocated so that their amino termini become accessible to the matrix peptidase. According to the first alternative, processed fumarase would be secreted out of mitochondria, possibly by a mechanism resembling protein secretion in Gram-negative bacteria. According to the second alternative, following processing, fumarase molecules destined for the cytosol return to this compartment by retrograde movement through the translocation pore.

In this report we present data suggesting that a cotranslational event actually appears to be required for the import and processing of the fumarase precursor, as well as for its distribution between mitochondrial matrix and cytosol. We also examine the probability of retrograde movement through the mitochondrial membranes as part of the targeting and distribution mechanism.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The S. cerevisiae strain used were DMM1-15A (leu2 ura3 ade2 his5) (2). Strains harboring the appropriate plasmids were grown overnight at 30 °C in SD 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 (100 μg/ml) (8). Plasmids pFT2, pFSE24, pGEMSu9 (1–86)-DHFR are described elsewhere (2, 9, 10). Plasmids pGEM-FUM, pGEM-FUM-M24(N25F), pGEM-FUM(M24S), and pGEMFUM-DHFRs are described in this study. 5′ (ACGACTGATAACATGTTGAAATTAC) and 3′ (AAGCTCTAGAGCGGGACCTTAGCGGAGGG) primers, respectively, were used to generate the fumarase coding sequence from plasmid pFT2 by polymerase chain reaction. The resulting fragment was cloned into plasmid pGEM3 between sites SalI and XbaI downstream of the SP6 promoter. The resulting plasmid was cleaved with

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1 The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DHFR, dihydrofolate reductase; MPP, mitochondrial processing peptidase; PEP, processing enhancing protein; PAGE, polyacrylamide gel electrophoresis.

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SpfI followed by DNA-polymerase I Klenow treatment and ligation to remove an in frame ATG codon and designated pGEM-FUM. Derivatives altering the 24th and 25th codons of fumarase from methionine, asparagin (wild type) to serine, phenylalanine (M24S) are described elsewhere. 2 To construct plasmid pGEM-FUM(1–94)-DHFR, a DHFR-encoding fragment from plasmid pVC60 was cloned in frame into the BglII and XbaI sites of pGEM-FUM. pFUM(1–71)-DHFR was constructed from pFUM(1–94)-DHFR cut with BamHI and SpfI, treated with DNA-polymerase I Klenow fragment followed by ligation. Plasmid pFUM(1–350)-DHFR was constructed from pFUM(1–94)-DHFR as follows. pFUM(1–94)-DHFR was cleaved with restriction endonucleases and the DHFR fragment cloned into pGEM-FUM cleaved with HpaI and XbaI. The resulting plasmid was cleaved with SmalI and XbaI and the DHFR fragment cloned into pGEM-FUM cleaved with HpaI and XbaI. To construct plasmid pGEM-FUM(1–350)-DHFR, pGEM-FUM(M24S/N25F) was cleaved with PstI and HpaI and the small fragment cloned into pFUM(1–350)-DHFR. pFUM(1–71)-DHFR was constructed by cleavage of pFUM(1–350)-DHFR with SpfI and HpaI followed by klenow and ligation.

In Vitro Translation and Import into Mitochondria—Transcription of pGEM plasmids in vitro was carried out according to the manufacturer’s instructions (Promega) using SP6 polymerase. Isolated mitochondria were prepared from yeast as described previously (11). Protein synthesis was performed in the presence of [35S]methionine in rabbit reticulocyte lysate (Promega). Coupled translation/ import reactions of 20 μl contained 16.5 μl of reticulocyte lysate, 0.5 μl of ribonuclease inhibitor, 1 μl of transcription reaction, and 10 μl of [35S]methionine. In all experiments, translation was allowed to proceed for 2 min (unless otherwise indicated) before the addition of mitochondria (10–20 μg) and the reaction further incubated at 30 °C for 60 min. In experiments involving dissection of the mitochondrial membrane potential, mitochondria were pretreated for 2–3 min with 10 μM valinomycin and then added to the reaction mixture. In experiments involving fusion proteins, methotrexate (1 μM) was added to the reaction mixture prior to addition of mitochondria. Following translation, all samples were diluted into SH-KCl buffer. Samples were denatured by boiling in 1% urea, 3% sorbitol, 50 mM Hepes, pH 7.2, 80 mM KCl. Selected reactions were treated with 70 μg/ml proteinase K for 15 min on ice. Centrifugation at 9000 × g for 10 min at 4 °C yielded a supernatant fraction, and a mitochondrial pellet, which was washed once before being resuspended in SHKCl buffer. Samples were denatured by boiling in loading buffer containing 1% SDS, 4% β-mercaptoethanol, 1% diethio- triol and analyzed on 10% SDS-polyacrylamide gel electrophoresis followed by visualization with the image-analyzing system BAS2000 (Fuji Corp.).

Import of Urea-denatured Precursors—Translation reactions of 20 μl in reticulocyte lysate were incubated for 60 min at 30 °C. Precursors were diluted 10-fold in water, treated with saturated (NH4)2SO4, and resuspended in 20 ml of fresh medium and labeled for 5 min with 2.7 Ci/ml [35S]methionine. Labeling was stopped by the addition of 2 μl methionine and 0.1 mg/ml cycloheximide. Aliquots were removed at 0, 10, and 30 min after labeling ended and subjected to standard subcellular fractionation (2) to obtain mitochondrial and cytosolic fractions. Fumarase was assayed by the method of Kanarek and Hill (12) at 250 nm with l-malic acid as substrate. Protein was determined by the method of Bradford (13).

RESULTS

Import of Fumarase into Isolated Mitochondria—We examined in vitro translated FUM1 products in conventional post-translational import reactions (14) and found essentially negligible import (see below). Since in vivo fumarase appears to be imported cotranslationally (2), we developed an in vitro assay for coupled translation and import. As a control in these experiments we used an Su9-DHFR hybrid gene, which encodes the NH2-terminal 86 amino acids of Neurospora crassa Fp1-ATPase fused in frame to murine DHFR. This protein has been shown previously to be efficiently targeted to the yeast mitochondrial matrix and processed in vivo and in vitro (9). As shown in Fig. 1, both the fumarase and the Su9-DHFR precursor (pFum and pSu9, respectively) were efficiently converted to mature-sized proteins (mFum and mSu9). To show that processed fumarase associated with mitochondria is the product of a true import reaction, valinomycin (which dissipates membrane potential) was used to block import. This treatment led to a block in processing of both pFum and pSu9-DHFR (Fig. 1, lane 3). Only a small fraction of the mFum molecules was fully protected and exposed on the external membrane. In contrast to mFum, mSu9-DHFR in the in vivo import reaction was fully protected against protease (Fig. 1, compare lanes 2 and 6) at 30 °C for 60 min. Supernatant and pellet fractions were denatured by boiling in 1% SDS, immunoprecipitated with antifumarase rabbit antisera and protein A-Sepharose (Amersham Pharmacia Biotech), and then analyzed by SDS-PAGE.

For pulse chase experiments yeast cells harboring PFT2 were grown overnight at 30 °C in 300 ml of galactose medium, centrifuged, and resuspended in 20 ml of fresh medium and labeled for 5 min with 2.7 mCi of [35S]methionine. Labeling was stopped by the addition of 2 μl methionine and 0.1 mg/ml cycloheximide. Aliquots were removed at 0, 10, and 30 min after labeling ended and subjected to standard subcellular fractionation (2) to obtain mitochondrial and cytosolic fractions. Wild type fumarase and Su9-DHFR mRNAs were translated together in reticulocyte lysate (Promega). Coupled translation/ import reactions were performed in the presence of [35S]methionine. Mitochondria were added 2 min after initiation of translation (lanes 2–7) and the reactions further incubated at 30 °C for 60 min. One import reaction (lanes 3 and 6) contained 0.1 μM valinomycin (Val), whereas a second reaction (lanes 4 and 7) was treated with proteinase K (PK, 70 μg/ml) following import. Samples were centrifuged at 9000 × g for 10 min to obtain mitochondrial pellet (P) and supernatant (S) fractions, which were analyzed by SDS-PAGE. Lane 1 contains mRNAs translated in the absence of mitochondria (L, reticulocyte lysate). Arrows indicate the positions of the fumarase precursor (pFUM) and mature mRNAs translated and the Su9-DHFR precursor (pSu9) and mature forms (mSu9).

FIG. 1. Import in vitro of fumarase into mitochondria. Wild type fumarase and Su9-DHFR mRNAs were translated together in reticulocyte lysate (Promega). Coupled translation/ import reactions were performed in the presence of [35S]methionine. Mitochondria were added 2 min after initiation of translation (lanes 2–7) and the reactions further incubated at 30 °C for 60 min. One import reaction (lanes 3 and 6) contained 0.1 μM valinomycin (Val), whereas a second reaction (lanes 4 and 7) was treated with proteinase K (PK, 70 μg/ml) following import. Samples were centrifuged at 9000 × g for 10 min to obtain mitochondrial pellet (P) and supernatant (S) fractions, which were analyzed by SDS-PAGE. Lane 1 contains mRNAs translated in the absence of mitochondria (L, reticulocyte lysate). Arrows indicate the positions of the fumarase precursor (pFUM) and mature m transcripts and the Su9-DHFR precursor (pSu9) and mature forms (mSu9).

PK 1 2 3 4 5 6 7
Val - - + - + - +
Fraction L P P P S S S
pFUM mFUM
pSu9 mSu9

2 E. Sass and O. Pines, manuscript in preparation.
4), indicating that it was fully imported. The absence of mSu9-DHFR in the supernatant (Fig. 1, lane 5) provides evidence for the integrity of the mitochondrial membranes in these experiments, since Su9-DHFR is a soluble matrix protein.

A small amount of product similar in size to mFum was present in the supernatant of import reactions (Fig. 1, lane 5), which originated from a small amount of translation product that initiated from the second ATG (codon 24). This is not a released form, as it was missing from import reactions using mutant fumarases in which the second ATG was changed to a serine (pFum-M24S and pFum-M24SN25F). On the other hand it cannot be excluded that methionine 24 is part of the fumarase sorting sequence, and its elimination may cause a defect in release of the mature mFum from the membrane.

Co-Versus Posttranslational Import—Standard coupled translation/import reactions were performed by translating the mRNAs of pFum and pSu9-DHFR together (in the same reaction) in reticulocyte lysate for 2 min before adding mitochondria for an additional 60 min. pFum and the control protein, pSu9-DHFR, were both imported and processed to mature forms (Fig. 2A, compare lane 1 with lane 2 and lane 10 with lane 11, respectively). If, on the other hand, translation was performed for 60 min prior to addition of mitochondria, processing of pFum was minimal, whereas pSu9-DHFR was completely processed (lanes 1 and 3 and 10 and 12). The requirement for simultaneous translation and import was more clearly observed with the mutant fumarases (pFum(M24SN25F), lanes 4–6, and pFum(M24S), lanes 7–9) due to the absence of a second band of the primary translation. These results indicate that for efficient translocation of the NH2 terminus and processing, pFum must be imported cotranslationally or that the import competence of the newly synthesized pFum is maintained for a very short time.

Precursor proteins, artificially unfolded by urea denaturation, can be translocated into the mitochondrial matrix, circumventing the need for externally added factors such as Hsp70, MSF, and ATP, which may otherwise be required to hold the protein in an unfolded translocation-competent state (15–18). These considerations prompted us to examine whether fumarase precursors that had been denatured in urea may be capable of being imported posttranslationally in vitro. Denatured Su9-DHFR precursor was imported as detected by its processing (Fig. 2B, compare lanes 7 and 8) and protection of the processed species from externally added proteinase K (not shown). In contrast, pFum and its mutant derivatives were neither processed (Fig. 2B, compare lanes 1 with 2, 3 with 4, and 5 with 6, respectively) nor protected from proteinase K (not shown), supporting a mechanism of cotranslational import.

Fumarase Processing and Conformation—The cleavage site of pFum for the MPP has been determined recently9 and shown to be between methionine residue 24 and asparagine residue 25. Consistent with the consensus (19), yeast fumarase includes arginines at positions −2 and −3 and a lysine at position −11. The processing of pFum in vitro closely follows its import, which exposes the protein to the peptidase upon translocation (2). Here we used susceptibility of fumarase to cleavage in vitro by purified MPP as an assay for the protein’s conformation. The pFum(M24SN25F) was used, since in contrast to wild type pFum, its in vitro translation lacks a second band (Fig. 2A), and it is processed more efficiently by MPP than pFum(M24S) (Fig. 2A, compare lanes 4 and 5 with lanes 7 and 8). When pFum(M24SN25F) and pSu9-DHFR were translated in reticulocyte lysate in the presence of MPP, processing to the mature-sized proteins was observed (Fig. 3, lane 2). However, if translation was allowed to proceed for 60 min prior to addition of MPP, processing of pFum to mFum was abolished, whereas significant pSu9-DHFR processing was still seen (Fig. 3, lane 3). The same observation was made with urea-denatured pFum, which was barely processed when compared with Su9-DHFR (lane 4). These results suggest that pFum acquires a conformation in which its signal peptide is not accessible to MPP after translation or renaturation.

Fumarase translation and processing in reticulocyte lysate containing MPP, as described above, was followed as a function of time. Translation as determined by trichloroacetic acid precipitation of 35S-methionine-labeled protein, leveled off after approximately 15–20 min (Fig. 4A). Translation of full-length 35S-methionine-labeled pFum and pSu9-DHFR analyzed by SDS-PAGE...
was observed after 10 min, but did not continue after 20 min (Fig. 4B). To examine the timing of fumarase processing, pFum mRNA was translated in reticulocyte lysate in the presence of MPP, and aliquots were removed and analyzed by SDS-PAGE to determine the percentage of mFum as related to total fumarase (mFum + pFum). This ratio was between 50 and 60% regardless of the time period of translation in the presence of MPP (Fig. 4C). Taken together these results indicate that pFum is efficiently processed only while the reticulocyte lysate is capable of translating protein and in particular pFum.

The view that fumarase precursors fold into a conformation incompatible with import is supported by the behavior of accumulated precursor in vivo. We examined extracts of cells expressing pFum with an exchange of methionine for valine (pFum-M24V, data not shown). This mutant precursor was very weakly processed in vivo, presumably due to alteration of the MPP cleavage site (2). In contrast to wild type fumarase or fumarase expressed without a signal peptide, mutant unprocessed fumarases were fully sedimented from cell extracts by ultracentrifugation (270,000 x g for 60 min). The same was true for wild type pFum accumulated in the cytosol in the presence of CCCP (data not shown). Consistent with the notion of misfolding and aggregation of fumarase precursors in vivo was the fact that extracts of cells expressing weakly processed fumarases, referred to above, exhibited a 10-fold lower fumarase enzymatic activity (2). Thus fumarase precursors misfold and aggregate very quickly if not processed. This explains our inability to detect posttranslational import and processing in vivo and in vitro.

Time-dependent Mitochondrial Association and Import—Considering the fact that in vivo processing of all FUM1 products takes place in mitochondria, the question arose as to how 80% of the protein end up in the cytosol (2). Either these molecules are partially translocated across the membranes so that their amino termini become accessible to the matrix peptidase, or full translocation takes place followed by processing and export back into the cytosol. If fumarase was first fully imported and exported, one might expect to detect the accumulation in mitochondria of processed molecules before export back into the cytosol. Such accumulation would not be consistent with the alternative model stipulating partial import followed by release back into the cytosol.

In an attempt to examine the two possible routes of fumarase distribution in vivo, yeast cells expressing fumarase (from plasmid pFT2) were pulse-labeled with [35S]methionine for 5 min and then treated with a surplus of cold methionine and cycloheximide to block further translation of labeled proteins. The distribution of fumarase was determined at various time points after the pulse by subjecting aliquots of the culture to subcellular fractionation and immunoprecipitation. The distribution of fumarase as a function of time did not change significantly throughout the experiment (Fig. 5, compare lanes 1 and 4 with lanes 2 and 5 and lanes 3 and 6). This result is consistent with a short lived association of fumarase with mitochondria, which in turn is consistent with partial import and release from the organelle, rather than full translocation into mitochondria followed by export. Since the minimal pulse time that provided reproducible results was 5 min, we cannot rule out a very fast import and export.

The same question was examined in vitro by following a mitochondrial import reaction as a function of time. Since, in vitro, most of the processed fumarase remains associated with the mitochondria, rather than being released, proteinase K treatment was used to determine distribution between fully imported fumarase and mature molecules outside mitochondria. To be able to present the very different amounts of translation products at the different time points, aliquots of decreasing volume were taken for PAGE (see Fig. 6 legend). The integrity of the mitochondrial import process was demonstrated by the time-dependent processing (Fig. 6, lanes 2, 4, 6, and 8, compare the two bottom p and m bands) and protection (Fig. 6, lanes 3, 5, 7, and 9) of Su9-DHFR, which was also true

Figure 3. Processing of pFum(M24SN25F) by purified matrix protease in vitro. pFum(M24SN25F) and pSu9-DHFR were synthesized as above and incubated with 10% trichloroacetic acid onto filters and counted by scintillation (A) or (ii) analyzed by PAGE (B). C describes a similar translation reaction of pFum(M24SN25F), but with MPP present, in which samples were taken at the same time points as above and analyzed by SDS-PAGE to determine the level of pFum processing (mFum relative to total Fum) as a function of time.
For fumarase processing (lanes 2, 4, 6, and 8, compare the two top p and m bands). There was also a time-dependent accumulation of fumarase protected against added protease (Fig. 6, lanes 3, 5, 7, and 9). Only a fraction of the protein was ultimately protected, indicating that most of the processed fumarase remained associated with the surface of the outer mitochondrial membrane (e.g. Fig. 1). These results correspond with the in vivo results and strongly support the notion that full import is not required for obtaining processed fumarase outside the mitochondria. Under the premise that cytosolic targeting of fumarase does not include full import, we suggest that following processing by matrix protease, fumarase undergoes retrograde movement across the mitochondrial membranes.

Import of Fumarase-DHFR Hybrid Proteins—Methotrexate, a folate analog, can stabilize DHFR and thereby inhibit translocation of fusion proteins containing a DHFR domain fused to a mitochondrial preprotein, or parts thereof, across the mitochondrial membranes (20, 21). Consequently, it can be used to examine the import of fumarase and test whether fumarase completely enters the mitochondrial matrix before ending up on the outer membrane. We constructed vectors encoding fusion proteins consisting of the amino terminus of pFum attached to DHFR, designated pFum71-DHFR, pFum94-DHFR, and pFum350-DHFR according to the number of fumarase amino-terminal residues in the fusion. When translation of these constructs was performed for 60 min prior to addition of mitochondria, processing was minimal when compared with a coupled translation/translocation reaction (Fig. 7A, compare 2-(2') and 60-min (60') reactions).

Methotrexate, added to a coupled translation/translocation reaction, has no significant effect on pFum71-DHFR, pFum94-DHFR, and pFum350-DHFR processing and protection (Fig. 7B, compare lanes 2 and 4 and lanes 3 and 5). In contrast, there is a significant inhibition of pSu9-DHFR processing (lanes 2 and 4, compare the two bottom bands). Inhibition of pSu9-DHFR processing in the presence of methotrexate has been reported (9) and explained by the fact that approximately 50 amino acid residues are required to span both outer and inner mitochondrial membranes (21). Under these conditions, the first cleavage site (following residue 35) is not readily accessi-

### Table: Fraction and Mitochondrial Fraction

| Fraction | Mito | Cyto |
|----------|------|------|
| Chase (min) | 0 | 0 | 1 | 10 | 20 | 0 | 10 | 20 |
| mFUM | - | - | + | + | + | + | + | + |

This table shows the distribution of mitochondria across different fractions. The chase periods are indicated, and the presence of mFUM is noted. The mitochondrial fractions were analyzed by SDS-PAGE.

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**Fig. 5.** Immunodetection of fumarase in subcellular fractions of pulse-labeled cells. Induced cultures of yeast expressing wild type fumarase (from plasmid pFT2) were labeled for 5 min, and unlabeled methionine (2 mM) and cycloheximide (0.1 mg/ml) were added to initiate the chase. Aliquots taken at 0, 10, and 30 min from the end of the pulse labeling were subjected to subcellular fractionation, and the fractions were analyzed by SDS-PAGE.

**Fig. 6.** Import and processing of fumarase in vitro as a function of time. pFum(M245N25F) and pSu9-DHFR were synthesized in reticulocyte lysate. As in Fig. 2A, mitochondria were added 2 (lanes 2, 5, 8) and 60 (lanes 3, 6, 9) min after initiation of translation, and the reactions were further incubated at 30 °C for 60 min. Translation in the absence of mitochondria is shown in lanes 1, 4, and 7 (L). Samples with mitochondria were centrifuged to obtain the mitochondrial fractions that were analyzed by SDS-PAGE. B, methotrexate does not inhibit the import of Fum-DHFR fusion proteins. pFum350-DHFR, pFum71-DHFR, and pFum94-DHFR fusion proteins were synthesized in the presence of mitochondria. Duplicate import reactions were carried out for each mRNA species with (+, lanes 4 and 5) and without (−, lanes 2 and 3) 1 μM methotrexate. After import, samples were split and one-half was treated with proteinase K. Pellet fractions were analyzed by SDS-PAGE.

**Fig. 7.** A, fumarase-DHFR fusions exhibit a preference for coupled translation/import. Fum350-DHFR, Fum94-DHFR, and Fum71-DHFR proteins were synthesized in reticulocyte lysate. As in Fig. 2A, mitochondria were added 2 (lanes 2, 5, 8) and 60 (lanes 3, 6, 9) min after initiation of translation, and the reactions were further incubated at 30 °C for 60 min. Translational in the absence of mitochondria is shown in lanes 1, 4, and 7 (L). Samples with mitochondria were centrifuged to obtain the mitochondrial fractions that were analyzed by SDS-PAGE. B, methotrexate does not inhibit the import of Fum-DHFR fusion proteins. pFum350-DHFR, pFum71-DHFR, and pFum94-DHFR fusion proteins were synthesized in the presence of mitochondria. Duplicate import reactions were carried out for each mRNA species with (+, lanes 4 and 5) and without (−, lanes 2 and 3) 1 μM methotrexate. After import, samples were split and one-half was treated with proteinase K. Pellet fractions were analyzed by SDS-PAGE.
Import into Mitochondria of Fumarase in Yeast

Import of proteins into mitochondria has been generally considered, for many years, not to require cotranslational insertion across the organellar membranes. Virtually all preproteins examined can be imported in vitro after completion of translation. Although it has been suggested previously that import may be affected by inhibiting translation (3, 4), a good example of a protein that obeys a cotranslational requirement has not been described. Fumarase is unique in this sense, since it clearly exhibits a distinct requirement for coupling of translation and translocation into mitochondria. Precursors accumulated in the cytosol in vivo (by blocking import) cannot be chased into the organelle (2), and pFum, synthesized in vitro in reticulocyte lysate, is efficiently imported when coupled to translation, whereas freshly translated or urea-denatured precursors are not. Furthermore, fumarase-DHFR fusions are not blocked for import in vitro by methotrexate (in coupled translation/translocation experiments). Interestingly, the import of adenylate kinase 2, which is distributed between the mitochondrial intermembrane space and the cytosol, has also been suggested to be coupled with translation and may follow a similar mechanism of distribution (23).

What is the molecular basis of this phenomenon? One possibility is that the rapid folding of the fumarase precursor determines the coupling between translation and import. Processing of precursor by purified mitochondrial matrix protease in reticulocyte lysate indicates that pFum cleavage sites rapidly become inaccessible. This may render precursors incompetent for translocation during or immediately following translation. Furthermore, fumarase precursors blocked for processing (by mutation or physiologically) aggregate in vivo. One may envisage the fumarase peptide chain emerging from the ribosome and interacting with the mitochondrial translocation machinery prior to translation termination. Other scenarios for the coupling of fumarase translation and import could include participation of molecular chaperones and direct interaction between the ribosome and the translocation apparatus. Notably, yeast strains depleted of the SRP or SRP receptor cotranslational secretory system not only show defects in protein translocation into the endoplasmic reticulum but also minor defects in mitochondrial import (24, 25). It will be interesting to examine fumarase import and distribution in such mutant strains.

Another unique feature of fumarase expression is that the protein is processed by the mitochondrial matrix protease prior to its distribution between the cytosol and mitochondria (2). There is no accumulation of fumarase in mitochondria prior to its ultimate distribution in vivo and in vitro, and fumarase-DHFR fusions are not trapped in mitochondria by methotrexate in vitro. Our data in vitro and in vivo are consistent with partial insertion of the fumarase polypeptide chain across the mitochondrial membranes rather than full import followed by export. In vitro, most of the fumarase and fumarase-DHFR hybrids (in contrast to the control Su9-DHFR) accumulate on the mitochondrial outer membrane in processed form, whereas the minority (as found in vivo) is fully imported. In this sense, distribution in vivo between the cytosol and mitochondria parallels the distribution in vitro between the mitochondrial outer membrane and the mitochondrial matrix. Thus, in vitro the protein remains externally attached to the mitochondria as though unnaturally obstructed at the final release step. At present, the factors and/or physical conditions (apparently missing in the in vitro system), which are responsible, in vivo, for release of processed fumarase from the mitochondrial outer membrane into the cytosol are unknown.

Based on the above results and rationale, we suggest that following processing, fumarase molecules destined for the cytosol re-enter this compartment by retrograde movement through the mitochondrial translocation pore. The phenomenon of retrograde movement has been described in a limited number of studies, mainly with respect to vectorial movement of preproteins through membranes in vitro. In such experiments, forward movement of the translocating protein through the translocation apparatus is artificially restricted favoring retrograde movement. For example, experiments have been carried out with mitochondria where the DHFR domain of different fusions with Su9 (Su9(1–48)/DHFR and Su9(1–94)/DHFR) were stabilized by methotrexate, thereby arresting import (9, 17). Such proteins were not long enough in the extended conformation to be maintained in the import apparatus and diffused out. Retrograde movement has also been observed in connection with transport across the endoplasmic reticulum (26, 27). In one case, backward movement of nascent polypeptides was artificially induced by eliminating the stop codon such that these proteins remained associated with the ribosome, thereby restricting forward movement. It has been suggested that the degradation of misfolded proteins in the endoplasmic reticulum may generally occur by retrograde transport of polypeptides through the protein conducting channel, followed by their degradation in the cytosol by the proteasome. Such a mechanism was suggested to describe the process by which the human cytomegalovirus eludes cytolytic T cells by triggering destruction of newly synthesized major histocompatibility class I molecules (28). The human cytomegalovirus gene product US2 interacts with these molecules causing them to move back into the cytosol by a reaction that involves the Sec61 complex, a major component of the endoplasmic reticulum translocation apparatus. Thus, the movement of proteins back and forth through the membrane may be a normal cellular...
function rather than a process that can be observed only in vitro by disturbing the normal translocation process.

Is there a connection between the presumed retrograde movement of fumarase and its cotranslational mode of import? One possibility is that fumarase has unique folding properties. In the full protein, we see that rapid folding conceals the mitochondrial targeting sequence within its structure. During translocation, rapid folding of domains of the translating fumarase could restrict its forward motion and cause retrograde movement into the cytosol. Alternatively or additionally, a so far unrecognized sequence after the targeting signal close to the NH2 terminus might retard translocation (e.g., by lacking high affinity binding sites for mitochondrial Hsp70) and thereby favor retrograde movement. We speculate that the following chain of events explain the subcellular distribution of fumarase in the cell (Fig. 8). A single translation product (including the signal peptide) of the FUM1 gene can either be fully translocated cotranslationally into the mitochondrial matrix, or it can be folded into an import incompetent state and released by retrograde movement through the translocation pore into the cytosol. The folding of the initial translation product may be determined by folding information within the protein sequence and participation of molecular chaperones or the translating ribosome. Future experiments will have to determine the specific targeting and sorting information within the fumarase primary sequence and the cellular components that interact with these sequence elements.

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REFERENCES
1. Wu, M. & Tzagoloff, A. (1987) J. Biol. Chem. 262, 12275–12282
2. Stein, I., Peleg, Y., Even-Ram, S. & Pines, O. (1994) Mol. Cell. Biol. 14, 4770–4776
3. Fujiki, M. & Verner, K. (1993) J. Biol. Chem. 268, 1914–1920
4. Fujiki, M. & Verner, K. (1991) J. Biol. Chem. 266, 6841–6847
5. Nelson, N. & Schatz, G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4365–4369
6. Suisa, M. & Schatz, G. (1982) J. Biol. Chem. 257, 13048–13055
7. Ades, I. Z. & Butow, R. A. (1980) J. Biol. Chem. 255, 9918–9924
8. Sherman, F., Fink, G. R. & Hicks, J.B. (1984) Methods in Yeast Genetics: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Ungermann, C., Neupert, W. & Cyrl, D. (1994) Science 266, 1250–1253
10. Pines, O., Even-Ram, S., Elnathan, N., Battat, E., Aharonov, O., Gibson, D. & Goldberg, I. (1996) Appl. Microbiol. Biotechnol. 46, 393–399
11. Daum, G., Bohni, P. C. & Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033
12. Kanarek, L. & Hill, R. L. (1964) J. Biol. Chem. 239, 2402–2406
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. Yaffe, M. P. (1991) Methods Enzymol. 194, 627–643
15. Wachter, C., Schatz, G. & Glick, B. S. (1994) Mol. Biol. Cell 5, 465–474
16. Stuart, R. A., Cyr, D. M., Craig, E. A. & Neupert, W. (1994) Trends Biochem. Sci. 19, 87–92
17. Schneider, H. C., Bertold, J., Bauer, M. F., Dietmer, K., Gudiard, B., Brunner, M. & Neupert, W. (1994) Nature 321, 768–774
18. Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G. & Lithgow, T. (1995) Nature 376, 705–709
19. Gavel, Y. & van Heijen, G. (1990) Protein Eng. 4, 33–37
20. Eilers, M. & Schatz, G. (1986) Nature 322, 228–232
21. Rassow, J., Hartl, F.-U., Guider, B., Pfanner, N. & Neupert, W. (1990) FEBS Lett. 275, 190–194
22. Horn, D. W., Holloway, R. S. & Said, H. M. (1993) J. Nutr. 123, 2204–2209
23. Bandow, W., Stoebel, G. & Schricker, R. (1998) Biochem. J. 329, 359–367
24. Ogg, S. C., Poritz, M. A. & Walter, P. (1992) Mol. Cell. Biol. 3, 895–911
25. Hann, B. C. & Walter, P. (1990) Cell 67, 131–144
26. Ooi, C. E. & Weiss, J. (1992) Cell 71, 87–96
27. Garcia, P. D., Ou, J. H., Rutter, W. J. & Walter, P. (1988) J. Cell Biol. 106, 1093–1104
28. Wachtel, E. J. H., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A. & Ploegh, H. L. (1996) Nature 384, 432–438