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

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Mammalian Mitochondria*

Protein synthesis involves two methionine-accepting 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<sup>Met</sup>. In Escherichia coli, it has been shown that the specific action of methionyl-tRNA transformylase on Met-tRNA<sup>Met</sup> mainly involves a set of nucleotides in the acceptor stem, particularly a C<sup>3</sup>A<sup>72</sup> mismatch. In animal mitochondria, only one tRNA<sup>Met</sup> 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<sup>Met</sup> 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<sup>Met</sup> an A<sup>1</sup>U<sup>72</sup> 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 aminocylated 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 gives its full functional identity to initiator Met-tRNA<sup>Met</sup>. It has been shown that the presence of a formyl group is essential for the recognition of tRNAs by methionyl-tRNA transformylase from E. coli, as a consequence of the specificity of FMT for the binding of Met-tRNA<sup>Met</sup> to the A site of the ribosome. This 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<sup>Met</sup> species in animal mitochondria.

MATERIALS AND METHODS

Expression and Purification of Bovine Mitochondrial FMT—The NdeI-BamHI fragment from pET19b-FMT<sup>mt</sup> (10) carrying the gene coding for the bovine mitochondrial formylase was cloned into the corresponding sites of pET15b. The resulting plasmid, pET15b-FMT<sup>mt</sup>, 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, 2.5 ml/min). The protein was homogenous as judged by SDS-polyacrylamide gel electrophoresis. FMT<sup>mt</sup> 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 were synthesized as described (11). The following four mutant tRNAs were used: tRNA<sup>Met</sup>-<sup>3</sup>U<sup>30</sup> ('<sup>3</sup>U' tRNA<sup>Met</sup>); tRNA<sup>Met</sup>-<sup>1</sup>C<sup>30</sup> ('<sup>1</sup>C' tRNA<sup>Met</sup>); tRNA<sup>Met</sup>-<sup>1</sup>C<sup>30</sup>-<sup>2</sup>C<sup>43</sup> ('<sup>1</sup>C<sup>2</sup>C' tRNA<sup>Met</sup>); tRNA<sup>Met</sup>-<sup>1</sup>C<sup>30</sup>-<sup>2</sup>C<sup>43</sup>-<sup>3</sup>C<sup>62</sup> ('<sup>1</sup>C<sup>3</sup>C' tRNA<sup>Met</sup>).
Recognition of tRNAs by Methionyl-tRNA Formylase 20065

were expressed in JM101Tr (12) from plasmid pBSTNAV2 (7). The gene
coding for rRNA<sub>Met</sub>G11-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
features were recolored in the pBSTNAV3S vector, to ensure full matura-
tion by RNase P (14). In three cases, however (tRNA<sub>Met</sub>G11-CG71, tRNA<sub>Met</sub>G11-GG71, and tRNA<sub>Met</sub>G11(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 ac-
cepting 1200–1700 pmol of amino acid per A<sub>260</sub> unit were purified as
described (7).

Measurement of Catalytic Parameters of FMT—Initial rates of ami-
noacylation-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<sub>2</sub>) con-
taining 125 μM 10-formyltetradecylphosphate 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 μM KCl, 0.5% CHAPS, 5 mM MgCl<sub>2</sub> Ref. (7)).
Homogeneous preparations of E. coli M274 methionyl-tRNA synthetase (17), vafyl-tRNA synthetase (18), or isoeucyl-tRNA synthetase (19) were used for the aminoclaylation of tRNAs.

Limit 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<sub>2</sub>, 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 position52LE/VV55, as previously demon-
strated (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<sub>Met</sub>.

RESULTS

Influence of the Nucleotidic Composition of the Acceptor Stem
of tRNA on the Formylation Reaction Catalyzed by FMTmt—

The sequence of tRNA<sub>Met</sub> from bovine mitochondria is shown in
Fig. 1, together with that of E. coli tRNA<sub>Met</sub>. Mitochondrial
tRNA<sub>Met</sub> 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-purimidine pair at position 11-24, and (5) aminoacylation with methionine. Indeed, initiator Met-tRNA<sub>Met</sub> from E. coli is as good a substrate of FMTmt as the authentic Met-tRNA<sub>Met</sub> 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<sub>Met</sub> in vitro. The efficiency of this reaction is
only 500-fold lower than that measured with E. coli initiator
Met-tRNA<sub>Met</sub>. Such a behavior contrasts with that of bacterial
FMT, which acts on elongator tRNA<sub>Met</sub> 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<sub>Met</sub> de-

Derivative, the acceptor stem of which had been replaced by that
of tRNA<sub>Met</sub>-<sub>Met</sub> (tRNA<sub>Met</sub>). This grafting was shown to increase the
catalytic efficiency of the bacterial formylation 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<sub>Met</sub>
is compared with tRNA<sub>Met</sub>. In the same manner, the introduction
of a CG or a GC pair at position 1-72 of tRNA<sub>Met</sub> (A12G or
C12T 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<sub>M</sub> of tRNA<sub>Met</sub> 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 G12C/T17G and C3G/G70T 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 Puro<sup>11</sup>-Py7<sup>24</sup> Pair in the D-stem of
tRNA—Like most initiator tRNAs, tRNA<sub>Met</sub> 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-pyrimi-
dine pair contributes to the recognition of its substrate by the
bacterial formylase (6, 9). Mutation of the A<sub>11</sub>T<sub>24</sub> pair
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 com-
plexed protein indicates that the C-terminal domain of FMTec
directly contacts the U<sub>24</sub> base (9).

With FMTmt as the formylating enzyme, the above-men-
tioned mutation in tRNA<sub>Met</sub> 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 mito-
chondrial enzyme than any mutation in the acceptor stem.
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$^{\text{Met}}$ tRNA$^{\text{Met}}_{\text{cat}}$ is a tRNA$^{\text{Met}}$ derivative carrying the acceptor stem of tRNA$^{\text{Met}}_{\text{cat}}$. Mutations are indicated in the name of each tRNA. All tRNAs were methionylated, unless otherwise indicated. Val-tRNA$^{\text{Met}}$ (GAC) means tRNA$^{\text{Met}}$ carrying a GAC anticodon and aminoacylated with valine. Ile-tRNA$^{\text{Met}}$ (GAU) means tRNA$^{\text{Met}}$ carrying a GAU anticodon and aminoacylated with isoleucine. Met-tRNA$^{\text{Met}}_{\text{Val}}$ (CAU) means tRNA$^{\text{Met}}_{\text{Val}}$ carrying a CAU anticodon and aminoacylated with methionine, nm, not measurable.

### Table I

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<td>&gt;10</td>
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### Discussion

**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$^{\text{Met}}$ 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), variations in 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$^{\text{Met}}_{\text{Val}}$; the anticodon of which had been changed into a CAU. As shown in Table I, upon methionylation, tRNA$^{\text{Met}}_{\text{Val}}$(CAU) can be formylated by FMTmt to an efficiency only 600-fold smaller than that measured with the natural methionylated substrate (Table I). The results obtained with FMTmt and iso- leucyl-tRNA$^{\text{Met}}_{\text{Val}}$(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 FMTmt (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 base pair at position 11-24. Only two tRNA species (Leu and Trp), in addition to tRNA$^{\text{Met}}_{\text{Val}}$ and tRNA$^{\text{Met}}_{\text{Val}}$(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$^{\text{Met}}_{\text{Val}}$ 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$^{\text{Met}}_{\text{Val}}$ 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 dominant identity element for mitochondrial formylation. 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$^{\text{Met}}_{\text{Val}}$ 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-
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$^{\text{Met}}$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$^{\text{Met}}$ 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 $\beta$-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 A11-U24 base pair with the C-terminal domain. Close to the active site, a loop of the enzyme...
Recognizes of tRNAs by Methionyl-tRNA Transformylase

makes numerous interactions in the major groove of the acceptor stem. Such a positioning results in the splitting of the C1-A2 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 Puo11-Pyd24 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 Lys291, Lys292, and Asn301 (11). In the E. coli system, Asn301 binds the O2 atom of U24 of tRNAfMet. Probably, the corresponding Asn318 of FMTmt binds the O2 group of C24 of mitochondrial tRNAfMet.

In the bacterial enzyme, loop 1 establishes many contacts with the acceptor stem of bound fMet-tRNAfMet. This interaction protects the loop from trypsin cleavage. FMTmt contains a single tRNAMet, also putatively lack loop 1 in the mitochondrial system (mouse, human, fruit fly), all of which appear to contain a single tRNAfMet, 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.

REFERENCES

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