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Evolution of aminoacyl-tRNA synthetase quaternary structure and activity: *Saccharomyces cerevisiae* mitochondrial phenylalanyl-tRNA synthetase

(operon construction/protein sequence/mitochondria/evolution)

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ABSTRACT **Phenylalanyl-tRNA synthetases [L-phenylalanine:tRNA^{Phe} ligase (AMP-forming), EC 6.1.1.20] from *Escherichia coli*, yeast cytoplasm, and mammalian cytoplasm have an unusual conserved $\alpha_2\beta_2$ quaternary structure that is shared by only one other aminoacyl-tRNA synthetase. Both subunits are required for activity. We show here that a single mitochondrial polypeptide from *Saccharomyces cerevisiae* is an active phenylalanyl-tRNA synthetase. This protein (the *MSF1* gene product) is active as a monomer. It has all three characteristic sequence motifs of the class II aminoacyl-tRNA synthetases, and its activity may result from the recruitment of additional sequences into an α-subunit-like structure.**

Aminoacyl-tRNA synthetases constitute a family of twenty functionally homologous enzymes displaying a large diversity in molecular weights and subunit structures (1). Unlike the majority of aminoacyl-tRNA synthetases, which are monomers or oligomers made up of identical subunits, glycyl- and phenylalanyl-tRNA synthetases exhibit an $\alpha_2\beta_2$ structure. With a few exceptions, homologous aminoacyl-tRNA synthetases display the same quaternary structure in prokaryotes and eukaryotes. The bacterial alanyl- (α_4) and glycyl- ($\alpha_2\beta_2$) tRNA synthetases (2, 3) occur, respectively, as monomeric (4) and dimeric (5) forms in yeast (*Saccharomyces cerevisiae*). However, it is not excluded that these forms may have arisen through uncontrolled proteolysis during the course of their purification. A third example, the transition of methionyl-tRNA synthetase from dimer (*Escherichia coli*) to monomer (yeast cytoplasmic or mitochondrial), could be confirmed at the molecular level. It arose by the loss of a dimerization domain at the C terminus of the yeast enzymes and reflects a situation that could be obtained *in vitro* by mild proteolysis of the dimeric *E. coli* enzyme (6). It results in a monomeric active fragment whose C terminus coincides with that of the native yeast cytoplasmic and mitochondrial methionyl-tRNA synthetases (7, 8). The conservation of quaternary structure is particularly exemplified in the case of phenylalanyl-tRNA synthetase [L-phenylalanine:tRNA^{Phe} ligase (AMP-forming), EC 6.1.1.20], which occurs as a tetramer of the $\alpha_2\beta_2$ type in *E. coli* (9), *Bacillus subtilis* (10), yeast cytoplasm (11), and mammalian cytoplasm (12, 13). All these forms have a similar molecular weight in the range of 250,000. However, the molecular weights for the large (β; M_r, 87,000) and small (α; M_r, 37,000) subunits of *E. coli* phenylalanyl-tRNA synthetase (9) differ markedly from those determined for the corresponding enzyme from yeast cytoplasm [respectively, 70,000 and 60,000‡ (5)]. The genes coding for the α and β subunits have been cloned and sequenced in the case of the *E. coli* (14, 15) and yeast (16) cytoplasmic phenylalanyl-tRNA synthetases. Sequence comparisons did not show any strong homology between individual subunits and excluded the possibility that the dissimilarity in subunit sizes between *E. coli* and yeast could have arisen through intramolecular rearrangements (16).

An $\alpha_2\beta_2$ structure was proposed, too, for the yeast mitochondrial phenylalanyl-tRNA synthetase, which has a molecular weight and subunit sizes very close to those of its cytoplasmic counterpart (17). Recently, the gene *MSF1* encoding the mitochondrial α subunit was isolated and sequenced (18). Despite considerable sequence divergence between the mitochondrial and cytoplasmic phenylalanyl-tRNA synthetases as far as the α subunit is concerned, their striking similarity in size was taken into consideration to examine the possibility that the mitochondrial α subunit could combine with the cytoplasmic β subunit to build up a functional chimera. To test this idea, we created an operon with the corresponding *MSF1* and *FRS1* structural genes, using the *E. coli pheST* intergenic region (15) to produce a polycistronic mRNA. A construction with the *MSF1* gene alone fused to the *lacZ* gene was used as a control, assuming that an isolated mitochondrial subunit would be inactive, as is the case for individual subunits of the yeast and *E. coli* phenylalanine enzymes. To our surprise the MSF1 protein by itself was found to be active. We purified the MSF1 protein and measured its catalytic properties. Turnover rates in the aminoacylation reaction were found to be comparable to those of monomeric or oligomeric aminoacyl-tRNA synthetases. Similarly, the same fully active monomeric protein was obtained when the MSF1 protein was expressed from the *MSF1–FRS1* operon, indicating that no heterologous association of the mitochondrial and cytoplasmic subunits had occurred. Therefore, we focused our attention on the quaternary structure and tRNA specificity of the MSF1 protein; the data reported here support the model of a monomeric mitochondrial phenylalanyl-tRNA synthetase.

MATERIALS AND METHODS

***lacZ–MSF1* Gene Fusion.** *MSF1* was fused to *lacZ* by using procedures and vectors similar to those used in the construction of the phenylalanyl-tRNA synthetase operon (19). Plasmid pG120/ST10 (18) was the source of the *MSF1* structural gene. An *Xba* I restriction site in the *MSF1* gene was created by using oligonucleotide AACATTGAAGTTACCTCCAT-

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‡In our previous work, α and β designated the large and small subunits of yeast phenylalanyl-tRNA synthetase, respectively. To comply with the rule adopted for the other cited aminoacyl-tRNA synthetase subunits, we have changed the order of designation: α, small, and β, large.

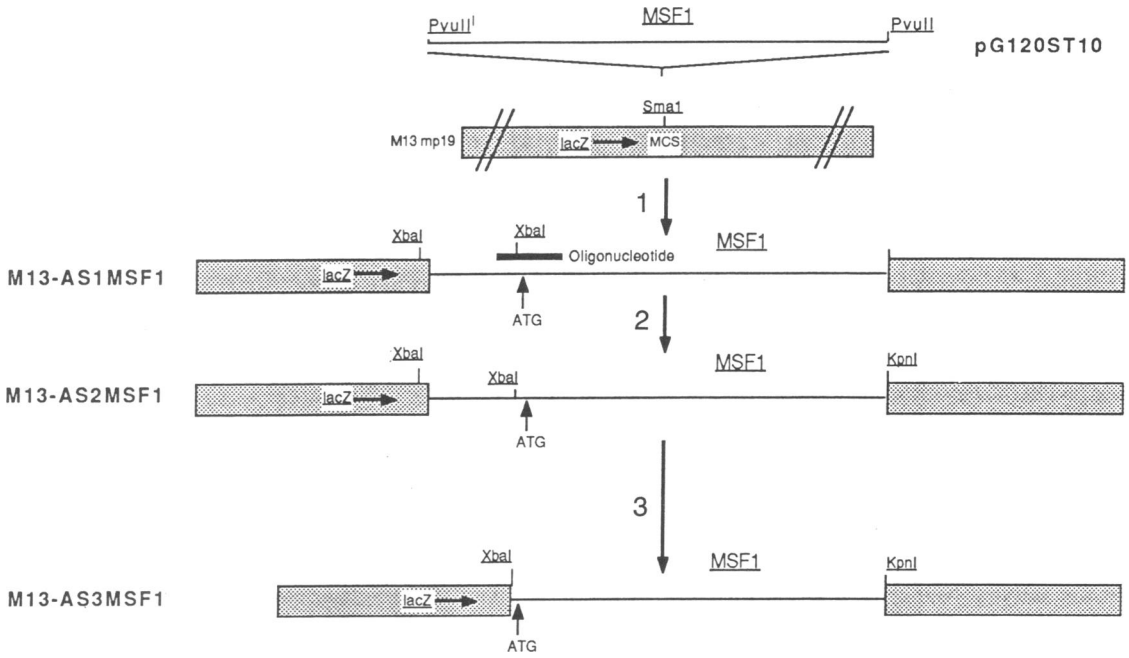

FIG. 1. Construction of the MSF1 protein–β-galactosidase fusion. The sequences of the M13 vector and *MSF1* gene are represented respectively by stippled boxes and thin lines. The direction of *lacZ* transcription is indicated by an arrow. Restriction sites of the polylinker region (MCS) used in the construction are indicated. Step 1: A 2-kilobase-pair *Pvu* II fragment bearing the entire *MSF1* structural gene was isolated from the plasmid pG120/ST10 and cloned in the *Sma* I site of M13mp19. The construct M13-AS1MSF1 has the *MSF1* gene in the right orientation with respect to the *lacZ* promoter. Step 2: *In vitro* mutagenesis using the mutant oligonucleotide 5′-AACATTGAAGTTACCTC-CATCTAGATATTGTTTTATCTCTTCTCT-3′ and single-stranded M13-AS1MSF1 form to create a *Xba* I site one base upstream from the methionine codon of the *MSF1* gene. The presence of the *Xba* I site was verified by dideoxy sequencing. Step 3: Digestion of M13-AS2MSF1 by *Xba* I restriction enzyme. The linearized vector was recircularized after a 10-fold dilution of the digestion mix in the presence of T4 DNA ligase. The *Xba* I–*Kpn* I fragment of M13-AS3MSF1 DNA was subcloned in pUC19 to yield pAS3MSF1.

CTAGATATTGTTTTATCTCTTCTCT (mismatched nucleotides are underlined). The initiator methionine codon of *MSF1* is indicated in boldface.

Determination of Enzymatic Activities. Heterologous yeast cytoplasmic tRNA was used as a substrate for the MSF1 protein. Aminoacylation was carried out at 37°C under the following conditions: 144 mM Tris·HCl at pH 7.8, 5 mM dithiothreitol, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM [^{14}C]phenylalanine (25 μCi/μmol; 1 Ci = 37 GBq), and 6 mg of unfractionated yeast tRNA (tRNA^{Phe} is 2.5% of the total) per ml, or 0.8–40 μmol of purified tRNA^{Phe}. At various time intervals 40-μl aliquots from a 200-μl reaction mixture were spotted onto Whatman paper discs and quenched by 5% trichloroacetic acid. Radioactivity was measured by using a toluene-based scintillant. One unit of activity is defined as the amount of enzyme aminoacylating 1 nmol of phenylalanine in 1 min on tRNA^{Phe}.

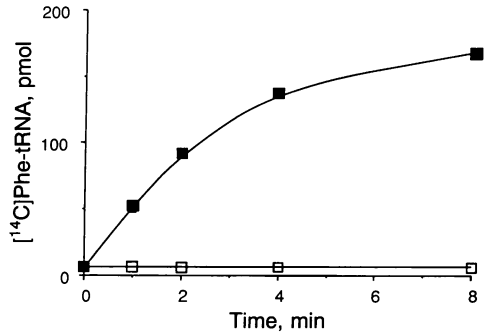

FIG. 2. Expression of the MSF1 protein. *E. coli* cells were transformed with pAS3MSF1 and pAS5MSF1 constructs and 10 μg of crude protein extract was used to measure the aminoacylation of yeast tRNA^{Phe}. □, Control with the vector alone; ■, pAS3MSF1-transformed cells.

Purification of the MSF1 Protein. The MSF1 protein was purified to homogeneity from cultures of JM101 cells carrying the plasmid pAS3MSF1. Overnight cultures (2 liters in LB medium containing isopropyl β-D-thiogalactoside and ampicillin) were harvested by centrifugation, resuspended in 2 ml of 100 mM Tris·HCl, pH 7.8/1 mM dithiothreitol/1 mM EDTA and submitted to ultrasonic disintegration. Nucleic acids were eliminated by precipitation with protamine sulfate (final concentration, 3.5 g/liter) after digestion with DNase 1. Proteins were fractionated by polyethylene glycol (5–17.5%; vol/vol), redissolved in 20 ml of 50 mM potassium phosphate, pH 7.2/1 mM dithiothreitol/0.1 mM EDTA/10% (vol/vol) glycerol, and loaded onto a hydroxyapatite/

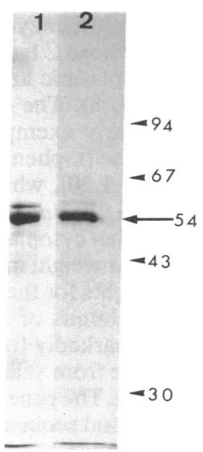

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of the purified MSF1 protein. Lane 1, 1.5 μg of hydroxyapatite/Ultrogel fraction; lane 2, 0.5 μg of TSK 3000 SW fraction. The sizes of reference molecular weight markers are indicated × 10^{-3}.

Table 1. Steady-state kinetic data for the MFS1 protein and yeast cytoplasmic phenylalanyl-tRNA synthetase in the aminoacylation reaction

Enzyme	*E. coli* tRNA^{Phe}			Yeast cytoplasmic tRNA^{Phe}			Yeast mitochondrial tRNA^{Phe}			Phenylalanine		
	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $s^{-1}\cdot\mu M^{-1}$	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $s^{-1}\cdot\mu M^{-1}$	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $s^{-1}\cdot\mu M^{-1}$	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $s^{-1}\cdot\mu M^{-1}$
MSF1 protein	0.4	16	0.026	1.25	15	0.083	ND	ND	0.35	0.82	40	0.025
Phe-tRNA synthetase	ND	ND	ND	5	0.15	33	ND	ND	ND	3, 6*	6, 30*	0.5, 0.2*

The individual K_m and k_{cat} values of the MSF1 protein were obtained by measuring the aminoacylation of purified yeast cytoplasmic or *E. coli* tRNA^{Phe}. Therefore, the values indicated represent apparent values. The values reported in this table are extrapolations of double-reciprocal plots of $1/v$ versus $1/S$. The values were obtained under the following conditions: K_m for phenylalanine, 5–100 μM phenylalanine (40 cpm/pmol), 2 mM ATP, 15 μM tRNA^{Phe} (cytoplasmic), and 67 nM enzyme; K_m for tRNA^{Phe}, 0.8–40 μM tRNA^{Phe}, 2 mM ATP, 100 μM phenylalanine (320 cpm/pmol), and 22 nM enzyme. k_{cat}/K_m was also measured by using unfractionated mitochondrial tRNA from the kinetics obtained with 6.2 and 12.5 μM tRNA (tRNA^{Phe} is 2.5% of total tRNA). The data with the cytoplasmic enzyme are taken from our previous published results (21). ND, not determined.

*Double-reciprocal plots are biphasic, indicating two sets of K_m and k_{cat} values.

Ultrogel column (3.5 cm × 11 cm; HA Ultrogel). Elution was with a 50–500 mM linear phosphate gradient.

RESULTS

Expression of the MSF1 Protein in *E. coli*. The *MSF1* gene was fused with *lacZ* to produce a fusion protein with 16 extra amino acid residues from β-galactosidase. Fig. 1 summarizes the steps in the construction. The plasmid derivative of M13-AS3MSF1 construct was obtained by subcloning the *Xba* I–*Kpn* I fragment in pUC19. This plasmid was used to transform *E. coli* cells, and the activity was tested with yeast cytoplasmic tRNA^{Phe} (Fig. 2). Cytoplasmic yeast tRNA^{Phe} was previously shown to be a good substrate for aminoacylation by the mitochondrial phenylalanine enzyme (20), and it allows discrimination of the endogenous *E. coli* phenylalanyl-tRNA synthetase activity (ref. 19 and Fig. 2). Extracts from pAS3MSF1-transformed *E. coli* cells were found to be active in charging cytoplasmic tRNA^{Phe}, with a specific activity of 5 units/mg. The same value was obtained for the MSF1 protein expressed from the *MSF1–FRS1* operon (not shown).

Purification of the MSF1 Protein and Determination of the Subunit Structure. The overproduced MSF1 protein was purified in one chromatographic step on a hydroxyapatite/Ultrogel column, starting from a polyethylene glycol-fractionated crude protein extract. The enzyme was estimated to be 90% pure on a Coomassie blue-stained NaDodSO$_4$/polyacrylamide gel (Fig. 3, lane 1) and displayed a specific activity of 700 units/mg. This enzyme preparation was used to measure K_m and k_{cat} for phenylalanine and tRNA^{Phe} in the aminoacylation reaction using purified cytoplasmic tRNA^{Phe} (Table 1). Further purification was achieved on an analytical scale by using fast protein liquid chromatography molecular sieving [TSK 3000 SW (Beckman) or Protein-Pak 300 (Waters)] that removed most of the contaminating bands (Fig. 3, lane 2). The upper minor band visible on the gel is not related to the β-galactosidase–MSF1 fusion protein containing the 16 amino acid tail from the *lacZ* fusion, since it did not react with the antibodies directed against the MSF1 protein (not shown). The enzyme eluted as a peak fraction of M_r 44,000 and had a specific activity of 900 units/mg. We conducted an equilibrium sedimentation experiment to confirm the low molecular weight of the MSF1 protein, using the meniscus depletion method with schlieren optics. A molecular weight of 56,800 was calculated from a 24-hr sedimentation run at 25,000 rpm. The molecular weight determinations of the MSF1 protein in the native and NaDodSO$_4$-denatured state indicate that the protein is monomeric. The discrepancy in molecular weights estimated by gel filtration versus sedimentation and polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$ was also noted for other aminoacyl-tRNA synthetases purified in the laboratory and may result from retardation effects due to hydrophobic or hydrophilic interactions with the gel matrix.

Because the MSF1 protein was expressed in *E. coli*, we wished to rule out the possibility that the observed activity results from an association with the β subunit of the *E. coli* phenylalanyl-tRNA synthetase. *A priori*, this hypothesis is unlikely, because the increase in specific activity follows the purification state of the protein. We could definitely rule out any contamination (or association) by Western blot experiments showing the absence of cross-reaction between the MSF1 protein and polyclonal antibodies raised against the *E. coli* phenylalanyl-tRNA synthetase (or β-specific antibodies; results not shown). All these results indicate that the MSF1 protein is active by itself.

N-Terminal Sequence Analysis of the MSF1 Protein Expressed by *E. coli*. To confirm that the purified protein is the *MSF1* gene product, we sequenced the N terminus by Edman degradation using an automatic gas-phase sequencer. Analyses revealed that two amino acids were simultaneously liberated at each step in equimolar amounts. However, the residues could easily be assigned with the help of the gene-derived sequence. They corresponded to the peptide region 11–17 and 21–27 of the N terminus (Fig. 4), indicating that internal proteolysis has removed the portion of the β-galactosidase together with 10 or 20 residues from the N terminus of the MSF1 protein. These sites of proteolysis may define a mitochondrial import presequence.

DISCUSSION

In the cases where aminoacyl-tRNA synthetases of the $\alpha_2\beta_2$ type have been isolated, neither subunit alone was active in

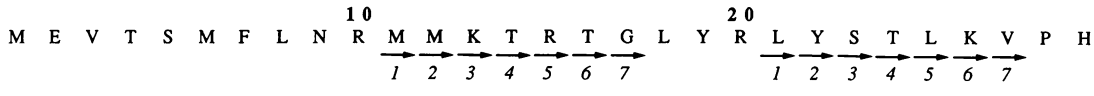

FIG. 4. N-terminal amino acid sequence of the purified MSF1 protein. The protein (1 nmol) was subjected to automated N-terminal degradation with phenylisothiocyanate on a gas-phase sequencer (Applied Biosystems model 470A). Samples were applied to a Polybrene-treated and precycled glass-fiber filter. Amino acid phenylthiohydantoin derivatives were identified by chromatography on a C_{18} Brownlee column (2.1 mm × 200 mm) coupled with the phenylthiohydantoin analyzer. The known sequences are indicated by the one-letter code. The numbers below the arrows identify the amino acids released at each step. The yield was 60–80 pmol of amino acids liberated at each step.

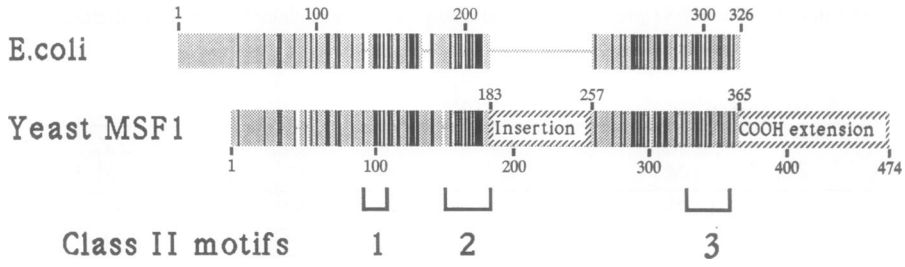

FIG. 5. Schematic diagram of the MSF1 protein, showing a possible evolutionary relationship to the *E. coli* phenylalanyl-tRNA synthetase α subunit. Vertical lines denote strict amino acid identities to the *E. coli* α-subunit sequence. The total amino acid identity is 30%. Class II motifs are indicated by the brackets. The alignment with the *E. coli* α-subunit sequence defines an insertion and a C-terminal extension in the MSF1 protein. Numbering is from the N terminus.

the ATP–PP_i exchange or tRNA aminoacylation. This has been particularly well documented for the bacterial glycyl- and phenylalanyl-tRNA synthetases and the yeast cytoplasmic phenylalanyl-tRNA synthetase, although a dissimilar tRNA binding site distribution was noted. Much of the interaction with the tRNA appears to be confined to the β subunit of the *E. coli* glycine and phenylalanine enzymes (3, 22, 23) and to the α subunit in the case of the yeast cytoplasmic phenylalanyl-tRNA synthetase (24). The combination of the two subunits to form an active species strongly suggests that the active site is at the subunit interface. It is shown here that the MSF1 protein, a mitochondrial α-subunit-like monomer of 474 residues, charges yeast cytoplasmic $tRNA^{Phe}$ with a high turnover number (k_{cat} value in the range of 1 s^{-1}, Table 1). The catalytic efficiency (k_{cat}/K_m) of the MSF1 protein for tRNA is low compared with the cytoplasmic enzyme but is in the range of other yeast cytoplasmic enzymes (i.e., the methionyl-tRNA synthetase has a value of 0.3 $s^{-1}\cdot\mu M^{-1}$). This suggests that all the determinants of the active site and probably tRNA binding are borne by a single subunit and that the quaternary $\alpha_2\beta_2$ structure may not be conserved for the mitochondrial phenylalanine enzyme. In fact, the existence of two different types of subunits for the latter was based on biochemical data (17) which now look weak: indeed, the protein band pattern shown in ref. 25 does not meet the criteria of a homogeneous protein that dissociates into two equimolar subunits under denaturing conditions. Furthermore, large differences in amino acid composition can be noted between the gene-derived MSF1 protein sequence (18) and that published for the so-called mitochondrial subunit (26): these differences vary from 2- to 10-fold for Gly, Ala, Met, His, and Trp. All these discrepancies cast serious doubts on the data published in ref. 17. It is worth stressing that all respiratory-deficient mutants isolated so far, whose defect is in the mitochondrial $tRNA^{Phe}$ aminoacylation, are alleles of the *msf1* gene (18). However, these genetic data are not sufficient to exclude the existence of a second gene for a dissimilar subunit.

Compared with the cytoplasmic phenylalanyl-tRNA synthetase, which can be viewed as a functional dimer of 1098 residues per $\alpha\beta$ promoter (16), the MSF1 protein has tolerated a drastic size reduction of more than 50%, giving rise to a catalytically active protein core. Evidence for a core structure has already been demonstrated for the tetrameric alanyl-tRNA synthetase from *E. coli* by means of a gene deletion that removed dispensable parts from the C terminus (2). The full-length protein of 875 residues per subunit can be progressively shortened into a catalytic domain of 461 residues. This fragment is a monomer and contains an independent N-terminal activation domain of 368 amino acids to which the addition of 93 residues at the C terminus is necessary for tRNA binding and aminoacylation (27). It was also demonstrated for glycyl-tRNA synthetase that the two subunits need not be organized in a tetrameric $\alpha_2\beta_2$ structure to generate activity: when the genes encoding the α and β subunits were fused, the resulting $(\alpha\text{-}\beta)_2$ dimer retained 10% of the aminoacylation activity (28). Activities comparable to the activity of the wild-type enzyme are achieved only in the unfused derivative, providing a catalytic advantage to the oligomeric $\alpha_2\beta_2$ structure. Such an advantage seems to be lost for the mitochondrial phenylalanyl-tRNA synthetase, since the MSF1 protein has a turnover rate that resembles that of the cytoplasmic isoenzyme. A plausible role for a second subunit would be to improve tRNA specificity by providing negative interactions with noncognate tRNAs.

The discovery that the mitochondrial phenylalanyl-tRNA synthetase can efficiently charge $tRNA^{Phe}$ as a single polypeptide chain raises some questions from a functional and evolutionary point of view. A closer sequence resemblance of the MSF1 protein with the *E. coli* α subunit (16) suggested that the *MSF1* ancestral gene was most probably prokaryotic. One possibility is that the mitochondrial phenylalanine enzyme evolved from the *E. coli* subunit (α = 327 residues) by insertion of a polypeptide of approximately 60 amino acid residues and an extension of 109 residues at the C terminus (Fig. 5). Experimental evidence for a functional role of the C-terminal stretch comes from the loss of the mitochondrial phenylalanyl-tRNA synthetase activity when a deletion in the *MSF1* gene removes 94 amino acid residues from the C terminus (18). This result supports the idea that functional regions are conserved between the MSF1 insertions and the yeast or bacterial β subunits. After extended computer analysis, no significant homology could be identified with the yeast cytoplasmic protein. However, there is considerable sequence identity (30%) between the C terminus of MSF1 and that of the β subunits from *E. coli* and *B. subtilis* (Fig. 6). The lack of homology with the cytoplasmic sequences is not clearly understood. It may reflect a greater sequence divergence compared with the prokaryotic sequences. The "primitive" MSF1 protein has the three sequence motifs that characterize the recently defined class II of aminoacyl-tRNA synthetases (29, 30). This class does not have the mononu-

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FRSBBS 707 taIpkYPsvtRDIAlVtdktVtsgqLeSVIKeaGGkLlkeVtVFDVYeGeRmeEGkKSvAFSLqYvnpEqTLTEKEVtkahsKVlkALeDtYQAvLRg 804
FRSTEC 698 geIsRFPanrRDIAvVvaenVpaaDiLSecKkVGvnqVvgVnLFDVYrGkgvaEGyKSLAiSLilQdtsRTLeEKEIaatvaKcveALkErFQAsLRd 794
MSF1SC 385 dvafWLPedkpDI.....hqVbenDLMeIIrnIaGdLVesVkLvDsF..tHpktGrKSMcYrinYQssDRnLTnaEVntlqdnVcskLvkeYsveLR 474
CONSENSUS  -I---P---RDIA-V----V---DL-S-IK--GG-LV--V-LFDVY-G-H--EG-KS-A-SL-YQ---RTLTEKEV-----KV--AL---YQA-LR-
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FIG. 6. Sequence alignment of the MSF1 protein and the β subunits of *E. coli* and *B. subtilis* in the C-terminal region. To validate this alignment we used the PROFILESEARCH program of Gribskov (33). A profile generated by the alignment of the two sequences from *E. coli* (FRSTEC) and *B. subtilis* (FRSBBS) was used to screen the complete protein data base Swiss-Prot. The C-terminal extension of the MSF1 protein was the only additional sequence found with a significant score (33).

cleotide-binding fold that interacts with ATP in class I enzymes. Instead, motif 3, which is composed of two antiparallel β strands connected by a disordered loop in seryl-tRNA synthetase, is thought to participate in the active site (30).

One particularity of the mitochondrial phenylalanyl-tRNA synthetase is that it charges both mitochondrial and nonmitochondrial $tRNA^{Phe}$ [yeast cytoplasmic or *E. coli* (20)], and this is also true for the MSF1 protein (see Table 1). Both yeast cytoplasmic and *E. coli* phenylalanyl-tRNA synthetases make contacts with discriminatory bases in $tRNA^{Phe}$ that are located in three topologically distinct single-stranded regions, the D and anticodon loops and the ACCA- end, implying a large covering of the tRNA by the pseudodimeric phenylalanyl-tRNA synthetases (31, 32). The MSF1 protein, which is much smaller, probably interacts with the tRNA in a simpler or different mode. Determination of the nucleotide sites that specify $tRNA^{Phe}$ identity and analysis of the topology of the MSF1·$tRNA^{Phe}$ complex may reveal the particular features in tRNA recognition by the MSF1 protein.

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