SPECIFICITY OF THE INITIATOR METHIONINE tRNA FOR TERMINAL AND INTERNAL RECOGNITION

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Summary: Initiator methionyl-tRNA's from E. coli and wheat embryo can also transfer methionine into internal positions of a polypeptide chain. This transfer depends on the protein synthesizing system used, an E. coli system being about 10-times more active than a wheat embryo system. At low Mg^{2+} concentration this incorporation depends on the presence of both initiation factors and fmet-$tRNA_f^{Met}$, but the rate is slower than that from met-$tRNA_m^{Met}$. Wheat embryo initiator met-$tRNA_1^{Met}$ can initiate protein synthesis in an E. coli system only when it is chemically formylated, but fmet-$tRNA_2^{Met}$ (non-initiator) is inactive in this reaction.

A special species of methionine tRNA is involved in the initiation of protein synthesis in both prokaryotic (1-4) and eukaryotic (5-13) systems. Formylation of the prokaryotic $tRNA_f^{Met}$ is necessary for its initiator function (14,15) but initiation in the eukaryotic system does not require any blocking of the initiator met-tRNA (6-13). In eukaryotic systems initiator $tRNA^{Met}$ ($tRNA_{f*}^{Met}$) species are not formylated (5-8, 11-13) but they can be charged and formylated by E. coli enzymes (5-8). An exception to this is wheat embryo initiator $tRNA_1^{Met}$, which is charged by E. coli synthetase but is not recognized by the E. coli transformylase (11-13). It has been reported that prokaryotic and eukaryotic initiator met-tRNA species are unable to transfer methionine into internal position of a polypeptide chain (3, 6-8, 11-13, 16; 17). A suggested explanation for this is the failure of initiator $tRNA^{Met}$ species to form a ternary complex with the elongation factor (T or TI) and GTP (6, 15, 18-20). However, two eukaryotic initiator tRNA's, yeast $tRNA_{f*}^{Met}$ and wheat embryo $tRNA_1^{Met}$ form ternary complexes with elongation factor T and GTP (21, 22). Furthermore, it was recently

reported that in mammalian systems initiator met-tRNA's can insert methionine internally (23-25).

We previously reported that in an E. coli system either E. coli fmet-$tRNA_f^{Met}$ or yeast fmet-$tRNA_{f*}^{Met}$ can initiate polymethionine synthesis from E. coli met-$tRNA_m^{Met}$ as directed by poly r (A-U-G) (4, 5). We also showed that in a wheat embryo system polymethionine synthesis from wheat embryo met-$tRNA_2^{Met}$ is dependent on the N-terminal incorporation of methionine from met-$tRNA_1^{Met}$ (13). In the present communication we report that the initiator met-tRNA's can transfer methionine into internal positions. The efficiency of this transfer depends on the initiator tRNA species and the ribosomal system used. Also eukaryotic initiator met-tRNA's can initiate in prokaryotic system only when it is formylated, but formylated non-initiator met-tRNA's are inactive.

Materials and Methods:

Wheat embryo $tRNA_1^{Met}$ (1810 pmoles/A_{260} unit) and $tRNA_2^{Met}$ (960 pmoles/A_{260} unit) were prepared as described earlier (13). E. coli $tRNA_f^{Met}$ (1650 pmoles/A_{260}) and $tRNA_m^{Met}$ (1420 pmoles/A_{260}) were prepared from E. coli B tRNA by the procedure of Nishimura (26). Purified $tRNA_f^{Met}$ was free from $tRNA_m^{Met}$ since alkaline hydrolysis of f-[^{35}S]met-$tRNA_f^{Met}$ yielded less than 0.1% free [^{35}S] met. Chemical formylation of methionyl-tRNA's was done with formyl-N-hydroxy-succinimide (15). The procedures for polypeptide synthesis using ribopolynucleotides of repeating sequences were previously described (13, 27). The polypeptide synthesizing system from E. coli contained three times washed ribosomes and purified elongation factors T and G (27). The unfractionated initiation factors d S-100 used were passed through a DEAE-cellulose column in presence of 0.25 M NH_4Cl to remove contaminating nucleic acids (5). Polymethionine synthesis as directed by poly r (A-U-G) in this system was dependent on the presence of added tRNA.

Table I

Polymethionine synthesis from initiator methionyl-tRNA's

in prokaryotic and eukaryotic systems

Ribosomal System Used	Condition	[^{35}S] Met polymerized (pmoles/ml)					
		E. coli Met-tRNA$_f^{Met}$		W. embryo Met-tRNA$_1^{Met}$		W. embryo Met-tRNA$_2^{Met}$	
			% of Input		% of Input		% of Input
E. coli	-poly(A-U-G)	2.4		2.3		3.0	
	+poly(A-U-G)	50.3	60	47.7	21	67.1	40
Reticulocyte	-poly(A-U-G)	1.2		1.6		0.7	
	+poly(A-U-G)	13.8	16	7.8	3.5	72.8	45
Wheat embryo	-poly(A-U-G)	0.6		0.8		1.0	
	+poly(A-U-G)	5.1	6	3.1	1.4	60.8	37

The *E. coli* system contained per ml 18 A_{260} units of washed ribosomes, 0.18 mg of purified T and G proteins and necessary components (27). Wheat embryo system contained per ml 25 A_{260} units of washed ribosomes, 0.65 mg of a partially purified wheat embryo T and G factor free from nucleic acids and other necessary components (13). 6 A_{260} units/ml of previously incubated reticulocyte ribosomes and other necessary components were used in the reticulocyte system (24). Poly r(A-U-G) present was 72 nmoles/ml. The Mg^{2+} concentrations used in *E. coli* and wheat embryo systems were 12 mM and in the previously incubated reticulocyte system was 6 mM. Incubation time was 45 minutes. (1 pmoles = 4500 cpm).

Results:

Table I shows that both met-tRNA$_f^{Met}$ and met-tRNA$_1^{Met}$ can form polymethionine directed by poly r (A-U-G) in cell-free systems from *E. coli*, reticulocytes, and wheat embryo. Edman degradation of the polymethionine synthesized (13) showed that about 90% of the [^{35}S] methionine is incorporated internally. The possibility of labelled methionine from met-tRNA$_f^{Met}$ being transferred to contaminating tRNA$_m^{Met}$

Table II

Effect of initiation factor and formylmethionine on the polymerization of methionine from initiator methionyl-tRNA's

Conditions	[^{35}S] Met polymerized (pmoles/ml)			
	4 mM Mg^{2+}		6 mM Mg^{2+}	
	[^{35}S] Met-tRNA		[^{35}S] Met-tRNA	
	W. embryo Met_1	E. coli Met_f	W. embryo Met_1	E. coli Met_f
-poly(A-U-G)	1.7	1.0	2.1	1.7
+poly(A-U-G)	1.9	1.1	4.4	10.6
+poly(A-U-G) + I.F.	2.4	1.7	13.8	33.0
+poly(A-U-G) + I.F. + fmet-$tRNA_f^{Met}$	3.4	4.5	17.1	34.6

The conditions are the same as described in Table I. The concentrations of unfractionated E. coli initiation factors and f[^{35}S] met-$tRNA_f^{Met}$ were 0.2 mg/ml and 11 pmoles/ml respectively. Time of incubation was 10 min.

which is subsequently incorporated internally was ruled out since presence of an excess of unlabelled methionine (10^{-4}M) did not decrease the incorporation of [^{35}S] met-tRNA's. Also addition of [^{3}H] methionine (2 nmole) in the reaction mixture did not reveal any incorporation of [^{3}H] methionine in the polymethionine synthesized.

The efficiency of polymerization varies with the cell-free system, and the $tRNA_1^{Met}$ used: the E. coli system and $tRNA_f^{Met}$ gives the greatest internal insertion of methionine, while the wheat embryo system and $tRNA_1^{Met}$ is least efficient. Polymerization from the non-initiator Met-$tRNA_2^{Met}$ shows that the three systems tested have similar polymerizing capacity.

We showed earlier that polymethionine synthesis from met-$tRNA_m^{Met}$ in an E. coli system at low Mg^{2+} concentration depends on the presence

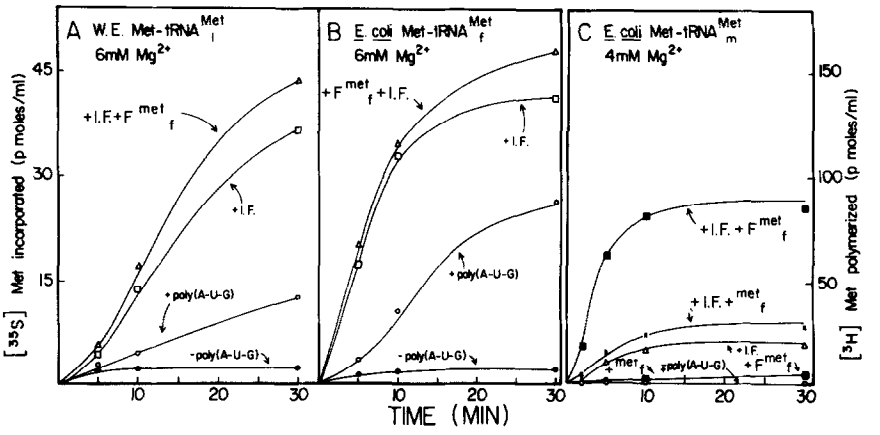

Fig. 1 Kinetics of polymethionine synthesis from met-tRNA$^{Met}_1$, met-tRNA$^{Met}_f$ and met-tRNA$^{Met}_m$. The conditions for 1A and 1B are the same as in Table II. In experiment 1C 290 p moles/ml of [^{3}H] met-tRNA$^{Met}_m$ (1 pmole ≡ 1050 cpm) and 11 pmoles/ml of f[^{35}S] met-tRNA$^{Met}_f$ or 8 pmoles/ml of [^{35}S] met-tRNA$^{Met}_f$ were used. Hot CCl_3COOH insoluble labeled materials were filtered on Millipore filter and counted. The contribution of ^{35}S was about 2%. $Fmet_f$ is fmet-tRNA$^{Met}_f$ and met_f is met-tRNA$^{Met}_f$.

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of fmet-tRNA$^{Met}_f$ and initiation factors (4). Table II shows that presence of both initiation factors and fmet-tRNA$^{Met}_f$ at 4 mM Mg^{2+} stimulates small amount of polymethionine synthesis from tRNA$^{Met}_f$ and tRNA$^{Met}_1$. However, at 6 mM Mg^{2+}, the addition of initiation factors alone is sufficient to give significant incorporation. The kinetics of incorporation of both met-tRNA$^{Met}_1$ and met-tRNA$^{Met}_f$ at 6 mM Mg^{2+} are shown in Fig. 1A and B respectively. Figure 1C shows that at 4 mM Mg^{2+} presence of both fmet-tRNA$^{Met}_f$ and initiation factors are needed for polymethionine synthesis from met-tRNA$^{Met}_m$. Unformylated met-tRNA$^{Met}_f$ could not replace fmet-tRNA$^{Met}_f$. Polymerization from the non-initiator met-tRNA was faster than that from the initiator met-tRNA's. Thus at 4 mM Mg^{2+} polymethionine synthesis from tRNA$^{Met}_m$ was almost complete at 10 min while the same from tRNA$^{Met}_f$ or tRNA$^{Met}_1$ was still continuing.

To find out if tRNA$^{Met}_1$ can act as an initiator in E. coli system we formylated met-tRNA$^{Met}_1$ chemically and tested for poly-

Table III

Stimulation of polymethionine synthesis by formylated wheat embryo methionyl-tRNA's in an E. coli system

Conditions	[^{14}C] Met polymerized (pmoles/ml)	
	15 min	30 min
-poly(A-U-G)	17	22
+poly(A-U-G)	46	54
+poly(A-U-G) + W. embryo fmet-tRNA$_1^{Met}$	113	140
+poly(A-U-G) + W. embryo fmet-tRNA$_2^{Met}$	41	42
+poly(A-U-G) + E. coli fmet-tRNA$_1^{Met}$	250	300

Mg^{2+} concentration was 5 mM. Concentrations of f[^{35}S] met-tRNA$_f^{Met}$, f[^{35}S] met-tRNA$_1^{Met}$ and f[^{35}S] met-tRNA$_2^{Met}$ used were 11, 10, and 8.5 pmoles/ml respectively. The system contained washed E. coli ribosomes, initiation factors, 0.7 mg/ml of an E. coli S-100 fraction depleted of its nucleic acids by DEAE-cellulose chromatography, unfractionated E. coli B tRNA (16 A_{260} units/ml) and [^{14}C] methionine (specific activity 60 µci/µmole) and other necessary components (4, 5).

methionine synthesis in an E. coli system at 5 mM Mg^{2+}. The results in Table III show that f*met-tRNA$_1^{Met}$ stimulates incorporation of methionine in presence of initiation factors. The extent of stimulation is about 50% of that obtained with fmet-tRNA$_f^{Met}$. Chemically prepared f*met-tRNA$_2^{Met}$ could not replace f*met-tRNA$_1^{Met}$ under identical conditions. Like the unformylated met-tRNA$_f^{Met}$, met-tRNA$_1^{Met}$ also failed to stimulate polymerization. Binding experiments also showed that only f*met-tRNA$_1^{Met}$ and not f*met-tRNA$_2^{Met}$ could be specifically bound to salt-washed E. coli ribosomes at 5 mM Mg^{2+} in

the presence of GTP and initiation factors and AUG (data not presented).

Discussion:

Our finding that initiator met-tRNA's can form polymethionine in both prokaryotic and eukaryotic systems shows that like the non-initiator aminoacyl-tRNA's they are also recognized by elongation factors and bind to ribosomal sites accepting aminoacyl-tRNA's. Gupta and co-workers have made similar observations with reticulocyte system (23, 24) and recently in an E. coli system (personal communications). The kinetics of polymerization, however, suggest that the recognition of initiator met-tRNA by polymerizing enzymes and internal codons are less efficient than that for the propagator $tRNA_m^{Met}$. In fact, the presence of met-$tRNA_m^{Met}$ decreases the polymerization of met-$tRNA_f^{Met}$ (unpublished observation).

Our results further suggest that the specificity of recognition of an initiator or internal methionine codon depends on both the ribosomal system and the structure of the $tRNA^{Met}$. The fact that E. coli system can add methionine internally from initiator met-tRNA's more easily than the eukaryotic system may explain its requirement for formylation of initiator met-tRNA's.

Although neither met-$tRNA_1^{Met}$ nor met-$tRNA_2^{Met}$ is recognized by E. coli transformylase, our observation that after chemical formylation met-$tRNA_1^{Met}$ is recognized by E. coli initiation factors while met-$tRNA_2^{Met}$ is not, suggests that there are structural differences between them. These structural features allows initiator tRNA's to be recognized by both initiation factors and elongation factors, but restricts the recognition of non-initiator tRNA's to elongation factors.

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