UTILIZATION OF ISOACCEPTING LEUCYL-tRNA IN THE SOLUBLE INCORPORATION SYSTEM AND PROTEIN SYNTHESIZING SYSTEMS FROM *E.COLI*

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1. Introduction

Aminoacyl tRNA's which play a key role in protein synthesis have been shown to participate in other types of peptide bond synthesis not involving ribosomes, GTP or Mg^{2+} ions. This reaction catalyzed by soluble incorporation system is present in the ribosome free supernatant fluid and is specific for leucine, phenylalanine, and tryptophane in bacteria [1–3] and for arginine in eukaryotic cells [4,5]. In each case a single amino acid is added to the amino terminus of a specific preformed acceptor molecule(s). The existence of five isoaccepting species of tRNA for leucine raises the question whether there is a specificity in the utilization of different leu-tRNA's in the soluble incorporation system. The present study is an attempt to answer this question. Four of the leu-tRNA species were prepared free from cross contamination of each other and were found to transfer leucine to proteins in the system. However, the soluble incorporation systems appeared to have a much higher affinity for leu-$tRNA_1$, and leu-$tRNA_5$ than for leu-$tRNA_3$ and -$tRNA_4$. On the other hand, in MS_2RNA directed protein synthesis, leu-$tRNA_3$ and leu-$tRNA_4$ were better utilized.

2. Materials and methods

^{3}H-Leucine (sp. act. 59.1 ci/mmole) was purchased from New England Nuclear. The soluble system and partially purified aminoacyl-tRNA synthetase were prepared from *E. coli* K_{12}-P678-54 as described previously [2]. Soluble incorporation system isolated from the Streptomycin concentrate fraction (SM fraction) [2] which had most of the leucine transferring activity was used in these studies. Transfer RNA of *E. coli* B (obtained from General Biochemicals) was used to prepare ^{3}H-leu-tRNA in the presence of all other amino acids and leucovorin according to the procedures described [6]. The isoaccepting leu-tRNA species were separated by the reverse phase chromatography (RPC) according to Kelmers and Heatherly whose nomenclature of leu-tRNA was adopted [7]. As shown in fig. 1 the leu-tRNA fractions from each peak were pooled and recovered by ethanol precipitation and slow filtration on millipore filters (HA 0.45μ). The retained labeled tRNA was eluted in a minimum volume of water and stored frozen at −80°C. *E. coli* Q13 ribosomes, initiation factors, elongation factors EFT, EFG, and MS_2RNA were prepared as described [8].

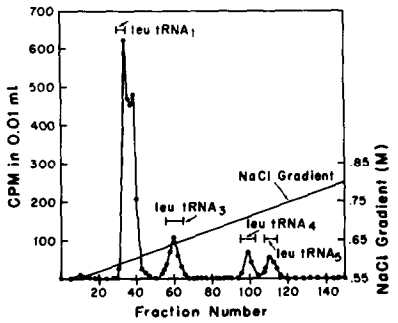

Fig. 1. Separation of isoaccepting leu-tRNA by reverse phase column (RPC) chromatography. A total of 355 μg of ^{3}H leu-tRNA (1735 cpm/μg tRNA) was applied on RPC in two batches and the fractions corresponding to each peak were pooled as described in Materials and methods.

The reaction mixture for transfer of leucine from leu-tRNA into protein contained (in μmoles/0.5 ml): Tris–HCl pH 7.8, 40; KCl, 25; 2-mercaptoethanol, 3; in addition to the SM fraction and leu-tRNA's as indicated in the figure legends. The incorporation of amino acids into protein was measured as radioactivity precipitable with hot TCA by the filter paper disc method [9]. Incorporation of leucine from leu-tRNA into protein by the ribosomal system was carried out at 37°C for 30 min in the reaction mixture containing the following in μmoles/0.1 ml, Tris–HCl pH 7.8, 5; $MgCl_2$ 0,75, NH_4Cl 2.5, KCl 1 5, 2-mercaptoethanol 0.7, and GTP 0.04; in addition ribosomes 320 μg, initiation factors 62.5 μg, EFT 59.5 μg, EFG 8.3 μg, ^{12}C-aminoacyl tRNA (-leu tRNA) 23.5 μg, MS_2RNA 20 μg, and leu-tRNA species as indicated. Protein was determined by the method of Lowry [10].

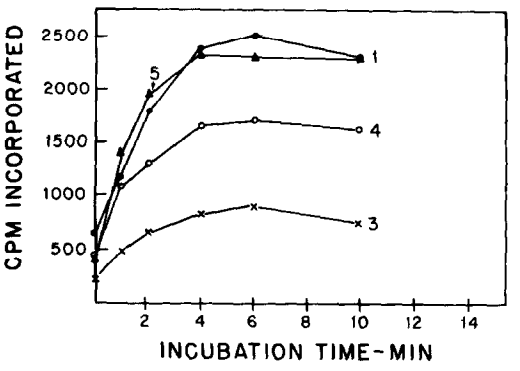

Fig. 2. Time course of incorporation of leucine from leu-$tRNA_1$, -$tRNA_3$, -$tRNA_4$ and -$tRNA_5$. The reaction mixture (134 μl) contained 292 μg SM fraction and leu-$tRNA_1$ (5670 cpm) leu-$tRNA_3$ (4800 cpm), leu-$tRNA_4$ (6620 cpm) or leu-$tRNA_5$ (5870 cpm) in buffered salts as described in Materials and methods. Aliquots of 20 μl were withdrawn for assay.

3. Results

The separation profile of four isoaccepting leu-tRNA's is shown in fig. 1. Rechromatography of the first large peak of leu-tRNA separated two components, leu-$tRNA_1$ and leu-$tRNA_2$. Thus, pooled fraction from this peak leu-$tRNA_1$ indicated in fig. 1 was rechromatographed on RPC before use. The purity of each leu-tRNA species was established by ribosomal binding experiments in the presence of polynucleotides containing codons for leucine (data not shown) [11]. The time course of the transfer of leucine from four leu-tRNA species to protein fraction is shown in fig 2. Leu-$tRNA_2$ could not be prepared free from leu-$tRNA_1$ in sufficient amounts and was not tested. The kinetics of leucine transfer from leu-$tRNA_1$ and leu-$tRNA_5$ were very similar while leu-$tRNA_3$ was less efficiently utilized. The plateau after 6 min was most probably due to deacylation of the leu-tRNA's. Table 1 shows that the incorporation of leucine from leu-$tRNA_1$ and leu-$tRNA_5$ was at least twice that from leu-$tRNA_3$ or leu-$tRNA_4$. These values represent the final level of incorporation with the various leu-tRNA's. The different rates at which each of the species is utilized could be due to the different affinities of the enzyme for the leu-tRNA's. That this is the case is shown in the Lineweaver–Burk plots for the four leu-tRNA's in fig. 3. The apparent K_m value for each of the leu-tRNA's calculated from these plots is given in table 1.

It can be seen that the affinities of the enzymes for leu-$tRNA_1$ and leu-$tRNA_5$ are 7 and 5 times greater than for leu-$tRNA_3$ and the efficiency of utilization of leu-tRNA's expressed as % utilized in 6 min approximately corresponds to affinity of each leu-tRNA for the system. On the other hand phenylalanyl tRNA which is also a substrate had a low affinity for the system but is efficiently utilized in the transfer reaction.

Since all the four leu-tRNA species tested functioned in the transfer reaction, albeit with different efficiencies, it was of interest to study and compare their utilization in in vitro protein synthesis involving ribosomes and messenger RNA. Thus, the cell free *E. coli* ribosomal system programmed with MS_2RNA was used to study the incorporation of leucine from ^{3}H leu-tRNA species into phage related proteins. Incorporation of leucine in this system was dependent upon added messenger RNA, initiation and elongation factors. The percentage of leucine incorporated into protein from each of the four species is shown in table 1. In contrast to the results obtained in the soluble system, leu-$tRNA_3$ was utilized best in the ribosomal system and leu-$tRNA_1$ and leu-$tRNA_5$ to a lesser extent. Since in the ribosomal system the utilization of each species is dependent on the presence of the corresponding codons in the messenger, the results suggest that the codons for leu-$tRNA_1$ and leu-$tRNA_5$ are present less frequently in the MS_2RNA sequence. In the in vitro protein synthesis programmed by MS_2RNA the main product is a

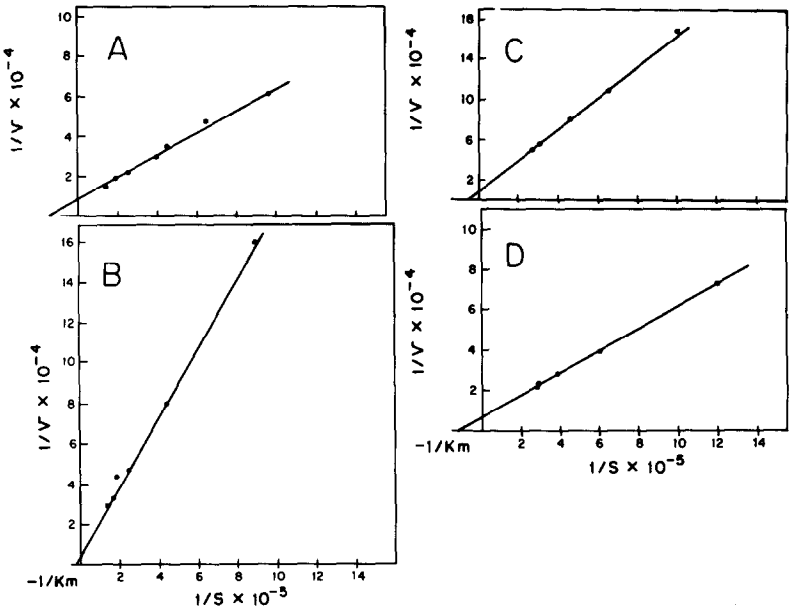

Fig. 3. Double reciprocal plots of initial rates of leucine incorporation versus leu-tRNA concentration. The total reaction mixture (200 μl) containing 109.5 μg of SM fraction and varying amounts of labeled leu-tRNA was incubated at 37°C for 1 min. Initial rates of incorporation at different substrate concentrations were measured as given in text. A) leu-tRNA$_1$, B) leu-tRNA$_3$, C) leu-tRNA$_4$ and D) leu-tRNA$_5$.

phage coat protein [12] which has seven leucine residues none of which are coded by the codons for leu-tRNA$_1$ or leu-tRNA$_5$ [13]. The observed limited incorporation of leu-tRNA$_1$ and leu-tRNA$_5$ may be due to the presence of codons for these two tRNA's in the message coding for the phage RNA synthetase. The ribosomal binding[11] and incorporation experiments [14] with isoaccepting leucyl-tRNA's and synthetic polynucleotides have shown clear codon specificities only for leu-tRNA$_1$ (CUG) and leu-tRNA$_5$ (UUG) while the others in general respond to nucleotides having high U and C content.

Table 1

Apparent K_m values for isoaccepting leu-tRNA's and comparison of their utilization in the soluble system and by *E. coli* ribosomes programmed with MS$_2$ RNA

tRNA Species	K_m* values (pmoles/0.2 ml)	% Utilized	
		Soluble system	Ribosomal system
Unfractionated	24.7	28.2	6.8
leu-tRNA$_1$	21.3	34.0	5.1
leu-tRNA$_3$	159.7	13.3	13.2
leu-tRNA$_4$	63.9	17.7	8.7
leu-tRNA$_5$	31.9	29.7	5.9
phe-tRNA	312.5**	50.4	not determined

*K_m values calculated from data in fig. 3 a,b,c, and d.
**Lineweaver–Burk plot not shown. Experimental conditions as described in text.

4. Discussion

Although no absolute specificity in the utilization of the four species could be demonstrated, the data suggest that leu-tRNA$_1$ and leu-tRNA$_5$ which account for nearly 50% and 10% of the total leucine acceptor activity respectively are better utilized in the soluble incorporation system while the two relatively minor species participate to a greater extent in MS$_2$ RNA directed ribosomal protein synthesis.

It has been shown that in T$_2$ infected *E. coli*, leu-tRNA$_1$ is poorly utilized suggesting that T$_2$ mRNA rarely contains the leucine codon CUG [15]. Wettstein found that leu-tRNA$_1$ is much less frequently bound to ribosomes intracellularly than the minor components and concluded that leu-tRNA$_1$ might have a special function [16]. Incorporation of leucine from leu-tRNA's into hemoglobin however seems to indicate that both minor and major leu-tRNA species are utilized in the synthesis of α- and β–chains [17].

It is tempting to speculate that the presence of isoaccepting aminoacyl-tRNA's is not just a reflection of the degeneracy of the code, but provides a mechanism for the use of the tRNA's in more than one biological function. The use of two specific glycyl-tRNA's in the synthesis of protein and all three species in the synthesis of cell wall peptides is a good example of this case [18]. In studies of incorporation of serine from seryl-tRNA similar findings have been made [19]. In contrast, however the isoaccepting species of threonyl-tRNA's were all utilized to varying degrees in peptidoglycan synthesis as well as in polypeptide synthesis using synthetic nucleotides as messengers [20]. An investigation of their use in ribosomal systems programmed with homologous natural messengers would be of interest to determine whether the species of tRNA used for the synthesis of peptidoglycan is less used for protein synthesis. While in ribosomal systems translational specificity involves codon–anticodon recognition, in aminoacylphosphatidylglycerol synthesis involving lysyl- and alanyl-tRNA's the enzymes show specificity both for the amino acid and the polynucleotide chain [21,22]. It appears possible that the transfer enzyme which is responsible for the amino terminal addition of the acceptor protein can distinguish the nucleotide sequence of various leu-tRNA's in the same manner as in the case of aminoacylphosphatidylglycerol synthesis. On the other hand the preference of leu-tRNA$_1$ for the soluble system may reside in the acceptor molecule. It is therefore possible that with an artificial acceptor molecule such as bovine serum albumin [3] the preference for certain leu-tRNA's may vary.

Acknowledgements

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