Rapid Communication *J. Biochem.* 124, 1069-1071 (1998)

Expression and Characterization of Bovine Mitochondrial Methionyl-tRNA Transformylase

Nono Takeuchi, Takuya Ueda, and Kimitsuna Watanabe[1]

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8656

Received for publication, September 8, 1998

Translational initiation in bacteria and some organelles such as mitochondria and chloroplasts requires formyl-methionyl-tRNA (fMet-tRNA). Methionyl-tRNA (Met-tRNA) undergoes formylation by methionyl-tRNA transformylase (MTF), and the resulting fMet-tRNA is utilized exclusively in the initiation process. The gene encoding mammalian mitochondrial MTF (MTFmt) was cloned recently. When the cDNA corresponding to mature MTFmt was cloned into an expression vector, no expression of MTFmt was observed. However, if the cDNA was fused with the histidine-tag sequence at the N-terminus, MTFmt could be expressed in *Escherichia coli*. The recombinant enzyme was purified by a single step on a histidine-binding metal affinity column. We previously found that native MTFmt is able to formylate *E. coli* elongator Met-tRNA as well as the initiator Met-tRNA. The specific formylation of the initiator Met-tRNA by *E. coli* MTF is quite important in bacterial translational initiation. The purified recombinant MTFmt with the histidine-tag showed almost identical kinetic parameters to those of native MTFmt. This expression system is suitable for the rapid, efficient production of MTFmt in amounts adequate for further biophysical studies, which will provide another approach for elucidating the formylation mechanism, in addition to studies on *E. coli* MTF.

Key words: expression, mammalian mitochondria, MTF, substrate specificity, translational initiation.

The initiation of translation in most prokaryotic organisms requires the formylation of the initiator methionyl-tRNA (Met-tRNA) by methionyl-tRNA transformylase (MTF) (*1*). In *Escherichia coli*, the formyl group is a positive determinant for the specific interaction of initiation factor 2 (IF-2) with the initiator tRNA. It also serves as a negative determinant that prevents the binding of elongation factor Tu (EF-Tu) to the initiator tRNA (*2*). Bacterial MTF does not formylate the elongator Met-tRNA species used for chain elongation (*3*). The strict substrate specificity of *E. coli* MTF is essential for ensuring the accuracy and efficiency of the initiation process. In contrast to all other systems, animal mitochondria do not contain two distinct Met-tRNA species that are used exclusively for either the initiation or elongation phase of protein synthesis. Mammalian mitochondria have a single methionine tRNA ($tRNA^{Met}$) gene which is encoded in the organelle genome (*4*). There is no evidence that cytoplasmic tRNAs are imported into animal mitochondria (*5*). Thus, the single $tRNA^{Met}$ gene must, in some unknown manner, give rise to both an initiator tRNA (fMet-tRNA) and an elongator tRNA (Met-tRNA).

The unique presence of a single Met-tRNA species in mammalian mitochondria makes it of considerable interest to determine the substrate specificity of mitochondrial MTF (MTFmt). We previously demonstrated the kinetic parameters of native MTFmt governing the formylation of various tRNAs (*6*). Bovine MTFmt formylates its homologous mitochondrial Met-tRNA as well as the *E. coli* initiator Met-tRNA with essentially equal efficiency. Surprisingly, the *E. coli* elongator Met-tRNA was also formylated by this bovine MTFmt, although with somewhat less favorable kinetics. These results suggest that the substrate specificity of MTFmt is not as rigid as that of the *E. coli* MTF, which clearly discriminates between the bacterial initiator and elongator Met-tRNAs. This observation is compatible with the fact that there is a single Met-tRNA species in mammalian mitochondria.

The structural and sequence elements important for the recognition of tRNA by *E. coli* MTF have been well elucidated (*7, 8*). The structure of *E. coli* MTF was recently determined by X-ray analysis (*9*). However, it remains unclear how the mitochondrial MTF is able to exhibit such a unique specificity. The expression system of MTFmt presented here will allow us to carry out further biophysical studies to elucidate the formylation mechanism of MTFmt. Furthermore, this expression system should also facilitate the structural study of recombinant MTFmt, which will contribute to elucidation of the structural basis of MTF governing its substrate specificity, combined with studies

[1] To whom correspondence should be addressed. Tel/Fax: +81-3-5800-6950, E-mail: kw@kwl.t.u-tokyo.ac.jp

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction.

on *E. coli* MTF.

Materials—Folinic acid was purchased from Sigma. [^{35}S]Methionine (37 TBq/mmol) and [^{14}C]methionine (1.85 GBq/mmol) were obtained from Amersham. The Hi Trap Chelating column was purchased from Pharmacia. Enterokinase was obtained from Novagen.

Strains and Vectors—*E. coli* JM109 was used for the propagation of the recombinant plasmids. BL21(DE3) was used in the expression study. The pET11a and pET19b vectors were purchased from Novagen.

Buffers—Binding Buffer comprised 50 mM Tris-HCl (pH 7.6), 500 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, and 3 mM 2-mercaptoethanol. Buffer TM comprised 20 mM Tris-HCl (pH 7.6) and 3 mM 2-mercaptoethanol.

Analytical Methods—Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (*10*).

Construction of Expression Plasmids—A primer corresponding to the NH_2-terminal end of the mature form of bovine MTFmt (5′-GGCATATGGCGTCCCCTGGCTGGGA-3′) was synthesized with the *Nde*I restriction site at the 5′ end. A primer for the COOH-terminal end of MTFmt (5′-GGGGATCCGGTGCACTATTGCATAGCAAC-3′) was synthesized carrying a *Bam*HI cutting site. PCR amplification using these primers generated a product containing the mature form of bovine MTFmt. This cDNA was then cloned into either pET11a or pET19b.

Expression and Purification of MTFmt—*E. coli* BL21(DE3) was used as the host for expression. For purification of the mature form under native conditions, two 1 liter of a 2×YT culture were grown to the mid-log phase at 37°C and then induced by exposure of the cells to 0.1 mM IPTG for 16 h at 18°C. The cells were harvested by centrifugation at 5,000×*g* for 30 min at 4°C. The cell pellet was washed with Binding Buffer described above, frozen quickly in a liquid nitrogen bath and stored at −70°C. The cells were resuspended in the Binding Buffer containing 0.1 mM PMSF (10 ml per g of wet cells), and then disrupted by 4 min sonication (repeated 1 s bursts following 1 s cooling periods) at 100 W at 4°C. The homogenate was subjected to centrifugation at 100,000×*g* for 180 min. An FPLC system was used for the following purification procedure. The supernatant fraction (S100) was loaded immediately onto the 5 ml Hi Trap Chelating column (Pharmacia) chelated with nickel and equilibrated with Binding Buffer, at the flow rate of 4 ml/min. After washing with about 500 ml of Binding Buffer at 4 ml/min, the column was developed with a 80 ml linear gradient from 0.01 to 0.250 M imidazole in Binding Buffer. Fractions (1 ml) were collected at the flow rate of 4 ml/min. MTFmt was eluted with about 0.2 M imidazole. The MTFmt fractions were pooled and then dialyzed against 1 liter of Buffer TM described above containing 0.1 M KCl (TM.1) for 4 h, with one change of the buffer. Samples were frozen quickly in the liquid nitrogen bath and stored at −70°C. When necessary, the histidine tag was cleaved from the recombinant MTFmt with enterokinase (Novagen) according to the manufacturer's instructions.

Enzymatic Assaying of MTFmt—The assaying of the formylation activity was carried out according to Ref. *11* with a slight modification, as follows. The reaction mixtures (50 μl) comprised 20 mM Tris-HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.5% (w/v) CHAPS, 1 mM dithiothreitol, 5 μM *E. coli* [^{14}C]Met-tRNA^{fMet}, 0.3 mM N^{10}-formyltetrahydrofolate, and the indicated amounts of MTFmt. *E. coli* and mitochondrial Met-tRNAs were prepared as described in Ref. *6*.

Expression and Purification of MTFmt in E. coli—Sequences corresponding to the mature form of bovine MTFmt (amino acid residues 1 to 357) have been cloned into either the pET19b or pET11a expression vector. The pET19b construct (pET19b-MTFmt) has an extra MGH_{10} SSGHIDDDDKHM at the N-terminus, which should permit the expression in *E. coli* of a protein with a mass of 43 kDa designated as recH-MTFmt. The pET11a construct (pET11a-MTFmt) should express the mature form of MTFmt, which has a protein mass of 40 kDa. As shown in Fig. 1A, the expression of the recombinant MTFmt was only observed in the case of pET19b-MTFmt. Enzymatic assaying of MTFmt also confirmed that rec-MTFmt was not expressed significantly in the case of pET11a-MTFmt (Fig. 1B). The optimal level of expression of the soluble recH-MTFmt using the pET19b-MTFmt vector was observed

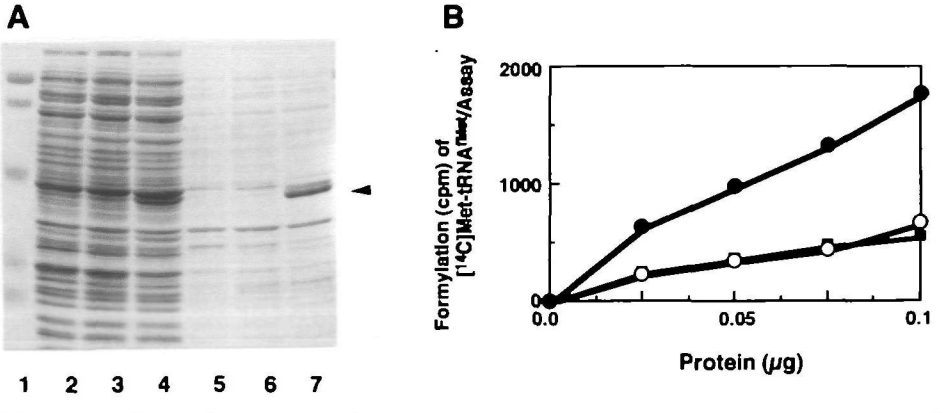

Fig. 1. **Analysis of the expression of the recombinant MTFmt.** A: After disruption of the host cells by sonication, either the soluble or insoluble fractions were analyzed by SDS-PAGE. Lanes 2-4: soluble fractions from the host cells carrying pET11a (lane 2), pET11a-MTFmt (lane 3), and pET19b-MTFmt (lane 4). Lanes 5-7: insoluble fractions from the host cells carrying pET11a (lane 5), pET11a-MTFmt (lane 6), and pET19b-MTFmt (lane 7). The molecular weight markers were phosphorylase B (102,000), bovine serum albumin (81,000), ovalbumin (46,900), carbonic anhydrase (32,700), and soybean trypsin inhibitor (30,200) (Lane 1). The arrow indicates the protein band corresponding to recH-MTFmt. B: The enzymatic activity of MTFmt was analyzed using soluble fractions from the host cells carrying pET11a (black squares), pET11a-MTFmt (open circles), and pET19b-MTFmt (closed circles). The assay was carried out as described under "MATERIALS AND METHODS." The slight formylation activity detected in the case of pET11a is due to the endogeneous MTF.

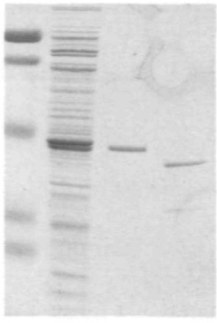

Fig. 2. **SDS-PAGE analysis of the recombinant MTFmt.** The molecular weight markers were phosphorylase B (102,000), bovine serum albumin (81,000), ovalbumin (46,900), carbonic anhydrase (32,700), and soybean trypsin inhibitor (30,200) (Lane 1). 10 μg of cell extract (s-100) (lane 2), 200 ng of recH-MTFmt purified by affinity chromatography (lane 3), and 200 ng of rec-MTFmt digested with enterokinase (lane 4) were analyzed. Nonspecific digestion by the enterokinase was slightly observed.

with about 16 h induction at 18°C in the presence of 0.1 mM IPTG. About 80% of the expressed recH-MTFmt remains in the insoluble fraction under these conditions. Although it is unclear why pET11a-MTFmt does not produce MTFmt, one possibility is that the beginning peptide sequence of the mature form of MTFmt is not suitable for expression in *E. coli*, for example, the nascent product might be susceptible to proteolysis.

Bovine recH-MTFmt derived from the pET19b construct was purified from *E. coli* cell extracts under the native conditions as described under "MATERIALS AND METHODS." After affinity chromatography, the overexpressed recH-MTFmt was purified almost to homogeneity (Fig. 2, lane 3). The yield from 1 liter of cell culture was about 3.8 mg. When the histidine-tag was cleaved with enterokinase (Fig. 2, lane 4), the product showed almost the same enzymatic activity as that of recH-MTFmt (data not shown).

Substrate Specificity of recH-MTFmt—Kinetic parameters were measured using the recH-MTFmt purified by affinity chromatography. As summarized in Table I, the purified recombinant MTFmt showed almost identical kinetic parameters to those of native MTFmt (*6*), indicating that the recH-MTFmt maintained the unique substrate specificity of native MTFmt.

Although recH-MTFmt may also formylate the *E. coli* elongator Met-tRNA *in vivo*, recH-MTFmt can be overexpressed in *E. coli* without any special problems. Peptidyl-tRNA hydrolase (PTH) in *E. coli* might be involved in the hydrolysis of such elongator fMet-tRNAs, even if they are produced in the cells. PTH removes the peptide moieties from the immature peptidyl-tRNAs released from the ribosome in the case of abortive protein synthesis. An *N*-blocked aminoacyl-tRNA can be a substrate for PTH, except for the initiator *N*-formyl or *N*-acetyl-Met-tRNA, which are protected from the action of PTH, since the C_1A_{72} unpairing in their acceptor stems is a negative determinant for PTH (*12*).

TABLE I. **Kinetic parameters of the recombinant MTFmt for the formylation of various Met-tRNAs.**

Met-tRNA	V_{max} ($\times 10^{-2}$ μM/min)	K_m (μM)	Relative V_{max}/K_m[a]
E. coli (f)	2.0	0.10	0.67
E. coli (m)	0.30	0.097	0.10
Bovine mitochondria	1.2	0.040	1

[a]Relative V_{max}/K_m is the ratio of V_{max}/K_m of mitochondrial Met-tRNA to V_{max}/K_m of each Met-tRNA.

This expression system will be very useful for biochemical or biophysical studies to elucidate the substrate specificity of MTFmt using various substrates. We previously proposed a hypothesis to explain the unique substrate specificity of MTFmt, based on comparison of the primary sequences of the *E. coli* and bovine mitochondrial MTFs (*6*). This expression system is also expected to facilitate the structural study of recombinant MTFmt to evaluate our hypothesis. It will also contribute to elucidation of the structural basis of MTF governing its substrate specificity, in combination with studies on *E. coli* MTF.

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