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Protein Chain-Initiating Methionine tRNAs in Chloroplasts and Cytoplasm of Wheat Leaves*

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Abstract. The role of methionine in protein chain initiation in wheat has been studied. Two chain-initiating methionine tRNAs have been found. One of these is located in the cytoplasm of the wheat cell. This methionyl-tRNA is not formylated by wheat extracts and appears to function in protein chain initiation in the cytoplasm without prior formylation. The other initiating tRNA is from chloroplasts. This methionyl-tRNA is formylated by a transformylase present in wheat extracts and functions in chain initiation in chloroplasts as formylmethionyl-tRNA.

Protein synthesis in bacteria is initiated by *N*-formylmethionyl–tRNA (fMet–tRNA).[1–3] There is evidence that fMet-tRNA also functions in chain initiation in the mitochondria[4,5] and plastids[6,7] of higher organisms but not in the cytoplasm.[4,5] Evidence has been presented recently that protein chain initiation in the cytoplasm of eukaryotes can be brought about by a methionyl-tRNA (Met-tRNA) that is unformylated.[8–12]

In a study of protein chain initiation in wheat germ,[10] three methionine tRNAs ($tRNA^{Met}$) were separated chromatographically; two were found to be chain-initiating tRNAs. One of these was a minor species that could be formylated by a wheat germ enzyme after charging with methionine. This tRNA was designated $tRNA_f^{Met}$. The other initiating $tRNA^{Met}$ was a major species. It was not formylated by wheat germ extracts and appeared to function in chain initiation without formylation.[10,12] It was designated $tRNA_i^{Met}$. There was also a major species, designated $tRNA_m^{Met}$, which inserted methionine internally into growing chains.[10,12] It was assumed in these studies that the two major species were cytoplasmic and the minor species was derived from plastids.

In order to determine the cellular localization of these methionine tRNAs, these studies have been extended to the wheat leaf. A tRNA comparable to the wheat germ $tRNA_f^{Met}$ was found in the chloroplasts. The cytoplasm contained a $tRNA_i^{Met}$, which could not be formylated after charging, that could function for protein chain initiation without formylation in the cytoplasm of wheat cells.

Materials. Yorkstar Seed Wheat treated with Panogen was obtained from Agway Inc., Syracuse, N.Y. *Escherichia coli*, grown to late log phase in an enriched medium, was obtained from Grain Processing, Inc., Muscatine, Ia. Puromycin was from Nutritional Biochemicals Corp., mycostatin (B grade) from Calbiochem, penicillin G from Eli Lilly and Co., tetrahydrofolate from Sigma Chemical Co., Ficol and

Dextran T 40 from Pharmacia, AUG from Miles Laboratories, [^{14}C]methionine from New England Nuclear, and [^{14}C]formate, sodium salt, from Amersham Searle. Formyltetrahydrofolate synthetase (*Costridium acidi-urici*) was a gift of Dr. Jesse C. Rabinowitz (U. of Calif., Berkeley). Benzoylated DEAE-cellulose (BD-cellulose) was a gift of Dr. B. S. Dudock. Other materials were as described.[10]

Growth of wheat plants: Wheat seeds were soaked for 4 hr in running tap water. The seeds were then sterilized by bathing in Clorox, diluted 1:20 with water for 6 min, and rinsed 9–10 times with sterile distilled water. The seeds were transferred, in a sterile hood, to sterile pans containing blotting paper saturated with a solution of mycostatin (0.1 mg/ml) and penicillin G (50 μg/ml). The pans were covered with sterile aluminum foil, and the seeds were germinated overnight in the dark. After the foil was replaced with sterile transparent plastic covers, the wheat was grown under fluorescent light (18 hr/day) for 5 days. Only one to two mold colonies were observed in each crop (about 2 m^2 of seedlings). Wheat leaves prepared in this fashion were tested for bacterial contamination by bathing leaves in a salts solution and plating aliquots of the solution on nutrient agar. The average bacterial contamination was 2×10^{-7} g bacteria/g wet weight of leaf, assuming 1×10^{-12} g/bacterium.

Preparation of bulk tRNA from wheat leaves: Wheat leaves were cut into 1–2 cm pieces and homogenized in 2 ml of 0.1 M Tris·HCl, pH 7.5, per g of tissue in a Waring Blendor for 1 min at high speed. The homogenate was passed through several layers of gauze. Crude RNA was then isolated from the extract by phenol treatment and alcohol precipitation. tRNA represents only about one-sixth of the crude RNA preparation. The tRNA was isolated by adsorption of the crude RNA on a column of DEAE-cellulose and elution with a linear NaCl gradient from 0 to 1 M in 0.1 M Tris·HCl, pH 7.5. The method is a modification of that of Holley.[13]

Isolation of chloroplast tRNA: A modification of the method of Jensen and Bassham[14] was used to isolate intact chloroplasts. This procedure results in a low yield, but is mild, and the chloroplasts isolated have intact membranes. All operations were at 4°C. Wheat leaves were cut into small pieces (2–3 mm) with a razor blade. The cut leaves were ground for about a minute in a mortar and pestle with 1–2 ml/g leaf of modified Honda medium[15] with 4 mM dithiothreitol in place of mercaptoethanol. The ground material was rapidly passed through several layers of gauze and the extract *immediately* centrifuged at 5000 $\times$ *g* for 2 min. The supernatant was decanted. The chloroplast pellet, which contained 40% of the chlorophyll in the extract, was suspended in Tris buffer, and the bulk tRNA was isolated as above.

Acceptor assay for methionine tRNAs: Methionine acceptor activity was assayed in a 0.2-ml volume with [^{14}C]methionine (20 Ci/mol) by the method of Loehr and Keller[16] except that 70 mM KCl was added to the incubation.[17] In assaying column fractions the tRNA, in 0.1 or 0.2 ml of each fraction, was first precipitated in an assay tube with 3 volumes of ethanol, centrifuged, and dried under reduced pressure. The source of the activating enzyme was the soluble fraction from wheat germ or *E. coli* that had been passed through Sephadex G-25 and DEAE-cellulose columns.[18] The charged tRNAs were precipitated with 8% trichloroacetic acid (pH 1.0), collected on glass fiber filters, and counted in a Nuclear Chicago thin-window counter (counting efficiency 31%).

Preparation of [^{14}C]Met-tRNA: tRNA^{Met} was charged with [^{14}C]methionine (200 Ci/mol) on a large scale using the wheat germ enzyme, with the assay conditions described above, except that the incubation volume was 10 ml. The reaction mixture was incubated for 40 min at 37°C, and 0.1 volume of 20% potassium acetate (pH 5.0) was added to stop the reaction. The charged tRNAs were isolated by phenol treatment, Sephadex G-25 chromatography in 0.1 M potassium acetate (pH 5.0), and ethanol precipitation. They were stored under reduced pressure in small portions.

Assay for [^{14}C]fMet-tRNA synthesis: The assay contained the following in a volume of 0.1 ml: 50 mM Tris·HCl (pH 7.5), 70 mM KCl, 20 mM $MgCl_2$, 6 mM dithiothreitol, 2.5 mM ATP, 0.5 mM EDTA, 0.4 mM [^{12}C]methionine, 11 μM [^{14}C]-formyltetrahydrofolate[19] (23.6 Ci/mol), and wheat-germ soluble enzyme preparation.

For the assay of column fractions, the tRNA in 0.2 ml of each fraction was first precipitated and dried as above. After incubation for 20 min at 37°C, the tRNAs were collected and counted as above.

Preparation of f-[^{14}C]Met-tRNA$_f$: fMet-tRNA$_f$ (200 Ci/mol), labeled in the methionine, was prepared as was [^{14}C]Met-tRNA but with the addition of 55 μM unlabeled formyltetrahydrofolate[20] to the incubation. Transformylase is present in the wheat-germ soluble enzyme preparation.

Results. The methionine tRNAs of wheat leaf chloroplasts: Chloroplasts were isolated from wheat leaves grown under sterile conditions. The chloroplast tRNA was extracted and chromatographed on BD-cellulose[23] in the presence of 1 mM EDTA and 10 mM sodium acetate (pH 4.5). Two major and one minor tRNA^{Met} species were detected (Fig. 1*A*). The column fractions were assayed

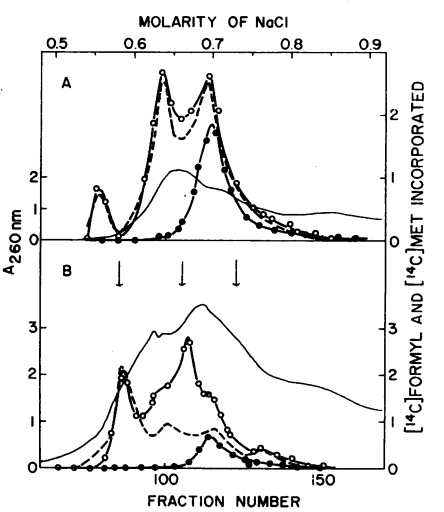


FIG. 1. Chromatography of wheat leaf tRNAs on BD-cellulose.

(*A*) Chloroplast tRNAs. 10 mg of tRNA isolated from unwashed wheat-leaf chloroplasts was adsorbed to BD-cellulose in a column (0.8 × 20 cm) equilibrated with 0.3 M NaCl–1 mM EDTA–10 mM sodium acetate (pH 4.5). The column was developed with a 200-ml linear gradient from 0.3 to 1.0 M NaCl, containing 1 mM EDTA and 10 mM sodium acetate (pH 4.5). Fractions of 1 ml were collected, at a flow rate of 0.08 ml/min. The collection tubes contained a concentrated solution of Tris·HCl (pH 7.5) and magnesium acetate to bring the pH of the fractions to 7 and the concentration of Mg^{++} to 10 mM.

(*B*) Leaf tRNAs. 130 mg of bulk tRNA isolated from wheat leaves was adsorbed to BD-cellulose in a column (1 × 85 cm) equilibrated as in *A*. The column was developed with a 1400 ml linear NaCl gradient as in *A*. Fractions of 7 ml were collected, at a flow rate of 0.36 ml/min.

After chromatography, aliquots were precipitated with ethanol and assayed for methionine acceptor activity or formate incorporation in the presence of methionine. ——, A_{260} nm; —○—, Met acceptor activity assayed with wheat germ enzyme (cpm/0.2 ml × 10^{-2} in *A*, and cpm/0.1 ml × 10^{-2} in *B*); —●—, formate incorporation assayed with wheat germ enzyme (cpm/0.2 ml × 10^{-2}); - - -, Met acceptor activity assayed with *E. coli* enzyme (cpm/0.2 ml × 10^{-2} in *A* and cpm/0.1 ml × 10^{-2} in *B*). The *arrows* in 1*B* indicate the position of elution of wheat germ tRNA^{Met} species on the same column.

for formate incorporation, after charging with methionine, with a wheat germ enzyme preparation. Only the tRNA^{Met} which eluted at 0.70 M NaCl was formylated after charging. This tRNA$_f^{Met}$ was one of the two major tRNA^{Met} species of chloroplasts, constituting about 50% of all of the chloroplast tRNA^{Met}. The chloroplast tRNA$_f^{Met}$ appears identical to the wheat germ tRNA$_f^{Met}$ (ref. 10), substantiating the suggestion that the wheat germ tRNA$_f^{Met}$ is derived from plastids.

The second major tRNA^{Met} in chloroplasts eluted at 0.64 M NaCl. Since it was the only other major tRNA^{Met} in the chloroplasts, it is probably a tRNA$_m^{Met}$, functioning in chain elongation in these organelles. The very small peak of tRNA^{Met} eluting at 0.56 M NaCl could be due to contamination of the prepara-

tion by cytoplasmic tRNAs (see below), as the chloroplasts were not washed. However, the two major $tRNA^{Met}$ species in the chloroplasts cannot be accounted for by cytoplasmic contamination since they do not constitute major peaks in the total leaf pattern (see below).

The methionine tRNAs of wheat leaf: Chloroplasts are extremely fragile. Even though a very mild isolation procedure was used,[14,15] 60% of the chloroplasts were broken and released their contents into the soluble fraction. As a consequence, the soluble fraction could not be used for an analysis of cytoplasmic tRNAs. However, an analysis could be made of the total $tRNA^{Met}$ from wheat leaves that would include the cytoplasmic and mitochondrial as well as the chloroplast $tRNA^{Met}$. The tRNAs from wheat leaves were isolated, fractionated, and analyzed as above (Fig. 1*B*). Five peaks of $tRNA^{Met}$ were detected, first a single major peak, followed by a composite peak made up of three species, and finally a small peak. Two of these could be identified as chloroplast $tRNA^{Met}$ species. On assay for formylation of the leaf Met-tRNAs, a $tRNA_f^{Met}$ was detected. Since it was the only formylatable species in the leaf pattern, and since it eluted at the same concentration of NaCl (0.70 M) as the chloroplast $tRNA_f^{Met}$, it must be this chloroplast tRNA. The chloroplast $tRNA_m^{Met}$, which eluted at 0.64 M NaCl, was present in the leaf pattern as a shoulder at 0.64 M NaCl. The identification was supported by the fact that the tRNA forming the shoulder was charged by *E. coli* Met-tRNA synthetase, as was the chloroplast $tRNA_m^{Met}$ (Fig. 1*A* and *B*). This $tRNA_m^{Met}$ was present in equal amounts to the $tRNA_f^{Met}$, as in the chloroplast pattern. This identification of the chloroplast $tRNA^{Met}$ species in the leaf pattern does not exclude the presence of a small amount of these chloroplast tRNAs in the cytoplasm of the wheat cell.

The remaining peaks in the leaf pattern did not correspond to chloroplast $tRNA^{Met}$ species. There were two major peaks, eluting at 0.59 M NaCl and 0.66 M NaCl. Each of these peaks was three times as large as the peaks of chloroplast tRNA; they were therefore considered to be cytoplasmic. The first of these eluted at a position similar to the $tRNA_i^{Met}$ from wheat germ.[10] (The position of elution of wheat germ $tRNA^{Met}$ species when they were fractionated on this same column is indicated by the arrows in Fig. 1*B*). Evidence that this leaf cytoplasmic $tRNA^{Met}$ is identical to wheat germ $tRNA_i^{Met}$, and functions as an initiating tRNA without formylation, will be presented below.

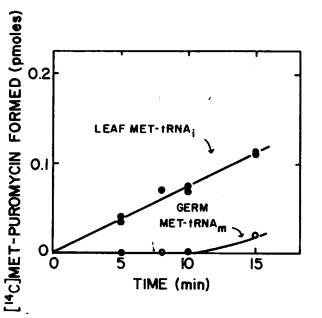
The other major $tRNA^{Met}$, eluting at 0.66 M NaCl, was tentatively identified as the cytoplasmic $tRNA_m^{Met}$. It eluted at the same salt concentration (0.66 M) as the major wheat germ $tRNA^{Met}$, which was identified as a $tRNA_m^{Met}$ (ref. 10, 12). In the earlier study of wheat germ $tRNA^{Met}$, it was found that the large peak of $tRNA_m^{Met}$ was not charged by the *E. coli* Met-tRNA synthetase. The leaf $tRNA^{Met}$ eluting at 0.66 M NaCl was also not charged by *E. coli* Met-tRNA synthetase (Fig. 1*B*) supporting its identification as the cytoplasmic $tRNA_m^{Met}$.

The four major $tRNA^{Met}$ species of the leaf pattern have thus been identified. The fifth peak, which eluted at 0.76 M NaCl, has not yet been characterized. It was a small peak, present in only one third the amount of a chloroplast $tRNA^{Met}$. A mitochondrial tRNA would be present in smaller amount than a chloroplast

tRNA in a green leaf. Whether the small peak is a mitochondrial tRNA is presently being investigated.

The puromycin reaction with leaf $tRNA_i^{Met}$: In order to firmly establish that the first peak in the leaf pattern was $tRNA_i^{Met}$, identical to wheat germ $tRNA_i^{Met}$, it was tested for binding to the initiation site on wheat-germ ribosomes by its reaction with puromycin.[21] Previously, we showed that germ Met-$tRNA_i$ was bound to the initiation site on wheat germ ribosomes, in the presence of AUG, by its reaction with puromycin.[10] In the same system, leaf Met-tRNA from the first peak reacted with puromycin (Fig. 2). The reaction was linear from zero

FIG. 2. Formation of methionyl-puromycin in the presence of wheat germ ribosomes. Wheat leaf [^{14}C]Met-$tRNA_i$ (21 pmol) or germ [^{14}C]Met-$tRNA_m$ (28 pmol) was tested in the two-step puromycin assay (0.05 ml) as described.[10] 57 pmol of uncharged germ $tRNA_m^{Met}$ was added to the incubation with Met-$tRNA_m$ but not to the incubation with Met-$tRNA_i$. In a separate experiment, uncharged $tRNA_m^{Met}$ was added to a charged initiating tRNA without any effect on the reaction with puromycin.



time. The products from the puromycin reaction that could be extracted at pH 8.1 into ethyl acetate were subjected to paper electrophoresis at pH 3.5. The major radioactive product was identified as methionyl-puromycin by its mobility relative to puromycin.[21] There were no detectable amounts of either fMet-puromycin or fMet on the paper. We conclude from these experiments that the first peak of leaf $tRNA^{Met}$ is an initiating tRNA, identical to $tRNA_i^{Met}$ from germ. Leaf Met-$tRNA_i$ is not formylated by the wheat transformylase (Table 1). Since it is cytoplasmic, it could function in protein chain initiation without formylation in the cytoplasm of the wheat leaf cell.

While Met-$tRNA_i$ gave a good reaction with puromycin, the noninitiating Met-$tRNA_m$ did not (Fig. 2). The wheat germ $tRNA_m^{Met}$ was used as the control since the corresponding $tRNA_m^{Met}$ from leaf was contaminated by the

TABLE 1. *Specificity of wheat germ and E. coli transformylases.*

tRNA	Wheat germ enzyme		*E. coli* enzyme	
	[^{14}C]Met-tRNA formed* (pmol)	[^{14}C]fMet-tRNA formed (pmol)	[^{14}C]Met-tRNA formed* (pmol)	[^{14}C]fMet-tRNA formed (pmol)
$tRNA_i^{Met}$ (wheat germ)	66.3	0	64	0
$tRNA_i^{Met}$ (wheat leaf)	14.5	0	50	0
$tRNA_f^{Met}$ (wheat leaf)	19.3	19.7	31.6	20.5
$tRNA_f^{Met}$ (*E. coli*)	112	101	39.6	37.0

Charging and formylation of a particular tRNA, with a given enzyme preparation, were performed at the same time under identical conditions. In the charging assay, [^{14}C]Met was used, in the formylation assay, [^{14}C]formyltetrahydrofolate. Since the amount of tRNA in the two assays was the same, the data may be compared to see what fraction of the $tRNA^{Met}$ present was formylated after it was charged with methionine.

* The methionine acceptor assay was as described in *Methods* except that 11 μM unlabeled formyltetrahydrofalate was added.

chloroplast initiating tRNA. Uncharged $tRNA_m^{Met}$ was added with the [^{14}C]Met-$tRNA_m$ to prevent any transfer of [^{14}C]methionine from the $tRNA_m^{Met}$ to any contaminating initiating $tRNA^{Met}$. Direct transfer, via enzyme-bound methionyl-AMP, could occur in this system and may account for the delayed methionyl-puromycin synthesis in our previous study where uncharged $tRNA_m^{Met}$ was not added. The delayed reaction was not due to release of [^{14}C]methionine, followed by charging of an initiating $tRNA^{Met}$, as it was not reduced by adding [^{12}C]methionine.

When the initiating $tRNA^{Met}$ species were tested for reaction with puromycin, only a partial dependence upon added AUG was observed.[10] When these same tRNAs were tested for binding to ribosomes,[22] they gave considerable binding in the absence of added AUG. On the other hand, the binding of the non-initiating $tRNA_m^{Met}$ was strongly dependent upon added AUG. These results could be explained by endogenous mRNAs on the ribosomes, if we assume that initiator regions are more available for binding than internal codons.

Met-$tRNA_i$ is not formylated by *E. coli* transformylase: The Met-$tRNA_i$ that is present in wheat germ and leaf appears to serve the same function as the so-called $tRNA_f^{Met}$ of yeast,[17,24] guinea pig,[25] and rabbit liver,[26] and the $tRNA_{f*}^{Met}$ of mouse liver.[8,9] These Met-tRNAs were designated in this fashion because they were all formylatable by the *E. coli* transformylase. In contrast, as shown in Table 1, wheat cytoplasmic Met-$tRNA_i$ is not formylated by the *E. coli* transformylase. In the wheat system, it is only the chloroplast Met-$tRNA_f$ which is formylated by the *E. coli* transformylase. These results show that not all initiating Met-tRNAs are formylatable by *E. coli* transformylase.

Discussion. There are four major methionine tRNAs present in wheat leaf. Two of these are localized in the chloroplasts, $tRNA_f^{Met}$ and a $tRNA_m^{Met}$. $tRNA_f^{Met}$ is an initiating tRNA and when charged is formylated by the wheat transformylase. It probably functions in protein chain initiation in the chloroplasts of wheat as fMet-$tRNA_f$. No difference has been detected between the chloroplast and germ $tRNA_f^{Met}$ species. However, there is about 2–3 times as much $tRNA_f^{Met}$ in leaf as in germ due to the presence of mature chloroplasts in the leaf.

The two leaf tRNAs present in largest amount, $tRNA_i^{Met}$ and a $tRNA_m^{Met}$, are cytoplasmic in origin. Met-$tRNA_i$ is not formylated by the transformylase present in wheat. It binds to the initiating site on germ ribosomes and reacts with puromycin to give methionyl-puromycin. So far no difference has been detected between this tRNA and $tRNA_i^{Met}$ isolated from wheat germ. The germ Met-$tRNA_i$ has been shown to transfer its methionine directly into the N-terminal position of peptides coded for by TMV-RNA in a cell-free amino acid incorporating system from wheat embryo.[12] From all these facts, we conclude that Met-$tRNA_i$ functions for protein initiation without prior formylation in the cytoplasm of the wheat cell.

Evidence has been presented for protein chain initiation by unblocked methionine in a variety of other organisms. *Streptococcus faecium*, which normally utilize s *N*-formylmethionine for chain initiation, can use unblocked methionine when folate metabolism is inhibited by trimethoprim (Pine *et al.*[27]). An initiat-

ing $tRNA^{Met}$, which may be cytoplasmic, has been found in yeast (Takeishi *et al.*[24] and RajBhandary and Ghosh[17]), guinea pig liver (Caskey *et al.*[25]), rabbit liver (Bhaduri *et al.*[26]), and mouse liver and ascites tumor (Smith and Marcker[8]). Methionine is used for chain initiation in the cytoplasm of yeast (Sherman *et al.*[28]), and for the initiation of globin synthesis in rabbit reticulocytes (Wilson and Dintzis[11]). Brown and Smith[9] have presented evidence that the initiating $tRNA^{Met}$ of mouse liver functions without formylation.

Though the list of organisms showing initiation by unblocked methionine is short, it contains examples from a wide phylogenetic spectrum. Where formylmethionine is not used, methionine appears to take its place. Methionine, therefore, appears to be the predominant initiating amino acid in nature.

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