Conservation in Evolution for a Small Monomeric Phenylalanyl-tRNA Synthetase of the tRNA^{Phe} Recognition Nucleotides and Initial Aminoacylation Site†

Ruslan Aphasizhev,‡,§,|| Bruno Senger,‡,|| Jens-Uwe Rengers,⊥ Mathias Sprinzl,⊥ Philippe Walter,‡ Gaby Nussbaum,‡ and Franco Fasiolo*,‡

CNRS, UPR 9002 Structure des Macromolécules Biologiques et Mécanismes de Reconnaissance, Institut de Biologie Moléculaire et Cellulaire, 15 Rue René Descartes, F-67084 Strasbourg, France, Institute of Molecular Biology, Russian Academy of Sciences, Vavilov, street 32, 117984, Russia, and Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany

Received August 2, 1995; Revised Manuscript Received October 9, 1995⊗

ABSTRACT: We previously showed that yeast mitochondrial phenylalanyl-tRNA synthetase (MSF protein) is evolutionarily distant to the cytoplasmic counterpart based on a high degree of divergence in protein sequence, molecular mass, and quaternary structure. Using yeast cytoplasmic tRNA^{Phe} which is efficiently aminoacylated by MSF protein, we report here the tRNA^{Phe} primary site of aminoacylation and the identity determinants for MSF protein. As for the cytoplasmic phenylalanyl-tRNA synthetase (Sampson, J. R., Di Renzo, A. B., Behlen, L. S., & Uhlenbeck, O. C. (1989) *Science 243*, 1363−1366), MSF protein recognizes nucleotides from the anticodon and the acceptor end including base A_{73} and, as shown here, adjacent G_1-C_{72} base pair or at least C_{72} base. This indicates that the way of tRNA^{Phe} binding for the two phenylalanine enzymes is conserved in evolution. However, tRNA^{Phe} tertiary structure seems more critical for the interaction with the cytoplasmic enzyme than with MSF protein, and unlike cytoplasmic phenylalanyl-tRNA synthetase, the small size of the monomeric MSF protein probably does not allow contacts with residue 20 at the top corner of the L molecule. We also show that MSF protein preferentially aminoacylates the terminal 2′-OH group of tRNA^{Phe} but with a catalytic efficiency for tRNA^{Phe}-CC-3′-deoxyadenosine reduced 100-fold from that of native tRNA^{Phe}, suggesting a role of the terminal 3′-OH in catalysis. The loss is only 1.5-fold when tRNA^{Phe}-CC-3′-deoxyadenosine is aminoacylated by yeast cytoplasmic PheRS (Sprinzl, M., & Cramer, F. (1973) *Nature 245*, 3−5), indicating mechanistic differences between the two PheRS's active sites for the amino acid transfer step.

Based on the presence of two sets of signature sequences within two types of nucleotide binding fold, the 20 aminoacyl-tRNA synthetases can be divided into two groups of 10 (Eriani *et al.*, 1990; Cusack *et al.*, 1990). In *Escherichia coli*, the majority of the class II tRNA synthetases are α_2 dimers with the exception of the alanine enzyme (α_4) and glycyl- or phenylalanyl-tRNA synthetase which are of the $\alpha_2\beta_2$ type (Schimmel & Söll, 1979). Dimerization has been attributed to motif 1 of the class II synthetases (Eriani *et al.*, 1990), although the intersubunit interface includes many other contacts between the two polypeptide chains of aspartyl- (Ruff *et al.*, 1991) or seryl-tRNA synthetases (Cusak *et al.*, 1990).

The cytoplasmic forms of PheRS occur as high molecular weights $\alpha_2\beta_2$ tetramers (M_r 250 000) in various organisms including mammalian cells, but the size of the individual subunits vary considerably from prokaryotes to eukaryotes (for references see Sanni *et al.*, 1988). Neither subunit alone is active *per se* (Baltzinger *et al.*, 1979), suggesting that the active site is at the subunit interface, and binding of 2 mol of each substrate indicates that the tetrameric enzyme behaves as a functional dimer (Fasiolo *et al.*, 1974). The same conclusion applies to the $\alpha_2\beta_2$ glycyl-tRNA synthetase from *E. coli* (McDonald *et al.*, 1984). The existence of heterodimeric interfaces for two members of class II, composed in the majority of homodimers, is still a matter of conjecture. The case of phenylalanyl-tRNA synthetase (PheRS) even complicates the puzzle, since this enzyme, in contrast to the other members of the class II, charges the terminal adenosine of tRNA at the 2′-OH group (Sprinzl & Cramer, 1973), a functional property that is only shared by the class I structures (Eriani *et al.*, 1990). A correlation between the way each class of enzymes approaches the tRNA and the primary site of tRNA aminoacylation was first proposed by Ruff *et al.* (1991).

Changes in quaternary structures for homologous synthetases in different organisms are rare. The only well documented cases, at the molecular level, are methionyl-tRNA synthetase (MetRS) which is a dimer in *E. coli* (Dardel *et al.*, 1984) and a monomer in yeast (Fasiolo *et al.*, 1985); glycyl-tRNA synthetase (GlyRS) which is of the $\alpha_2\beta_2$ type in *E. coli* and a dimer in *Bombyx mori* (Nada *et al.*, 1993) as well as in *Thermus thermophilus* (D. Kern, personal communication); and alanyl-tRNA synthetase which is a tetramer in *E. coli* (Jasin *et al.*, 1983) and a monomer in human (Shiba *et al.*, 1995). These examples can be

† This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS). R.A. was supported by a fellowship from the French-Russian scientific cooperation program (PICS) and FEBS (short-term).

* Corresponding author, at IBMC, 15 Rue Descartes, F-67084 Strasbourg, France. Tel: (33) 88 41 70 60; Fax: (33) 88 60 22 18.

‡ Institut de Biologie Moléculaire et Cellulaire.

§ Institute of Molecular Biology, Russian Academy of Sciences.

|| Both authors contributed equally to this work.

⊥ Universität Bayreuth.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

considered as a dimerization (or multimerization) of a catalytic core formed by a single polypeptide chain (MetRS) or an intersubunit interface (GlyRS). In this context, the change we noted for the yeast mitochondrial PheRS (MSF protein), which is monomeric (Sanni *et al*., 1991) whereas the cytoplasmic PheRS is an $\alpha_2\beta_2$ enzyme, is rather exceptional in the sense that dimerization of a protomer is no longer a prerequisite for activity. The yeast mitochondrial PheRS (MSF protein) is an α-like subunit having all three sequence motifs of the class II enzymes. Its activity may result from the recruitment of an internal poypeptide and a C-terminal extension. The latter domain has convincing homology with the C-terminal end of the large subunit of the bacterial PheRS (Sanni *et al.*, 1991).

The differences in the quaternary structure and activity between yeast cytoplasmic and mitochondrial PheRS prompted us to analyze further the biochemical properties of the MSF protein. We investigated the following: (i) the initial site of $tRNA^{Phe}$ aminoacylation at the terminal adenosine in order to see if there is a switch in the positional specificity from the 2′-OH to the 3′-OH group and (ii) the set of $tRNA^{Phe}$ identity nucleotides for the MSF protein using the collection of yeast cytoplasmic $tRNA^{Phe}$ mutants from Uhlenbeck's laboratory. Although quantitative differences may exist with similar mutants made in the mitochondrial $tRNA^{Phe}$ background, the results obtained with the heterologous $tRNA^{Phe}$ allow a direct comparison with the existing data of the yeast cytoplasmic and also *E. coli* PheRS-$tRNA^{Phe}$ interactions (Peterson & Uhlenbeck, 1992). To discriminate their cognate $tRNA^{Phe}$, the cytoplasmic yeast or *E. coli* PheRS use determinants located mainly in three distal parts of the L structure: the anticodon, the acceptor end, and the so-called "variable pocket" resulting from the spatial proximity of nucleotides from the D- and T-loop (Sampson & Uhlenbeck, 1988; McClain & Foss, 1988; Sampson *et al*., 1989, 1990a, 1992; Peterson & Uhlenbeck, 1992). Studies with the $\alpha_2\beta_2$ PheRS from human origin (Nazarenko *et al*., 1992) also favor a "three-point attachment" model of interaction between the tRNA and the synthetase while the "variable pocket" seems not to be a discriminatory site for the tetrameric *Thermus thermophilus* PheRS (Moor *et al*., 1992). One question raised by the existence of a small monomeric PheRS is as follows: does the mitochondrial PheRS contact only acceptor stem nucleotides or simultaneously acceptor stem and anticodon nucleotides? The answer to this question can bring some interesting information about the evolutionary relationship in the various PheRS-$tRNA^{Phe}$ recognition systems.

MATERIALS AND METHODS

Preparation of Yeast Cytoplasmic $tRNA^{Phe}$ 2′-dA and 3′-dA. Yeast $tRNA^{Phe}$ from bakers' yeast was isolated by chromatography from bulk tRNA (Boehringer, Mannheim) on BD-cellulose (1450 pmol/A_{260}). It was composed of about 85% $tRNA^{Phe}CpC_{75}$ and 15% $tRNA^{Phe}CpCpA$. Separation of $tRNA^{Phe}CpC_{75}$ was achieved by chromatography on DEAE-Sephadex A25 using a linear gradient from 450 to 650 NaCl as previously described in (Sprinzl *et al.*, 1977).

For the incorporation of 2′- and 3′-deoxyadenosine, 50 μM $tRNA^{Phe}CpC_{75}$ was incubated in 300 μL of a solution containing 100 mM Tris-HCl, pH 9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 3 mM of the nucleoside 5′-triphosphate (dATP and cordycepine triphosphate for 2′- and 3′-deoxyadenosine, respectively) with 50 nM ATP(CTP): tRNA nucleotidyltranferase for 15 h at 25 °C. rATP was also used as substrate to test the integrity of the $tRNA^{Phe}CpC_{75}$ species. The pH of the mixture was lowered by adding 30 μL of 2 M sodium acetate buffer, pH 4.5. After addition of 300 μL of water, the solution was applied to a column of DEAE-Sephadex A25 (1 cm × 2 cm), equilibrated with 10 mM $MgCl_2$ and 200 mM NaCl in 20 mM sodium acetate buffer, pH 5.2. Excess nucleoside 5′-triphosphate and the enzyme were washed off with the same buffer. tRNA was finally eluted with the same buffer containing 1 M NaCl and desalted on a Bio-Gel P6 column.

Contaminating $tRNA^{Phe}CpCpA$ in $tRNA^{Phe}Cp2′dA$ and $tRNA^{Phe}Cp3′dA$ preparations was deactivated by periodate oxidation. 75 μM tRNA was incubated in the dark with 0.8 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 6.5, for 2 h at room temperature. Excess periodate was destroyed by addition of glucose to a final concentration of 0.8 mM.

In Vitro Transcription. $tRNA^{Phe}$ transcripts from plasmid encoded tRNA genes were enzymatically synthesized using T7 RNA polymerase as described in Sampson and Uhlenbeck (1988). Runoff transcription was obtained by creating a *Bst*NI restriction site at the 3′-terminal end of tRNA variants. T7 RNA polymerase was isolated from *E. coli* strain BL 21 containing the plasmid pAR1219 and purified according to Grodberg and Dunn (1987).

Forty micrograms of linearized plasmid DNA was transcribed in a 0.5−1 mL reaction mixture under the following conditions: 60 mM Tris-HCl, pH 8, 30 mM $MgCl_2$, 7 mM dithiothreitol, 1. 4 mM spermidine, 14 mM Triton X-100, 20 mM GMP, 4 mM of each ribonucleotide, and 0. 1 mg/mL T7 RNA polymerase. After 4 h of incubation at 37 °C, the reaction was stopped by addition of 100 μl of EDTA 0.5 M and phenol extraction. The RNA was precipitated by ethanol, resuspended in 250 μL of 50% formamide, and purified by 12% polyacrylamide gel electrophoresis under denaturing conditions. The bands were visualized by UV shadowing, excised, and electroeluted using a Biotrap BT 1000 system (Schleicher & Schuell) according to the manufacturer's instruction.

Aminoacylation Assays. Transcripts were submitted to a renaturation step under the following conditions: heating in a 90 °C water bath for 1 min, cooling at room temperature for 5 min, and incubation at 30 °C for 5 min after addition of the reaction mixture containing magnesium, ATP, and phenylalanine. Aminoacylation was carried out at 37 °C under the following conditions: 144 mM Tris-HCl, pH 7.8, 5 mM dithiothreitol, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM [^{14}C]phenylalanine (125 μCi/μmol; 80 cpm/pmol), 5−80 mM $tRNA^{Phe}$ wild-type or transcript, and 10−300 nM MSF protein. At various time intervals 10 μL aliquots from a 50 μL reaction mixture were spotted onto Whatman paper discs and quenched by 5% trichloroacetic acid. Initial rates from 5 to 6 tRNA concentrations were used to determine K_m and k_{cat} from Lineweaver−Burk plots. Radioactivity was counted using a toluene-based scintillant.

Purification of MSF Protein. MSF protein was overexpressed in yeast as a fusion protein with gluthatione *S*-transferase (GST) and purified in one affinity chromatography step on a glutathione-agarose bed. The N-terminal added GST tag was subsequently removed by thrombin cleavage and repassage over the agarose bed followed by HPLC chromatography on a TSK-hydroxyapatite column.

Two variants of MSF protein were prepared in this way: one corresponding to the complete structural gene of MSF gene as well as a mutant deleted from the 11 N-terminal end. Both showed similar k_{cat} values (45−50 min^{-1}). We submitted the full-length MSF protein to a dynamic light scattering study according to Phillies *et al.* (1976). In the presence of tRNA, the apparent hydrodynamic radius of the enzyme−tRNA complex corresponds to the sum of the diffusion coefficients of the individual macromolecules and not to that of a dimeric MSF−$tRNA^{Phe}$ complex, indicating that MSF protein does not dimerize in the presence tRNA (unpublished results). The enzyme used in this study was the N-terminally deleted mutant form.

RESULTS

$tRNA^{Phe}$ Recognition Nucleotides for MSF Protein. MSF protein aminoacylates native yeast cytoplasmic $tRNA^{Phe}$ and the corresponding T7 transcript with a similar catalytic efficiency, k_{cat}/K_m (Table 1). Mutant forms derived from the heterologous yeast $tRNA^{Phe}$ transcripts can therefore be used to determine the set of identity nucleotides for MSF protein. To validate the study with the cytoplasmic $tRNA^{Phe}$, we also measured the activity of the homologous yeast mitochondrial $tRNA^{Phe}$ transcript. The yield of this RNA transcript by T7 RNA polymerase is very low, perhaps because the DNA sequence encoding the 5′ end of the tRNA might contain sequences which can act as a potential terminator of transcription. The mitochondrial $tRNA^{Phe}$ sequence has most of the tertiary nucleotides conserved as those seen in cytoplasmic $tRNA^{Phe}$ (Figure 1), suggesting a similar tertiary folding, but differs in 9 stem positions and 6 single-stranded regions including the nucleotide at position 20 (Figure 1). The catalytic efficiency of the mitochondrial $tRNA^{Phe}$ transcript is 15 times higher than that of cytoplasmic $tRNA^{Phe}$ transcript mainly because the K_m is decreased (Table 1).

Table 1 shows the result of the mutation of 6 single-stranded nucleotides and 5 tertiary interactions from the central core of cytoplasmic $tRNA^{Phe}$ (15−48, 9−23−12, 13−22−46, 10−25−45, and 26−44). The impaired activity or minor modification in the catalytic efficiency of the tertiary mutants tested, which corresponded to conservative changes found in other tRNAs ($G_{15}A$, $C_{48}U$; A_9G, $A_{23}C$, $U_{12}G$; $C_{13}U$, $G_{22}A$, $G_{46}A$; $G_{10}C$, $C_{25}G$; $G_{45}U$; $G_{26}A$, $A_{44}G$), correlated with the absence of disruptive features in the structure of these mutants (Behlen *et al*., 1990; Sampson *et al*., 1992; Peterson & Uhlenbeck, 1992). Therefore, these tertiary nucleotides do not participate in sequence specific contacts with the mitochondrial PheRS as reported for the cytoplasmic PheRS (Sampson *et al*., 1990). However, MSF protein seems sensitive to the particular pairing geometry (propeller twist) of G_{26}-A_{44} which contributes to the coaxial stack between the anticodon and the D-stem (Quigley & Rich, 1976). While the inversion of G_{26}-A_{44} base pair (as found in *E. coli* $tRNA^{Phe}$, see Figure 1) or $A_{44}G$ and $A_{44}U$ single mutations affect marginally the activity of the tRNA, the k_{cat}/K_m of the $G_{26}A$ mutant is reduced 6-fold (Table 1). Model building studies (Sampson *et al*., 1990) indicate that the number of hydrogen bonding interactions is maintained for the A_{26}-G_{44} base pair or $A_{44}G$ and A44U mutants but not for the $G_{26}A$ mutant, which may explain the 6-fold loss of activity of the resulting A_{26}-A_{44} base pair.

In contrast to the modest effects seen with the tertiary mutants, $A_{73}U$ and $A_{73}C$ mutations reduce the k_{cat}/K_m 33- and 17-fold, respectively, whereas each anticodon substitution ($G_{34}A$, $A_{35}U$, $A_{36}C$) practically inactivates the mutant tRNA (Table 1). The anticodon substitutions have much larger effects on k_{cat}/K_m when tested with MSF protein than with the homologous PheRS (Table 1), suggesting that two negative effects are additive: an initial nonoptimal apposition between the heterologous $tRNA^{Phe}$ and MSF protein, and the loss of contacts when identity determinants are mutated. A similar result is observed when *E. coli* PheRS recognition nucleotides are analyzed in the yeast cytoplasmic $tRNA^{Phe}$ background (Peterson & Uhlenbeck, 1992).

The tetrameric yeast cytoplasmic and *E. coli* PheRS use the "variable pocket" as a site for tRNA discrimination, particularly nucleotide at position 20 (Sampson & Uhlenbeck,

Table 1: Determination of $tRNA^{Phe}$ Recognition Nucleotides for MSF Protein and Comparison with That of Yeast Cytoplasmic PheRS

	K_m (μM)	k_{cat} (min^{-1})	normalized k_{cat}/K_m	
			MSF	yeast PheRS
Modified tRNAs				
yeast $tRNA^{Phe}$ ($\alpha_4\beta_2$)	12	56	1	1
E. coli $tRNA^{Phe}$ ($\alpha_4\beta_2$)	20	40	0.44	0.006[a]
$tRNA^{Phe}$ Transcripts				
yeast cytoplasmic ($\alpha_4\beta_2$)	15	50	1	1
yeast mitochondrial ($\alpha_3\beta_2$)	1. 8	86	14.3	
variable pocket				
$G_{20}U$	33	104	1	0.083
anticodon				
$G_{34}A$			<0.01	0.019
$A_{35}U$			<0.01	0.0038
$A_{36}C$			<0.01	1
acceptor arm				
$A_{73}U$	24	2.5	0.030	0.091
$A_{73}C$	25	5	0.059	0.125
$C_{72}U$	10	5	0.140	0.03[b]
C_2G, $G_{71}C$	30	10	0.710	0.14[b]
tertiary interactions				
$G_{15}A$, $C_{48}U$	30	104	1.0	1.67
A_9G, $A_{23}C$, $U_{12}G$	50	243	1.4	0.50
$C_{13}U$, $G_{22}A$, $G_{46}A$	45	121	0.80	1.21
$G_{10}C$, $C_{25}G$	20	20	0.30	0.91
$G_{45}U$	15	43	0.80	1
$G_{26}A$, $A_{44}G$	40	63	0.50	1.43
$A_{44}G$	30	166	1.67	1.67
$A_{44}U$	20	81	1.25	1.25
$G_{26}A$	40	21	0.16	1.25
U_8C			0.10	0.013[b]
identity switches				
Tyr $\alpha_3\beta_3$→Phe ($\alpha_4\beta_2$)	15	20	0.4	1.43
Met $\alpha_4\beta_2$→Phe ($\alpha_4\beta_2$)	nm	nm	nm	0.71
Arg $\alpha_3\beta_2$→Phe ($\alpha_4\beta_2$)	33	108	1.0	0.62
Ile $\alpha_4\beta_3$→Phe ($\alpha_4\beta_3$)	200	27	0.04	0.0002[b]
Asp $\alpha_3\beta_3$→Phe 2 ($\alpha_3\beta_3$) (D/1c/F)	21	216	3.12	0.0091[c]
Asp $\alpha_3\beta_3$→Phe 1 ($\alpha_4\beta_2$) (D/4c1/F)	11	92	2.50	0.10[c]

[a] Value normalized to our own measurements of kinetic constants of yeast PheRS for the cognate native $tRNA^{Phe}$ (k_{cat}: 190 min^{-1}; K_m: 0. 4 μM). [b] Values normalized to our own measurements of kinetic constants of yeast PheRS for the cognate $tRNA^{Phe}$ transcript (k_{cat}: 6. 84 min^{-1}; K_m: 0. 5 μM). [c] Values taken from Perret *et al*. (1992). The other normalized values for yeast cytoplasmic PheRS are taken from Sampson and Uhlenbeck (1988), Sampson *et al*. (1989a,b, 1991), and Nazarenko *et al*. (1992). The α/β D-loop arrangement for each tRNA species is indicated in parentheses. Concentration of $tRNA^{Phe}$ transcript varied from 1 to 80 μM. The usual concentration of MSF protein was 25 nM and was raised to 100 nM when the activity of the mutant $tRNA^{Phe}$ is very low. The concentration of cytoplasmic PheRS was 10 nM for wild-type $tRNA^{Phe}$ transcript and 50−100 nM for $tRNA^{Phe}$ mutant forms.

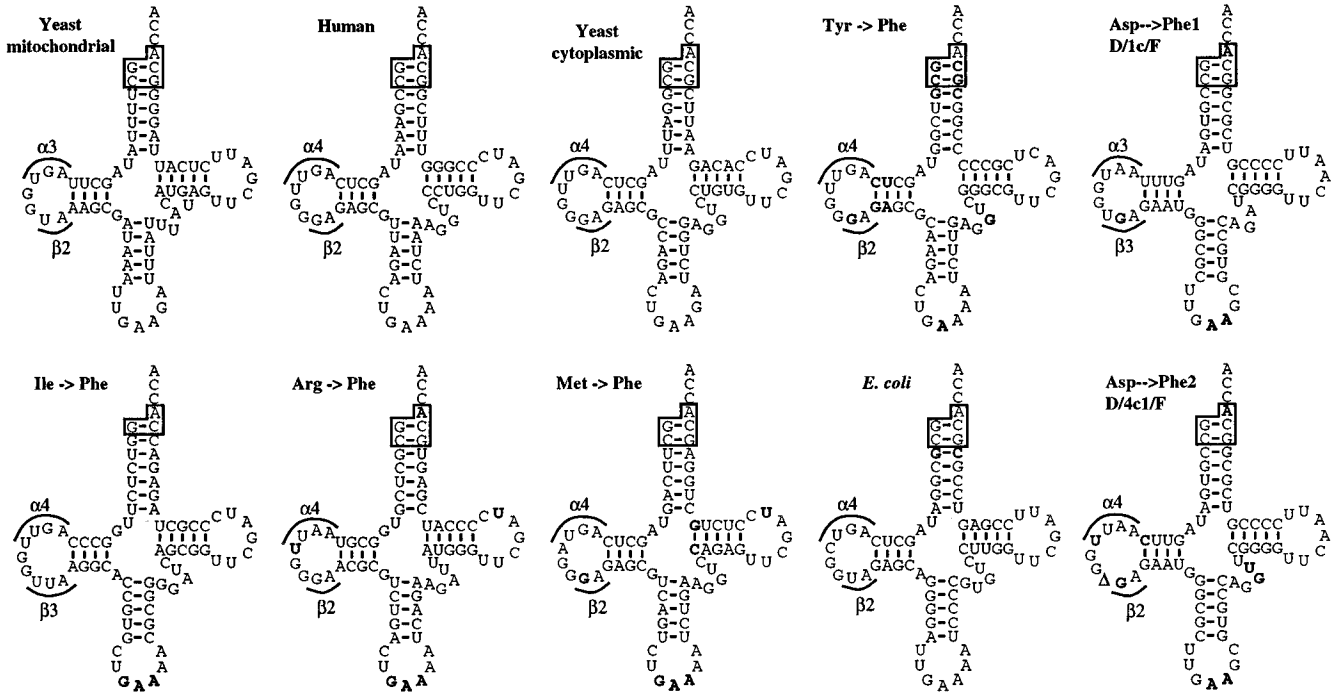

FIGURE 1: Sequence of various $tRNA^{Phe}$ used as substrates for the cytoplasmic and mitochondrial PheRS (MSF protein). tRNA identity switches are indicated by the change of the original amino acid acceptance into phenylalanine acceptance. Letters in bold indicate the substitutions made in the original noncognate $tRNA^{Phe}$. Variants D/1c/F and D/4c1/F were derived from yeast $tRNA^{Asp}$ and their synthesis described in Perret *et al*. (1992). With the exception of the yeast mitochondrial $tRNA^{Phe}$ and Ile→Phe variant, the other $tRNA^{Phe}$ sequences were already tested for aminoacylation by the human and yeast cytoplasmic PheRS (Nazarenko *et al*., 1992; Sampson *et al*., 1989a,b). The complete set of changes made in Tyr→Phe, Arg→Phe, and Met→Phe are described in Sampson *et al*. (1989a). Boxed nucleotides underline the occurrence of common nucleotides in the acceptor stem. The α/β D-loop arrangement for each tRNA is indicated.

1988; McLain & Foss, 1988). However, the level at which these two enzymes discriminate this nucleotide is not the same. Whereas G20 is required to convert *E. coli* $tRNA^{Phe}$ to a good substrate for yeast PheRS, the *E. coli* enzyme tolerates the presence of G_{20} and A_{20} in addition to the naturally occurring U_{20} in *E. coli* $tRNA^{Phe}$ (Roe *et al*., 1973; Peterson & Uhlenbeck, 1992). The $G_{20}U$ replacement in yeast $tRNA^{Phe}$ does not influence the interaction with MSF protein either. This result suggests either an absence of contacts with the mitochondrial protein due to its small size (M_r 52 000) or the insensitivity of MSF protein to the chemical nature of base 20. Further studies are necessary to distinguish between the two possibilities.

MSF Protein Is Less Sensitive to the tRNA Framework Than Yeast Cytoplasmic PheRS. Comparison of the crystallographic structures of yeast $tRNA^{Phe}$ (Holbrook *et al*., 1978) and $tRNA^{Asp}$ (Moras *et al*., 1980) revealed conformational differences between these two molecules (Romby *et al*., 1985). Yeast $tRNA^{Phe}$ has a greater structural rigidity due to the additional hydrogen bonding of nucleotide-47 with phosphate-50 and a larger angle between the two arms of the tRNA due to the position of the conserved tertiary nucleotides $G_{18}G_{19}$ within the D-loop. In $tRNA^{Asp}$, the α and β domains that surround the $G_{18}G_{19}$ sequence comprise 3 nucleotides, respectively, whereas $tRNA^{Phe}$ has an $\alpha_4\beta_2$ nucleotide arrangement. To explore the sensitivity of the MSF−tRNA interaction to subtle changes in the tRNA conformation, we tested the phenylalanine acceptance of two yeast $tRNA^{Asp}$ variants which have been converted to substrates for yeast cytoplasmic PheRS (Perret *et al*., 1992). In the first construction (D/1c/F), only the identity elements of yeast $tRNA^{Phe}$ were introduced in the yeast $tRNA^{Asp}$ framework, whereas in the second (D/4c1/F), the D- and variable loops were modified to yield a $tRNA^{Asp}$ variant having the same nucleotide number as yeast $tRNA^{Phe}$ (Figure 1). These two variants have phenylalanylation activities that differ by a factor of 110 and 10, respectively, from that of yeast cytoplasmic $tRNA^{Phe}$ when tested with the homologous PheRS (Perret *et al*., 1992). In contrast, D/1c/F and D/4c1/F variants are aminoacylated with the same efficiency by MSF protein and have k_{cat}/K_m values increased 2.5- to 3-fold over that of yeast cytoplasmic $tRNA^{Phe}$ (Table 1).

Aminoacylation of yeast $tRNA^{Ile}$ with anticodon GAA (Figure 1) further emphasizes the differences between yeast cytoplasmic and mitochondrial PheRS with respect to subtle changes in the tRNA conformation. The mutant $tRNA^{Ile}$ is aminoacylated with an efficiency reduced 25-fold by MSF protein and 5000-fold by yeast cytoplasmic PheRS (Table 1). In the latter case, a combination of multiple negative interactions may severely hamper the interaction with enzyme. One of them is the presence of U_{20} nucleotide which only contributes to a 12-fold loss of k_{cat}/K_m (Sampson *et al*., 1989a), suggesting that the tRNA framework is the main block in aminoacylation by cytoplasmic PheRS. Indeed, yeast $tRNA^{Ile}$ has an additional nucleotide in the D-loop yielding an $\alpha_4\beta_3$ D-loop arrangement and a triple base 13−22−46 involving a $C_{13}A_{22}$ mismatch which probably dictate a tRNA conformation different from that of yeast $tRNA^{Phe}$.

The different sensitivities of the cytoplasmic and mitochondrial PheRS's toward tRNA conformation is also seen when the highly conserved reverse Hoogsteen base pair U_8A_{14} of yeast $tRNA^{Phe}$ is disrupted. It is generally believed that this critical base pair plays a role in the folding of the L-shaped molecule. The change of U_8 to C_8 reduces yeast $tRNA^{Phe}$ aminoacylation by 10- and 77-fold when tested with

the mitochondrial and cytoplasmic PheRS, respectively (Table 1). A model of tRNA−enzyme complex, involving only two distal ends of the tRNA (the anticodon and the acceptor end for MSF protein) instead of three (the three distal ends of the L molecule) for the cytoplasmic PheRS, may explain the ability of MSF protein to match different tRNA conformations.

The Set of Recognition Nucleotides Includes the C72 Acceptor Nucleotide for both Yeast Cytoplasmic and Mitochondrial PheRS. Three tRNAs, initially poorly or not aminoacylated by cytoplasmic PheRS, were converted to good substrates for this enzyme by multiple changes that recreate the identity set and the Pb^{2+} cleavage domain of yeast $tRNA^{Phe}$ (Sampson *et al*., 1989a). These are $tRNA^{Arg\rightarrow Phe}$, elongator $tRNA^{Met\rightarrow Phe}$, and $tRNA^{Tyr\rightarrow Phe}$ from yeast (Figure 1). When tested with MSF protein, $tRNA^{Arg\rightarrow Phe}$ and $tRNA^{Tyr\rightarrow Phe}$ exhibit a relative catalytic efficiency of 1 and 0.43, respectively. Interestingly, as noted for human PheRS (Nazarenko *et al*., 1992), the yeast elongator $tRNA^{Met\rightarrow Phe}$ is a poor substrate for MSF protein. A plateau of 8% could be measured with MSF protein, but no initial rates were available, indicating an unfavorable balance between acylation and deacylation rates (enzymatic and/or chemical). Inefficient aminoacylation of *E. coli* $tRNA^{fMet\rightarrow Phe}$ by *E. coli* PheRS has also been reported and was correlated, at least partly, to the anticodon stem dependent conformation of the corresponding loop (Peterson & Uhlenbeck, 1992).

Based on $tRNA^{Phe}$ sequences that are aminoacylated by the cytoplasmic and mitochondrial PheRS (Figure 1), the consensus of common nucleotides in the acceptor stem suggests the last two base pairs G_1C_{72} and C_2G_{71} as potential recognition nucleotides for both tRNA synthetases. Because G_1 nucleotide could not easily be replaced without affecting the yield of T7 RNA transcription, we only tested $G_{72}U$ mutant. The results indicate a 7- and 33-fold reduction in the catalytic efficiency by MSF protein and yeast cytoplasmic PheRS, respectively (Table 1). The inversion of the base pair at positions 2−71 (G_2C_{71}mutant) seems not to influence dramatically aminoacylation either by MSF protein (1.4-fold) or by cytoplasmic PheRS (7-fold) (Table 1), in agreement with the observation that yeast cytoplasmic PheRS cross-aminoacylates wheat germ $tRNA^{Phe}$ with U_2-A_{71} base pair (Sampson *et al*., 1989a). From these results, we conclude that interaction of yeast mitochondrial and cytoplasmic PheRS with the acceptor end includes the G_1C_{72} base pair or at least C_{72} nucleotide adjacent to A_{73} (Figure 2).

$tRNA^{Phe}$ Site of Aminoacylation by MSF Protein. Cytoplasmic yeast $tRNA^{Phe}$ is aminoacylated by yeast cytoplasmic PheRS at the 2′-OH of the terminal adenosine (Sprinzl & Cramer, 1973). This result could be unequivocally demonstrated using $tRNA^{Phe}$ species modified at the 3′-terminal end: whereas $tRNA^{Phe}$-CC-3′dA (3′-deoxyadenosine) is an efficient substrate for yeast PheRS (the catalytic efficiency is 0.64 that of native tRNA), $tRNA^{Phe}$-CC-2′dA(2′-deoxyadenosine) is not charged by PheRS.

We tested the aminoacylation of the same $tRNA^{Phe}$ derivatives using MSF protein. In conditions where $tRNA^{Phe}$-CC-3′dA is charged at a level of 60% by PheRS, neither $tRNA^{Phe}$-CC-3′dA nor $tRNA^{Phe}$-CC-2′dA is aminoacylated by the equivalent concentration (50 nM) of MSF protein. The aminoacylation of $tRNA^{Phe}$-CC-3′dA requires a concentration of MSF protein in the range of 300 nM. Under these conditions, the reaction proceeds linearly with time to yield 15% of $tRNA^{Phe}$-CC-3′dA charging within 15 min, whereas aminoacylation values for $tRNA^{Phe}$-CC-2′dA remain at a background level (Figure 3). The kinetic parameters of $tRNA^{Phe}$-CC-3′dA for MSF protein were determined and compared to that obtained with native $tRNA^{Phe}$. The K_m is maintained while k_{cat} is reduced 100-fold (Table 2), indicating that an efficient aminoacylation of $tRNA^{Phe}$ by MSF protein requires both hydroxyl groups of the terminal adenosine.

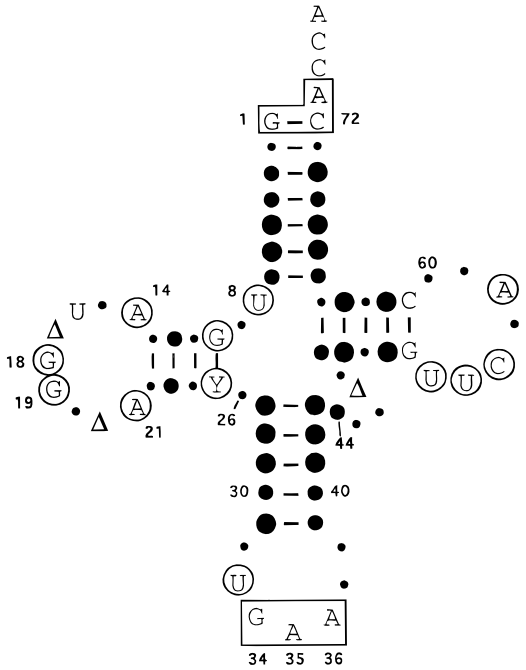

FIGURE 2: Consensus sequences of common nucleotides recognized by cytoplasmic PheRS and MSF protein. Nucleotides involved in tertiary interactions and conserved or semiconserved in all tRNAs are circled. Y stands for pyrimidine residues. The other nucleotides indicate nucleotides common to all $tRNA^{Phe}$ sequences used in this study. Small, medium, and large dots indicate positions in yeast $tRNA^{Phe}$ where nucleotides have been changed to one, two, or three different nucleotides, respectively. Δ refers to a nucleotide deletion at the indicated position. Key bases are numbered based on the convention for tRNAs. Identity nucleotides for both cytoplasmic PheRS and MSF protein are boxed.

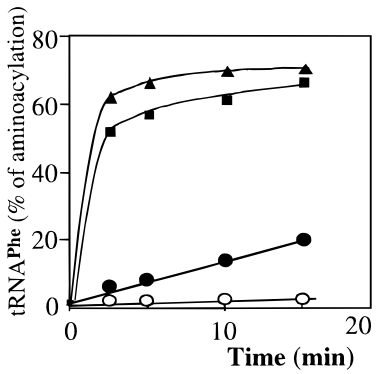

FIGURE 3: Aminoacylation kinetics of wild-type and terminal adenosine modified yeast $tRNA^{Phe}$. ATP, dATP, and cordyceptin are enzymatically incorporated into yeast $tRNA^{Phe}$ -CC to yield respectively the wild-type $tRNA^{Phe}$-CC-riboA and modified $tRNA^{Phe}$-CC-2′dA and $tRNA^{Phe}$-CC-3′dA species as described in Materials and Methods. Aminoacylation kinetics were measured using 300 nM MSF protein and 5 μM tRNA. (▲) Native $tRNA^{Phe}$; (■) $tRNA^{Phe}$-CC-riboA; (●) $tRNA^{Phe}$-CC-3′dA; (○) $tRNA^{Phe}$-CC-2′dA.

Table 2: Kinetic Parameters of $tRNA^{Phe}$-CC-3′dA Derivative Measured with Cytoplasmic and Mitochondrial PheRS

	PheRS[a]			MSF		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (relative)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (relative)
$tRNA^{Phe}$ wild type	2.9	300	1	10.5	45	1
$tRNA^{Phe}$-CC-3′dA	2.9	192	0.64	12.5	0.54	0.01

[a] Values taken from Sprinzl and Cramer (1973). The concentration of modified $tRNA^{Phe}$ varied from 5 to 80 μM. MSF protein concentration was 300 nM.

DISCUSSION

Despite their distant relationship manifested at the level of the quaternary structure and protein sequences (Sanni *et al*., 1991), yeast cytoplasmic and mitochondrial PheRS bind $tRNA^{Phe}$ in the same way, as deduced from an analysis of the $tRNA^{Phe}$ identity nucleotides. To achieve $tRNA^{Phe}$ aminoacylation, both enzymes interact with the anticodon triplet and with the acceptor end comprising the last base pair of the stem and single-stranded A_{73} nucleotide. The similar mode of tRNA recognition is further confirmed by footprinting experiments showing that both PheRS protect the same $tRNA^{Phe}$ phosphate backbone regions inside the L structure (unpublished results). However, the $tRNA^{Phe}$ conformation seems more important for interaction with cytoplasmic PheRS than with MSF protein, as shown from tertiary mutants made in the $tRNA^{Phe}$ background and from anticodon swap experiments made with noncognate phenylalanine tRNAs. How does the 52 kDa monomeric MSF protein accommodate binding of the two distal $tRNA^{Phe}$ sites? MSF protein has a 100-residue C-terminal extension which is critical for tRNA aminoacylation (Koerner *et al*., 1987) and is separated by a 20-peptide segment from motif 3 of the catalytic domain. The modular arrangement between the two functional domains may be similar to that seen in the recent crystal structure of *E. coli* histidyl-tRNA synthetase where a flexible 8-amino acid peptide links a 100-residue C-terminal anticodon binding domain to the α-helix of motif 3 (Arnez *et al*., 1995). It may be worthy to mention, however, that Mosyak *et al.* (1995) have assigned the MSF C-terminal like domain of *Th. thermophilus* PheRS to play a role in binding the acceptor stem of $tRNA^{Phe}$.

Removal of the terminal 3′-OH group of adenosine in yeast $tRNA^{Phe}$ has little or no consequence for tRNA aminoacylation by yeast cytoplasmic PheRS (Sprinzl & Cramer, 1973), whereas the missing 3′-OH reduces k_{cat} 100-fold but not K_m with MSF protein. However, both enzymes use the 2′-OH of $tRNA^{Phe}$ as the initial site of aminoacylation. This indicates that the active sites of the two PheRS's differ. The quasi-exclusive k_{cat} effect of the missing OH group, observed with MSF protein, indicates that the 3′-OH group of $tRNA^{Phe}$ plays a role in catalysis either directly by providing a functional group or indirectly by contributing to a nucleophilic environment or to a specific conformation of the terminal adenosine. Interestingly, the tetrameric *E. coli* PheRS also shows a 100-fold reduced k_{cat} for $tRNA^{Phe}$-CC-3′dA aminoacylation (Sprinzl & Cramer, 1979), underlining similarities in the organization of the active sites of the prokaryotic and mitochondrial PheRS. Yeast cytoplasmic PheRS, like *E. coli* PheRS, has an active site made at the interface of the α and β subunits (Baltzinger *et al*., 1979; Khodyreva *et al*., 1985). Thus, the difference seen between yeast and mitochondrial PheRS as to the requirement or not for two terminal hydroxyl groups of tRNA cannot be related to the particular quaternary structure of these two enzymes.

The demonstration that the 2′-OH specificity of aminoacylation is maintained for two evolutionary distant PheRS's indicates that the preferential 2′-hydroxyl attachment of phenylalanine is a property of the ancestral protein biosynthesis system or at least appeared early in evolution. Considering that chemical esterification of 5′-AMP by *N*-acetylphenylalanine occurs predominantly at the 2′-OH position (Lacey *et al*., 1990), this chemical reactivity may also have occurred in the primitive translation system. If this were the case, then full understanding of the molecular switch noted for the majority of the class II aminoacyl-tRNA synthetases from a 2′-OH to a 3′-OH position requires the high crystallographic resolution of a phenylalanyl-tRNA synthetase active site structure in the presence of tRNA.

ACKNOWLEDGMENT

We are indebted to Prof. O. Uhlenbeck for making available his large collection of yeast $tRNA^{Phe}$ mutants which greatly facilitated this study. We thank R. Giégé and C. Florenz for their gift of $tRNA^{Asp}$ variants.

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BI9517998