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

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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 targeting signal and functionally inhibits its endoplasmic reticulum targeting signal. The NADH-cytochrome b₅ 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) (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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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 (lue2 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 (pLATG24), and pSFE24 (pFUM26SP) are described elsewhere (17). Plasmids pFUM24S25F, pFUM24S, pFUM24I, pFUM19, pFUM11, pFUM13, 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 5'-sites of the fumarase gene in pFT2 and using the following primers (HindIII site in pFT2 and using the following primers (HindIII site underlined): pM24S25F, 5'-CCCAAGCTTAATATAAGAAGATCGTTCTCC-3'; pM24S, 5'-CCCAAGCTTAATATAAGAAGATCGACCTG-3'; pM24I, 5'-CCCAAGCTTAATATAAGAAGATGCACGC-3'; pFUM24V, 5'-CACGT-TTACCAGCTTACACACGGTCCGCTC-3' and 5'-CTAGTATCAAACCTCTGCTGCAAGAC-3' (templates, pFT2, and pb-Δ191667-DHFR (18)); pFUM24S25F27 (Δ9), 5'-CTTTCTGTTTATACCTAGAGGATCGACCTG-3'; and 5'-CCCAAGCTTAATATAAGAAGATCGACCTG-3'; pFUM24S25F, pFUM24S, pFUM24I, and a double mutant was constructed that has, in addition to the Met-24 to serine substitution, an Asn-25 to phenylalanine substitution (Table I). Additional fumarase mutants were expressed in yeast under the galactose-inducible promoter, cell-free extracts were prepared, and the proteins were affinity-purified using Co3+ (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% trifluoroacetic acid at 1% per 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 was exchanged for valine is distributed between the cytosol and 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...
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). The absence or presence of CCCP affects the mitochondrial membrane potential 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 W7 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 (Mdhlp) 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 Mdhlp-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 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, 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 Phe-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...
Identification of fumarase products in the yeast cell was to use MALDI-TOF MS as a tool for examining the amino termini of the 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).

To rule out this possibility—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 Man3 on the basis of its amino-terminal sequence (MLRF, Refs. 28 and 29). The FumSP 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 MFNL 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 FumΔSP 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 FumΔSP, 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 FumΔSP 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 FumΔSP 23-amino acid fragment includes this same 14-Da modification caused by the BNPS-skatole treatment (23, 24). The FumΔSP amino terminus is most probably the lower mass from FumSP 23-amino acid fragment includes this same 14-Da modification caused by the BNPS-skatole treatment (23, 24). The FumΔSP amino terminus is most probably the lower mass from FumSP after taking the modifications into consideration corresponds to that of the amino acid methionine (131 Da), while the lower mass from FumΔSP (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 biologically signif.

**Fig. 3.** Determination of the fumarase amino-terminal protein sequence. A, cultures of yeast expressing Fum-6h (a), FumΔSP-6h (b), and Fumα24I-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.

**Fig. 4.** MALDI-TOF MS analysis of BNPS-skatole-cleaved fumarases. Fum-6h and FumΔSP-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 FumΔSP-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.
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 b2 (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 c1 and b2 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 (RTETDAGFEHIHPADK). 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
other Met-24 mutants, Fum24S25F and Fum24S, which are solution with mitochondria upon centrifugation. Nevertheless, shown that unprocessed fumarase has a tendency to precipitate be carefully reexamined. For example we have previously shown that expression of fumarase from the plasmid. The same results are obtained with mutants de-
tween the cytosol and mitochondria. In this regard, we have Fum24S, which are processed efficiently and distributed be-
plasmid. The same results are obtained with mutants de-
conclusions were based on detection of a single band on SDS-
precursor unavailable for cleavage by the protease (7). Our This was achieved either by directly inhibiting the MPP or 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-
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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 target-
A third type of experiment supporting a single fumarase distribution phenomenon.

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