Mitochondrial and Cytosolic Isoforms of Yeast Fumarase Are Derivatives of a Single Translation Product and Have Identical Amino Termi

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Ehud Sass, Eran Blachinsky, 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. Alternative models for fumarase distribution have been proposed that require more than one translation product. In the current work (i) we show by using sequential Edman degradation and mass spectrometry that fumarase cytosolic and mitochondrial isoenzymes have an identical amino terminus that is formed by cleavage by the mitochondrial processing peptidase, (ii) we have generated fumarase mutants in which the second potential translation initiation codon (Met-24) has been substituted, yet the protein is processed efficiently and retains its ability to be distributed between the cytosol and mitochondria, and (iii) we show that although a signal peptide is required for fumarase targeting to mitochondria the specific fumarase signal peptide and the sequence immediately downstream to the cleavage site are not required for the dual distribution phenomenon. Our results are discussed in light of our model of fumarase targeting and distribution that suggests rapid folding into an import-incompetent state and retrograde movement of the processed protein back to the cytosol through the translocation pore.

Dual targeting of a protein encoded by a single gene to different subcellular locations has been shown to occur by a number of mechanisms. There is a wealth of reports on situations where a single gene gives rise to a number of translation products that differ in the targeting information they bear, e.g. a signal sequence or lack of such a signal. This has been shown to be attained by multiple transcription initiation sites (1), by multiple translation initiation sites (2, 3), and more recently by splicing out of such signals (4–6). On the other hand there are only a limited number of examples in which a single translation product has been shown to be distributed between two subcellular locations (7–11). The molecular mechanisms underlying these situations have not been fully elucidated. Dual targeting of cytochrome P450oxidB1 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 endoplasmic reticulum targeting signal. The NADH-cytochrome b oxidoreductase (Mcr1p) is sorted to the outer mitochondrial membrane or the mitochondrial intermembrane space in yeast due to what appears to be an incomplete translocation through the outer membrane.

Cytosolic and mitochondrial fumarase isoenzymes are encoded by the same gene (FUM1) in Saccharomyces cerevisiae (11). We have previously suggested that these proteins follow a unique mechanism of subcellular localization and distribution. There is only one translation product of FUM1, which is targeted to mitochondria by an amino-terminal presequence and which is then removed by the mitochondrial processing peptidase (MPP) (7). This notion is based on previous work (7): (i) the appearance of single precursor or mature fumarase bands on SDS-polyacrylamide gels and (ii) mutagenesis of potential translation initiation codons. Our working model proposes that a subset of the processed fumarase molecules are fully imported into the matrix, whereas the majority (~70%) are partially translocated so that their amino termini become accessible to MPP. These latter molecules are released back into the cytosol as soluble active enzyme by retrograde movement through the translocation pore (13). Another unique feature of fumarase is that in vivo its translocation into the mitochondrial matrix occurs only while it is being translated and in vitro it requires the presence of mitochondria during translation (7, 13).

Alternative models for fumarase distribution have been proposed. Wu and colleagues (12, 14) detected in S. cerevisiae a number of RNA transcripts of the FUM1 gene. They suggested that these transcripts encode two fumarase products, one harboring a mitochondrial signal peptide and the other lacking this sequence, thereby encoding a cytosolic protein. In rat liver Tuboi and colleagues (15) proposed that the transcript of the single fumarase gene can be translated from two in-frame AUGs thereby giving rise to two products, one harboring and one lacking a mitochondrial signal peptide. Importantly both of the alternative models of distribution predict the existence of two fumarase primary translation products.

Since the amino termini of fumarase isoenzymes, according to our model and the alternative models above, are predicted to differ, we have made an effort to characterize the fumarase amino terminus and determine its role in fumarase processing and subcellular distribution. In this report we have determined the MPP cleavage site of fumarase and fumarase mutants in vivo. We conclude that for wild type fumarase this cleavage site

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† To whom correspondence should be addressed. Tel.: 972-2-6757203; Fax: 972-2-6758918; E-mail: ophry@md.huj.ac.il.
‡ To whom correspondence should be addressed. Tel.: 972-2-6757203; Fax: 972-2-6758918; E-mail: ophry@md.huj.ac.il.

The abbreviations used are: MPP, mitochondrial processing peptidase; CCCP, carbonyl cyanide p-chlorophenylhydrazone; PAGE, polyacrylamide gel electrophoresis; BNPS, 3-bromo-3-methyl-2-(2-nitrophenoxy)indole; MALDI-TOF, matrix-assisted laser-desorption time-of-flight; MS, mass spectrometry; HPLC, high pressure liquid chromatography.
determines the single amino terminus for all fumarase molecules in mitochondria and the cytosol, thus providing evidence for our single translation product model. In addition, we found 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.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids—* The *S. cerevisiae* strain used was DMM1-15A (lei2 ura3 ade2 his3) (7). Strains harboring the appropriate plasmids were grown overnight at 30°C in SD (synthetic depleted) medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose or galactose (w/v) supplemented with the appropriate amino acids (50 µg/ml). Plasmids pFT2 (pFUM), pFUM24V (pLIATG24), and pFSE24 (pFUM31G33G) are described elsewhere (7, 16).

**Activity and Processing of Fumarase Mutants—** The fumarase amino terminus contains the mitochondrial targeting signal, and the deletion of the signal peptide results, as expected, in the release of the fumarase primary, and the deletion of the signal peptide results, as expected, in the release of the fumarase primary.

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<table>
<thead>
<tr>
<th>Protein</th>
<th>Variation</th>
<th>Predicted precursor protein sequence</th>
<th>Specific activity</th>
<th>Percentage of GAL10-FUM1</th>
</tr>
</thead>
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<td>100</td>
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<tr>
<td>FumΔSP</td>
<td>ΔAs(1–23)</td>
<td>Met. Met. Met. Asn.</td>
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<tr>
<td>Fum24S25F</td>
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<td>Met. Ser-Phe</td>
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<td>Fum24S</td>
<td>Ser-24</td>
<td>Met. Ser-Asn</td>
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<tr>
<td>Fum24I</td>
<td>Ile-24</td>
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<tr>
<td>Fum24V</td>
<td>Val-24</td>
<td>Met. Val-Asn</td>
<td>1.9</td>
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<td>Chromosomal Fum1</td>
<td>Wild type</td>
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<td>0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

*aa, amino acids.*

**Table I** Activity of mutant fumarases in yeast cell extracts

For matrix-assisted laser-desorption time-of-flight (MALDI-TOF), peptides were deposited on a metal target as co-crystals with α-cyano-4-hydroxycinnamic acid (Aldrich). The mass spectrometry analysis was done using MALDI-TOF (2E, Micromass UK) in the positive ion mode.

For electrospray ionization-mass spectrometry (MS) the peptides were resolved by reverse-phase chromatography on a 1 × 150-mm Vydac C-18 column. The peptides were separated by a linear gradient of 4–65% acetonitrile in 0.25% trifluoroacetic acid A at 1%/min and a flow rate of 40 µl/min. The liquid from the column was electrospayed into an ion-trap mass spectrometer (LCQ, Finnigan, San Jose, CA).

**Fumarase derivatives were cleaved by adding 20 µl of the purified protein sample to 80 µl of BNPS-skatole (1.3 mg/ml of acetic acid), and the solution was incubated at 47°C for 1 h (23, 24).** The reaction was stopped by precipitation with 10% trichloroacetic acid, and the precipitate was washed three times with acetone and dried.

For mass spectrometry, dissociation of the most dominant ion selected from the first MS scan. The mass spectrometry data was compared with simulated proteolysis and collision-induced dissociation of the proteins in the "genepet" using the Sequest software (J. Eng and J. Yates, both from Washington and Finnigan, San Jose, CA).

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valine (Fum24V) or isoleucine (Fum24I), the specific activity was only about 10–15% of wild type fumarase.

The reason for this low activity of Fum24I and Fum24V became apparent when the processing of these derivatives was examined. Cultures of yeast expressing the appropriate proteins were induced in galactose medium and labeled with [35S]methionine in the absence or presence of CCCP (proton ionophore). Existence of the mitochondrial membrane potential is required for fumarase mitochondrial import, and accordingly this ionophore blocks processing by MPP (26). In the absence and presence of CCCP fumarase appears as a lower and a higher molecular weight band corresponding to mature (m) and unprocessed precursor (p) fumarases, respectively (Fig. 1B, compare WT – and + CCCP). The size of FumΔSP does not change upon treatment with CCCP, and the same is true for Fum24V and Fum24I (Fig. 1B, compare – and + CCCP of ΔSP, 24V, and 24I, respectively). The interpretation of the results is that these proteins are not processed due to the lack of a signal peptide (FumΔSP, lower molecular weight band, bottom arrow) or the lack of a MPP cleavage site (Fum24V and Fum24I, higher molecular weight bands, top arrow).

To be able to draw firm conclusions as to the role of the second potential initiation codon in fumarase distribution it was important to eliminate the Met-24 codon without destroying the MPP cleavage site (as occurs in the case of Fum24I and Fum24V). In examining known MPP cleavage sites of mitochondrial proteins, that of rat malate dehydrogenase I (MdhIp) turned out to be the most useful for planning fumarase mutations (27). By substituting fumarase Met-24 and Asn-25 with serine and phenylalanine, respectively, we essentially constructed the MdhIp MPP site within the fumarase precursor sequence (see illustration in Fig. 1A). This fumarase mutant and a mutant with only the Met-24 to serine substitution exhibited more than 85% of the wild type enzyme activity in cell extracts (Table I). More important was the finding that both mutants were processed efficiently as shown by labeling experiments with and without CCCP (Fig. 1B, compare lanes – and + CCCP of Fum24S25F and Fum24S). Fum24V and Fum24I remain unprocessed and exhibit low specific activities, whereas wild type and mutant enzymes Fum2452SF and Fum24S, which are processed efficiently, exhibit high specific activities (Table I). This indicates that the amino-terminal signal peptide is inhibitory to fumarase activity as has been shown for many other precursor proteins.

**Subcellular Distribution of Fumarase Mutant Proteins in Yeast Cells**—To examine the distribution of mutant fumarases, induced cells expressing these proteins were subjected to subcellular fractionation. The distribution of enzymatic activity is shown in Fig. 2A, and the distribution of fumarase derivatives as detected by Western blot analysis is shown in Fig. 2B. As previously reported (7, 12) about 70% of the fumarase activity (Fig. 2A) and protein (Fig. 2B) is localized in mitochondria, whereas fumarase lacking the signal peptide is found exclusively in the cytosol. For the enzymatic activity, mitochondrial citrate synthase and cytosolic glucose-6-phosphate dehydrogenase were routinely used to determine cross-contamination of fractions, and mitochondrial Hsp60 served as our control for Western analysis (Fig. 2B). For fumarase mutants defective for processing (Fum24I and Fum24V), the majority of the activity and protein was detected in the cytosolic fraction even though a reproducible small amount (about 8–10%) is fully imported into mitochondria. Fumarase mutants that are processed even though Met-24 is eliminated (Fum24S25F and Fum24S) are distributed in the cell with about 50–60% in mitochondria. These results indicate that although substitution of Met-24 can cause a significant change in the distribution of fumarase in the cell it does not eliminate the dual targeting phenomenon itself.

**Sequential Edman Degradation of Fumarase**—To characterize fumarase products we constructed genes encoding histidinyl-tagged fumarase derivatives. Fum-6h was essentially identical to the nontagged form in its specific activity, processing by MPP, tetramerization, and distribution in the cell (30). Fum-6h and tagged versions of fumarase derivatives, FumΔSP-6h, FumS24F25-6h, FumI24-6h, were purified from yeast cell extracts by affinity chromatography. As shown in Fig. 3A as with the nontagged versions FumΔSP-6h migrated faster and FumI24-6h migrated slower than Fum-6h on SDS-PAGE. The purified proteins were subjected to sequential Edman degradation, and the results are summarized in Fig. 3B. Mature fumarase whose sequence starts with asparagine is cleaved between amino acids Met-24 and Asn-25. If Met-24 and Asn-25 are exchanged for Ser-24 and Asn-25 the derivative protein is cleaved efficiently by MPP at the corresponding site between Ser-24 and Phe-25.

For FumI24-6h and FumΔSP-6h, which are not processed by MPP, the methionine from which translation was initiated is
expected to remain at the amino terminus of the protein. These two proteins even at high concentrations were resistant to Edman degradation and did not provide any sequence information. The interpretation of these results is that these proteins have a modified amino-terminal amino acid. The modification of these polypeptides is not surprising, and in fact the fumarase precursor has been predicted to be N-acetylated by the N-acetyltransferase Mak3 on the basis of its amino-terminal sequence (MLRF, Refs. 28 and 29). The FumASP amino terminus (MNSS) is most probably N-acetylated by the N-acetyltransferase Nat3, which has been shown to N-acetylate among other sequences MNNS and MNFL of CYC-872 and CYC-849, respectively (29). This blocking raises the theoretical possibility that strains expressing Fum-6h may express, in addition to processed Fum-6h, a product whose translation started from Met-24, which would be blocked and undetected by Edman degradation.

**Mass Spectrometry of Amino- and Carboxyl-terminal Peptides Generated by BNPS-Skatole—**To rule out this possibility of a blocked fumarase species and to fully identify the products, we subjected Fum-6h products to mass spectrometry analysis. The strategy for identification of fumarase products in the yeast cell was to use MALDI-TOF MS as a tool for examining short peptides from the termini of fumarase. Unmutated Fum and FumASP both tagged with six histidines were analyzed. The reagent BNPS-skatole was chosen for this analysis since it responds to that of the amino acid methionine (131 Da), while the lower mass from FumSP (2714 Da) is most probably the terminal tryptophan of the cleaved peptide, which is a known modification caused by the BNPS-skatole treatment (23, 24).

The mass spectra of the peptides generated by BNPS-skatole are characteristic of that of the amino acid methionine and are double and triple ions of this peptide, and shown in panels a and c of Fig. 4B. As shown in panels a and c of Fig. 4B and as predicted for Fum and FumASP, a single fragment corresponding to the carboxyl-terminal 16 amino acids of these proteins is identical with a molecular mass of 1945 Da (includes a 16-Da addition due to oxidation of the single methionine). In contrast the amino-terminal fragments of Fum and FumASP differ showing molecular masses corresponding to 22 amino acids (2584 Da) and 23 amino acids (2775 Da), respectively (panels b and d of Fig. 4B, respectively). The Fum 22-amino acid fragment includes a 14-Da addition due to formation of an oxolactone by oxidative halogenation on the terminal tryptophan of the cleaved peptide, which is a known modification caused by the BNPS-skatole treatment (23, 24). The FumASP 23-amino acid fragment includes this same 14-Da modification, a 16-Da addition due to acetylation of the amino-terminal methionine, and a 42-Da addition due to acetylation of the amino-terminal methionine. The difference in mass between Fum and FumASP after taking the modifications into consideration corresponds to that of the amino acid methionine (131 Da), while the lower mass from FumASP (2714 Da) is most probably the result of different undetermined modifications.

To fully characterize the amino-terminal peptide of Fum-6h (2586 Da) this product was identified and analyzed by electrospray mass spectrometry, and fragmentation of this peptide was undertaken by collision-induced dissociation. Shown in Fig. 5A are double and triple ions of this peptide, and shown in Fig. 5B are characteristic internal fragment ions confirming the identity of the peptide. These analyses indicate that the wild type yeast produces fumarase molecules with a single amino-terminal sequence and distributes them within the cell. This amino terminus of Fum is determined by the MPP cleavage of the fumarase precursor between amino acids Met-24 and Asn-25 (Figs. 3B and 4A).
to be cotranslational in vivo and in vitro (7, 13). Although Met-24 is not required for fumarase distribution certain substitutions of the 24th amino acid residue appear to have significant effects on distribution; thus, it seemed reasonable that secondary targeting information may be found within the amino terminus of fumarase. The first element we examined was the fumarase mitochondrial targeting sequence. We fused the cytochrome b$_2$ (Cyb2p) mitochondrial targeting signal to mature fumarase. For this a DNA sequence encoding 39 amino acids (which includes the Cyb2 signal peptide and its MPP cleavage site) was fused to a DNA sequence encoding Fum1 starting from amino acid Asn-25 (Fig. 6A). This hybrid Cyb2-Fum exhibited more than 90% of the wild type Fum specific enzymatic activity (Fig. 6C, compare − and + CCCP). As shown in Fig. 6, subcellular fractionation experiments reveal that about 60% of the Cyb2-Fum enzymatic activity (Fig. 6C) and protein (Fig. 6D) are fully localized to mitochondria, and the rest are cytosolic, a pattern that is reminiscent of Fum24S25F and Fum24S. These results indicate that although a signal peptide is crucial for mitochondrial targeting, the specific fumarase-targeting signal is not crucial for maintaining the fumarase dual targeting phenomenon.

Many secondary targeting signals appear in the protein sequence immediately following the amino-terminal primary signal of mitochondrial proteins. For example, the c$_1$ and b$_2$ cytochromes in yeast contain a mitochondrial intermembrane space hydrophobic-sorting sequence immediately following the amino-terminal matrix targeting sequence. The fumarase signal sequence is followed by a highly charged and conserved sequence [RTETDFAEIHVPADK]. In fact we had speculated that this sequence may weaken the interaction of the translocating protein with mHsp70 and thereby delay completion of the import process. We have examined this by specific substitutions and deletions of various portions of this charged sequence. We have constructed three deletions within the fumarase open reading frame which remove 9, 11, and 13 codons from the sequence following the MPP cleavage site (Δ25–37, Δ27–37, and Δ29–37). As shown in Fig. 7A these deletions include the first four charged amino acids following the cleavage site. In another construct, the acidic amino acids Glu-31 and Asp-33 were substituted by glycines. All of these mutant proteins were detected in yeast extracts of induced cells by Western blot analysis but were devoid of fumarase enzymatic activity. FumΔ29–37 (∆9) is processed efficiently as detected by labeling experiments in the presence and absence of CCCP (Fig. 7B, compare − and + CCCP). The same is true for Fum31G33G (not shown). In contrast, FumΔ25–37 (∆13) shows a significant defect in processing (Fig. 7B, compare − and + CCCP).
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C CCCP of Δ13), which is not very surprising since in this mutant Asn-25 is replaced by isoleucine at the MPP cleavage site. FumΔ27–37 displays a very minor defect in processing, which is not always detected (not shown). As shown in Fig. 7C, subcellular fractionation experiments reveal that more of the FumΔ29–37 (Δ9) than the wild type molecules are fully imported into mitochondria (60% compared with 30%, respectively). In contrast for FumΔ25–37 (Δ13) only a small portion of the molecules is fully imported (Fig. 7C), which is similar to the situation with other mutant fumarases, which show a defect in processing. Taken together these results strongly suggest that the 11-amino acid sequence immediately following the MPP cleavage site is not crucial for fulfillment of the fumarase dual targeting phenomenon.

DISCUSSION

Our working model is that the FUM1 gene directs the expression of a single translation product. In the present study full identification of fumarase products with respect to their amino-terminal sequences was achieved. Wild type fumarase is cleaved by MPP between Met-24 and Asn-25, whereas a mutant fumarase lacking the Met-1 codon and a signal peptide initiates translation at the Met-24 codon and retains methionine at its amino terminus. Edman degradation followed by mass spectrometry shows that all the fumarase molecules in the yeast cell have an identical amino terminus starting with Asn-25 without traces of the Met-24 variant. Since this fumarase distributes normally between the cytosol and mitochondria in yeast, these results provide evidence for the single translation product hypothesis.

A second type of experiment to show the existence of a single fumarase translation product has used mutants in which the Met-24 codon was eliminated by mutagenesis. Stein et al. (7) and Wu et al. (14) have previously substituted Met-24 with valine and isoleucine, respectively. Wu et al. (14) claimed on the basis of enzyme activity alone that a Met-24 to isoleucine mutant is targeted exclusively to mitochondria, yet we find that most of the protein is located in the cytosol. As shown in this study both these mutant fumarases are not processed by MPP in vivo. Thus conclusions based on these mutants should be carefully reexamined. For example we have previously shown that unprocessed fumarase has a tendency to precipitate out of solution and in the case of Fum24I may have come out of solution with mitochondria upon centrifugation. Nevertheless, in this study we have successfully constructed and expressed other Met-24 mutants, Fum24S25F and Fum24S, which are processed in vivo by MPP. These mutant fumarases when expressed in yeast are distributed between the cytosol and mitochondria ruling out alternative distribution mechanisms that require the second Met-24 codon for translation initiation of a second fumarase product (see the Introduction) and support the single translation product model.

A third type of experiment supporting a single fumarase translation product was based on blocking processing by MPP. This was achieved either by directly inhibiting the MPP or by blocking fumarase import into mitochondria thereby making the precursor unavailable for cleavage by the protease (7). Our conclusions were based on detection of a single band on SDS-PAGE corresponding to mature fumarase in yeast cells and when fumarase processing is blocked detection of a single band corresponding to the precursor. These results hold true for fumarase expressed from the chromosome as well as from a plasmid. The same results are obtained with mutants described in this study and in particular with Fum24S25F and Fum24S, which are processed efficiently and distributed between the cytosol and mitochondria. In this regard, we have previously shown that expression of fumarase from the GAL10 promoter, which initiates transcription upstream to the first potential translation initiation codon, allows distribution of the enzyme in the cell similarly to the chromosomally expressed gene. This finding and the fact that a ASP-fumarase like protein cannot be detected in wild type yeast cells indicates that the minority of the shorter mRNA molecules detected by Wu and colleagues (12, 14) do not appear to direct the translation of fumarase (starting from Met-24). This appears to be true under the conditions of our experiments, yet such RNAs may be expressed in other circumstances. As pointed out above the scenario suggested by Tuboi (15) for rat fumarase in which translation can initiate from either of the two 5’ proximal methionine codons does not apply to yeast fumarase (Met-1 and Met-24) since a product starting from Met-24 cannot be detected.

Essential information required for fumarase distribution in the cell does not appear to reside within the first 37 amino acids of the precursor since (i) exchange of the fumarase mitochondrial targeting peptide for that of cytochrome b5 and (ii) deletion of amino acids immediately following the MPP cleavage site (through Glu-37) do not eliminate the fumarase dual targeting phenomenon. Thus, essential information required for fumarase distribution is expected to be found further downstream inside the mature protein sequence. This notion fits our working model that suggests that targeting and distribution involves rapid fumarase folding (outside mitochondria) into a conformation incompatible with further import, which in turn leads to retrograde movement of the processed protein back through the translocation pore. The hypothesis of retrograde movement of the fumarase single translation product is supported by previously published data in vivo and in vitro (13). The notion that folding outside mitochondria is an important factor in its final subcellular location is supported by the apparent rapid folding of fumarase in vitro (13) and other preliminary data. In this regard, experiments currently in progress show that a number of differently located deletions within the core of the fumarase subunit causes nearly full import of fumarase into mitochondria. In addition, overexpression of SSA1, a yeast cytosolic Hsp70 homolog, causes the localization of 2-fold more fumarase in mitochondria. It is interesting to note that the strongest effects of modifications of the amino terminus on fumarase distribution were mutations of the MPP cleavage site. While mutations in which the fumarase precursor is still efficiently processed (Fum24S25F and Fum24S) exhibit only a minor change in distribution, mutants defective in processing (Fum24I, Fum24V, and Fum24S37–37) are primarily targeted to the cytosol. These results can most easily be explained by assuming that noncleavage of the precursor may slow down import providing additional time for more of the polypeptide to fold outside mitochondria into an import-incompetent conformation. Future studies will have to determine whether posttranslational modifications occur in the downstream polypeptide sequence. Such posttranslational modifications and/or molecular chaperones may in fact affect the fumarase conformation and determine its distribution in the cell.

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