METHIONYL-tRNAs AND INITIATION OF PROTEIN SYNTHESIS IN VICIA FABA (L.)

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Summary: Developing seeds of Vicia faba (L.) contain two major and one minor $tRNA^{Met}$ species. Only one of the major species ($tRNA_1^{Met}$) is charged by E. coli enzyme and neither can be formylated. The minor species ($tRNA_3^{Met}$) is charged and formylated by bean or E. coli enzyme. Results of AUG dependent binding, release of methionyl-puromycin, and N-terminal analysis of the products of endogenous messenger, poly-AUG and poly-UG directed incorporation, all implicate $tRNA_1^{Met}$ in protein chain initiation; $tRNA_3^{Met}$ is possibly the initiator tRNA of cell organelles

Initiation of the synthesis of polypeptide chains involves N-formylmethionyl-tRNA (f-met-$tRNA_F$) in bacterial systems (1,2,3), and in mitochondria and chloroplasts of eukaryotes (4-8). It has been proposed therefore, that this tRNA species is the universal initiator of protein synthesis on 70s ribosomes (4,5). f-met-$tRNA_F$ has not been found in the cytoplasm of eukaryotes, but cytoplasmic tRNA preparations from mammals (9,10,11) and yeast (12) contain two major methionyl-accepting tRNAs. Although an active transformylase has not been detected in the cytoplasm, one of the $tRNA^{Met}$ species ($tRNA_{F*}^{Met}$) is formylated by Escherischia coli transformylase, and the product is indistinguishable from E. coli f-met-$tRNA_F$ and can substitute for it in the E. coli cell-free system (5,12), indicating that initiation on 80s ribosomes involves an unformylated met-$tRNA_F$*.

So far, information is available from only one 80s plant system, that of wheat germ (13,14,15,23). Wheat germ contains

two major and one minor $tRNA^{Met}$ species; the minor and one of the major tRNAs appear to function as initiators as shown by AUG-dependent binding (13), puromycin release (14) and N-terminal analysis of the products of TMV-RNA-directed incorporation (15). Unlike the other eukaryote systems described, wheat germ has an active cytoplasmic transformylase. This enzyme formylates the minor met-tRNA, which is thought to occur in plastids and/or mitochondria, but not the major initiating $tRNA^{Met}$, which is also not formylated by E. coli transformylase.

The present results obtained using an 80s system from V. faba (Dicotyledon), confirm those of wheat (Monocotyledon). Two major and one minor $tRNA^{Met}$ species have been isolated and neither of the two major tRNAs are formylated by homologous or heterologous transformylases. The major tRNAs differ, (a) in their nucleotide sequence near the 3' end; (b) in that only one is charged by the E. coli enzyme; (c) in their capacities to donate methionine into N-terminal and internal positions of newly synthesised polypeptides in the bean cell-free system (16); (d) in their capacity for AUG dependent binding at low Mg^{++} concentration, and (e) in that met-puromycin release experiments indicate that only one of them is bound to the peptidyl site.

Methods:

Ribosomes were prepared from the plumules of 3-day germinated beans as described previously (16), and were washed twice by repelleting from the resuspending medium. The bean enzyme was a high-speed supernatant fraction from developing beans (16) which was chromatographed on a DE52 column to remove

RNA. Deacylated tRNA prepared from developing beans was bulk purified by chromatography on DEAE-cellulose (16). Chromatographic separation of $tRNA^{Met}$ species was carried out on columns of DEAE-sephadex and BD-cellulose (see legends for details of these and other procedures used).

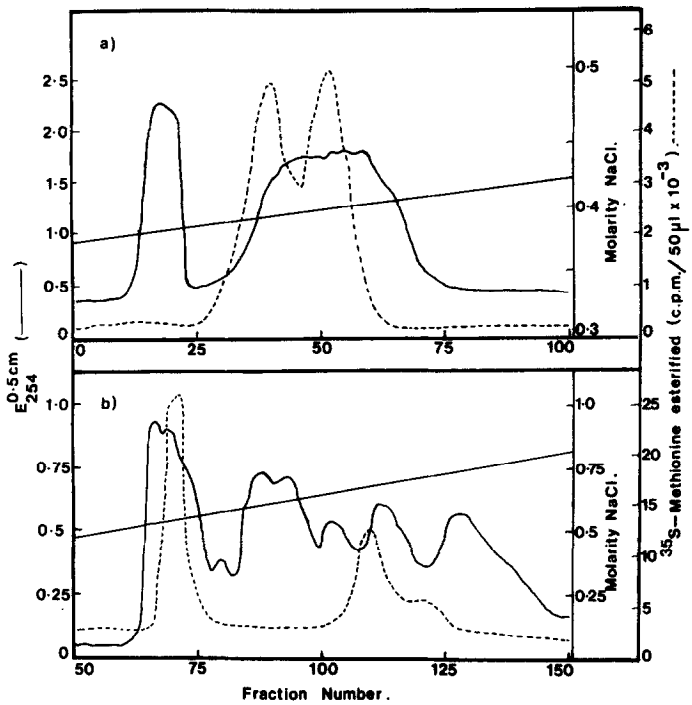

Fig. 1. Chromatography of bean tRNA on (a) DEAE-sephadex and (b) BD-cellulose (Schwartz Bioresearch).

(a) A DEAE-sephadex column (1 x 50 cm) was prepared by the method of Nishimura et al. (18). 18 mg bean tRNA in buffer A (0.375 M NaCl, 0.008 M $MgCl_2$, 0.02 M Tris-HCl pH 7.5) was applied and eluted with a linear gradient of 250 ml buffer A and 250 ml buffer B (0.475 M NaCl, 0.016 M $MgCl_2$, 0.02 M Tris-HCl pH 7.5). The flow rate was 12 ml/hr and 2.4 ml fractions were collected. Methionine acceptor activity was determined on 50 µl aliquots in a system containing 0.1 M Tris-HCl pH 7.6 at 30°, 5 mM ATP, 15 mM $MgCl_2$, 10 mM DTT, 70 mM KCl, 0.02 mM ^{35}S-methionine (sp. act. 150 mC/mM) and bean enzyme in a total volume of 200 µl.

(b) BD-cellulose chromatography (28 mg bean tRNA) was carried out on a 1 x 32 cm column by the method of Leis & Keller (14), except that elution buffers contained 1 mM mercaptoethanol and a 600 ml gradient was used. Fractions were assayed as above, using ^{35}S-methionine (sp. act. 510 mC/mM).

Results and Discussion:

The elution profiles and methionine acceptor capacities of bean tRNA after chromatography on DEAE-sephadex and BD-cellulose are shown in fig. 1. Two major and one minor $tRNA^{Met}$ species were separated on BD-cellulose, and in keeping with the nomenclature used by the wheat germ workers (13,14, 15), these will be referred to as $tRNA_1^{Met}$, $tRNA_2^{Met}$ and $tRNA_3^{Met}$, in order of their emergence from the column. Sephadex chromatography of similar preparations incompletely resolved two peaks (fig. 1). Using the fractions separated by either method, only one of the major fractions ($tRNA_1^{Met}$ and sephadex peak 2) was chargeable with E. coli enzyme (Table 1), and neither were formylatable since only spots corresponding to methionyl adenosine and free methionine were visible on autoradiographs of electrophoretograms of pancreatic ribonuclease digests of the charged tRNAs (not shown). Controls using

Table 1. Fractions were assayed as in Fig. 1, except that where appropriate bean enzyme was replaced by E. coli enzyme and 20 µg of formyl donor (F.D.) was added. Results are expressed as c.p.m. esterified/0.05 ml column fraction. Sp. act. Methionine was 510 mC/mM. The unfractionated E. coli tRNA was obtained from Calbiochem.

	BD cellulose			DEAE sephadex		E. coli tRNA
	$tRNA_1$	$tRNA_2$	$tRNA_3$	Peak 1	Peak 2	c.p.m./ 0.01 mg.
Bean Enz.	15564	7510	2875	19620	19421	4976
Bean Enz. + F.D.	16345	7885	2315	18650	13460	6372
E. coli Enz.	14275	-	3245	2005	16496	8892
E. coli Enz. + F.D.	14921	-	3415	2145	22264	11310

unfractionated E. coli tRNA showed in addition, a spot corresponding to f-met-adenosine after incubations with either bean or E. coli enzyme preparations showing that these contain an active transformylase. A similar f-met-adenosine spot was obtained with $tRNA_3^{Met}$. Fig. 2 is an autoradiograph of an electrophoretogram of ribonuclease T_1 digests of the two major

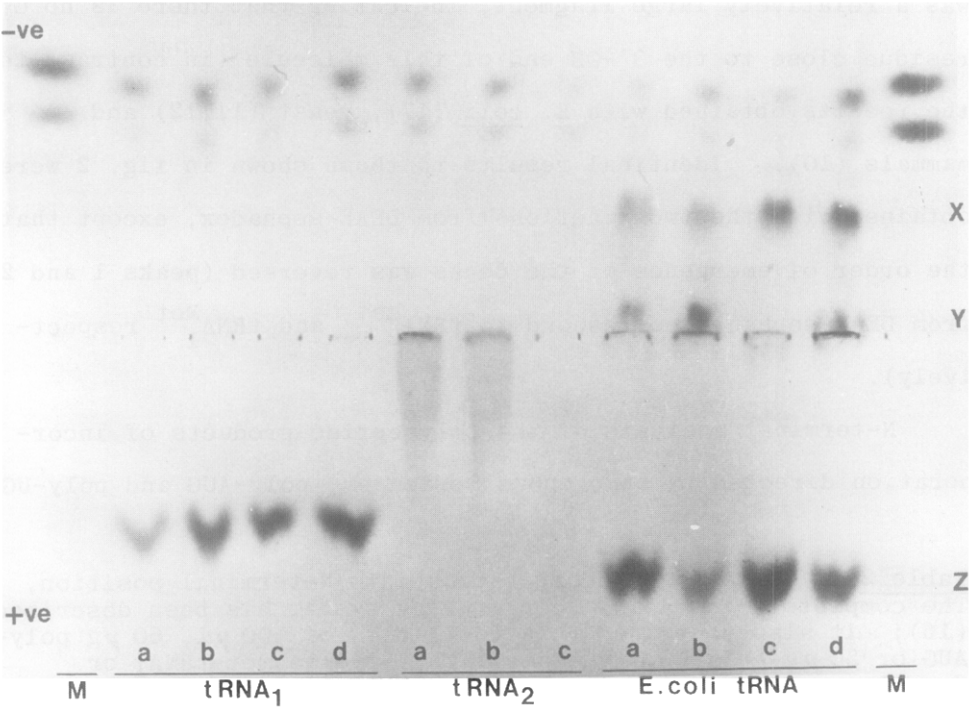

Fig. 2. Autoradiograph of T_1 ribonuclease digests of charged tRNA fractions. Met-$tRNA_1$, met-$tRNA_2$ and infractionated E. coli tRNA were charged using, (a) bean enzyme + formyl donor (F.D.); (b) bean enzyme - F.D.; (c) E. coli enzyme + F.D.; (d) E. coli enzyme - F.D. The charged tRNA was isolated, washed, and subjected to T_1 ribonuclease digestion and electrophoresis as described by Marcker (19,10), followed by autoradiography. $tRNA_2$ did not charge under conditions (c) and (d) and only one sample has been electrophorised.
X = met-$tRNA_M$ fragment; Y = met-$tRNA_F$ fragment;
Z = f-met-$tRNA_F$ fragment (17); M = methionine and methionine sulphoxide.

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bean ^{35}S-methionyl tRNAs, and shows that these differ in nucleotide composition from the corresponding E. coli tRNAs. Thus,

the ^{35}S-met-oligonucleotide from met-tRNA$_1$ (later shown to be the initiator tRNA), runs as a discrete spot. Its mobility was greater than that of the corresponding fragment from E. coli met-tRNA$_F$ being almost the same as that from f-met-tRNA$_F$. The labelled oligonucleotide from met-tRNA$_2$ streaked badly from the origin towards the positive electrode, presumably because it was a relatively large fragment, indicating that there is no G residue close to the 3'-OH end of this molecule (in contrast to the results obtained with E. coli (17), yeast (11,12) and mammals (10). Identical results to those shown in fig. 2 were obtained with the two fractions from DEAE-sephadex, except that the order of emergence of the peaks was reversed (peaks 1 and 2 from DEAE-sephadex correspond to tRNA$_2^{Met}$ and tRNA$_1^{Met}$ respectively).

N-terminal analysis of the polypeptide products of incorporation directed by endogenous messenger, poly-AUG and poly-UG

Table 2. Percentage incorporation into N-terminal position. The complete amino acid incorporating system has been described (16); it also contained in a total vol. of 100 µl, 60 µg poly-AUG or 50 µg poly-UG, together with 7 pmoles met-tRNA$_1$ or 6 pmoles met-tRNA$_2$ from DEAE sephadex (1 pmole = 22500 c.p.m.). Mg^{2+} was 4 mM. After 30 min., 10 µg pancreatic RNase in 100 µl 100 mM EDTA was added and incubated for 2 min. The reaction was terminated by the addition of 5 ml ice cold 5% (w/v) TCA, 0.1 M methionine and the precipitation washed x3 with the same solution. N-terminal analysis was by the method of Blombäck et al. (20).

Messenger	% Methionine in N-terminus	
	met-tRNA$_1$	met-tRNA$_2$
Endogenous	69%	13%
poly-AUG	59%	14%
poly-UG	82%	20%

(Table 2), shows that met-tRNA$_1$ is much more effective than met-tRNA$_2$ at donating methionine into the N-terminal position.

The ability of the met-tRNAs to bind to ribosomes in response to the triplet AUG has been investigated. Table 3 shows that met-tRNA$_1$ and met-tRNA$_3$ are bound at low Mg^{2+} concentrations, whereas 20 mM Mg^{2+} is required for significant binding of met-tRNA$_2$. In an identical set of incubations the reaction of the bound met-tRNAs with puromycin (14,22) has been used to distinguish between binding at the peptidyl (initiating) site and the amino acid site. The results (Table 3) show that only methionine bound as met-tRNA$_1$ or as met-tRNA$_3$ is released as

Table 3. AUG dependent binding of met-tRNA species to bean ribosomes and release with puromycin.

AUG dependent binding was determined by the method of Nirenberg & Leder (21). Reaction mixtures contained in a total volume of 45 µl, 88 mM Tris-HCl pH 7.6 at 25°, 55 mM KCl, 2.8 mM DTT, 0.66 mM GTP, 0.25 mg bean ribosomes, 0.10 units AUG, and either 40 pmoles met-tRNA$_1$, 20 pmoles met-tRNA$_2$ or 7 pmoles met-tRNA$_3$, together with the indicated Mg^{2+} concentration. Incubations were for 15 min. at 25°. The puromycin release assay was carried out on an identical set of incubations by adding 5 µl of 10 mM puromycin (neutralised) at the end of the incubation period and reincubating for 15 min. Methionyl puromycin was extracted with ethyl acetate (22) and determined by scintillation counting. All figures have been corrected for binding or release in the absence of AUG at the relevant Mg^{2+} concentration.

mM Mg^{2+}	2.2	4.4	6.6	11.0	20
met-tRNA$_1$					
pmoles bound	0.05	0.15	0.19	0.17	0.76
pmoles released	0.08	0.14	0.12	0.18	0.21
met-tRNA$_2$					
pmoles bound	-	-	-	0.04	0.23
pmoles released	-	-	-	-	-
met-tRNA$_3$					
pmoles bound	0.06	0.05	0.08	0.10	0.13
pmoles released	0.03	0.04	0.05	0.04	0.05

methionyl puromycin, i.e. was bound at the peptidyl site. In contrast, met-$tRNA_2$ and the additional molecules of the other met-tRNAs bound at high Mg^{2+} concentrations, were not released on treatment with puromycin indicating binding at the amino acid site. It is concluded therefore, that bean $tRNA_1^{Met}$ is probably a cytoplasmic initiating tRNA equivalent to $tRNA_{F*}^{Met}$ of mammalian systems (10) and $tRNA_i^{Met}$ (14) or $tRNA_1^{Met}$ (13) of wheat germ, and that $tRNA_2^{Met}$ donates methionine internally in polypeptide chains. In agreement with the views of Leis and Keller (14), $tRNA_3^{Met}$ is tentatively ascribed the role of initiator tRNA in 70s ribosome containing organelles.

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