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Recognition of tRNAs by Methionyl-tRNA Transformylase from Mammalian Mitochondria*

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Protein synthesis involves two methionine-isoaccepting tRNAs, an initiator and an elongator. In eubacteria, mitochondria, and chloroplasts, the addition of a formyl group gives its full functional identity to initiator Met-tRNA^{Met}. In *Escherichia coli*, it has been shown that the specific action of methionyl-tRNA transformylase on Met-tRNA$_f^{Met}$ mainly involves a set of nucleotides in the acceptor stem, particularly a C^{1}A^{72} mismatch. In animal mitochondria, only one tRNA^{Met} species has yet been described. It is admitted that this species can engage itself either in initiation or elongation of translation, depending on the presence or absence of a formyl group. In the present study, we searched for the identity elements of tRNA^{Met} that govern its formylation by bovine mitochondrial transformylase. The main conclusion is that the mitochondrial formylase preferentially recognizes the methionyl moiety of its tRNA substrate. Moreover, the relatively small importance of the tRNA acceptor stem in the recognition process accounts for the protection against formylation of the mitochondrial tRNAs that share with tRNA^{Met} an A^{1}U^{72} motif.

In protein biosynthesis, methionine is universally used as the starting amino acid, and a particular initiator methionine tRNA ensures initiation of translation. Cells also possess an elongator methionine tRNA dedicated to the incorporation of internal methionines. Once aminoacylated by methionyl-tRNA synthetase, the two methionine tRNAs have distinct fates. The elongator tRNA is carried by an elongation factor to the A site of the ribosome, whereas the initiator tRNA enters the ribosomal P site with the help of several initiation factors.

In eubacteria, a decisive step in the acquisition of an initiator identity of the tRNA is an *N*-formylation of the esterified methionine (reviewed in Refs. 1 and 2). The added formyl group reinforces the binding of the initiator tRNA to initiation factor 2 and impairs its binding to elongation factor Tu (3).

In mitochondria and chloroplasts, the translational system also uses a formyl-methionyl-tRNA^{Met} for initiation of translation. However, the rule of the occurrence of two distinct methionine tRNAs breaks down in the mitochondria of many animals, such as mammals and insects. In these organelles, only a single tRNA^{Met}, believed to participate in both the initiation and the elongation of protein biosynthesis, has yet been evidenced (4, 5). The current working hypothesis assumes that this Met-tRNA^{Met} molecule can either bind elongation factor Tu and further participate in chain elongation or undergo formylation through the action of mitochondrial methionyl-tRNA^{Met} transformylase (FMTmt)[1] and then be directed to the initiation machinery. Thus, competition between elongation factor Tu and FMT for the binding of Met-tRNA^{Met} would ensure a correct balance between the elongator and initiator functions of mitochondrial tRNA^{Met}.

Specificity of formylase toward tRNA$_f^{Met}$ has been thoroughly documented in the *Escherichia coli* system (6, 7). The main determinant selected by the bacterial enzyme is a mismatch at position 1-72 in the initiator tRNA structure. The crystalline structure of FMT, free (8) or complexed with fMet-tRNA$_f^{Met}$ (9), reveals that an opening of base pairs 1–72 allows the esterified methionine to reach the active site of the enzyme.

Bovine mitochondrial FMT has recently been characterized (10, 11). This enzyme was found to be able to formylate the *E. coli* elongator Met-tRNA$_m^{Met}$ at a significant rate. This property indicates that the specificity of FMTmt is broader than that of its bacterial counterpart. The present study extends the examination of the substrate specificity of FMT from bovine liver. The specificity of the mitochondrial enzyme is shown to be preferentially governed by the methionyl moiety of its substrate. The choice of such an identity element appears to be compatible with the occurrence of a single initiator/elongator tRNA^{Met} species in animal mitochondria.

MATERIALS AND METHODS

Expression and Purification of Bovine Mitochondrial FMT—The *Nde*I-*Bam*HI fragment from pET19b-FMTmt (10) carrying the gene coding for the bovine mitochondrial formylase was cloned into the corresponding sites of pET15b. The resulting plasmid, pET15b-FMTmt, expressed a 6-His-tagged version of the formylase with a thrombin cleavage site. The tagged protein was purified by affinity chromatography on a nickel Hi-Trap column (Amersham Pharmacia Biotech), as described (10). After this step, the His tag was cleaved by using thrombin (0.5 unit per mg of protein). Remaining tagged protein was removed by a second pass on the affinity column. A final step of purification consisted of ion exchange chromatography on SP-Sepharose HP (Amersham Pharmacia Biotech, 1.6 × 20 cm, 0.2 M KCl/h, 2.5 ml/min). The protein was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. FMTmt was further concentrated by dialysis against 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM 2-mercaptoethanol, 55% glycerol and stored at −20 °C.

Production of Mutant tRNAs—E. coli tRNAs and their derivatives

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[1] The abbreviations used are: FMT, methionyl-tRNA^{Met} transformylase; FMTmt, mitochondrial FMT; FMTec, *Escherichia coli* FMT; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

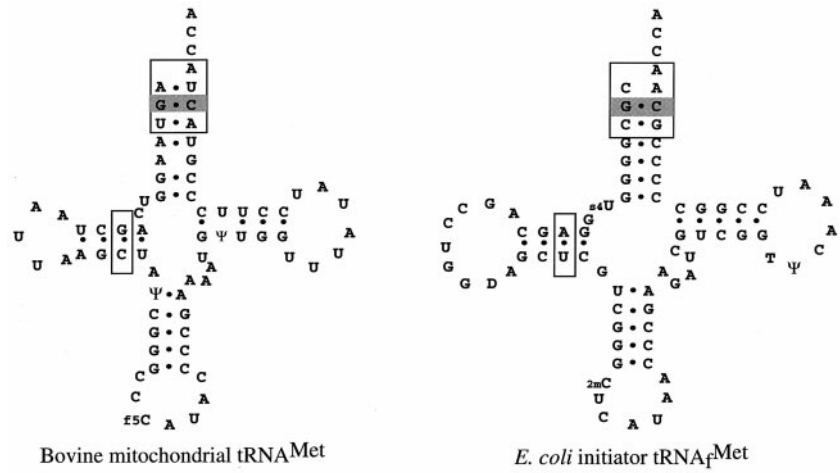


FIG. 1. **Cloverleaf representations of bovine mitochondrial tRNA^{Met} (*left*) and *E. coli* initiator tRNA$_f^{Met}$ (*right*).** Main determinants of the bacterial tRNA governing its formylation are *boxed*. For comparison, the same positions are also *boxed* in the mitochondrial tRNA sequence. Within these regions, nucleotides shared by the two tRNAs are *shaded*. Note that there are 22 bovine mitochondrial tRNAs, among which 12 possess a non-GC or CG pair in position 1-72, and 2 tRNAs have a purine-pyrimidine pair at position 11-24.

were expressed in JM101Tr (12) from plasmid pBSTNAV2 (7). The gene coding for tRNA$_f^{Met}$C11-G24 was constructed by assembling six overlapping oligonucleotides as described (13) and cloned into pBSTNAV3S (14). 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 (14). In three cases, however (tRNA$_f^{Met}$C^{2}G^{71}, tRNA$_f^{Met}$G^{3}C^{70}, 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 any significant difference in the formylation by either FMTec or FMTmt. tRNAs accepting 1200–1700 pmol of amino acid per A_{260} unit were purified as described (7).

Measurement of Catalytic Parameters of FMT—Initial rates of aminoacyl-tRNA formylation in the presence of catalytic amounts of the studied enzyme (0.01 nM to 1 μM for FMTec, depending on the studied tRNA, and 0.1 nM to 1 μM for FMTmt, depending on the studied tRNA) were measured as described (15, 16) in a buffer (20 mM Tris (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. For the sake of homogeneity, identical assay conditions were used for the two formylases. Note that these conditions differ from those previously used for FMTmt (10 mM KCl, 0.5% CHAPS, 5 mM $MgCl_2$; Ref. 11). Homogeneous preparations of *E. coli* M547 methionyl-tRNA synthetase (17), valyl-tRNA synthetase (18), or isoleucyl-tRNA synthetase (19) were used for the aminoacylation of tRNAs.

Limited Proteolysis of FMTmt—FMTmt (2 mg/ml, *i.e.* 50 μM) was digested at 37 °C in 50 μl of buffer (100 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol) by adding 1 μl of protease V8 (0.125 mg/ml from Roche Molecular Biochemicals). Aliquots were analyzed by electrophoresis on a 12% polyacrylamide SDS gel following the time course (5–50 min). A 35-kDa peptide fragment accumulated, as the result of cleavage at the position ^{52}LE/VV^{55}, as previously demonstrated (11). To assay a protection from proteolytic cleavage by bound tRNA, the experiment was repeated in the presence of a saturating concentration (65 μM) of *E. coli* f-Met-tRNA$_f^{Met}$.

RESULTS

Influence of the Nucleotidic Composition of the Acceptor Stem of tRNA on the Formylation Reaction Catalyzed by FMTmt—The sequence of tRNA^{Met} from bovine mitochondria is shown in Fig. 1, together with that of *E. coli* tRNA$_f^{Met}$. Mitochondrial tRNA^{Met} shares with the bacterial tRNA the most important features governing formylation by the *E. coli* enzyme. These features are as follows: (1) absence of a GC or CG base pair at position 1-72, (2) GC at position 2-71, (3) A as the discriminator base at position 73, (4) a purine-pyrimidine pair at position 11-24, and (5) aminoacylation with methionine. Indeed, initiator Met-tRNA$_f^{Met}$ from *E. coli* is as good a substrate of FMTmt as the authentic Met-tRNA^{Met} from bovine mitochondria (11). Therefore, the bacterial initiator tRNA and mutant derivatives were used as model substrates to study the specificity of FMTmt.

As previously reported (11), FMTmt can formylate *E. coli* elongator Met-tRNA$_m^{Met}$ *in vitro*. The efficiency of this reaction is only 500-fold lower than that measured with *E. coli* initiator Met-tRNA$_f^{Met}$. Such a behavior contrasts with that of bacterial FMT, which acts on elongator tRNA^{Met} with an efficiency at least 6 orders of magnitude smaller than that observed with initiator tRNA. To evaluate the influence of the acceptor stem on the catalytic efficiency of FMTmt, we used a tRNA$_m^{Met}$ derivative, the acceptor stem of which had been replaced by that of tRNA$_f^{Met}$ (tRNA$_{fasm}^{Met}$). This grafting was shown to increase the catalytic efficiency of the bacterial formylase by at least 5 orders of magnitude (Refs. 6 and 7 and Table I). With FMTmt, this change increases the catalytic efficiency by a factor of only 3. Therefore, the acceptor stem alone is unable to account for the 500-fold difference in catalytic efficiency when tRNA$_m^{Met}$ is compared with tRNA$_f^{Met}$. In the same manner, the introduction of a CG or a GC pair at position 1-72 of tRNA$_f^{Met}$ (A72G or C1G/A72C mutations) has a small effect on the activity of the mitochondrial enzyme. Catalytic efficiency decreases by a factor of less than 10-fold, whereas the consequences of the same mutations measured with the bacterial enzyme are dramatic (6, 7). Moreover, substitution of A^{73} of tRNA$_f^{Met}$ by a G reduces the catalytic efficiency by a factor greater than 20 in the case of FMTec, as compared with a factor of only 2 with FMTmt (Table I). Finally, the G2C/C71G and C3G/G70C mutations, which affect nucleotidic positions important for recognition by the bacterial enzyme (6, 7), only slightly decrease the efficiency of FMTmt (Table I). As a whole, the results in this paragraph clearly indicate that the acceptor stem of tRNA has less weight in the bovine mitochondrial formylation reaction than in the bacterial one.

The Important Role of the Puo^{11}-Pyd^{24} Pair in the D-stem of tRNA—Like most initiator tRNAs, tRNA^{Met} from bovine liver mitochondria possesses a purine-pyrimidine pair at position 11-24. Instead, elongator tRNAs usually have a pyrimidine^{11}-purine^{24} pair. As previously demonstrated, the purine-pyrimidine pair contributes to the recognition of its substrate by the bacterial formylase (6, 9). Mutation of the A^{11}-U^{24} pair of tRNA$_f^{Met}$ into a CG one was shown to impair the catalytic efficiency of formylation by a factor of 22 (Ref. 6 and Table I). In agreement with this result, the crystal structure of the complexed protein indicates that the C-terminal domain of FMTec directly contacts the U^{24} base (9).

With FMTmt as the formylating enzyme, the above-mentioned mutation in tRNA$_f^{Met}$ caused a loss of catalytic efficiency by a factor of 83 (Table I). This decrease is of the same order as that measured with FMTec. Notably, a mutation at position 11-24 has more effect on the catalytic efficiency of the mitochondrial enzyme than any mutation in the acceptor stem.

TABLE I

Catalytic parameters of the studied E. coli tRNA derivatives in the formylation reaction catalyzed by methionyl-tRNA transformylases of the indicated origins

Standard errors on measurements did not exceed 20%. In the case where K_m value was too high to be determined, the k_{cat}/K_m value was obtained from the slope of the plot of initial rates against substrate concentrations. k_{cat}/K_m values are relative to the values measured with wild-type $tRNA_f^{Met}$. $tRNA_{fasm}^{Met}$ is a $tRNA_m^{Met}$ derivative carrying the acceptor stem of $tRNA_f^{Met}$ (7). 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. nm, not measurable.

	E. coli			Beef mitochondria		
	k_{cat}	K_m	Relative k_{cat}/K_m	k_{cat}	K_m	Relative k_{cat}/K_m
	s^{-1}	μM	%	s^{-1}	μM	%
$tRNA_f^{Met}$	28	0.2	100	0.93	0.5	100
$tRNA_m^{Met}$	nm	nm	<0.0001	>0.04	>10	0.21
$tRNA_{fasm}^{Met}$	24	1.2	14	0.07	5	0.74
$tRNA_f^{Met}$ C^1G^{72}	>1	>15	0.02	0.55	3	9.6
$tRNA_f^{Met}$ G^1C^{72}	>0.2	>15	0.005	0.7	3	12
$tRNA_f^{Met}$ C^2G^{71}	0.6	3.6	0.12	0.25	0.5	26
$tRNA_f^{Met}$ G^3C^{70}	0.9	1.8	0.4	0.4	1.5	15
$tRNA_f^{Met}$ G^{73}	18	2.9	4.4	1.4	1.4	53
$tRNA_f^{Met}$ $C^{11}G^{24}$	18	2.8	4.6	0.16	7	1.2
Val-$tRNA_f^{Met}$ (GAC)	0.08	2	0.03	0.0008	1.4	0.03
Ile-$tRNA_f^{Met}$ (GAU)	0.075	0.9	0.06	0.001	0.3	0.18
Met-$tRNA_f^{Val}$ (CAU)	nm	nm	<0.0001	>0.03	>10	0.16

Specificity toward the Aminoacyl Group Esterified to tRNA—In the case of the FMTec, the side chain of the aminoacyl group attached to tRNA modulates the efficiency of the formylation reaction. Methionine provides the highest efficiency of formylation, as compared with the other tested amino acids, Gln, Phe, Val, or Lys (7, 20, 21).

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 render the tRNAs aminoacylatable with valine and isoleucine, respectively (7, 22). As previously reported (7), valyl-$tRNA_f^{Met}$(GAC) can be formylated by FMTec, although the efficiency of the reaction is reduced by a factor of 3000 as compared with that measured with the natural methionylated substrate (Table I). The results obtained with FMTec and isoleucyl-$tRNA_f^{Met}$(GAU) are shown in Table I. Upon isoleucylation instead of methionylation, the catalytic efficiency is reduced by a factor of 1500.

When using FMTmt as the formylating enzyme, the changes in catalytic efficiency upon changing methionine to valine or isoleucine resembled those measured with FMTec (Table I). However, the relative consequences of these two changes on the FMTmt reaction are greater than any of those measured upon varying the tRNA nucleotidic structure. We suspected, therefore, that the presence of a cognate methionyl group could be the predominant parameter specifying formylability of its substrate by FMTmt. To probe this idea, we used an *E. coli* $tRNA_1^{Val}$, the anticodon of which had been changed into a CAU. As shown in Table I, upon methionylation, $tRNA_1^{Val}$(CAU) can be formylated by FMTmt with an efficiency only 600-fold smaller than that measured with Met-$tRNA_f^{Met}$. Valyl-$tRNA_1^{Met}$ is not a substrate of FMTmt ($k_{cat}/K_m < 10^{-6}\ s^{-1}\ M^{-1}$). With the bacterial enzyme, Met-$tRNA_1^{Val}$(CAU) is much less efficient than Met-$tRNA_f^{Met}$ (by 6 orders of magnitude).

Mild Proteolysis of FMTmt—In the *E. coli* system, the opening of the acceptor stem of $tRNA^{Met}$ involves an enzyme loop, called loop 1 (see Fig. 3). In the free enzyme, loop 1 is disordered and highly sensitive to trypsin cleavage, with a cut between Arg^{42} and Gly^{43}. In the presence of bound f-Met-$tRNA_f^{Met}$, the loop establishes many contacts with the acceptor stem (9) and becomes protected from trypsin cleavage (8). Similar experiments were performed with FMTmt. No trypsin cut was observed under mild conditions. However, upon using the V8 endoprotease under mild conditions, a single cleavage site between Glu^{53} and Val^{54} is evidenced. This region is thought from previous sequence alignments to correspond to loop 1 of the *E. coli* enzyme (11). In the presence of saturating formylmethionyl-$tRNA_f^{Met}$ (65 μM tRNA and 50 μM FMTmt), the efficiency of cleavage by the V8 protease did not vary. The latter result suggests that the interaction of the tRNA substrate with FMTmt is different from that occurring with the bacterial enzyme.

DISCUSSION

Discrimination among tRNAs by FMTec or FMTmt—Fig. 2 summarizes the relative effects of the various changes performed in $tRNA_f^{Met}$ on the efficiency of the formylation reaction catalyzed by either FMTec or FMTmt. In the *E. coli* system, the acceptor stem plays the most important role. The absence of CG or GC at position 1-72 is crucial for the reaction. The other determinants, in decreasing order of importance, are the side chain of the attached amino acid and the Puo-Pyd pair at position 11-24.

The results obtained in this study show that FMTmt is much less sensitive than its bacterial counterpart to the nucleotides composing the tRNA substrate. This difference enables the side chain of the methionyl group attached to tRNA to become a dominant identity element for mitochondrial formylation (Fig. 2). In agreement with this conclusion, once aminoacylated with methionine, *E. coli* $tRNA_m^{Met}$ and $tRNA^{Val}$(CAU) behave as reasonably efficient substrates of FMTmt, but not of FMTec.

Bovine mitochondria contain 22 tRNA species, all encoded by the mitochondrial genome, among which a single $tRNA^{Met}$ appears to be responsible for both initiation and elongation of translation. Consequently, to ensure a correct formylation reaction, recognition of the methionyl group carried by this single $tRNA^{Met}$ species may be sufficient. Moreover, in the organelle, 12 tRNA species of 22 lack a GC or CG pair at the top of their acceptor stem. This may be another reason to explain why the base pair at position 1-72 has become a marginal determinant for the selection by the mitochondrial formylating enzyme. More important for the recognition by FMTmt is the purine-pyrimidine pair at position 11-24. Only two tRNA species (Leu and Trp), in addition to $tRNA^{Met}$, display this feature in bovine mitochondria. We therefore conclude that the combination of a methionyl group and the 11-24 pair is enough to unambigu-

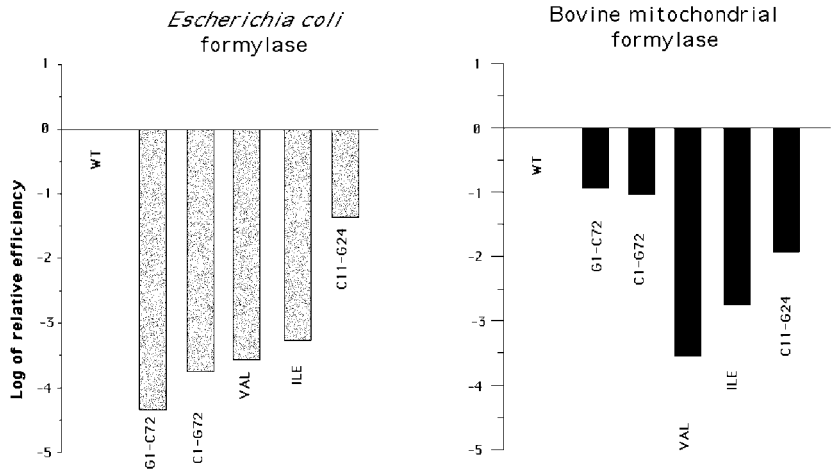
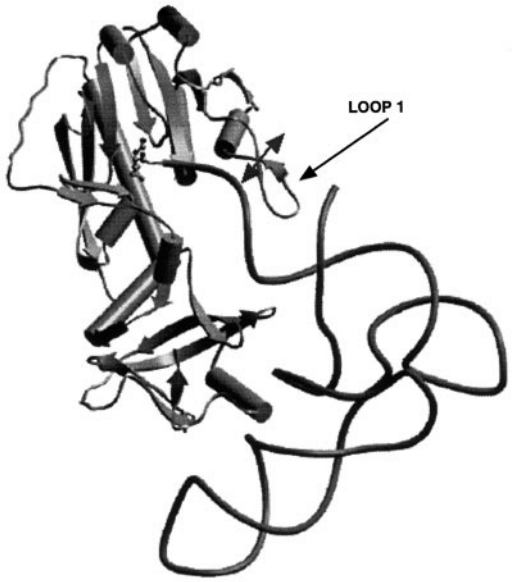


FIG. 2. **Comparison of the weight of substrate determinants in the formylation reaction catalyzed by either *E. coli* or bovine mitochondrial formylase.** The *bars* represent the logarithm of the ratio of the k_{cat}/K_m parameter for the studied tRNA over that for the authentic $tRNA_f^{Met}$. *Gray bars* represent the values for the bacterial enzyme, and *black bars* represent the values for the bovine mitochondrial enzyme.



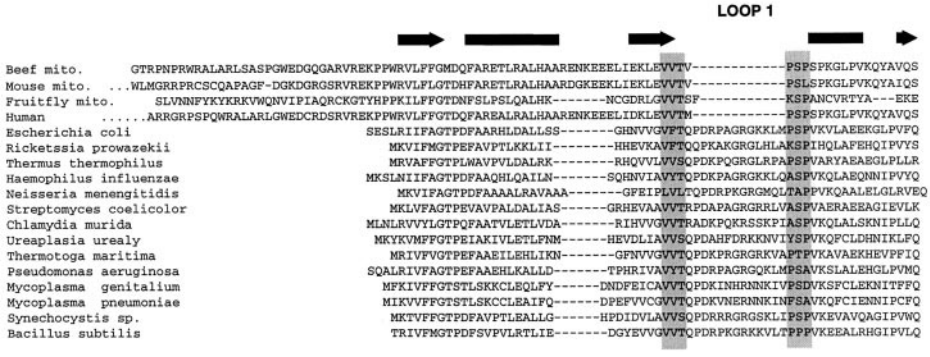


FIG. 3. *Top*, schematic representation of the three-dimensional structure of FMTec complexed to formyl-met-$tRNA_f^{Met}$. Loop 1 is indicated by an *arrow*. *Bottom*, sequence alignments of FMT from various origins in the region of loop 1. Alignments were performed using CLUSTAL V (23), and refined manually. The positions of the secondary structure elements of the *E. coli* protein are indicated, with *bars* representing α helices and *arrows* representing β strands. The position of loop 1 is indicated. Two conserved sequences bordering loop 1 are shaded in *gray*.

ously direct specific formylation in animal mitochondria. Notably, mutations in the nucleotidic part of the tRNA substrate mainly affect the K_m of the formylation reaction, whereas the changing of esterified amino acid influences the catalytic rate (Table I). The use of the 11-24 pair as an identity element can thus prevent excessive competition of non-methionine tRNAs for the binding to FMTmt. Finally, some FMTmt molecules can be assumed to accidentally escape the mitochondrial import system and promote erroneous formylation of cytoplasmic tRNAs. However, the occurrence of a pyrimidine-purine pair at position 11-24 of all mammalian cytoplasmic $tRNA^{Met}$s could help them to escape such an undesired action of FMTmt.

Mechanism of Action of FMTmt—The crystallographic structure of FMTec complexed with formyl-methionyl-$tRNA_f^{Met}$ offers a structural basis to the recognition mechanism. FMTec is built up of two domains, connected by an elongated linker. The N-terminal domain consists of a Rossmann fold containing the catalytic center, whereas the C-terminal domain is made of a β-barrel (Fig. 3). The tRNA substrate binds on its D-stem side at the surface of the enzyme. This allows the formation of a base-specific interaction of the A^{11}-U^{24} base pair with the C-terminal domain. Close to the active site, a loop of the enzyme

makes numerous interactions in the major groove of the acceptor stem. Such a positioning results in the splitting of the C^{1}-A^{72} mismatch, with typical bending of the 3′ arm toward the active center, where the methionyl moiety fits in a specific pocket.

The present study establishes that a Puo^{11}-Pyd^{24} pair is important for the formylation reaction in the mitochondrial system. Therefore, the initial positioning of the tRNA substrate at the surface of the C-terminal domain of FMTmt is likely to occur in the same way as in the bacterial system. This idea is favored by the conservation in the FMTmt sequence of residues of FMTec involved in tRNA binding, like Lys^{291}, Lys^{292}, and Asn^{301} (11). In the *E. coli* system, Asn^{301} binds the O_2 atom of U^{24} of $tRNA_f^{Met}$. Probably, the corresponding Asn^{318} of FMTmt binds the O_2 group of C^{24} of mitochondrial $tRNA^{Met}$.

In the bacterial enzyme, loop 1 establishes many contacts with the acceptor stem of bound fMet-$tRNA_f^{Met}$. This interaction protects the loop from trypsin cleavage. FMTmt contains a V8 endoprotease-sensitive site corresponding to the trypsin cutting site of FMTec. However, binding of f-Met-$tRNA_f^{Met}$ does not affect the efficiency of the V8 digestion. The sequence alignment in Fig. 3 indicates that, in the bovine mitochondrial enzyme, loop 1 is replaced by a short turn. We may therefore speculate that this short turn does not make contacts with the tRNA acceptor stem. Such a conclusion would account for the reduced sensitivity of FMTmt to the composition of the acceptor stem of its substrate. In agreement with this idea, other animal mitochondria (mouse, human, fruit fly), all of which appear to contain a single $tRNA^{Met}$, also putatively lack loop 1 in the sequence of their formylase. Therefore, mitochondrial formylases might not need to open the 1-72 base pair to allow the methionyl group to reach the active site. However, the possibility that despite the lack of loop 1, FMTmt opens the 1-72 base pair, even if it is a GC one, cannot be ruled out. Further structural investigations are required to answer these questions.

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