Mitochondrial Methionyl-tRNA$_f^{Met}$ Formyltransferase from *Saccharomyces cerevisiae*: Gene Disruption and tRNA Substrate Specificity

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ABSTRACT: Initiation of protein synthesis in bacteria, mitochondria, and chloroplasts involves a formylated methionyl-tRNA species. Formylation of this tRNA is catalyzed by a methionyl-tRNA$_f^{Met}$ formyltransferase (formylase). Upon inactivation of the gene encoding formylase, the growth rate of *Escherichia coli* is severely decreased. This behavior underlines the importance of formylation to give tRNA^{Met} an initiator identity. Surprisingly, however, recent data [Li, Y., Holmes, W. B., Appling, D. R., and RajBhandary, U. L. (2000) *J. Bacteriol. 182*, 2886−2892] showed that the respiratory growth of *Saccharomyces cerevisiae* was not sensitive to deprivation of the mitochondrial formylase. In the present study, we report conditions of temperature or of growth medium composition in which inactivation of the formylase gene indeed impairs the growth of a *S. cerevisiae* haploid strain. Therefore, some selective advantage can eventually be associated to the existence of a formylating activity in the fungal mitochondrion under severe growth conditions. Finally, the specificity toward tRNA of *S. cerevisiae* mitochondrial formylase was studied using *E. coli* initiator tRNA and mutants derived from it. Like its bacterial counterpart, this formylase recognizes nucleotidic features in the acceptor stem of mitochondrial initiator tRNA. This behavior markedly distinguishes the mitochondrial formylase of yeast from that of animals. Indeed, it was shown that bovine mitochondrial formylase mainly recognizes the side chain of the esterified methionine plus a purine−pyrimidine base pair in the D-stem of tRNA [Takeuchi, N., Vial, L., Panvert, M., Schmitt, E., Watanabe, K., Mechulam, Y., and Blanquet, S. (2001) *J. Biol. Chem. 276*, 20064−20068]. Distinct tRNA recognition mechanisms adopted by the formylases of prokaryotic, fungal, or mammalian origins are likely to reflect coevolution of these enzymes with their tRNA substrate. Each mechanism appears well suited to an efficient selection of the substrate within the pool of all tRNAs.

Initiation of translation requires precise selection of a start codon on mRNA. To achieve this selection, a specialized initiator tRNA, universally aminoacylated with methionine, occurs in any cell (*1*). However, features of this initiator tRNA enabling its appropriation by the translation start apparatus depend on the considered organism. In bacteria, a key step in the acquisition of its initiator identity by tRNA is the N-formylation of the esterified methionine (*2*, *3*). Introduction of the formyl group reinforces the binding of the initiator tRNA to initiation factor 2 (IF2)[1] and impairs its binding to elongation factor Tu (*4*, *5*). In archaea and in the cytoplasm of eukarya, there is no formylation of the initiator tRNA. Met-tRNA$_i^{Met}$ is recruited by an heterotrimeric factor called e/aIF2 (*6*). Finally, in mitochondria and chloroplasts, formylation of the initiator tRNA is observed (*1*, *7*, *8*). This finding is in line with the idea that these organelles derive from bacterial intracellular symbiotic organisms.

Formylation of methionylated initiator tRNA is catalyzed by methionyl-tRNA$_f^{Met}$ formyltransferase (formylase or FMT). The importance of formylation for the initiation process is underlined by the large decreases in growth rate resulting from inactivation of the formylase gene in *Escherichia coli* (*9*) or in *Pseudomonas aeruginosa* (*10*). In both organisms, however, the gene was not found to be strictly essential.

The *E. coli* formylase is highly specific for the initiator tRNA, by mainly recognizing the top of the acceptor stem. In particular, the absence of strong pairing between bases 1 and 72 is a key feature in the formylation of bacterial Met-tRNA$_f^{Met}$ (*11*, *12*). Moreover, in the D-stem, the enzyme recognizes a Pu11-Py24 base pair that is also characteristic of bacterial initiator tRNAs (*11*). Finally, formylase is sensitive to the nature of the side chain of the amino acid esterified to tRNA (*12*−*14*). The crystallographic models of *E. coli* formylase, free or complexed with fMet-tRNA$_f^{Met}$, have shown that an opening of bases 1−72 bent the acceptor stem, allowing the esterified methionine to dip inside the active site of the enzyme (*15*, *16*).

In animal mitochondria, a single tRNA^{Met} is thought to be used in either initiation or elongation of translation (*17*, *18*). The balance between the initiator and elongator functions of this tRNA would be governed by the rate of its formylation. In contrast with the bacterial formylase, the bovine mitochondrial formylase is less sensitive to sequences in the acceptor stem of tRNA^{Met}. Instead, it recognizes the side

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[1] Abbreviations: FMTmt, mitochondrial methionyl-tRNA$_f^{Met}$ formyltransferase; FMTec, *Escherichia coli* methionyl-tRNA$_f^{Met}$ formyltransferase; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; IF2, initiation factor 2.

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chain of the esterified methionine plus the Pu11-Py24 pair (*19*, *20*).

Unlike the mitochondria of animals, those of single cell eukaryotes (*21*) or of plants (*22*) contain two distinct methionine tRNAs involved in initiation and elongation of translation, respectively. This raises the question of the rules of tRNA substrate recognition in such cases. For instance, does the formylase of a fungal mitochondrion resemble a bacterial enzyme or a mammalian mitochondrial one? To address this question, we expressed the mitochondrial formylase of *Saccharomyces cerevisiae* in *E. coli*. After purification to homogeneity, study of the tRNA specificity of the enzyme was undertaken using mutants of *E. coli* initiator tRNA. Like its bacterial counterpart, yeast mitochondrial formylase recognizes the acceptor stem of its substrate, thereby markedly distinguishing itself from an animal mitochondrial enzyme.

Another problem to solve deals with the functional role of formylation in yeast mitochondria. Recent studies showed that respiratory growth of *S. cerevisiae* could be maintained at a wild-type rate despite the absence of formylation (*23*). This result suggested that formylation in yeast mitochondria might only represent a relic of the bacterial origin of the organelle. However, the systematic presence of a formylase gene in all known eukaryotic genomes rather indicates a unique role of formylation in mitochondria. Therefore, to find some selective advantage associated to the conservation of formylase, we searched for growth conditions where the absence of mitochondrial formylation impairs the growing of yeast cells. Eventually, we show that, in minimal culture medium, several *S. cerevisiae* strains lacking the chromosomal formylase gene exhibit a thermosensitive respiratory growth and a slower growth rate.

MATERIALS AND METHODS

Disruption of the FMT1 Gene. The *S. cerevisiae* strains used in this work are summarized in Table 1. Rich media contained 1% peptone and 1% yeast extract (Difco) with either 2% glucose (YPD) or 2% glycerol (YPG). Minimal media contained 0.67% yeast nitrogen base (Difco) with either 2% glucose (MMYD) or 2% glycerol (MMYG). Required amino acids and nucleotides were added at final concentrations of 50 μg/mL each, except lysine which was used at 40 μg/mL. Disruptions of *FMT1* were achieved in haploid strains by using the PCR-based method of Wach et al. (*24*). The *kanMX* module was used as a marker giving resistance to geneticin. This module, carried by pFA6a-kanMX4 (*24*), was PCR amplified in order to produce a DNA fragment where the *kanMX* module is flanked by sequences corresponding to the two extremities of *FMT1*. The following primers were used for amplification: **CAAATGGTTAAAATGAGAAGAATAACACCTACACGCCTCCTAT**CGTACGCTGCAGGTCGAC and **GGGCGCCGCATCTTTTCCGCAGCGCGCCATGAACTGGCCA**ATCGATGAATTCGAGCTCG. The bold-faced sequences correspond to the two *FMT1* extremities. The PCR product was then used to transform the yeast cells by using the lithium chloride method (*33*), and disruptants were selected on YPD medium containing 200 μg/mL geneticin. Disruptions were controlled by PCR amplification of genomic DNA.

Cloning, Expression, and Purification of Yeast Mitochondrial FMT. The chromosomal gene coding for the yeast mitochondrial FMT (FMTmt) was PCR amplified from the yeast strain CMY214 (*34*). The resulting fragment was cloned between the *Nde*I and *Bam*HI sites of pET3a to introduce *E. coli* translation initiation signals. To improve the expression of the protein, an *Xba*I−*Hin*dIII fragment from this plasmid was cloned between the corresponding sites of pUC18Fatg (*15*). The resulting plasmid, called pUCFmt, therefore contains the gene encoding yeast mitochondrial FMT under the control of both the Lac promoter and the translation initiation signals of gene *10* of bacteriophage T7. Site-directed mutagenesis was then used to delete codons 2−26, thought to correspond to an N-terminal extension containing the mitochondrial import signal (the N-terminus of the protein becomes therefore VQPLNV, after removal of the initiator methionine). The presence of the resulting plasmid, pUCFmt2, proved sufficient to restore the normal growth of an *E. coli fmt* null strain PAL13Tr (*9*). This allowed the production of FMTmt completely devoid of contaminating *E. coli* formylase. For expression in *S. cerevisiae*, *FMT1* was PCR amplified from W303 chromosomal DNA and cloned between the *Bam*HI and *Sph*I sites of the pYES2 yeast expression vector (Invitrogen). The resulting plasmid was called pYES2-Form.

PAL13Tr-pUCFmt2 cells were grown at 37 °C in 2× TY medium (1 L) containing 50 μg/mL ampicillin. When the optical density at 650 nm reached a value of 1, IPTG was added at a final concentration of 1 mM, and the culture was continued for 12 h. Cells were harvested by centrifugation, resuspended in 40 mL of buffer A (10 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 M KCl), and disrupted by ultrasonic disintegration. Cell debris was removed by centrifugation, and nucleic acids were removed by precipitation with 3% (w/v) streptomycin sulfate. After fractionation with ammonium sulfate (35−60% saturation), proteins were dissolved in 4 mL of buffer A, dialyzed against the same buffer, and loaded at 0.2 mL/min onto a Superose 6 molecular sieving column (1.6 × 50 cm, Amersham Biosciences) equilibrated in buffer A. The pooled fractions were loaded onto an anion-exchange column (Q-Sepharose, 1.6 × 10 cm, Amersham Biosciences) equilibrated in buffer A, and FMTmt was recovered in the flow-through. Finally, a cation-exchange chromatographic step was performed (S-Sepharose high performance, 1.6 × 10 cm, Amersham Biosciences) by using a 0.1−1 M KCl gradient (0.2 M/h) at 2.5 mL/min in buffer A. The protein was homogeneous, as judged by SDS−PAGE analysis. FMTmt concentration was measured using a calculated absorbance at 280 nm of 0.449 for a 1 mg/mL protein solution.

Production of Mutant tRNAs. *E. coli* tRNAs and their derivatives were expressed in JM101Tr (*35*) from plasmid pBSTNAV2 (*12*). The gene coding for $tRNA_f^{Met}$ C11G24 was constructed by assembling six overlapping oligonucleotides as described (*36*) and cloned into pBSTNAV3S (*37*). In the cases of tRNAs with a mismatch at position 1−72, several genes were recloned in the pBSTNAV3S vector to ensure full maturation by RNase P (*37*). In three cases, however [$tRNA_f^{Met}$ C2G71, $tRNA_f^{Met}$ G3C70, and $tRNA_f^{Met}$-(GAU)], constructions in pBSTNAV2 were used directly. For these tRNAs, it was verified by polyacrylamide gel electrophoresis that more than 50% of the tRNA molecules had been correctly processed. In a few cases, both fully matured and partially matured tRNA preparations were

available. Comparison of the kinetics obtained with the two types of preparations did not show significant differences in the formylation reaction with either FMTec or FMTmt.

tRNAs were purified as described (*12*) to acceptances of 1200−1700 pmol of amino acid per A_{260} unit.

Measurement of Catalytic Parameters of Formylase. Initial rates of aminoacyl-tRNA formylation in the presence of catalytic amounts of the studied enzyme (0.01 nM to 1 μM for FMTec and 0.5 nM to 1 μM for FMTmt, depending on the studied tRNAs) were measured as described (*9*, *38*, *39*) in a buffer (20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 150 mM KCl, 7 mM $MgCl_2$) containing 125 μM 10-formyltetrahydrofolate and 0.05−10 μM aminoacyl-tRNA. Homogeneous preparations of *E. coli* M547 methionyl-tRNA synthetase (*40*), *E. coli* valyl-tRNA synthetase (*41*), or *E. coli* isoleucyl-tRNA synthetase (*42*) were used for the aminoacylation of tRNAs.

RESULTS

Initiator tRNA Formylation and Respiration of Yeast. To assess whether yeast growth was sensitive to the absence of formylation of initiator $tRNA^{Met}$, the *FMT1* gene in the *S. cerevisiae* haploid strain W303 was disrupted to give W303ΔForm. The disruption was achieved by replacing most of the *FMT1* gene sequence by that of *kanMX4* (*24*). Correct disruption was verified by PCR analysis of genomic DNA from W303ΔForm. Growth of the disrupted strain was tested under various conditions by spotting dilutions of a saturated culture on the desired medium. Mitochondrial function was first assessed by growing the cells on glycerol, a nonfermentable carbon source. In keeping with the results of Li et al. (*23*), no growth difference between W303 and its disrupted derivative could be observed on YPG medium at 30 °C (Figure 1, panel A). This confirms that, under these conditions, mitochondrial translation can proceed normally despite the absence of formylation of the initiator tRNA (*23*).

E. coli was reported to grow very slowly at 37 °C upon disruption of the *fmt* gene. At 42 °C, growth stopped (*9*). This behavior prompted us to evaluate the respiratory growth of W303ΔForm at 37 °C instead of 30 °C. Interestingly, the disrupted yeast cells were no more able to grow at the higher temperature, while clear growth was observed with the parental W303 strain (Figure 1, panel B). On minimal respiratory medium (MMYG) at 30 °C, a growth defect is clearly visible upon disruption of *FMT1* (Figure 1, panel C). To demonstrate that the observed growth defects were indeed caused by the absence of formylation activity, we verified that the phenotype of W303ΔForm could be reversed by expression of the formylase from a plasmid. For this purpose, the *FMT1* gene was cloned into the pYES2 yeast expression vector under control of the inducible *GAL1* promoter. The resulting plasmid was called pYES2-Form. As shown in Figure 1, the deficient respiratory growth phenotypes of the disrupted strain at either 37 °C (panel D) or 30 °C on minimal medium (panel E) were cured by the presence of the pYES2-Form plasmid. A residual growth of the disrupted strain (W303ΔForm) carrying the control plasmid pYES2 can be accounted for by the presence of 0.1% of the fermentable sugar galactose in the medium. Addition of this compound was necessary to induce expression of the plasmid-encoded formylase. The overall data indicate, therefore, that formylation of the initiator tRNA becomes important for initiation of translation in mitochondria, provided the yeast cells are exposed to relatively severe growth conditions.

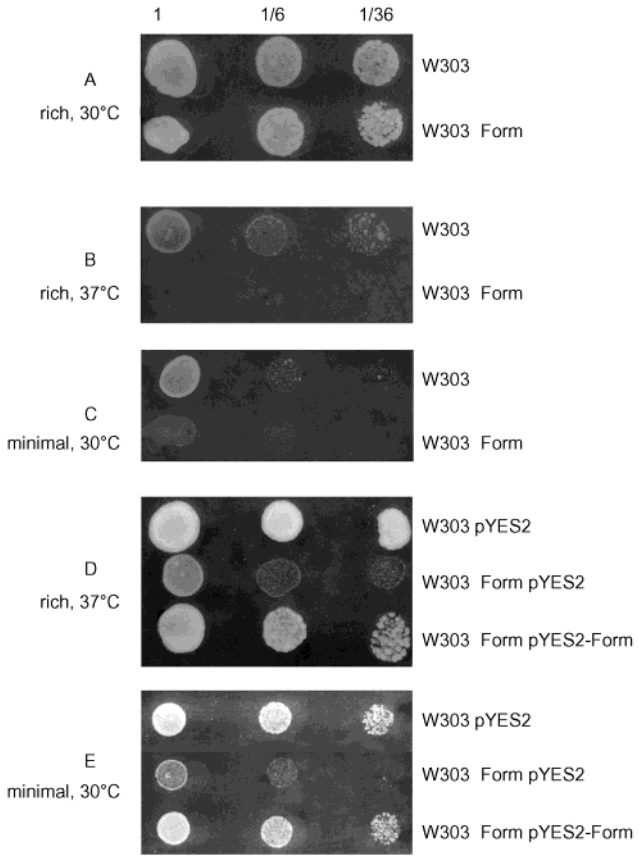

FIGURE 1: Growth of W303 and of its *FMT1* disrupted derivative W303ΔForm. Saturated cultures in YPD-rich fermentative medium were diluted in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to obtain suspensions having optical densities of 1, 1/6, or 1/36 at 650 nm. Each suspension (4 μL) was then spotted on the desired respiratory medium, and plates were incubated during 4 days. Panels: A, YPG medium, 30 °C; B, YPG medium, 37 °C; C, MMYG medium (plus Leu, His, Trp, Ade, and Ura), 30 °C; D, YPG medium plus 0.1% galactose, 37 °C; E, MMYG medium (plus Leu, His, Trp, Ade, and 0.1% galactose), 30 °C.

Table 1: *S. cerevisiae* Strains Used in this Study

strain	genotype	ref
W303	mat **a** *leu2-3,112 his3-11,15 ura3-1 trp1-1 ade2-1*	*44*
W303ΔForm	same as W303 but *fmt1::kanMX*	this study
YPALS	mat **a** *trp1*-Δ1 his3Δ200 ura3-52 ade2-101 lys2-801 can1	*45*
YPALSΔForm	same as YPALS but *fmt1::kanMX*	this study
DBY2057	mat **a** *ura3-52*	*46*
DBY2057ΔForm	same as DBY2057 but *fmt1::kanMX*	this study

Such a conclusion differs from that drawn by Li et al. (*23*), who showed that a haploid *fmt1* disruptant can grow with a nearly wild-type rate on a respiratory minimal medium. Since such a discrepancy might reflect different genetic backgrounds of the tested strains or be due to different cassettes (*kanMX4* and *URA3*) used for the disruption, we examined the behavior of various strains containing a disrupted *FMT1* gene. For this purpose, two additional yeast haploids, YPALS and DBY2057 (Table 1), were submitted to *FMT1* disruption by using the same method as that described above in the case of W303. The growths of the

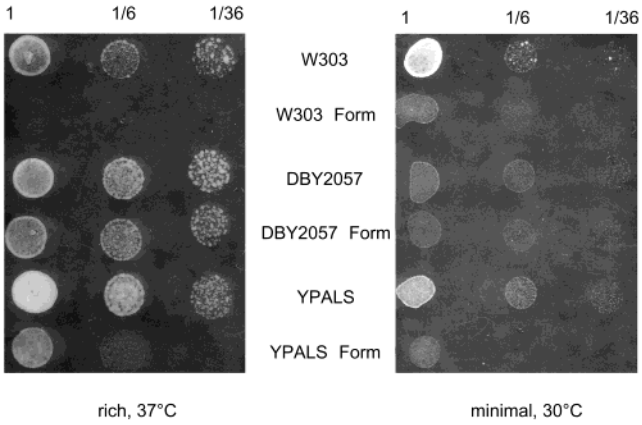

FIGURE 2: Growth of three *FMT1*-disrupted yeast strains. Cell suspensions were spotted at the top of agar plates, as described in the legend of Figure 1. Left: YPG medium, 37 °C. Right: MMYG medium (plus Leu, His, Trp, Lys, Ade, and Ura), 30 °C.

resulting strains were then assayed on respiratory media, either in minimal medium at 30 °C or in rich medium at 37 °C (Figure 2). The YPALS-derived *fmt1* disruptant showed a behavior similar to that of W303-ΔForm, whereas that of DBY2057ΔForm was only moderately affected by the inactivation of the *FMT1* gene. The genotype of W303, the strain used at the beginning of this study, is similar to that of the strain used by Li et al. (*23*), except that, in the latter case, the disruptant is *URA3*$^+$, because of the use of an *URA3* cassette. However, it can be excluded that the *ura3*$^-$ character of W303-ΔForm accounts for the specific behavior of this strain. Indeed, growth of the *ura3*$^-$ strain DBY2057 is only slightly affected by the *FMT1* disruption. Therefore, depending on the strain considered, yeast mitochondria respond differently to the absence of formylase activity. Nevertheless, examination of the genotypes of the studied strains has not yet allowed us to find out the origin of such discrepancies.

Yeast Mitochondrial Formylase Can Substitute for the E. coli Formylase in Vivo. To characterize the activity of *S. cerevisiae* mitochondrial formylase (FMTmt), we wished to overproduce the protein in *E. coli*. For this purpose, a vector was constructed where the expression of FMTmt is controlled by the Lac promoter and by translation start signals from gene *10* of bacteriophage T7 (see Materials and Methods). In the expression plasmid, called pUCFmt2, codons 2−26 of FMT1 were deleted in order to remove an N-terminal extension corresponding to the mitochondrial import signal. To facilitate the obtaining of FMTmt preparations devoid of *E. coli* formylase, an *E. coli fmt*$^-$ strain was used as a host. This strain, PAL13Tr-pMAF, has a disrupted chromosomal *fmt* gene complemented in trans by an intact copy present on a plasmid (pMAF) with a thermosensitive replicon (*9*). After transformation of this strain with pUCFmt2, the pMAF plasmid could be chased at 44 °C. The resulting strain, in which FMTmt has become the only source of formylase activity, grew normally both at 42 °C and at 37 °C. Therefore, pUCFmt2 can relieve the Fmt$^-$ phenotype, showing that the yeast mitochondrial enzyme ensures formylation of the *E. coli* initiator tRNA in vivo. Furthermore, after induction with 1 mM IPTG, PAL13Tr-pUCFmt2 expressed large amounts of FMTmt, which allowed easy purification of the protein to homogeneity (see Materials and Methods).

Role of the Nucleotide Composition of the Acceptor Stem of Initiator tRNA^{Met} on the Efficiency of the Formylation Reaction Catalyzed by FMTmt. The sequences of initiator and elongator tRNA^{Met} from yeast mitochondria are shown in Figure 3 together with that of *E. coli* initiator tRNA. Interestingly, base pairs G2-C71 and C3-G70 are conserved in the sequences of yeast mitochondrial and *E. coli* initiator tRNAs. Moreover, unpaired bases are encountered at positions 1−72 in both of the initiator tRNAs. We therefore decided to measure the catalytic efficiency of FMTmt in vitro by using *E. coli* initiator tRNA$_f^{Met}$ as a model substrate. To compare data with those already obtained with either *E. coli* or bovine mitochondrial formylase (*20*), the following buffer conditions were used: 150 mM KCl and 7 mM $MgCl_2$ in 20 mM Tris-HCl, pH 7.5.

As shown in Table 2, the yeast mitochondrial enzyme is active in the in vitro formylation of *E. coli* initiator tRNA, as expected from the ability of pUCFmt2 to relieve the Fmt$^-$ phenotype of bacterial cells. This activity is, however, 320-fold less efficient than that of the *E. coli* formylase. To investigate the role of the acceptor stem in substrate specificity, variants of *E. coli* initiator and elongator tRNA^{Met} were used (Table 2). With *E. coli* elongator tRNA$_m^{Met}$, the efficiency (k_{cat}/K_m) of the formylation reaction was decreased by 4 orders of magnitude, if compared to the reaction with *E. coli* tRNA$_f^{Met}$. This comparison establishes the specificity of FMTmt for an initiator tRNA species. When tRNA$_{fasm}^{Met}$ was used, the formylation capacity of an authentic tRNA$_f^{Met}$ was fully recovered. The tRNA$_{fasm}^{Met}$ variant contains the acceptor stem of tRNA$_f^{Met}$ in the context of the *E. coli* elongator tRNA$_m^{Met}$. Hence, the acceptor stem of *E. coli* tRNA$_f^{Met}$ is sufficient to confer full formylation properties. Moreover, as observed with *E. coli* formylase (FMTec), the single substitution in tRNA$_f^{Met}$ of the C1-A72 pair by a C1-G72 one was enough to decrease the efficiency of the formylation reaction by more than 2 orders of magnitude. Similarly, as shown by the study of a tRNA$_f^{Met}$C2G71 mutant, the G2-C71 second base pair of the acceptor stem contributes to the formylation efficiency by 2 orders of magnitude. Finally, base pair C3-G70 and the discriminator base A73 also govern the specificity of FMTmt. However, their contributions are limited since mutations at these positions each affected the catalytic efficiency of the yeast mitochondrial enzyme by a factor of at most 50 (Table 2). As a whole, the results in this paragraph indicate that the acceptor stem of its tRNA substrate has a very important weight in the capacity of FMTmt to achieve formylation.

Role of the 11-24 Pair in the D-Stem and Specificity toward the Esterified Amino Acid. In contrast with most initiator tRNAs of bacterial, archaeal, or organellar origin, initiator tRNA from *S. cerevisiae* mitochondria displays a pyrimidine−purine U11-A24 pair in the D-stem. Mutation of the purine−pyrimidine A11-U24 pair of *E. coli* initiator tRNA into C11-G24 had no consequence on the formylation activity of the yeast FMTmt (Table 2). Therefore, contrarily to the cases of FMT from bovine mitochondria or from *E. coli* (*11*, *20*), the 11-24 pair is not recognized as a nucleotidic determinant in the reaction catalyzed by the yeast enzyme. This conclusion is consistent with the occurrence of a same U11-A24 pair in both the initiator and the elongator tRNA^{Met} from yeast mitochondria. In FMTec, the N301 residue was shown to recognize the 11-24 pair (*16*). Interestingly,

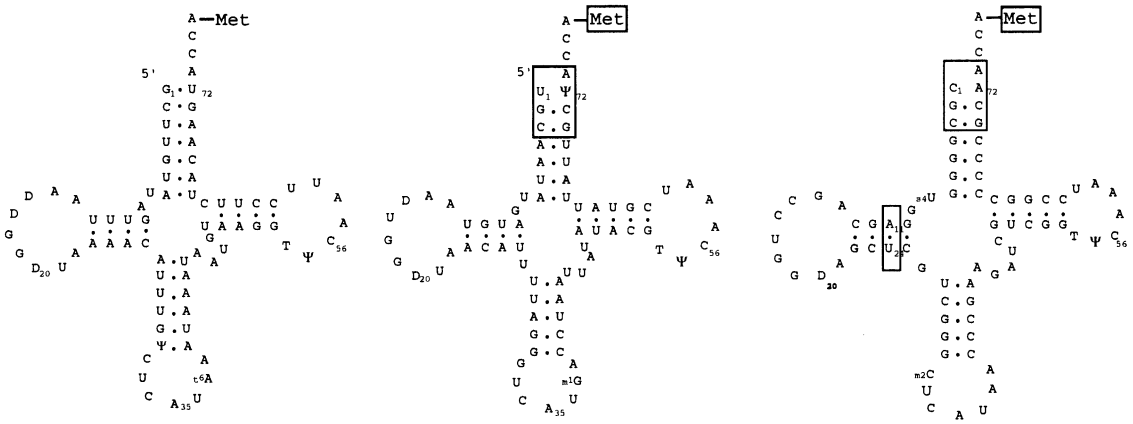

FIGURE 3: Cloverleaf representations of the two *S. cerevisiae* mitochondrial methionine tRNAs and of the *E. coli* initiator tRNA$_f^{Met}$. Crucial determinants in *E. coli* tRNA$_f^{Met}$ for the formylation reaction are boxed (*12*, *16*). Equivalent determinants in the yeast initiator tRNA are also boxed.

Table 2: Catalytic Parameters of the Reaction of Formylation of *E. coli* tRNA Derivatives by the Methionyl-tRNA Formyltransferases of Indicated Origins[a]

	E. coli			beef mitochondria			yeast mitochondria		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (%)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (%)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (%)
tRNA$_f^{Met}$	28	0.2	100	0.93	0.5	100	0.13	0.3	100
tRNA$_m^{Met}$	nm	nm	<0.0001	>0.04	>10	0.21	0.0002	5.7	0.008
tRNA$_{fasm}^{Met}$	24	1.2	14	0.07	5	0.74	0.11	0.3	85
tRNA$_f^{Met}$ C1G72	>1	>15	0.02	0.55	3	9.6	0.02	7.5	0.6
tRNA$_f^{Met}$ C2G71	0.6	3.6	0.12	0.25	0.5	26	0.012	3.5	0.3
tRNA$_f^{Met}$ G3C70	0.9	1.8	0.4	0.4	1.5	15	0.009	1	2.1
tRNA$_f^{Met}$ G73	18	2.9	4.4	1.4	1.4	53	0.1	2	11
tRNA$_f^{Met}$ C11G24	18	2.8	4.6	0.16	7	1.2	0.21	0.5	97
Val-tRNA$_f^{Met}$(GAC)	0.08	2	0.03	0.0008	1.4	0.03	0.009	0.5	4
Ile-tRNA$_f^{Met}$(GAU)	0.075	0.88	0.06	0.001	0.3	0.18	0.0005	0.3	0.4
Met-tRNA$_1^{Val}$(CAU)	nm	nm	<0.0001	>0.03	>10	0.16	0.0005	9	0.012

[a] Values with *E. coli* and bovine mitochondrial formylases are from ref *20*. Standard errors on measurements did not exceed 20%. nm: not measurable. Mutations are indicated in the name of each tRNA. All tRNAs were methionylated,unless otherwise indicated. Val-tRNA$_f^{Met}$(GAC) means tRNA$_f^{Met}$ carrying a GAC anticodon and aminoacylated with valine. Ile-tRNA$_f^{Met}$(GAU) means tRNA$_f^{Met}$ carrying a GAU anticodon and aminoacylated with isoleucine. Met-tRNA$_1^{Val}$(CAU) means tRNA$_1^{Val}$ carrying a CAU anticodon and aminoacylated with methionine. k_{cat} are expressed in s^{-1} and K_m in μM; k_{cat}/K_m are expressed as the percentage of the value measured with *E. coli* Met-tRNA$_f^{Met}$ as substrate.

sequence alignments between formylases from various species suggest that this residue is absent in yeast FMTmt (Figure 5).

With FMTec, the side chain of the aminoacyl group attached to tRNA modulates the efficiency of the formylation reaction (*12*−*14*). Methionine provides the highest efficiency of formylation, if compared to other esterified amino acids such as Gln, Phe, Val, or Lys. To study the importance of the amino acid moiety in the case of FMTmt, we used two derivatives of tRNA$_f^{Met}$ with the CAU anticodon modified into either a GAC or a GAU one. These changes give tRNA$_f^{Met}$ the capacity to be aminoacylated with valine and isoleucine, respectively (*12*, *25*). As shown in Table 2, esterification of *E. coli* initiator tRNA with valine or with isoleucine reduced the catalytic efficiency of yeast FMTmt by factors of 25 and of 250, respectively. These effects mainly result from a decrease in the k_{cat} values, showing the importance of the presence of the methionyl group for the efficiency of the catalytic step in the formylation reaction.

DISCUSSION

Importance of Initiator tRNA Formylation for the Respiratory Growth of S. cerevisiae. The formylase gene has now been disrupted in the cases of *E. coli* (*9*), *P. aeruginosa* (*10*), and yeast mitochondria (*23*; present study). In no case was the gene found strictly essential. However, in *E. coli*, the effects of the disruption are severe, with a lack of growth at 42 °C and a generation time of 215 min at 37 °C, as compared to 26 min with the nondisrupted control. With *P. aeruginosa*, the effect is less stringent, with a doubling time of 152 min for the *fmt*-disrupted strain as compared to 54 min for the control strain. Nevertheless, it remains that, in the absence of formylase, the doubling time of *P. aeruginosa* becomes very large. Formylation of Met-tRNA$_f^{Met}$ is believed to favor its complexation by IF2 and to impair its misappropriation by EF-Tu (*26*, *27*). Consequently, in the absence of formylation, the concentration of the IF2·Met-tRNA$_f^{Met}$ complex is likely to be limiting for initiation of translation.

In the case of yeast mitochondrion, one must admit that, in the absence of formylation, the rate of initiation remains sufficient to allow respiratory growth at 30 °C on a rich medium. However, as shown here, culture conditions can be found where the lack of formylase renders translation initiation growth-limiting. This observation suggests that mitochondrial translation generally benefits from the presence of a formylating enzyme. This idea agrees with that discussed

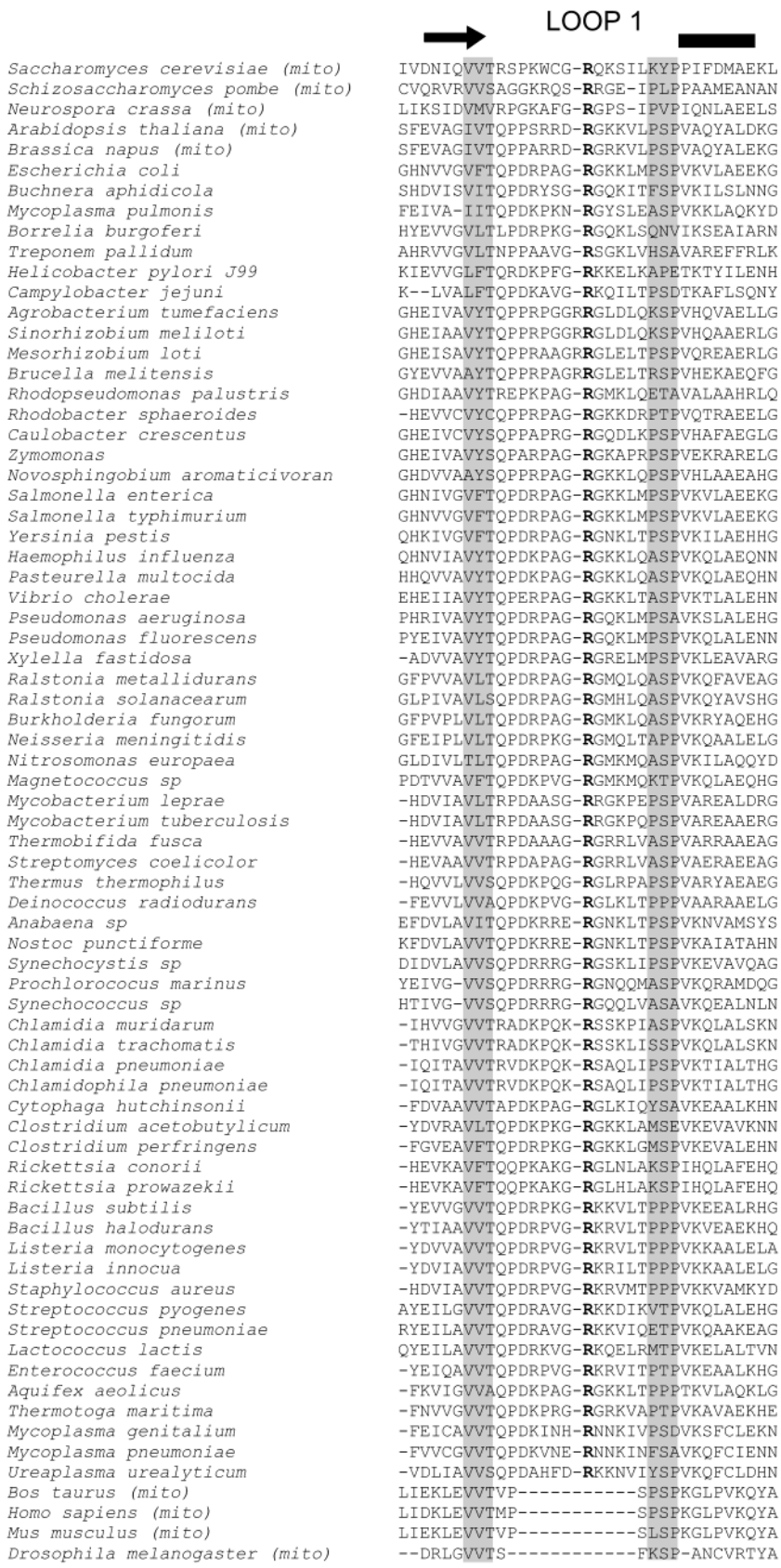

FIGURE 4: Alignments of the sequences of FMT from various origins in the region of loop 1. Alignments were performed using CLUSTAL V (*43*) and refined manually. The positions of the secondary structure elements of the *E. coli* protein are indicated, with the bar representing an α helix and the arrow representing a β strand. The position of loop 1 is indicated. Two conserved sequences bordering loop 1 are shaded in gray. The conserved arginine, corresponding to R42 in the *E. coli* enzyme, is boldfaced. Note that a single formylase homologue was found in the two genomes of plants sequenced so far. Therefore, the enzymes from *Arabidopsis thaliana*, *O. sativa*, and *B. napus* might serve in both the mitochondria and the chloroplasts.

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E.coli          PGTILEANKQGIQVATG-DGILN----LLSLQPAGKKAMSAQDLLNSRREWFVPGNRLV
Bovine mito     PGSVIYHKQSQILLVCCKDDWI------GVRSVMLKKTLTATDFYNGYLHPWYQKNSQAQPSQCRFQTLRLPPKKKQKKKIVAMQ
H. sapiens      PGSVIYHKQSQILLVYCKDGWI------GVRSVMLKKSLTATDFYNGYLHPWYQKNSQAQPSQCRFQTLRLPTKKKQKKTVAMQQCIE
M. musculus     PGFVVYHRPSQMLLVRCKDGWI------GVRSVMLKKTLTATDFYNGYLHAWYQKNSHAHPSQCRFQTLRLPTKMQQKTKLLLCNSAL
D. melanogasterPGAISYLRKSRSLIIGCAQQSQ---LEVLQLRVEGRKPMSAQDFNNGFLKQARSLSFTENKIASI
A. thaliana     SEQDYVTFKKGSLIFPCRGGTA---LEVLEVQLPGKKAINAAAFWNGLRGQKLKKL
B. napus        GEQDYVTFKKGSLVFSCGGGTA---LEVLEVQLPGKKAIDATAFWNGLRGQKLKKL
O. sativa       GEPDVLEIKVISTKACASCDKTGDGNEVVELQLPGKKVTTARDFWNGLRGQRLLKSP
C. elegans      HKQLLASYRLDDWKAPIIKHNPPLSSLNPSEQKVFRRFLFYM-MSNKRSVDKVLKHR
Yeast mito      PGMFKYDDIKDCILVTCRGNLLLCVSRLQFEGFAVERAGQFMARCGKDAAP
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FIGURE 5: Comparison of the C-terminal regions of mitochondrial FMT from various origins with that of FMTec. Conserved sequences are in bold.

by Li et al. (*23*), according to which the strong conservation of initiator tRNA formylation in mitochondria should be associated to at least an incremental advantage for the cell. The present study highlights such an advantage, provided the cell is submitted to severe growth conditions. Likely, the formyl group reinforces the binding of the tRNA to the yeast mitochondrial initiation factor 2 (IF2mt) as shown in the case of the bacterial system.

Since under favorable growth conditions formylation is dispensable, we may conclude that IF2mt can select unformylated initiator tRNA among all elongator tRNAs on the only bases of the methionyl group and of distinctive nucleotidic features. In the mitochondria of an *FMT1*-disrupted yeast cell, the concentration of Met-$tRNA_f^{Met}$ would be large enough to significantly saturate the initiation factor and not limit the rate of translation. Possibly, with only 23 elongator tRNA species in the yeast mitochondrion, competition between Met-$tRNA_f^{Met}$ and the other tRNAs to complex IF2mt is low. Examination of the genotypes of the studied strains did not allow us to find genetic characters explaining the different growth behaviors of the various *FMT1* yeast strains assayed at 37 °C or on minimal growth medium. However, it can be imagined that the observed discrepancies reflect variable amounts of $tRNA_f^{Met}$ and/or of IF2mt in the corresponding mitochondria. In agreement with this idea, overproduction of either $tRNA_f^{Met}$ or IF2 in an *fmt*$^-$ *E. coli* strain has been reported to partly compensate for the absence of formylase. It is likely that such overproductions favor the complexation of unformylated Met-$tRNA_f^{Met}$ with the bacterial IF2 and promote initiation of translation (*27*−*30*).

Comparison of the Specificities of Formylases from Various Species. The second part of this work deals with the study of the specificity of yeast FMTmt toward the *E. coli* $tRNA_f^{Met}$ as a model substrate. The obtained data enable a comparison of the nucleotidic determinants recognized by either FMTec (*12*), mitochondrial FMTmt from beef (*20*), or yeast mitochondrial FMTmt (this study). The results are summarized in Table 2. The crystallographic structure of FMTec complexed with formylmethionyl-$tRNA_f^{Met}$ offers a structural basis to discuss the recognition mechanisms (*16*). $tRNA_f^{Met}$ approaches FMTec through the minor groove of its acceptor stem and mainly interacts with two regions of the protein. Loop 1, in the catalytic N-terminal domain of the enzyme, interacts with the 3′ end of tRNA. In the complex, loop 1 is inserted between base 1 and base 72 of $tRNA_f^{Met}$ and rejects base 1 outside of the protein. R42 ensures selection of base pair C3-G70. The second region of the protein recognizing the tRNA corresponds to an α helix located within the C-terminal barrel. This helix contains N301, which is responsible for the recognition of U24 within the D-stem.

The present results show that, like its bacterial counterparts (*12*, *31*), yeast FMTmt is highly sensitive to the sequence of the acceptor stem of its substrate. The alignment of formylase sequences presented in Figure 4 indicates a well-conserved loop 1, in which a residue corresponding to R42 is systematically found. Such a conservation allows us to propose that FMT from all bacteria and from mitochondria of single cell eukarya or of plants recognize the acceptor end of the corresponding initiator tRNAs through loop 1, in a manner similar to that documented in the case of *E. coli* (*15*, *16*). In contrast, in formylases from animal mitochondria, loop 1 is lacking and appears to be replaced by a short turn (*20*) (Figure 4). This difference may reflect the property of the animal formylases to poorly recognize the acceptor stem of their tRNA substrate. Instead, the single $tRNA^{Met}$ species of animal mitochondria (*17*, *18*) is mainly recognized through the esterified methionyl group and the Pu11-Py24 base pair (*20*) (Table 2). In line with this view, bovine mitochondrial formylase indeed possesses the N residue corresponding to N301 of FMTec (N318, Figure 5). On the other hand, the sequence of the C-terminal region of yeast FMTmt diverges from those of the bacterial enzymes. In particular, the region in FMTec surrounding N301 cannot be satisfyingly aligned with the sequence of yeast FMTmt or with the sequences of FMTmt from any other unicellular eukaryote. Such a distinctive feature of *S. cerevisiae* FMTmt can explain the absence of recognition of the 11-24 pair in $tRNA_f^{Met}$. The recognition pattern of its substrate by yeast FMTmt appears therefore well adapted to the mitochondrial context, where both the initiator tRNA and the elongator tRNAs display a Py11-Pu24 pair.

To conclude, the rules of recognition of their initiator tRNA by the various formylases offer a good example of co-evolution. Indeed, in each case, the set of determinants recognized by the enzyme seems ideally suited to the features enabling the initiator tRNA to be distinguished from the pool of all elongator tRNAs. As a consequence, mitochondrial formylases do not form a homogeneous family. For instance, the *S. cerevisiae* enzyme has a functioning closer to that of a bacterial enzyme than to that of a formylase from animal mitochondria. From sequence analysis of initiator tRNAs and of the formylating enzymes (Figures 4 and 5), we propose that formylases in plant organelles recognize the acceptor stem and the Pu11-Py24 base pair of their substrate. Indeed, plant formylases display an N residue corresponding to N301 of FMTec (Figure 5). Therefore, formylases in plants should have a closer proximity to the bacterial enzymes than the fungal formylases. Such variability is also illustrated by the case of *Trypanosoma brucei* mitochondrial formylase, which differs from the canonical formylases. Additional structural domains might be related to the peculiar capacity of this

enzyme to aminoacylate the cytosolic elongator $tRNA^{Met}$ after its import in the mitochondrion (*32*).

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