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The Single Translation Product of the *FUM1* Gene (Fumarase) Is Processed in Mitochondria before Being Distributed between the Cytosol and Mitochondria in *Saccharomyces cerevisiae*

ILAN STEIN, YOAV PELEG, SHARONA EVEN-RAM, AND OPHRY PINES*

Department of Molecular Biology, Hebrew University—Hadassah Medical School, Jerusalem 91010, Israel

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The yeast mitochondrial and cytosolic isoenzymes of fumarase, which are encoded by a single nuclear gene (*FUM1*), follow a unique mechanism of protein subcellular localization and distribution. Translation of all *FUM1* messages initiates only from the 5′-proximal AUG codon and results in a single translation product that contains the targeting sequence located within the first 32 amino acids of the precursor. All fumarase molecules synthesized in the cell are processed by the mitochondrial matrix signal peptidase; nevertheless, most of the enzyme (80 to 90%) ends up in the cytosol. The translocation and processing of fumarase are cotranslational. We suggest that in *Saccharomyces cerevisiae*, the single type of initial translation product of the *FUM1* gene is first partially translocated, and then a subset of these molecules continues to be fully translocated into the organelle, whereas the rest are folded into an import-incompetent state and are released by the retrograde movement of fumarase into the cytosol.

The distribution of identical enzymatic activities between different subcellular compartments can be achieved by a number of routes. The existence of two or more genes is often used as a solution; however, a single gene, if specifically adapted, can also allow distribution between two subcellular compartments. For example, the localization of invertase (5, 28), histidyl-tRNA synthetase (25), and α-isopropylmalate synthase (1) is determined at the level of transcription for which, in all three cases, two different mRNAs are produced from the same gene. In each case, one of the mRNA molecules encodes a cleavable subcellular targeting signal peptide, whereas a second, shorter mRNA species lacks this coding sequence and therefore encodes a cytoplasmically located protein. It has been shown that the subcellular distribution of the MOD5 isoenzymes [tRNA (m^2G)dimethyltransferases] is determined at the level of translation (14, 34). The *MOD5* gene encodes mRNAs that contain two in-frame AUGs, either of which can be used for the initiation of translation. This fact results in two protein species which differ in size and, as in the preceding example, the peptide extension in one of the protein species determines the final location of the longer protein. Recently, it was proposed that the subcellular distribution of rat liver fumarase may also be determined by the initiation of translation at two alternative AUG codons on a single mRNA species (35, 36).

In *Saccharomyces cerevisiae*, a single nuclear gene (*FUM1*) has been shown to encode both cytosolic and mitochondrial fumarase isoenzymes (41). One study claimed that these isoenzymes have molecular weights roughly estimated at 48,000 and 53,000 for the mitochondrial and cytosolic species, respectively (2), while a more recent study detected only a single size of fumarase molecules in *S. cerevisiae* (20). The amino-terminal sequence of fumarase contains the mitochondrial signal sequence since (i) 92 of 463 amino acid residues from the amino terminus of fumarase are sufficient to localize *FUM1-lacZ* fusions to mitochondria (27) and (ii) fumarase and *FUM1-lacZ* fusions lacking 17 amino acids at the amino terminus are localized exclusively in the cytosol (27, 41).

We previously suggested that the translation of both mitochondrial and cytosolic fumarases initiates at the same AUG, consistent with the production of both isoenzymes from a *GAL10* promoter situated upstream of a promoterless *FUM1* gene (27). Our suggestion also explains the initial observations of Wu and Tzagoloff (42) showing that the vast majority of *FUM1* transcripts include the AUG upstream of the proposed signal peptide. This AUG is expected to be an efficient codon for translation initiation, since it is the first from the 5′ end of the FUM1 mRNA and since the surrounding nucleotides form a sequence which is similar to the yeast translation initiation consensus sequence (44). In particular, the third nucleotide upstream of this AUG codon (−3 position) is an A, and the first following this AUG codon (+4 position) is a U; these have been identified as optimal for initiation by yeast ribosomes (44). The possibility of a single *FUM1* translation product differs from the models of subcellular distribution of other isoenzymes described above.

In this study, we examined the role of the two potential *FUM1* initiation codons in fumarase expression and determined the capacity of fumarase amino-terminal sequences to target proteins into mitochondria. Our results revealed a unique mechanism of fumarase isoenzyme distribution in the cell which includes the processing of all fumarase molecules in the mitochondria before distribution between the cytosol and the mitochondria.

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. cerevisiae* strains used were DMM1-15A (*leu2 ura3 ade2 his5*) (31, 32) and the *mas1* mutant (*MAT***a** *leu2-3 leu2-112 his3-11 his3-15 phoC phoE*) (42). Plasmids YEp51, pFT2, pCG1, pFTS1, pMG2, and pBM690 have been described elsewhere (4, 6, 18, 27). Plasmids pIL-ATG1, pIL-ATG24, pIL-ATG1+24, pMG3, and pMG4 are described in this study. The growth medium (SD medium) used contained 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories) and 2% (wt/vol) glucose or

* Corresponding author. Phone: 972-2-758242. Fax: 972-2-784010. Electronic mail address: OPHRY@HUJIMD.

galactose and was supplemented with the appropriate amino acids (100 μg/ml). YPD medium, used for cultivating the *mas1* mutant, contained 1% (wt/vol) yeast extract, 2% (wt/vol) Bacto Peptone (Difco), and 2% (wt/vol) glucose. The lithium acetate method (33) was used for yeast transformation. The *Escherichia coli* strains used were JM109 and M15 (37). *E. coli* strains were grown on LB medium and transformed as described by Maniatis et al. (23).

Preparation of cell extracts and cell fractions. For enzyme assays, yeast cultures (2 ml) grown to 1.5 units of optical density at 600 nm (OD_{600}) in SD medium were harvested by centrifugation and suspended in 300 μl of TE buffer (10 mM Tris hydrochloride buffer [pH 8.0], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were broken by vigorous mixing with glass beads for 2 min and centrifuged. The supernatant fraction obtained was used for enzyme assays. Cells for the β-galactosidase assay were grown as described above in SD medium buffered to pH 7 (7). Intact cells were used for the β-galactosidase assay as described previously (7, 24).

For the preparation of mitochondrial and postmitochondrial supernatant (cytosolic) fractions, yeast cultures (300 ml) grown to 1.5 optical density units were used. Yeast mitochondria were prepared essentially by the method of Daum et al. (8). Spheroplasts were prepared from yeast cells in the presence of Zymolyase-20T (Seikagaku Kogyo). For enzyme assays, the mitochondrial pellet was broken by vigorous mixing with glass beads and centrifuging; the resulting supernatant was used for enzyme assays. The percentages of total enzyme activity found in the mitochondrial fraction were 90 to 100% for citrate synthase and isocitrate dehydrogenase and less than 0.1% for glucose-6-phosphate dehydrogenase and lactate dehydrogenase.

Enzyme assays. Fumarase was assayed by the method of Kanhrek and Hill (19) at 250 nm with L-malic acid as a substrate. Citrate synthase was measured by monitoring the reduction of acetyl coenzyme A in the presence of DTNB at 412 nm (39). Glucose-6-phosphate dehydrogenase was measured by monitoring the formation of NADH in the presence of glucose-6-phosphate at 340 nm (40). Lactate dehydrogenase was measured by monitoring the oxidation of NADH at 340 nm in the presence of pyruvate (40). Isocitrate dehydrogenase was measured by monitoring the formation of NADH at 340 nm (11). β-Galactosidase activity was assayed by the method of Miller (24). Protein was determined by the method of Bradford (3).

DNA manipulations. Standard DNA techniques and manipulation of *E. coli* and *S. cerevisiae* strains have been described elsewhere (23, 33). Site-specific mutagenesis was performed with double-stranded plasmid DNA as previously described (17). The sequences of the oligodeoxyribonucleotides used were as follows: for the elimination of the first methionine codon from the 5′ end of *FUM1* (ATG1) and the introduction of a *Cla*I site, TAAGAGATACAATCGATTTGAGATTTC CCAA; for the elimination of the second methionine codon (24th codon overall) from the 5′ end of *FUM1* (ATG24) and the introduction of an *Hpa*I site, TTAATATAAGAA GAGTTAACTCCTCGTTCAG; and for a frameshift between ATG1 and ATG24 and the introduction of an *Xho*I site, ATATAAGCTTAATCTCGAGAAGAATGAACT. The oligonucleotides introduced a novel restriction enzyme recognition site to facilitate screening for mutants. Mutagenesis was performed with plasmid pILSB, a Bluescript derivative that contains a *Sal*I-*Bam*HI fragment of the yeast *GAL10-FUM1* gene from pFT2 (27). A plasmid containing mutations in both initiation codons was constructed by use of a naturally occurring *Hin*dIII site located between ATG1 and ATG24, allowing their combination by simple ligation. After mutations were identified, the respective *FUM1* sequences were cloned back into pFT2.

Plasmids were constructed as follows. For pMG3, the small fragment of pMG2, cut with *Hin*dIII, converted to blunt ends (treated with the DNA polymerase I large fragment) and then cut with *Kpn*I, was ligated into the *Kpn*I and *Sma*I sites of Bluescript. The small *Kpn*I-*Bam*HI fragment of the resulting plasmid was cloned into the corresponding *Bam*HI-*Kpn*I fragment of pMG2. For pMG4, the small *Nsi*I (blunt ended) (by DNA polymerase I)-*Sal*I fragment of pMG2 was cloned into the *Sma*I and *Sal*I sites of Bluescript. The small *Sal*I-*Bam*HI fragment of the resulting plasmid was cloned into the corresponding *Sal*I-*Bam*HI fragment of pMG2.

In vivo labeling of yeast cells. *S. cerevisiae* DMM1-15A cells containing the appropriate plasmid were grown in SD medium containing glucose to an OD_{600} of 1 to 1.5. Cells were harvested, washed, and resuspended in 5 ml of SD medium containing galactose and lacking methionine and leucine. After incubation of the cells for 4 h at 30°C, 100 to 500 μCi of [^{35}S]methionine was added and the cells were incubated for the times indicated in the figure legends. Labeling was stopped by the addition of cycloheximide (0.1 mg/ml). When needed, 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was included in the reaction mixtures to disrupt the mitochondrial membrane potential, whereas β-mercaptoethanol was added to 0.05% (vol/vol) to reestablish the membrane potential after CCCP treatment (29). In pulse-chase experiments, labeling was terminated by the addition of excess unlabeled methionine (2 mM) and, as needed, cycloheximide as well (0.1 mg/ml).

The *mas1* temperature-sensitive (ts) mutant cells were grown in YPD medium to an OD_{600} of 2, washed twice, and resuspended in 5 ml of SD medium containing glucose and lacking methionine. The cells were then divided into two samples; the first was grown for 1 h at the permissive temperature (23°C), and the second was shifted to the nonpermissive temperature (37°C). Both samples were then radiolabeled for 5 min with 300 μCi of [^{35}S]methionine.

Total protein extraction, immunoprecipitation, and Western blot (immunoblot) analysis. The labeled cells were collected by centrifugation, washed once with 1 ml of distilled water, and suspended in 300 μl of TE buffer containing 1 mM PMSF. The cells were mixed vigorously with glass beads for 2 min and centrifuged to recover a clear supernatant fraction. Samples were boiled, immunoprecipitated with antifumarase rabbit antiserum and formaldehyde-treated *Staphylococcus aureus* A Cowan cells (21), and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide) as previously described (31, 32), except that the sample buffer contained 5% β-mercaptoethanol and 10 mM dithiothreitol. Radiolabeled proteins were detected by autoradiography. Quantitation of film darkening observed on autoradiograms was carried out with a Biomed Instruments Zeineh soft-laser scanning densitometer (model SL-TRFF).

For Western blot analysis, equal amounts of fumarases from total cell extracts and the mitochondrial and cytosolic fractions were electrophoresed on SDS–7.5% polyacrylamide gels and transferred to nitrocellulose filters by use of a TE 70 semidry transfer unit (Hoefer Scientific Instruments). Antifumarase antiserum was added, detected by use of ^{125}I-labeled protein A (Amersham Corp.), and exposed to X-ray film at −70°C.

Antisera. (i) Antigen preparation. The antiserum against fumarase was raised against a hybrid protein consisting of fumarase amino acids 92 to 488 preceded by six adjacent histidine residues, an affinity tag which facilitates purification

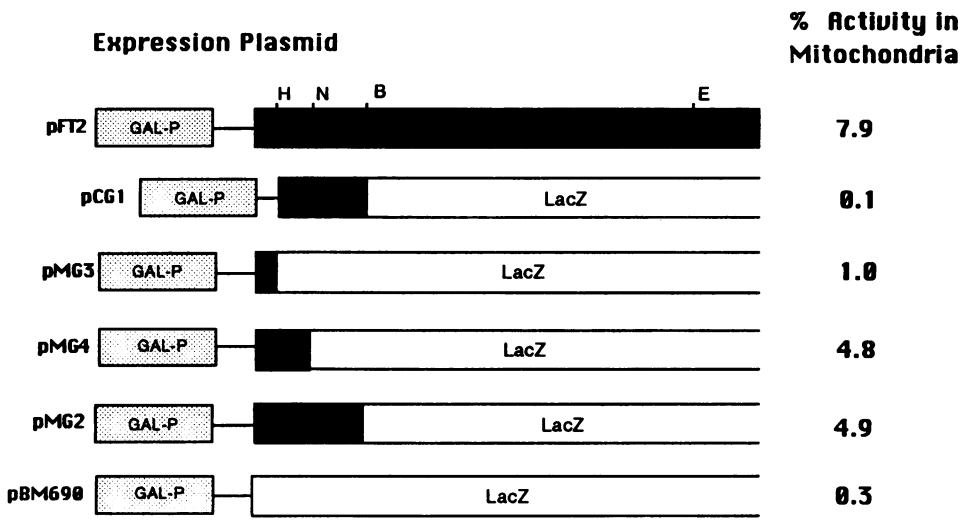

FIG. 1. Efficiency of targeting of fumarase–β-galactosidase fusions to mitochondria. Schematic representation of the *GAL10* promoter-*FUM1-lacZ* fusion (not to scale) in the different plasmids. *FUM1* gene sequences are aligned with the *FUM1* sequence of pFT2. Solid boxes represent *FUM1* protein-encoding sequences, open boxes represent the *lacZ* gene, and stippled boxes represent the galactose-regulated promoter. For strains harboring pCG1 (fumarase amino acids 18 to 92–β-galactosidase), pMG3 (fumarase amino acids 1 to 19–β-galactosidase), pMG4 (fumarase amino acids 1 to 32–β-galactosidase), pMG2 (fumarase amino acids 1 to 92–β-galactosidase), and pBM690 (β-galactosidase), the distribution of β-galactosidase activity was determined. For the strain harboring pFT2, which encodes wild-type fumarase, the distribution of fumarase activity was determined. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I.

of the protein by use of an Ni^{2+}-nitrilotriacetic acid column (Qiagen QIAexpressionist kit type IV) (16). The expression plasmid was created by cloning a *Bam*HI-*Hin*dIII fragment of plasmid pFT2 into plasmid pQE10 (16) downstream to the six histidine residues. A colony expressing the hybrid protein was grown to an OD_{600} of 0.7 of 0.9, isopropyl-β-D-thiogalactopyranoside (IPTG) (2 mM) was added, and the cells were incubated for an additional 5 h. The cells were harvested by centrifugation at 4,000 × *g* for 20 min and lysed in buffer A (6 M guanidine HCl, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8]). After centrifugation of the lysate at 10,000 × *g* for 15 min, the clear supernatant was applied to the Ni^{2+}-nitrilotriacetic acid column. The column was washed with buffer A, buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8]), and buffer C (same as buffer B, but with the pH adjusted to 6.3). The recombinant protein was eluted with buffer D (same as buffer B but with the pH adjusted to 5.9) and then with buffer E (same as buffer B but with the pH adjusted to 4.5). Fractions from the elution were collected and analyzed by SDS–12.5% PAGE and protein-containing fractions were pooled.

(ii) Antibody production. New Zealand White female rabbits were immunized with the 6× histidine–fumarase fusion protein. Preimmune serum was collected prior to the initial subcutaneous injection with 200 μg of antigen (500 μl) and Freund's complete adjuvant (500 μl; Sigma). The rabbits were given boosters of 100 μg of hybrid protein with Freund's incomplete adjuvant at 2- to 3-week intervals for 2 months. Sera were first collected 10 days after the initial injection and then 6 to 9 days after the next booster injection. Antibodies were checked for reaction with FUM1-specific proteins by immunoprecipitation and protein blotting (see Results).

RESULTS

Mitochondrial targeting information in *FUM1*. We previously showed that when a DNA fragment encoding the amino-terminal 92 amino acids of fumarase is fused to *lacZ* and expressed in *S. cerevisiae*, β-galactosidase activity is distributed between mitochondria and the cytosol (27). The proportion of β-galactosidase activity in mitochondria is lower than but similar to the distribution of wild-type fumarase in yeast cells. To delimit the targeting signal, fragments of the *FUM1* gene were fused to *lacZ* and cloned downstream of the strong inducible *GAL10* promoter in vector YEp51 (Fig. 1). Plasmids pMG2, pCG1, pMG3, and pMG4 harbor hybrid genes which encode amino acids 1 to 92, 18 to 92, 1 to 19, and 1 to 32 of fumarase, respectively, and which are fused to the β-galactosidase gene. To examine the subcellular location of the hybrid β-galactosidases, mitochondrial and cytosolic fractions were prepared from galactose-induced cells harboring the plasmids described above. As shown in Fig. 1, the amino-terminal 32 amino acids of fumarase (pMG4) can target about 5% of β-galactosidase to mitochondria. This distribution is essentially the same as the distribution of β-galactosidase in cells expressing a fusion containing the amino-terminal 92 amino acids of fumarase (pMG2) and is similar to the distribution of the entire fumarase protein (pFT2). These results indicate that the first 32 amino acids of fumarase contain the targeting information which is required for the distribution of the enzyme between mitochondria and the cytosol. In contrast, the amino-terminal 19 amino acids of fumarase (pMG3) target only 1% of β-galactosidase to mitochondria. Although this low level of import is significantly higher than that of fusion proteins lacking a signal peptide (pBM690 and pCG1), it is about fivefold lower than that of fusion proteins containing 32 or more amino acids from the fumarase amino terminus (pMG4 and pMG2). The first 17 amino acids of fumarase are essential for signal peptide function, since their deletion abolished mitochondrial import of the β-galactosidase fusion (pCG1; Fig. 1) and fumarase (pFST1; data not shown).

Fumarase activity and its distribution in translation initiation codon mutants. Our hypothesis that a single translation product of the *FUM1* gene is distributed between the cytosol and mitochondria in *S. cerevisiae* was based on several results

TABLE 1. Effect of initiation codon mutations on the production and subcellular distribution of fumarase

Plasmid	ATG1	ATG24	Sp act (μmol/min/mg of protein)[a] with:		% Activity in mitochondria[b] with galactose
			Galactose	Glucose	
pFT2	ATG	ATG	39.00	0.98	8.0
pIL-ATG1	X[c]	ATG	36.50	1.09	0.2
pIL-ATG24	ATG	X[d]	4.92	0.48	6.5
pIL-ATG(1+24)	X[c]	X[d]	0.34	0.27	9.7
pSE-SHIFT	ATG FS[e]	ATG	0.39	0.32	9.8
YEp51[f]			0.44	0.37	10.4

[a] Fumarase specific activity in total extracts of cells grown with glucose or galactose and expressing fumarase from a *GAL10* promoter. These cells also contain an unaltered chromosomal *FUM1* gene which is not regulated by galactose.
[b] Percentage of the total cellular fumarase activity in preparations of mitochondrial subcellular fractions from cells induced with galactose.
[c] ATG1 is changed to GAT (becomes a noncoding region).
[d] ATG24 is changed to GTT (becomes a valine codon).
[e] pSE-SHIFT contains a frameshift (FS) mutation (net addition of 1 nucleotide) between ATG1 and ATG24, causing them not to be in frame.
[f] YEp51 contains no *FUM1* sequences.

obtained by our group and others (see above and references 27 and 41). In the present study, this hypothesis was directly examined by the elimination of *FUM1* translation initiation codons by site-specific mutagenesis. Mutagenesis of the first ATG of *FUM1* (changed to GAT) was accompanied by the creation of a *Cla*I restriction endonuclease site, whereas mutagenesis of the second ATG (forming a valine codon; GTT) was accompanied by the creation of an *Hpa*I site. The reading frame between ATG1 and ATG24 was changed by a net addition of 1 bp, and an *Xho*I site was created. The mutant sequences were verified by DNA sequence analysis and then incorporated into the fumarase expression vector pFT2 (27) downstream of the inducible yeast *GAL10* promoter. The plasmids created, designated pIL-ATG1, pIL-ATG24, pIL-ATG1+24 (containing both ATG mutations), and pSE-SHIFT, are described in Table 1.

Elimination of the first two ATGs (ATG1 and ATG24) from the 5′ end of the *FUM1* RNA coding region essentially blocks the galactose-regulated expression of fumarase activity in yeast cells (pIL-ATG1+24; Table 1). The strain harboring the pIL-ATG1+24 plasmid and induced with galactose retains less than 1% of the fumarase specific activity in the pFT2-containing strain expressing the nonmutated gene, an activity similar to that in a strain harboring control plasmid YEp51 (containing no *FUM1* sequences). Hence, this residual activity is due to the expression of the chromosomal *FUM1* gene, which is not regulated by galactose (Table 1). These results indicate that the translation of fumarase molecules is initiated from either the ATG1 or the ATG24 codon. These and the following experiments were not done with a *fum1* deletion strain to eliminate possible complications of a petite phenotype due to a fumarase deficiency. Thus, we allowed the expression of a low background level of wild-type fumarase in all strains.

When the first ATG (ATG1) was mutated in the *GAL10-FUM1* coding region, the galactose-induced fumarase specific activity was about 90% of the activity in the pFT2-containing strain expressing the nonmutated gene (Table 1). This result indicates that the translation of fumarase molecules can initiate from the ATG24 codon in the absence of ATG1. Translation initiation from ATG24 results in the localization of fumarase activity solely to the cytoplasm (Table 1). This result is expected, since the sequence upstream of this initiation codon includes a crucial part of the mitochondrial targeting sequence, as described above.

Elimination of the second ATG (ATG24) did not influence the subcellular distribution of galactose-induced molecules but did reduce the activity of the enzyme to about 13% of that from the nonmutated gene (pFT2). The reduced activity of fumarase exhibited by pIL-ATG24 is due to the substitution of the 24th codon, methionine, with valine, since the synthesis, stability, and level at which the *GAL10*-induced ATG24, ATG1, and wild-type fumarases are maintained are similar (see below and Fig. 2, 3, and 6). We can therefore conclude that the first ATG is not essential for fumarase expression but is essential for fumarase targeting to mitochondria, whereas the second ATG is important for obtaining a fully active protein. A frameshift mutation (+1 shift) between ATG1 and ATG24 eliminates the galactose-regulated expression of fumarase in yeast cells (pSE-SHIFT; Table 1). This frameshift mutation disturbs the reading frame of fumarase translation starting from ATG1 but does not alter the frame of translation starting from ATG24. The fact that we did not detect translation from ATG24 is therefore due to the presence of ATG1 and directly indicates that translation does not naturally initiate from ATG24.

Immunodetection of wild-type and mutant fumarases. To directly detect and characterize *FUM1* gene products, antibodies were raised against a hybrid protein containing amino acids 92 to 488 of fumarase preceded by six histidine residues. This hybrid protein was overexpressed in *E. coli* and affinity purified according to the Qiagen expression and purification protocols (16).

To identify fumarase-like proteins produced in yeast cells, the cells were grown and induced in galactose medium. Cultures were then labeled with [^{35}S]methionine, and cell extracts were prepared, immunoprecipitated with antifumarase antiserum, and analyzed by SDS-PAGE. A single band was specifically immunoprecipitated from extracts of cells harboring plasmid pFT2, which encodes the wild-type protein (Fig. 2, lane 3, middle arrow). Single bands were also detected in strains expressing the mutant fumarases encoded by plasmids pIL-ATG24 and pIL-ATG1 (lanes 1 and 2, respectively). When the second ATG is mutated, a product larger than the wild-type protein is detected (lane 1, top arrow), whereas a mutated first ATG results in the detection of a protein smaller than the wild-type protein (lane 2, bottom arrow).

The detection of a single immunoprecipitated protein species in yeast cells expressing nonmutated fumarase was surprising because of a previous report (2) claiming that yeast mitochondrial and cytoplasmic isoenzymes have different molecular masses detected by mobility in SDS-PAGE (48 and 53 kDa, respectively). To examine the mitochondrial and cytosolic

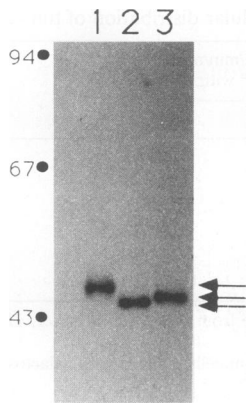

FIG. 2. Immunodetection of wild-type and mutant fumarases. Cultures of DMM1-I5A harboring the appropriate plasmid were induced in galactose medium and labeled with [^{35}S]methionine. Total cell extracts were prepared and immunoprecipitated with polyclonal antifumarase antiserum as described in Materials and Methods. The samples were analyzed by SDS–7.5% PAGE and autoradiography. Lanes 1 to 3 show immunoprecipitates of strains containing the following plasmids: 1, pIL-ATG24 (top arrow); 2, pIL-ATG1 (bottom arrow); 3, pFT2 (middle arrow; wild-type fumarase).

fumarases of yeast cells, induced cells were subjected to subcellular fractionation. Aliquots were analyzed by SDS-PAGE and then by Western blotting with antifumarase antiserum. For each strain, approximately 8- to 12-fold more of the total mitochondrial fraction than the total respective cytosolic fraction was applied to the gel to allow a comparison of similar amounts of the isoenzymes. As shown in Fig. 3, the sizes of the mitochondrial and cytosolic fumarases for each of the strains were identical (compare lanes C and lanes M for each strain). The same was true for fumarase which was expressed from the chromosomal gene in the yeast strain harboring plasmid YEp51 (containing no *FUM1* sequences) and which, as expected, exhibited the same gel mobility as the product expressed from plasmid pFT2 (overexpressing wild-type fumarase from a *GAL10-FUM1* hybrid gene). The reason for this discrepancy between our results and those of Boonyarat and Doonan (2) is not clear; however, a few points should be made. (i) Keruchenko et al., in a very recent study, also detected only a single fumarase species in *S. cerevisiae* by SDS-PAGE (20). (ii) The yeast strain (probably polyploid) used by Boonyarat and Doonan (2) was of an industrial source, whereas our

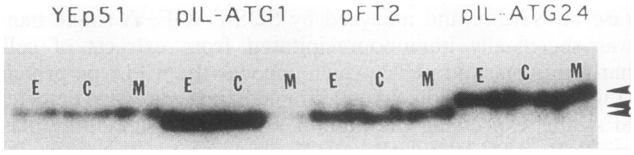

FIG. 3. The isoenzymes of fumarase in the mitochondria and cytosol are identical in size. Cultures of DMM1-15A harboring the appropriate plasmid were induced in galactose medium for 4 h, and the cells were harvested and subjected to subcellular fractionation. Aliquots from the subcellular fractions and total cell extracts were separated by SDS–7.5% PAGE, and the proteins were transferred to nitrocellulose and challenged with antifumarase antiserum. Bound antibody was detected by secondary binding of ^{125}I-protein A and autoradiography. E, extract; C, cytosol; M, mitochondria. The arrowheads show the positions of wild-type (middle), ATG1 mutant (bottom), and ATG24 mutant (top) fumarases.

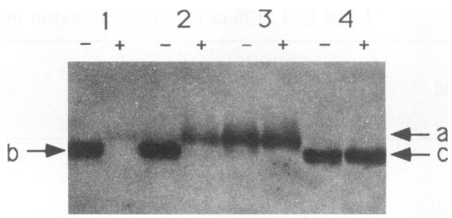

FIG. 4. Inhibition of import into mitochondria by CCCP blocks the processing of fumarase. Yeast cells harboring plasmids YEp51 (lanes 1), pFT2 (lanes 2), pIL-ATG24 (lanes 3), and pIL-ATG1 (lanes 4) were induced in galactose medium and labeled with [^{35}S]methionine for 10 min, either in the presence (+) or in the absence (−) of 20 μM CCCP. Total cell extracts were prepared, and fumarase was then immunoprecipitated from each sample and analyzed by SDS–7.5% PAGE. Arrows show the positions of the fumarase precursor and the ATG24 mutant fumarase (a) and mature fumarase (b) and the ATG1 mutant fumarase (c).

strains are haploid and are derived from strains used for research. It is possible, but not very likely, that these different strains include differences with respect to fumarase isoenzyme molecular weights. (iii) We also detected in initial studies two immunoprecipitated or immunoblotted fumarase-like protein species. We soon realized that their appearance depended on the concentrations of reducing agents in the SDS-PAGE-analyzed aliquots. In our initial experiments, samples contained 2.5% β-mercaptoethanol (rather than 5% β-mercaptoethanol and 10 mM dithiothreitol, as in Fig. 3), and wild-type fumarase appeared on gels as two separate bands identical in size to the single bands of fully oxidized and reduced forms of the protein (data not shown). It is therefore possible that under conditions of incomplete reduction of fumarase, there may be confusion with respect to isoenzyme molecular masses.

Fumarase (cytoplasmic and mitochondrial) is processed in mitochondria. As described above, the amino terminus of fumarase encodes a mitochondrial targeting signal. In the vast majority of cases, such amino-terminal sequences are removed by a specific metalloprotease located in the mitochondrial matrix (13, 43). Our finding of a single fumarase species according to SDS-PAGE mobility prompts the question of whether mitochondrially targeted fumarase is processed by the matrix protease. The approach that we have taken is to block the processing of mitochondrial precursors in vivo and then examine the sizes of fumarase molecules in the cell. In one type of experiment, the import of mitochondrial precursors into the mitochondria was blocked, thereby making them unavailable for cleavage by the matrix protease. In the second type of experiment, the activity of the matrix protease was directly inhibited via a specific conditional mutation in the protease.

To examine the sizes of fumarase molecules in yeast cells following a block of protein import into mitochondria, galactose-induced cells were treated with CCCP. CCCP, a proton ionophore, dissipates the mitochondrial membrane potential which is required for the translocation of precursors into mitochondria (26). When cells expressing wild-type fumarase (from plasmid pFT2) are treated with CCCP and then labeled, they produce a higher-molecular-weight fumarase (Fig. 4, lane 2+, band a) than untreated cells (lane 2−, band b). This is also true for cells expressing fumarase from the chromosomal gene (lanes 1+ and 1−). This result is extremely surprising, since we cannot detect any of the lower-molecular-weight wild-type mature fumarase (band b) upon treatment with CCCP. The data are consistent with the notion that all fumarase molecules are processed in mitochondria, even though the majority of the

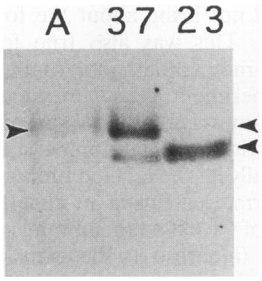

FIG. 5. A mutation in the *MAS1* gene blocks the processing of fumarase. *S. cerevisiae* AH 216 carrying a ts mutation in the *MAS1* gene was grown at 23°C in YPD medium to an OD_{600} of 2 to 3. The cells were washed and resuspended in SD medium containing glucose. The culture was divided and preincubated for 1 h, at either 37 or 23°C. The cells were labeled with [^{35}S]methionine for 5 min and analyzed by PAGE as described in the legend to Fig. 2. Lane 23 shows cells labeled at 23°C, lane 37 shows cells labeled at 37°C, and lane A shows cells harboring pIL-ATG24 and labeled at 30°C.

enzyme (80 to 90%) ends up in the cytoplasm. Also consistent with this course of events is the behavior of the initiation codon mutants. As expected, pIL-ATG1, which encodes fumarase that is not targeted to mitochondria because of the lack of a targeting sequence (see above), is not affected by CCCP treatment. The size of the ATG1 mutant fumarase remains the same in the presence or absence of CCCP (lanes 4+ and 4−, band c).

The size of the ATG24 mutant fumarase is also not affected by treatment of yeast cells expressing the mutant enzyme with CCCP (Fig. 4, compare lanes 3− and 3+). In fact, the size of this mutant fumarase appears identical to that of the CCCP-treated (unprocessed) form of wild-type fumarase. Taken together, these results indicate that the ATG24 mutation blocks processing of the mutant fumarase. The notion of an unprocessed ATG24 fumarase may also explain why yeast cells expressing the mutant enzyme exhibit less fumarase activity than cells expressing the wild-type protein.

The matrix metalloprotease responsible for processing imported mitochondrial precursors consists of two nonidentical subunits encoded by the *MAS1* and *MAS2* genes (43). Yeast *mas1* or *mas2* ts mutants accumulate mitochondrial precursor proteins at the nonpermissive temperature. To examine the effect of inhibiting the matrix processing protease on fumarase, exponentially growing *mas1* ts mutant cells grown in glucose medium were shifted to the nonpermissive temperature (37°C) and then labeled with [^{35}S]methionine. Cell extracts were prepared, immunoprecipitated, and subjected to SDS-PAGE. It is important to note that these cells express fumarase from the chromosomal gene only. At the permissive temperature (Fig. 5, 23°C), all the fumarase in the cell is processed. In sharp contrast, more than 85% of the fumarase accumulates in precursor form at the nonpermissive temperature (Fig. 5, 37°C). As found for the treatment with CCCP, the sizes of the unprocessed wild-type fumarase and the product of the ATG24 mutant gene are the same (Fig. 5, compare lanes A and 37, top arrowhead). The low level of processing of the wild-type fumarase at the nonpermissive temperature (Fig. 5, lane 37, bottom arrowhead) can be attributed to leakiness of the mutation. On the basis of these results and the results obtained with CCCP (see above), we conclude that both mitochondrial and cytosolic fumarases in yeast cells are processed by the matrix protease in mitochondria.

Cotranslational translocation of fumarase into mitochondria. To monitor the fate of the fumarase precursor as a function of time, pulse-labeling and pulse-chase experiments were performed. Induced exponentially growing yeast cells were labeled for 1 min with [^{35}S]methionine and then chased with cold methionine for various times. As shown in Fig. 6A, the fumarase precursor could be identified after a 1-min pulse (pFT2, 1 min, band a); however, the vast majority of fumarase appeared to be processed already (band b). At the following times, the small amount of precursor remaining was chased into the mature form. As expected, the size of the ATG1 mutant fumarase did not change (Fig. 6A, pIL-ATG1, band e), since this enzyme does not contain a signal peptide and therefore is not processed. The size of the ATG24 mutant fumarase did not change during the first 10 min of the pulse-chase (Fig. 6A, pIL-ATG24, band c), and only after 30 min could a very small amount of processed ATG24 fumarase be detected (band d). The size of this processed ATG24 form was identical to that of processed wild-type fumarase (Fig. 6A, compare band b with band d), whereas the unprocessed ATG24 form was identical in size to the wild-type precursor (Fig. 6A, compare band a with band c). This result indicates that the processing of the ATG24 mutant fumarase is very inefficient. The fact that nearly all the ATG24 mutant fuma-

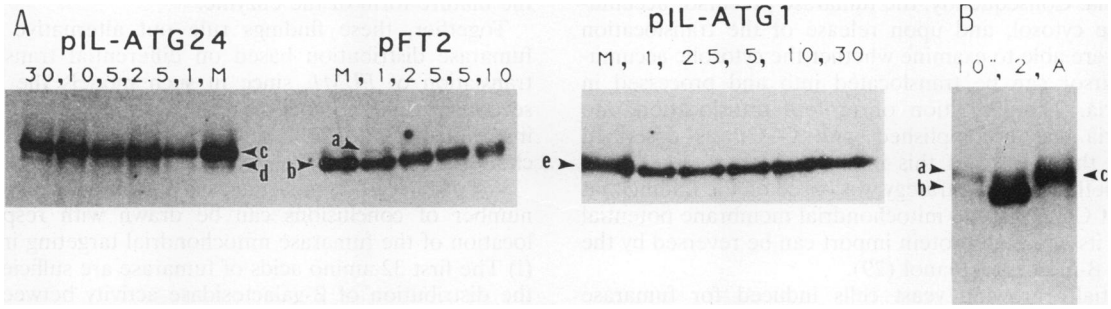

FIG. 6. Pulse-chase labeling of wild-type and mutant fumarases. (A) Induced cultures of yeast strains harboring plasmids pIL-ATG24, pFT2, and pIL-ATG1 were pulse-labeled for 1 min (lane 1 in each case), and cold methionine was added to initiate the chase. Aliquots were taken at 2.5, 5, 10, and 30 min from the start of the labeling procedure (as indicated at the top of each lane). Cell extracts were prepared and analyzed as described in the legend to Fig. 2. Arrowheads show the positions of the fumarase precursor (a) and mature (b) forms, the ATG24 mutant fumarase precursor (c) and mature (d) forms, and the ATG1 mutant fumarase (e). Lanes M contained metabolically labeled fumarases from the different strains. (B) Cultures of DMM1-15A harboring pFT2 were induced in galactose medium. Cells were pulse-labeled for 10 s (lane 10″) and chased for 2 min (lane 2) with cold methionine and cycloheximide (0.1 mg/ml). Samples were treated and analyzed as described above. Lane A shows the ATG24 mutant fumarase. Arrowheads are as in panel A.

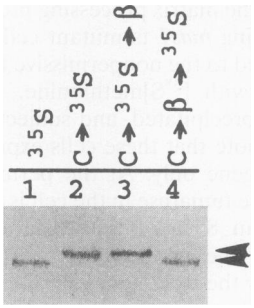

FIG. 7. The effect of CCCP on precursor accumulation is not reversed by β-mercaptoethanol. Induced cultures of yeast cells harboring plasmid pFT2 were (i) labeled with [^{35}S]methionine (lane 1); (ii) labeled with [^{35}S]methionine in the presence of CCCP (lane 2); (iii) labeled with [^{35}S]methionine in the presence of CCCP and then incubated for 10 min in the presence of β-mercaptoethanol (lane 3); and (iv) incubated for 10 min with CCCP, incubated for 10 min with β-mercaptoethanol, and then labeled with [^{35}S]methionine (lane 4). Arrowheads show the positions of unprocessed (top) and processed (bottom) fumarases.

rase retains the mitochondrial targeting signal can explain why cells expressing this protein produce a relatively lower level of fumarase activity (Table 1) even though they do not appear to produce less fumarase protein than cells expressing the wild-type enzyme (data not shown).

The finding that after a 1-min pulse of [^{35}S]methionine most of the wild-type fumarase in the cell was already processed suggests that removal of the targeting signal of fumarase in yeast cells occurs shortly after the synthesis of the protein. To examine the extent of this phenomenon, yeast cells expressing wild-type fumarase (from plasmid pFT2) were induced in galactose medium and heavily labeled for 10 s. Azide, cycloheximide, and cold methionine were simultaneously added to the culture, and cell extracts were immediately prepared. Even following this extremely short pulse, about 40% of the labeled protein was processed (Fig. 6B, lane 10 min), suggesting that fumarase is most probably cotranslationally translocated and possibly also cotranslationally processed.

To directly examine whether fumarase can be posttranslationally translocated into mitochondria, we employed a previously described approach (12) in which we allowed the translation of fumarase but blocked its translocation into mitochondria. Consequently, the fumarase precursor accumulated in the cytosol, and upon release of the translocation block, we were able to examine whether the cytosolic accumulated precursor can be translocated into and processed in mitochondria. The inhibition of protein translocation into mitochondria was accomplished with CCCP as described above, and the release of this inhibition was obtained with β-mercaptoethanol. This strategy was based on the finding that the effect of CCCP on the mitochondrial membrane potential in vivo and its effect on protein import can be reversed by the addition of β-mercaptoethanol (29).

Exponentially growing yeast cells induced for fumarase expression (from plasmid pFT2) were labeled in the presence of CCCP for 10 min. β-Mercaptoethanol and the translation inhibitor cycloheximide were added, and the cultures were incubated for an additional 10 min. As shown in Fig. 7, treatment with CCCP resulted in the production of the unprocessed precursor form of fumarase, which is larger than the processed form of the protein (compare lanes 2 and 1). Incubation of these CCCP-treated cells with β-mercaptoethanol for 10 min did not bring about the formation of mature fumarase (lane 3). This was also true for longer times of incubation with β-mercaptoethanol (data not shown). As a control for this experiment, cells were treated with CCCP for 10 min and with β-mercaptoethanol for an additional 10 min and then labeled with [^{35}S]methionine (lane 4). Under such conditions, essentially all the labeled fumarase synthesized was in a processed form, reassuring us that β-mercaptoethanol reverses the effect of CCCP on membrane potential and allows newly synthesized fumarase to be translocated. Taken together, these results indicate that the fumarase precursor is not posttranslationally imported into mitochondria.

DISCUSSION

This study uncovered a unique mechanism of protein subcellular localization and distribution in eukaryotes. The first aspect of this mechanism is that the same initial translation products of the *FUM1* gene are distributed between the cytosol and the mitochondria. Thus, the decision as to the final destination of fumarase is posttranslational. The understanding that all fumarase molecules are translated from a single methionine (ATG) codon was derived from several lines of evidence. (i) Elimination of the first ATG codon from the 5′ end of the *FUM1* gene results in the translation (from the second ATG codon) of a small product which cannot be detected under any conditions in wild-type cells and therefore is not a natural product of the yeast cell. The only reason that this codon is used for translation initiation in this mutant is the lack of the first natural methionine codon. (ii) A mutant fumarase gene lacking the second methionine codon (replaced by a valine) encodes fumarase, which is distributed in a manner similar to that of the wild-type enzyme between the cytosol and the mitochondria. Thus, a *FUM1* gene without the second ATG directs the production of fumarase which is correctly localized in yeast subcellular compartments. (iii) A frameshift mutation between ATG1 and ATG24 that disturbs the frame of translation initiating at ATG1 but not that initiating at ATG24 blocks fumarase expression from ATG24. Thus, translation initiation from ATG24 will not occur when the ATG1 codon is present, as one would expect according to the scanning model of translation initiation (44). (iv) Mature fumarase in *S. cerevisiae* appears to be a singly sized protein species, and upon blockage of processing, all fumarase molecules appear only in precursor form. This result is consistent with the processing of a single fumarase precursor species to the mature form of the enzyme.

Together, these findings rule out alternative models of fumarase distribution based on differential transcription or translation of *FUM1*, since in such models the use of the second methionine codon (ATG24) is crucial for translation initiation of a cytosolic fumarase product lacking the mitochondrial targeting sequence (see above).

With fumarase–β-galactosidase protein fusion technology, a number of conclusions can be drawn with respect to the location of the fumarase mitochondrial targeting information. (i) The first 32 amino acids of fumarase are sufficient to allow the distribution of β-galactosidase activity between cytosolic and mitochondrial fractions. This distribution is similar to that of wild-type fumarase (27). (ii) The first 17 amino acids are vital for mitochondrial targeting; however, they constitute only part of the signal, since even though this sequence by itself targets β-galactosidase to mitochondria, targeting is fivefold less efficient than that with the 32-amino-acid sequence.

A comparison between the fumarase mitochondrial targeting sequence and sequences of other mitochondrial proteins

predicts no features of either composition or structure (9, 30, 38) that can explain the relatively low level of fumarase import into mitochondria. Like other mitochondrial signals, the fumarase targeting sequence is enriched for basic, hydrophobic, and hydroxylated amino acid residues. Also, the calculated hydrophobic moment and maximal hydrophobicity parameters (which are a measure of predicted helical amphiphilicity) of the fumarase presequence are similar to those of many other mitochondrial sequences.

According to the analysis described above, the single translation product of *FUM1* is predicted to be efficiently targeted to mitochondria. Actually, the most striking feature of fumarase localization in *S. cerevisiae* is, in fact, the conclusion that all the fumarase molecules synthesized in the cell are targeted and processed by the matrix protease in mitochondria, even though most of the fumarase ends up in the cytosol. This interpretation is based on two types of independent experiments designed to block the processing of fumarase precursors: destruction of the membrane potential of mitochondria, which is required for the translocation of proteins into the organelle, thereby rendering these precursors unavailable to the matrix protease, and inhibition of the mitochondrial matrix processing activity via a ts mutation in the protease. In addition, mature fumarase isoenzymes in the mitochondria and cytoplasm are identical in size, consistent with the notion that the single *FUM1* translation product made in the cell is processed only in mitochondria.

Since both mitochondrial and cytoplasmic fumarases are processed in mitochondria, the question is (i) whether the fumarase molecules are only partially translocated across the mitochondrial membranes, thereby allowing the matrix protease to cleave their amino termini inserted through the mitochondrial inner membrane, or (ii) whether the fumarase molecules are fully translocated and processed in the matrix of mitochondria, with the majority of the molecules then being exported back into the cytosol. This dilemma is reminiscent of the unresolved question dealing with the sorting of cytochrome c_1 and cytochrome b_2 to the intermembrane space of mitochondria in yeast cells (15, 22). The presequences of these cytochromes are cleaved in two steps: the matrix targeting sequence is removed by the matrix processing enzyme, and the intermembrane hydrophobic sorting sequence is removed by a different protease located at the outer face of the inner membrane. With respect to fumarase, we cannot detect an intermediately sized protein species, implying that fumarase is cleaved only once. In addition, there is no significant stretch of hydrophobic amino acid residues following or in the vicinity of the fumarase matrix targeting sequence that can be considered a stop-transfer or bacterium-like signal sequence. Hence, in contrast to cytochrome c_1 and b_2 intermembrane localization, cytoplasmically located fumarase apparently follows a different pathway. The lack of a cleavable hydrophobic signal sequence following the fumarase mitochondrial targeting sequence suggests that fumarase is most probably not imported and then exported, even though this possibility cannot be ruled out.

It was recently demonstrated that many mitochondrially targeted protein precursors are cotranslationally translocated across membranes into mitochondria (12). Our finding that even after a very short pulse-label (10 s) of yeast cells more than 40% of the newly synthesized fumarase is already processed and the fact that fumarase precursors accumulating in the cytoplasm cannot be translocated suggest that this protein is cotranslationally translocated and processed. The most simple explanation is that fumarase precursors accumulating in the cytosol fold into a conformation that is noncompatible with protein translocation, whereas under normal conditions the coupling of translation with translocation prohibits the formation of such fumarase precursor conformations in the cytosol prior to processing.

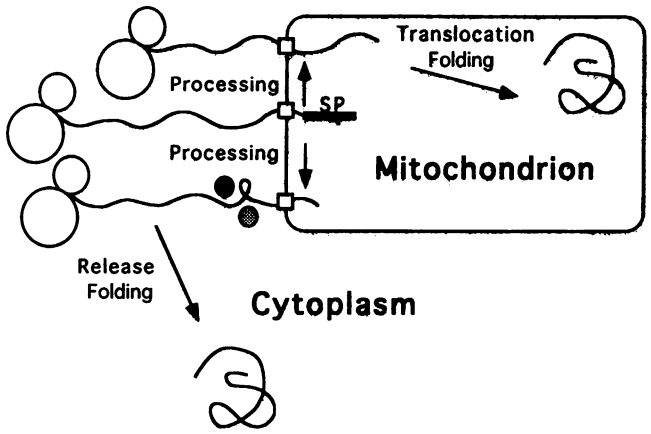

FIG. 8. Proposed mechanism of fumarase subcellular distribution in *S. cerevisiae*. The amino termini of initially identical translation products of the *FUM1* gene are cotranslationally translocated and processed in mitochondria by the matrix protease. The minority of these fumarase molecules are fully translocated into mitochondria, whereas the majority are released from the organelle into the cytosol. It is possible that differentiation between full translocation and release from the organelle is determined by the interaction of fumarase with different molecular chaperones (stippled circle), modification enzymes (solid circle), or mitochondrial receptors (open boxes). SP, mitochondrial signal peptide.

On the basis of this study, Fig. 8 presents a model describing the possible chain of events in the subcellular distribution of fumarase by use of partial translocation of fumarase as the scenario. A single translation product of the *FUM1* gene is cotranslationally partially translocated into mitochondria and cleaved by the matrix protease. A subset of the molecules are fully imported, whereas the rest of the protein is folded or situated in an import-incompetent state and released from the organelle. The proposed release from mitochondria can be explained by retrograde movement of the protein in the translocation pore back across mitochondrial membranes. Folding of the initial translation product and retrograde movement of fumarase may be determined and possibly regulated by the activity of molecular chaperones (Fig. 8, stippled circle), by the interaction of fumarase precursors with different receptors on the mitochondrial organelle surface (open boxes), by the modification of fumarase by specific enzymes (e.g., phosphorylation, acylation, or methylation; solid circle), or by pausing of the translating ribosome, which would then cause the partially translated fumarase to move backwards. The possibility of retrograde movement through the endoplasmic reticulum membrane was recently demonstrated in an in vitro system (10). Thus, the behavior of fumarase may be a naturally occurring example of this phenomenon. Future experiments will have to include cell-free translocation assays of fumarase and an investigation of the interaction of this protein with molecular chaperones, mitochondrial receptors, and modification systems in vivo and in vitro.

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