

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

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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 P4502B1 by two targeting signals to the endoplasmic reticulum and mitochondria is controlled by phosphorylation of the protein, which activates its mitochondrial tar-

geting signal and functionally inhibits its endoplasmic reticulum targeting signal. The NADH-cytochrome *b*₅ reductase (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* (12). 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)¹ (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 above 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.huji.ac.il.

¹ The abbreviations used are: MPP, mitochondrial processing peptidase; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; PAGE, polyacrylamide gel electrophoresis; BNPS, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole; MALDI-TOF, matrix-assisted laser-desorption time-of-flight; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

TABLE I
Activity of mutant fumarases in yeast cell extracts

Protein	Variation	Predicted precursor protein sequence			Specific activity ^a	Percentage of <i>GAL10-FUM1</i>
		1	24	25		
Fum1	Wild type	Met. . .	Met-Asn		15.2	100
FumΔSP	Δaa(1–23)		Met-Asn		15.9	105
Fum24S25F	Ser-24, Phe-25	Met. . .	Ser-Phe		13.0	85
Fum24S	Ser-24	Met. . .	Ser-Asn		14.9	98
Fum24I	Ile-24	Met. . .	Ile-Asn		2.4	16
Fum24V	Val-24	Met. . .	Val-Asn		1.9	13
Chromosomal Fum1	Wild type	Met. . .	Met-Asn		0.5	3

^a μmol/min/mg of protein.

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 (*leu2 ura3 ade2 his5*) (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 (pILATG24), and pFSE24 (pFUMΔSP) are described elsewhere (7, 16). Plasmids pFUM24S25F, pFUM24S, pFUM24I, pCYB2-FUM, pFUMΔ9, pFUMΔ11, pFUMΔ13, and pFUM31G33G and plasmids encoding six histidine-tagged versions of fumarase are described in this study.

pFum24S25F, pFum24S, and pFum24I were constructed by standard polymerase chain reaction using the naturally occurring *Hind*III site in pFT2 and using the following primers (*Hind*III site underlined): pM24S25F, 5'-CCCAAGCTTAAATATAAGAAGATCGTTCTCC-3'; pM24S, 5'-CCCAAGCTTAAATATAAGAAGATCGAACT-3'; pM24I, 5'-CCCAAGCTTAAATATAAGAAGAATCAAC-3'. pCyb-Fum, pFumΔ29–37 (Δ9), pFumΔ27–37 (Δ11), pFumΔ25–37 (Δ13), and pFum31G33G were constructed by overlap-extension polymerase chain reaction (17) with the following primers corresponding to site of mutation or fusion: pCyb-Fum, 5'-CGAGGAGTTTAGATCTAGGGTAGAACCGTACG-3' and 5'-CTAGATCTAAATCCTCGTTCAGAAC-3' (templates, pFT2, and pb₂-Δ19(167)-DHFR (18)); pFumΔ29–37 (Δ9), 5'-CTCCTCGTTCATACACGTGCCTGCTC-3' and 5'-GCACGTGTATGAACGAGGAGTTCATTC-3'; pFumΔ27–37 (Δ11), 5'-GAATGAATCCATACACGTGCCTGCTG-3' and 5'-CACGTGTATGGAATTCATTCCTTATATTAAGC-3'; pFumΔ25–37 (Δ13), 5'-GAAGAATGATACACGTGCCTGCTG-3' 5'-CACGTGTATCATTCCTTATATTAAGC-3'; pFum31G33G, 5'-CAGAAGTGTACCGGTGCATTTGG-3' and 5'-CCAAATGCACCGGTACCAGTTC-3'. Fumarase derivatives with six carboxyl-terminal consecutive histidine residues were constructed by standard polymerase chain reaction using naturally occurring 5'-sites of the fumarase gene in pFT2 and the following primer for the 3'-end of the *FUM1* open reading frame: 5'-CACGGGCCCTTAGTGATGGTGATGGTGATGTTTAGGACCTAGCATGTG-3'.

Labeling and Fractionation—Induced cultures (in galactose) were harvested and labeled with 10 μCi/ml [³⁵S]methionine and further incubated for 30 min at 30 °C. When required, 20 μM CCCP was added at the start of labeling. The labeled cells were collected by centrifugation, resuspended in Tris/EDTA buffer (pH 8.0) containing 1 μM phenylmethylsulfonyl fluoride, broken with glass beads for 2 min, and centrifuged to obtain the supernatant fraction. Supernatant and pellet fractions were denatured by boiling in 1% SDS, immunoprecipitated with anti-fumarase rabbit antiserum and protein A-Sepharose (Amersham Pharmacia Biotech), and then analyzed by SDS-PAGE.

Fumarase was assayed by the method of Kanarek and Hill (19) at 250 nm with L-malic acid as substrate. Citrate synthase was assayed by following the reduction of acetyl-CoA in the presence of 5,5'-dithiobis-(nitrobenzoic acid) at 412 nm (20). Glucose-6-phosphate dehydrogenase was assayed by following the formation of NADH in the presence of glucose 6-phosphate at 340 nm (21). Protein was determined by the method of Bradford (22).

Protein Sequencing and Mass Spectrometry—Histidine-tagged fuma-

rase derivatives were expressed in yeast under the *GAL10* promoter, cell-free extracts were prepared, and the proteins were affinity-purified using Co²⁺ (CLONTECH) or Ni²⁺ (Qiagen) columns. amino-terminal sequence analysis was performed by automated Edman degradation using standard chemistry on an Applied Biosystems Procise sequencer (Model 492).

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 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 0.025% trifluoroacetate A at 1%/min and a flow rate of 40 μl/min. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnigan, San Jose, CA). Mass spectrometry was performed in the positive ion mode using repetitively full MS scan followed by collision-induced 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 "genpept" using the Sequest software (J. Eng and J. Yates, both from University of Washington and Finnigan, San Jose, CA).

RESULTS

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 exclusive cytosolic localization (7, 12, 25). We previously reported that a mutant fumarase in which Met-24 was exchanged for valine is distributed between the cytosol and mitochondria even though less fumarase is targeted to mitochondria (7). In contrast, Wu *et al.* (14) have reported that a mutant fumarase in which Met-24 is exchanged for isoleucine is targeted exclusively to mitochondria. In the current study we have constructed a fumarase mutant gene with this Met-24 to isoleucine substitution (Table I). Additional fumarase mutants were constructed in which Met-24 was exchanged for serine, and a double mutant was constructed that has, in addition to the Met-24 to serine substitution, an Asn-25 to phenylalanine substitution (the rationale for this is provided below).

Table I summarizes the specific activity of fumarase and its derivatives in extracts of yeast cells. As previously described these proteins were expressed from the galactose-inducible *GAL10* promoter, which in the case of wild type fumarase controls high expression yet with full processing and a similar distribution in the cell as that obtained with expression from the chromosomal gene (7). Wild type fumarase (Fum1) and fumarase lacking a mitochondrial signal peptide (FumΔSP) exhibit similar high fumarase specific activity. In contrast, for mutants in which the second methionine was substituted for

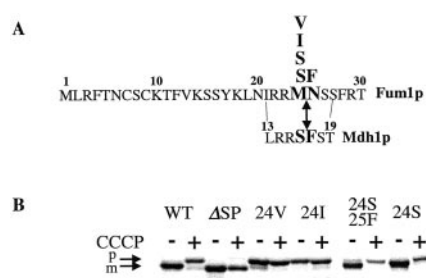


FIG. 1. **Processing of wild type and mutant fumarases.** *A*, partial sequence of the fumarase amino terminus presenting alterations that were made (shown above the Fum sequence) and the amino acid sequence surrounding the rat MdhIp MPP cleavage site (shown below the Fum sequence). The double-headed arrow indicates MPP cleavage sites. *B*, blocking of fumarase processing by inhibiting import into mitochondria. Yeast cells induced in galactose medium and expressing Fum, Fum Δ SP, Fum24V, Fum24I, Fum24S25F, and Fum24S were labeled with [³⁵S]methionine for 15 min either in the absence (–) or presence (+) of 20 μ M CCCP. Total cell extracts were prepared, immunoprecipitated with fumarase antiserum, and analyzed on SDS-PAGE. Arrows show positions of the precursor (*p*) (*top*) and mature fumarase (*m*) (*bottom*). WT, wild type.

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 (a 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 Fum Δ SP does not change upon treatment with CCCP, and the same is true for Fum24V and Fum24I (Fig. 1B, compare – and + CCCP of Δ SP, 24V, and 24I, respectively). The interpretation of the results is that these proteins are not processed due to the lack of a signal peptide (Fum Δ SP, 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 MdhI-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 Fum24S25F 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

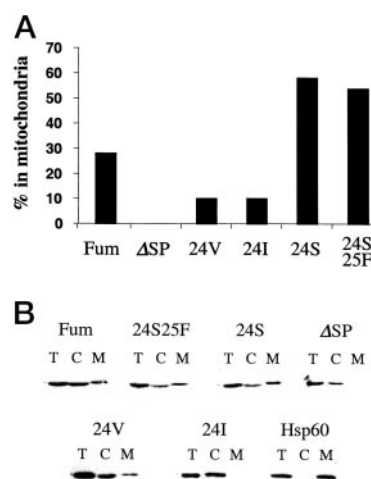


FIG. 2. **Distribution of fumarase activity and protein in subcellular fractions.** Induced cultures expressing the indicated fumarase derivatives were subjected to subcellular fractionation. *A*, fumarase enzymatic activity in fraction aliquots was determined, and the percentage of the total found in mitochondria is presented. *B*, fumarase was immunodetected by Western blot analysis using anti-fumarase antiserum. T, total extract; C, cytosol; M, mitochondria.

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 proteins 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 histidyl-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, Fum Δ SP-6h, FumS24F25-6h, FumI24-6h, were purified from yeast cell extracts by affinity chromatography. As shown in Fig. 3A as with the nontagged versions Fum Δ SP-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 Phe-25 the derivative protein is cleaved efficiently by MPP at the corresponding site between Ser-24 and Phe-25.

For FumI24-6h and Fum Δ SP-6h, which are not processed by MPP, the methionine from which translation was initiated is

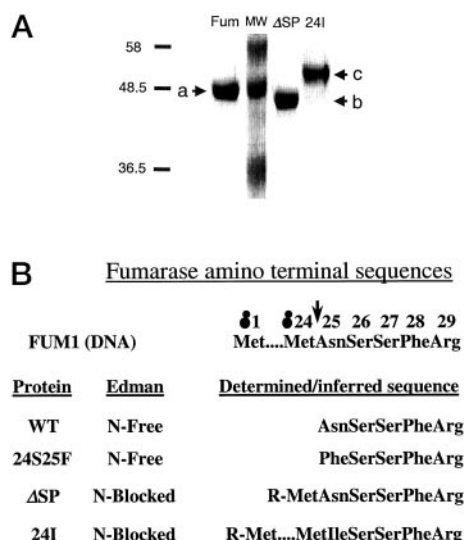


FIG. 3. Determination of the fumarase amino-terminal protein sequence. A, cultures of yeast expressing Fum-6h (a), Fum Δ SP-6h (b), and Fum24I-6h (c) and fumarase derivatives tagged with six histidines were purified by affinity chromatography. The clear difference in migration on SDS-PAGE between the fumarase derivatives is indicated by arrows. Molecular mass markers 58, 48.5, and 36.5 kDa are indicated. B, predicted (from DNA), determined (*N-Free*), and inferred (*N-Blocked*) fumarase sequences. Fumarase derivatives tagged with six histidines and affinity-purified were subjected to sequential Edman degradation. WT, wild type. Black stacked circles indicate potential ribosome translation initiation positions.

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 Fum Δ SP 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 Fum Δ SP both tagged with six histidines were analyzed. The reagent BNPS-skatole was chosen for this analysis since it cleaves fumarase only twice after the two tryptophans (Trp-46 and Trp-477) in this protein, thereby producing a predicted 16-amino acid carboxyl-terminal peptide and a predicted 22- or 23-amino acid amino-terminal fragment depending on whether the protein was processed by MPP (after Met-24) or whether translation initiated at Met-24 (Fig. 4A). As shown in panels a and c of Fig. 4B and as predicted for Fum and Fum Δ SP, 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

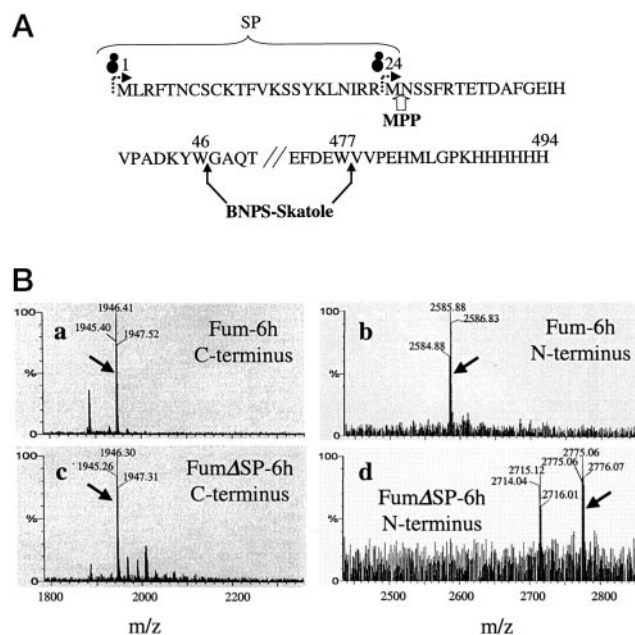


FIG. 4. MALDI-TOF MS analysis of BNPS-skatole-cleaved fumarases. Fum-6h and Fum Δ SP-6h expressed in yeast and purified as described in Fig. 3 were subjected to cleavage with BNPS-skatole and analyzed by mass spectrometry. A, partial sequence of the fumarase precursor showing (i) predicted BNPS-skatole cleavage sites (solid arrows), (ii) the determined MPP cleavage site (hollow arrow), and (iii) potential translation initiation sites (broken arrows). B, MALDI-TOF mass spectra detection of the Fum-6h (top panels) and Fum Δ SP-6h (bottom panels) carboxyl-terminal (a and c) and amino-terminal (b and d) fragments of BNPS-skatole-cleaved fumarases. Predicted masses are indicated by arrows. SP, signal peptide. Black stacked circles are as in Fig. 3B.

and Fum Δ SP 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 Fum Δ SP 23-amino acid fragment includes this same 14-Da modification, a 16-Da addition due to oxidation of the methionine, and a 42-Da addition due to acetylation of the amino-terminal methionine. The difference in mass between Fum and Fum Δ SP after taking the modifications into consideration corresponds to that of the amino acid methionine (131 Da), while the lower mass from Fum Δ SP (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).

Deletion and Swapping of Fumarase Amino-terminal Sequences—Our working model for fumarase distribution proposes that the fumarase amino terminus of cytosol-destined precursors are only partially translocated and then cleaved by MPP (see the Introduction and Refs. 7 and 13). In addition fumarase translocation into the mitochondrial matrix appears

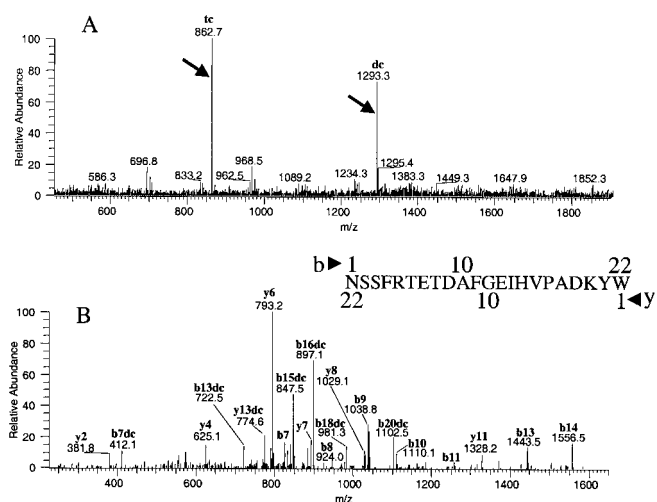


FIG. 5. Identification of the BNPS-skatole Fum amino-terminal peptide by electrospray ion trap mass spectrometry. Peptides were separated by reverse phase HPLC chromatography and either electrosprayed directly from the HPLC column into an electrospray ion trap mass spectrometer (panel A) or following collision-induced fragmentation (panel B). Doubly charged (*dc*) and triply charged (*tc*) ions are labeled accordingly. Masses corresponding to the amino-terminal peptide (residues 25–46) are indicated by arrows in panel A, and the major fragment ions attributed to b and y series ions are indicated in panel B.

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 activity and was processed efficiently as detected by labeling experiments in the presence and absence of CCCP (Fig. 6B, 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 (RTETDAFGEIHVPADK). In fact we had speculated that this sequence may weaken the interaction of the translocating protein with mtHsp70 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 ($\Delta 25$ –37, $\Delta 27$ –37, and $\Delta 29$ –37). As shown in Fig. 7A these deletions

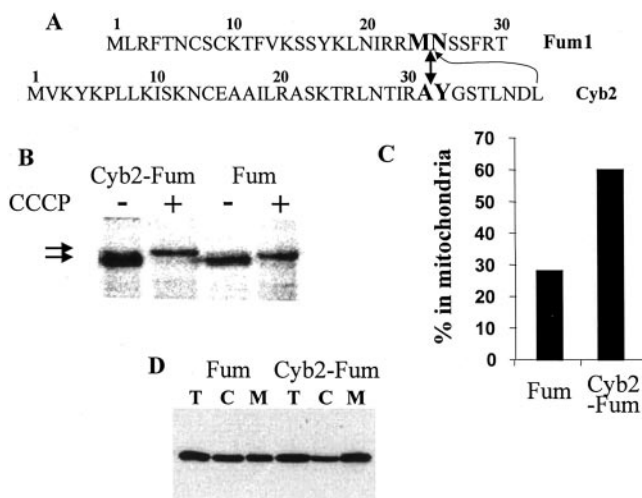


FIG. 6. Processing and subcellular distribution of Cyb2-Fum. A hybrid protein consisting of the Cyb2 signal peptide, the Cyb2 MPP cleavage site, and the mature wild type fumarase was expressed in yeast. A, the amino-terminal of Fum1 and Cyb2 is shown with the MPP cleavage sites (double-headed arrow) and the point of fusion between the two sequences (curved arrow) indicated. B, processing was determined by labeling in the presence and absence of CCCP as described in Fig. 1. C, subcellular distribution determination of enzymatic activity was as described in Fig. 2. D, Western blot analysis of subcellular fractions was as described in Fig. 2. T, total extract; C, cytosol; M, mitochondria.

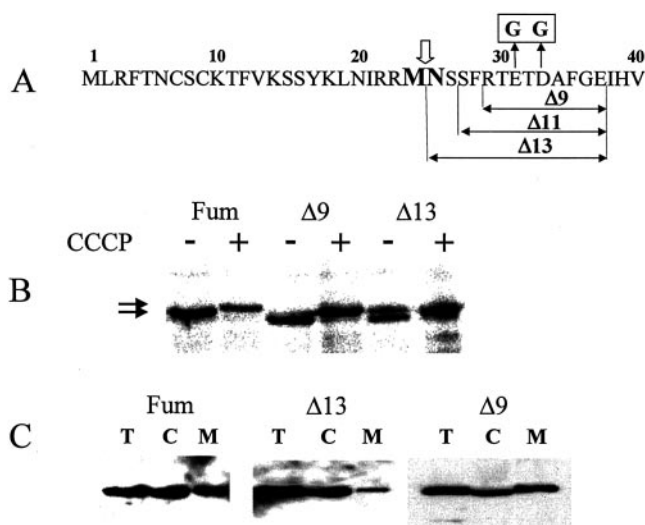


FIG. 7. Processing and subcellular distribution of fumarase deleted for sequences immediately downstream of the MPP cleavage site. Fum, Fum $\Delta 13$, and Fum $\Delta 9$ were expressed in yeast. A, the amino-terminal sequence of Fum1 is shown with the deletions ($\Delta 9$, $\Delta 11$, and $\Delta 13$) and the double substitution (Fum31G33G) indicated. The hollow arrow indicates the MPP cleavage site. B, processing was determined by labeling in the presence and absence of CCCP as described in Fig. 1. C, subcellular distribution was determined by cell fractionation and Western blotting as described in Fig. 2. T, total extract; C, cytosol; M, mitochondria.

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. Fum $\Delta 29$ –37 ($\Delta 9$) is processed efficiently as detected by labeling experiments in the presence and absence of CCCP (Fig. 7B, compare – and + CCCP of $\Delta 9$). The same is true for Fum31G33G (not shown). In contrast, Fum $\Delta 25$ –37 ($\Delta 13$) shows a significant defect in processing (Fig. 7B, compare – and +

CCCP of $\Delta 13$), which is not very surprising since in this mutant Asn-25 is replaced by isoleucine at the MPP cleavage site. Fum $\Delta 27-37$ 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 Fum $\Delta 29-37$ ($\Delta 9$) than the wild type molecules are fully imported into mitochondria (60% compared with 30%, respectively). In contrast for Fum $\Delta 25-37$ ($\Delta 13$) 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 held 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 ΔSP -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 b_2 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.² In addition, overexpression of SSA1, a yeast cytosolic Hsp70 homolog, causes the localization of 2-fold more fumarase in mitochondria.² 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 Fum $\Delta 25-37$) 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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REFERENCES

1. Natsoulis, G., Hilger, F., and Fink, G. R. (1986) *Cell* **46**, 235–243
2. Slusher, L. B., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9789–9793
3. Gillman, E. C., Slusher, L. B., Martin, N. C., and Hopper, A. (1991) *Mol. Cell.*

² E. Sass, S. Karniely, and O. Pines, unpublished.

- Biol.* **11**, 2382–2390
4. Tong, W. H., and Rouault, T. (2000) *EMBO J.* **19**, 5692–5700
 5. Kurys, G., Tagaya, Y., Bamford, R., Hanover, J. A., and Waldmann, T. A. (2000) *J. Biol. Chem.* **275**, 30653–30659
 6. Huang, L. J., Wang, L., Ma, Y., Durick, K., Perkins, G., Deerinck, T. J., Ellisman, M. H., and Taylor, S. S. (1999) *J. Cell Biol.* **145**, 951–959
 7. Stein, I., Peleg, Y., Even-Ram, S., and Pines, O. (1994) *Mol. Cell. Biol.* **14**, 4770–4778
 8. Haucke, V., Ocana, C. S., Honlinger, A., Tokatlidis, K., Pfanner, N., and Schatz, G. (1997) *Mol. Cell. Biol.* **17**, 4024–4032
 9. Anandatheerthavarada, H. K., Biswas, G., Mullick, J., Sepuri, N. B., Otvos, L., Pain, D., and Avadhani, N. G. (1998) *EMBO J.* **18**, 5494–5504
 10. Addya, S., Anandatheerthavarada, H. K., Biswas, G., Bhagwat, S. V., Mullick, J., and Avadhani, N. G. (1997) *Cell Biol.* **139**, 589–599
 11. Bhagwat, S. V., Biswas, G., Anandatheerthavarada, H. K., Addya, S., Pandak, W., and Avadhani, N. G. (1999) *J. Biol. Chem.* **274**, 24014–24022
 12. Wu, M., and Tzagoloff, A. (1987) *J. Biol. Chem.* **262**, 12275–12282
 13. Knox, C., Sass, E., Neupert, W., and Pines, O. (1998) *J. Biol. Chem.* **273**, 25587–25593
 14. Wu, M., Wong, S.-M., Tan, H.-M., and Ting, R. (1995) *Biochem. Biophys. Res. Commun.* **215**, 578–590
 15. Suzuki, T., Yoshida, T., and Tuboi, S. (1992) *Eur. J. Biochem.* **207**, 767–772
 16. Pines, O., Even-Ram, S., Elnathan, N., Battat, E., Aharonov, O., Gibson, D., and Goldberg, I. (1996) *Appl. Microbiol. Biotechnol.* **46**, 393–399
 17. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amst.)* **77**, 51–59
 18. Koll, H., Guiard, B., Rassow, J., Ostermann, J., Horwich, A. L., Neupert, W., and Hartl, F. U. (1992) *Cell* **68**, 1163–1175
 19. Kanarek, L., and Hill, R. L. (1964) *J. Biol. Chem.* **5**, 465–474
 20. Weitzman, P. D. J. (1969) *Methods Enzymol.* **13**, 22–24
 21. Worthington, C. C. (ed) (1988) *Worthington Manual*, Worthington Biochemical Corp., Freehold, NJ
 22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 23. Rahali, V., and Gueguen, J. (1999) *J. Protein Chem.* **18**, 1–12
 24. Vestling, M. M., Kelly, M. A., and Fenselau, C. (1994) *Rapid Commun. Mass Spectrom.* **8**, 786–790
 25. Peleg, Y., Rokem, J. S., Goldberg, I., and Pines, O. (1990) *Appl. Environ. Microbiol.* **56**, 2777–2783
 26. Nelson, N., and Schatz, G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4365–4369
 27. Ogishima, T., Niidome, T., Shimokata, K., Kitada, S., and Ito, A. (1995) *J. Biol. Chem.* **270**, 30322–30326
 28. Tercero, J. C., Dinman, J. D., and Wickner, R. B. (1993) *J. Bacteriol.* **175**, 3192–3194
 29. Polevoda, B., Norbeck, J., Takakura, H., Blomberg, A., and Sherman, F. (1999) *EMBO J.* **18**, 6155–6168
 30. Blachinsky, E. (2001) *Processing of the Single Translation Product of the FUM1 Gene (Fumarase) in Saccharomyces cerevisiae*. M.Sc. thesis, Hebrew University of Jerusalem