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Specific Transformylation of One Methionyl-tRNA from Cotton Seeding Chloroplasts by Endogenous and *Escherichia coli* Transformylases

WILLIAM C. MERRICK AND L. S. DURE III*

Department of Biochemistry, University of Georgia, Athens, Ga. 30601

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ABSTRACT Two isoaccepting chloroplastic and one cytoplasmic $tRNA^{Met}$ species have been separated from germinating cotton cotyledons. The methionylated form of one of the chloroplastic species (but none of the other or of the cytoplasmic $tRNA^{Met}$) can be formylated either by an endogenous transformylase or by *Escherichia coli* transformylase.

Accruing evidence suggests (1–10) that nonorganelle protein synthesis in eukaryotic organisms is initiated by a nonformylated methionyl-tRNA that is in some cases formylatable by an *Escherichia coli* transformylase (1–4, 8, 9), in other cases not (5). Participation of *N*-formylmethionyl-tRNA in the initiation of protein synthesis in chloroplasts was first demonstrated by Schwartz *et al.* (11), and recently the *in vitro* formylation of 35% of unfractionated chloroplast methionyl-tRNA by an endogenous enzyme has been reported (12).

We report here (*a*) the finding of chemically different cytoplasmic and chloroplastic $tRNA^{Met}$ species in germinating cotton seedlings, and (*b*) the finding that one, and only one, of these species is formylatable *in vitro* both by an endogenous transformylase and by *E. coli* transformylase.

Methods for determining cytoplasmic and chloroplastic tRNA species have been published (13). The experimental approach involves the *in vitro* enzymatic aminoacylation, with [^{14}C]methionine, of tRNA prepared from cotton cotyledons at various stages of development and from partially purified chloroplasts, accompanied in some cases by the *in vitro* enzymatic formylation of the methionyl-tRNA by transformylase from cotton or *E. coli*. The aminoacyl-tRNA is then separated from the reaction mixture and digested with ribonuclease T_1 to produce [^{14}C]aminoacyl-oligonucleotides, which are separated by DEAE-cellulose chromatography.

MATERIALS AND METHODS

[U-^{14}C]L-methionine (200–230 Ci/mol) and [methyl-^{14}C]-*N*-formylmethionine (14 Ci/mol) were obtained from New England Nuclear Corp. Ribonuclease T_1 (EC 2.7.7.26) was obtained from Worthington Biochemical Corp. N^{10}-formyltetrahydrofolate was prepared enzymatically by the method of Ljungdahl *et al.* (14).

Preparation of cotton tRNA

tRNA preparations were made by conventional phenol procedures from the cotyledons of dry seeds and of 5-day germinated cotton seedlings (both etiolated and greened), and from cotton chloroplasts that were purified by the nonaqueous method of Stocking (15). (This procedure provides a highly enriched rather than a pure chloroplast preparation, contaminated by adhering cytoplasmic constituents.) For quantitative extraction, 0.5% sodium deoxycholate is required in the homogenization medium. The nucleic acid preparation was enriched for tRNA by batchwise treatment on a DEAE-cellulose column (0.4–1.0 M NaCl fraction), and the amount of tRNA in this fraction was determined by polyacrylamide gel electrophoresis (16). tRNA prepared in this manner showed no loss or gain of amino acid acceptance after heating to 80°C for 5 min, more than 70% combined amino acid acceptance with 14 amino acids, and no loss of amino acid acceptance for one specific amino acid (^{14}C-labeled) when assayed in the presence of the 17 other, nonradioactive, amino acids. The percentage of total tRNA thus prepared that accepts methionine is given for each preparation in Table 1.

Preparation of aminoacyl-tRNA synthetases and transformylase

A partially purified transformylase from *E. coli* B was prepared by the method of Kelmers *et al.* (17). A crude cotton enzyme was prepared from a homogenate of green cotyledons by purification of a 17,000 × *g* supernatant by a batchwise fractionation on a DEAE-cellulose column (0.05–0.40 M NaCl fraction). This fraction was made 50% in glycerol after dialysis.

Preparation of [^{14}C]methionyl- and [^{14}C]formylmethionyl-tRNA

The optimum concentrations of reagents for the formation of methionyl-tRNA proved to be 0.002 M ATP and 4.0 × 10^{-5}

TABLE 1. *Distribution of $tRNA^{Met}$ species*

Source	% of total tRNA	% of $tRNA^{Met}$ in chloroplast		Col 3: Col 4	% $tRNA^{Met}$ in cytoplasm
		$tRNA^{Met}_m$	$tRNA^{Met}_f$		
Dry seed	3.9	9	6	1.50	85
Etiolated	4.6	30	23	1.30	47
Green	4.6	34	24	1.41	42
Chloroplast	4.2	46	35	1.31	19

$tRNA^{Met}_m$, first peak (to left) of Fig. 1*A*, *B*, or *C* (accepts methionine, not formylatable). $tRNA^{Met}_f$, second peak in Fig. 1*A*, *B*, or *C* (accepts methionine, which is formylatable).

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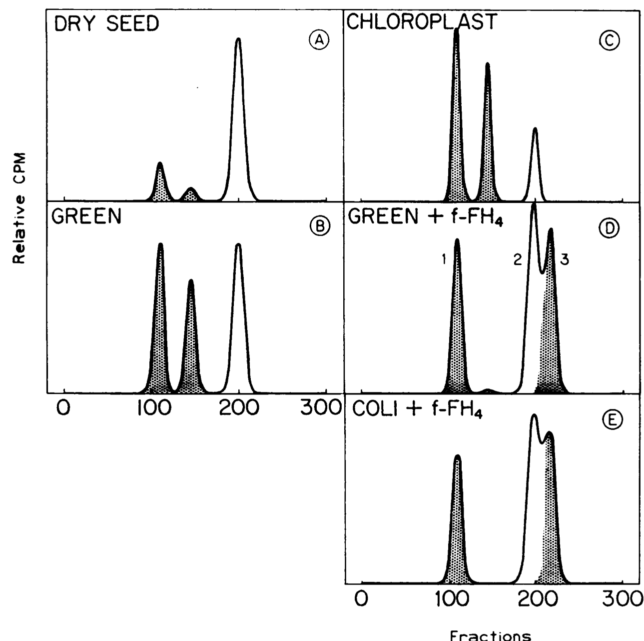

FIG. 1. Radioactivity elution profiles of [^{14}C]aminoacyl-oligonucleotides from DEAE-cellulose columns. Transfer RNA prepared from the cotyledons of dry cotton seeds (*A*), green seedlings (*B*, *D*, and *E*), or from chloroplasts (*C*) was charged with [^{14}C]methionine by a cotton cotyledon enzyme preparation, the charged tRNA was purified and digested with ribonuclease T_1, and the resultant oligonucleotide mixture was eluted from a DEAE-cellulose column with a 0.05–0.4 M NaCl gradient, pH 4.5. In *D*, the charging reaction mixture contained N^{10}-formyltetrahydrofolate (f-FH_4), and in *E* the methionyl-tRNA was incubated with N^{10}-formyltetrahydrofolate and *E. coli* transformylase prior to digestion with the ribonuclease T_1. Shaded areas denote tRNA species deduced to be chloroplastic.

M [^{14}C]L-methionine in the presence of 0.01 M $MgCl_2$, 0.005 M mercaptoethanol, 0.10 M Tris·HCl (pH 7.3), 0.01 M NH_4Cl, and 0.001 M EDTA. These reagents were incubated with tRNA and the cotton enzyme preparation for 60 min at 30°C. The methionyl-tRNA generated in the reaction mixture was purified, after aminoacylation was complete, by batchwise DEAE-cellulose chromatography at pH 4.5 (0.3–1.0 M NaCl fraction taken). This fraction was precipitated with ethanol after the addition of yeast nucleic acid as carrier.

When formylmethionyl-tRNA was generated with the endogenous transformylase, N^{10}-formyltetrahydrofolate was included in the acylation reaction mixture at a concentration of 3.7×10^{-5} M. When it was generated with the *E. coli* transformylase, the alcohol-precipitated methionyl-tRNA was dissolved in 0.1 M Tris buffer (pH 7.0) and incubated with *E. coli* transformylase and 3.7×10^{-5} M N^{10}-formyltetrahydrofolate for 20 min at 30°C. The aminoacylated tRNA was again purified by batchwise DEAE-cellulose chromatography and reprecipitated with alcohol.

Determination of $tRNA^{Met}$ species by chromatography of ribonuclease T_1 digests

The alcohol precipitates of methionyl-tRNA and formylmethionyl-tRNA were dissolved in 0.01 M sodium acetate (pH 5.5)–0.01 M EDTA and ribonuclease T_1 was added (200 units per A_{260} unit of nucleic acid). Digestion was complete in 90 min at 37°C. The digests were applied directly to DEAE-cellulose columns (1.0 × 24 cm) equilibrated with 0.05 M NaCl–0.01 M sodium acetate (pH 4.5) and the [^{14}C]aminoacyl-oligonucleotides were eluted with a linear 250–250 ml gradient from 0.05 to 0.40 M NaCl. The radioactivity elution profile was determined by means of an aqueous scintillation counting solution.

It should be noted that this procedure for determining the number of isoaccepting tRNA species is based on the possibility that each will have a characteristic nucleotide number and (or) base composition from the guanosine residue nearest its 3′-OH terminal adenosine. Since several isoaccepting tRNA species may generate identical aminoacyl-oligonucleotides, the total number of isoaccepting tRNA species determined by this procedure will be a minimum.

Determination of methionine and formylmethionine from aminoacyl-oligonucleotides

Aminoacyl-oligonucleotides produced by the ribonuclease T_1 digestion were adsorbed on activated charcoal and the amino acids were discharged from the charcoal-bound oligonucleotides with 0.1 M NH_4OH and 0.01 M mercaptoethanol for 3 hr at 40°C. The discharged [^{14}C]methionine and [^{14}C]formylmethionine were separated by electrophoresis (pH 3.7, 2000 V, 45 min) and measured with a Packard Radiochromatogram Scanner.

RESULTS

Fig. 1 (*A–C*) shows the radioactive peaks eluted from the DEAE-cellulose column of [^{14}C]methionyl-oligonucleotides produced by the ribonuclease T_1 digestion of [^{14}C]methionyl-

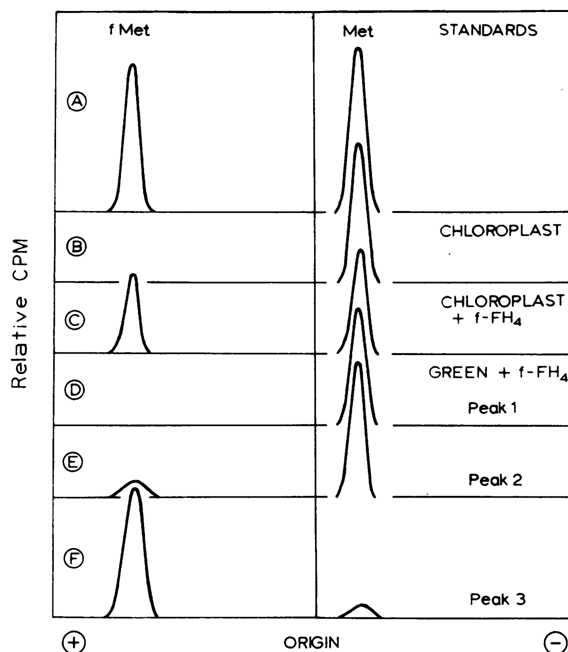

FIG. 2. Radioactivity scans of strip electrophoretograms of [^{14}C]amino acids. *A*, standards. *B* and *C*, [^{14}C]amino acids recovered from chloroplast tRNA that was charged with [^{14}C]-methionine by cotton cotyledon enzyme; in *C*, N^{10}-formyltetrahydrofolate (f-FH_4) was included in the charging reaction mixture. *D–F*, [^{14}C]amino acids recovered from the [^{14}C]aminoacyl-oligonucleotide peaks shown in Fig. 1*D* and numbered accordingly.

tRNA from tRNA of dry seed cotyledons (*A*), 5-day germinated green cotyledons (*B*), and partially purified chloroplasts (*C*). This procedure reveals that at least three species of tRNA^{Met} exist in this tissue, two of them at low concentrations in the dry seed cotyledons (and in embryo cotyledons and in roots, unpublished data). These two species increase relative to the third during germination (*B*, the same was found for 5-day germinated etiolated cotyledons). In the chloroplast tRNA these two species predominate; the third species probably represents cytoplasmic contamination.

This increase in certain isoaccepting tRNA^{Met} species during germination and their enrichment in nonaqueously prepared chloroplasts is found with valine- and isoleucine-isoaccepting tRNA species also (13). On this basis we tentatively conclude that the tRNA^{Met} species that increase in relative concentration are localized in the chloroplast.

To demonstrate transformylase activity in the crude preparation from cotton cotyledon, we aminoacylated chloroplast tRNA with [^{14}C]methionine in the presence of N^{10}-formyltetrahydrofolate. The aminoacyl-tRNA was purified and the esterified [^{14}C]amino acids were discharged and recovered. Fig. 2*C* shows the electrophoretic mobilities of the [^{14}C]amino acids and reveals that about 40% of the methionine bound to tRNA was converted to formylmethionine (compare the 35% of ref. 12). Fig. 2*B* shows that no formylmethionine is formed in the absence of N^{10}-formyltetrahydrofolate.

To determine which of the methionine-accepting tRNA species was formylated by the endogenous transformylase, we utilized tRNA from green cotyledons, since the amounts of the three tRNA^{Met} species in this preparation are roughly equal. This tRNA was acylated with [^{14}C]methionine in the presence of N^{10}-formyltetrahydrofolate and the aminoacyl-tRNA was purified and digested with ribonuclease T_1. The resulting aminoacyl-oligonucleotides were chromatographed and the radioactivity elution profile was determined. Formylmethionyl-oligonucleotides should be eluted from this column at higher salt concentrations than their nonformylated counterpart since the formylation increases their net negative charge. Fig. 1*D* shows that one of the chloroplast aminoacyl-oligonucleotides is now eluted at higher salt concentrations, while the position of the other peaks remains unchanged. This indicates that only one of the methionyl-tRNA species was formylated.

To substantiate this indication, we collected the radioactive aminoacyl-oligonucleotide from each peak and recovered the radioactive amino acids by the charcoal procedure. Their electrophoretic mobilities are shown in Fig. 2 (*D–F*). As can be seen, peak 3 contained [^{14}C]formylmethionine exclusively (when the overlap between peaks 2 and 3 is taken into consideration). Thus it is apparent that one of the chloroplast methionyl-tRNA species is formylated by an endogenous transformylase.

To test the ability of *E. coli* transformylase to formylate cotton methionyl-tRNA, we carried out the formylation reaction on [^{14}C]methionyl-tRNA formed by the cotton synthetase and separated from the acylation reaction mixture by DEAE-cellulose chromatography. The aminoacylated and formylated tRNA was then incubated with ribonuclease T_1 and the [^{14}C]aminoacyl-oligonucleotides were separated on the DEAE-cellulose column (Fig. 1*E*). It is apparent that the *E. coli* transformylase formylated the same chloroplast methionyl-tRNA species as did the cotton transformylase, and did not formylate the cytoplasmic species, since there is no transposition of radioactivity in the elution profile from the cytoplasmic species to regions of higher salt concentration.

DISCUSSION

The demonstration that one of the postulated chloroplastic methionyl-tRNA species can be formylated to produce formylmethionyl-tRNA by an endogenous enzyme strengthens our previous suggestion that the isoaccepting tRNA species that increase during the first 5 days of germination and are concentrated in an enriched chloroplast preparation are chloroplast species. Table 1 summarizes the distribution of these methionine-accepting species in each of the tRNA preparations. Cotton cotyledon tissue does not undergo cell division during this period of development, and the increased concentration of chloroplastic tRNA species constitutes an increase per cell. It is not known if this increase represents an increase in plastids per cell or in tRNA per plastid. Interestingly, the concentration of plastid rRNA also increases in this tissue during this developmental period, and to the same extent (unpublished data).

These data do not demonstrate a chloroplast DNA origin for these chloroplastic tRNA species. However, Williams and Williams have recently reported that about 35% of the total leucyl-tRNA from green bean leaves hybridizes with chloroplast DNA (18).

As has been pointed out (see *Methods*), this experimental approach involving the ion-exchange chromatography of aminoacyl-oligonucleotides produced by ribonuclease T_1 digestion of aminoacyl-tRNAs will not reveal isoaccepting tRNA species that are identical in base composition from the 3′-OH terminus to the first guanosine residue. We attribute our inability to demonstrate two cytoplasmic tRNA species to this limitation of the technique, although our attempts to demonstrate more than one cytoplasmic species by benzoylated DEAE-cellulose chromatography (19) and reversed-phase chromatography (20) of intact methionyl-tRNA molecules have also been unsuccessful. At least two species of cytoplasmic tRNA^{Met} would be expected since other plant tissues (5) and many animal tissues contain at least two nonorganelle species (2, 4). If nonorganelle protein synthesis in plants is initiated by a tRNA^{Met} species that is distinct from the tRNA^{Met} utilized in positioning methionine internally, as has been suggested (5), our data show that the two species in cotton have the same base composition from the 3′-OH terminus to the first guanosine residue.

It is interesting that, in cases where the formylation of nonorganelle methionyl-tRNA has been studied, yeast and animal tissues contain a nonorganelle methionyl-tRNA that can be formylated by *E. coli* transformylase (2), whereas *Neurospora* (22) and higher plants (wheat (5) and cotton) do not. Thus the methionyl-tRNA responsible for the initiation of nonorganelle protein synthesis in a eukaryote may or may not have structural features in common with prokaryotic methionyl-tRNA$_f^{Met}$, at least as judged by its interaction with *E. coli* transformylase.

NOTE ADDED IN PROOF

Leis and Keller have recently reported (23) that in wheat the initiating chloroplastic tRNA$_f^{Met}$ is formylatable by *E. coli* transformylase, whereas the initiating cytoplasmic tRNA$_f^{Met}$ is not.

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