mRNA TRANSLOCATION IN PROTEIN BIOSYNTHESIS: ASSOCIATION CONSTANTS RELATED TO THE TRANSLOCATION PROCESS

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1. Introduction

The EF-G and GTP promoted translocation of the mRNA in ribosome-dependent protein synthesis still represents a fascinating mechanistic problem [1]. During this process the mRNA is translocated by three nucleotides, i.e., 10 Å per GTP hydrolyzed. The translocation of the mRNA with respect to the ribosome requires the elongation factor EF-G, aminoacyl- and peptidyl-tRNA, GTP as an energy source and assorted cations, a small selection out of the roughly 130 components needed to make a peptide bond [2].

The movement of the mRNA along the ribosome during translocation can be experimentally demonstrated. It was shown that the mRNA fragment protected against ribonuclease by the attached ribosome extends three nucleotides further towards the 3′-end in the mRNA–ribosome complex that was treated with EF-G and GTP [3,4].

The models so far proposed for mRNA translocation can be divided into two groups. In one view the movement of a codon triplet is governed largely by the movement of the anticodon triplet of the tRNA, whereas the second model stresses the importance of conformational changes of the ribosome or the EF-G factor for mRNA translocation [5–7]. Supporting evidence for the first view includes the observation that a frameshift suppressor tRNA, which recognizes a tetranucleotide codon, moves the mRNA by four rather than three nucleotides [8].

Here we propose an experimental approach for the investigation of the mechanism of mRNA translocation, which originated from the following observation. In the $AUGU_3$-dependent binding of Phe-tRNA to 70 S ribosomes formation of a stoichiometric complex is only observed in the presence of $tRNA_f^{Met}$. This suggested to us, that it is the $tRNA_f^{Met}$ bound to the ribosomal P site which binds the $AUGU_3$ to the ribosome [9,10]. We have therefore studied the binding of the oligonucleotide $AUGU_3$ to the ribosome at equilibrium and its dependence on the presence of the cognate tRNAs, in an attempt to correlate the magnitude of the association constant of the hexanucleotide $AUGU_3$ to the 70 S ribosome with the functional state of the mRNA:

70 S · $AUGU_3$ no cognate tRNA present	'dissociable'
70 S · $AUGU_3$ + $tRNA^{Phe}$ one cognate tRNA present	'mobile'
70 S · $AUGU_3$ + $tRNA_f^{Met}$, $tRNA^{Phe}$ two cognate tRNAs present	'locked'

Here we show that removal of the deacylated tRNA from the peptidyl site of the ribosome allows the translocation of the mRNA and that the affinity of the ribosomal peptidyl site for the peptidyl moiety of the tRNA guides the mRNA · PP-tRNA complex from the aminoacyl to the peptidyl site [11].

Abbreviations: AA-tRNA, aminoacyl-tRNA; PP-tRNA, peptidyl-tRNA; $tRNA_f^{Met}$, initiator tRNA; $tRNA_m^{Met}$, elongator methionine tRNA; N-AcMet-$tRNA_m^{Met}$, methionine tRNA acetylated at the amino-group of Met residue

2. Materials and methods

[^{3}H]$AUGU_3$ (spec. act. 120 Ci/mol) was prepared from ApU by two consecutive elongation steps with polynucleotide phosphorylase [12]. 70 S ribosomes

(tight couples) and 30 S ribosomal subunits were isolated from *Escherichia coli* MRE 600 by zonal centrifugation [13]. These 70 S ribosomes showed no activity in poly(Phe) synthesis when assayed in the presence of poly(U), EF-Tu and an appropriate polymerisation mixture. This should indicate that the ribosomes were free of EF-G. $tRNA_f^{Met}$ (1500 pmol/A_{260} unit) and $tRNA^{Phe}$ (1200 pmol/A_{260} unit) were purchased from Boehringer, Mannheim; $tRNA_m^{Met}$ (1400 pmol/A_{260} unit) was a gift of Dr R. Buckingham, Paris, and $tRNA^{Lys}$ (1100 pmol/A_{260} unit) was prepared in the laboratory of Dr Leberman, Heidelberg. For the acetylation of the AA-tRNA the procedure in [14] was followed. Equilibrium dialysis was performed as in [15]; technical details and the calculation of the data were reported in [16].

The reaction mixture contained 30 pmol 30 S (reactivated [17]) 150 pmol tRNA, 10–300 pmol $AUGU_3$ (spec. act. 120 Ci/mol) and as a buffer system 50 mM Tris–HCl (pH 7.4), 20 mM $MgCl_2$, 200 mM NH_4Cl, 5 mM mercaptoethanol. One chamber was supplemented with only the buffer system; equilibrium was reached at 0°C after 12 h. At the highest $AUGU_3$ concentration, >90% of the ribosome existed as the 30 S · $AUGU_3$ complex. All association constants (K_a) were calculated from a Scatchard plot as documented in fig.1.

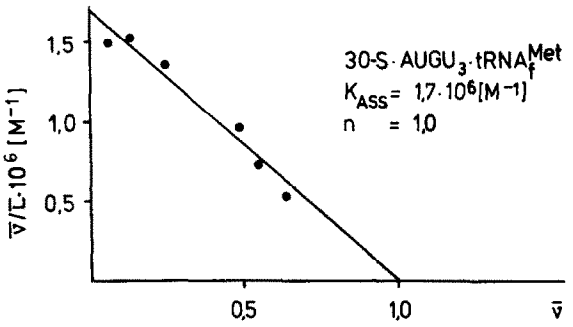

Fig.1. Scatchard plot of the 30 S · $AUGU_3$ complex in the presence of excess $tRNA_f^{Met}$. For the estimation of the best straight line the method of least squares (unweighted) was used. $\bar{v}$ = $AUGU_3$ bound/30 S subunit, $\bar{L}$ = concentration of free $AUGU_3$, n = number of binding sites for $AUGU_3$ on the 30 S subunit.

3. Results

The data in table 1 demonstrate the specific function of the initiation $tRNA_f^{Met}$ for the binding of the hexanucleotide [^{3}H]$AUGU_3$ to the 30 S ribosomal subunit. The presence of $tRNA_f^{Met}$ increases the K_a of the 30 S · $AUGU_3$ complex by a factor of 6.1. The Scatchard plot shown in fig.1 furthermore demonstrates that in the presence of $tRNA_f^{Met}$ the binding of $AUGU_3$ to the ribosomal subunit becomes specific with only one binding site (n = 1.0). Neither phenylalanine tRNA nor methionine $tRNA_m^{Met}$ can stabilize the 30 S · $AUGU_3$ complex to the same extent as $tRNA_f^{Met}$ (table 1). The presence of the second cognate tRNA does not further change the K_a of the 30 S · $AUGU_3$ · $tRNA_f^{Met}$ complex. This should indicate that only one specific tRNA binding site exists on the 30 S ribosomal subunit [18], and that this site has unique affinity for the initiator tRNA.

Table 1
Influence of the initiator $tRNA_f^{Met}$ on the stability of a 30 S · $AUGU_3$ complex as measured by equilibrium dialysis

Complex	tRNA present	K_a (M^{-1})	Number of binding sites
30 S · $AUGU_3$	–	2.8×10^5	1.4
30 S · $AUGU_3$	$tRNA_f^{Met}$	1.7×10^6	1.0
30 S · $AUGU_3$	$tRNA_m^{Met}$	3.9×10^5	1.1
30 S · $AUGU_3$	$tRNA^{Phe}$	4.7×10^5	1.2
30 S · $AUGU_3$	$tRNA_f^{Met}$ + $tRNA^{Phe}$	1.4×10^6	1.0
30 S · $AUGU_3$	$tRNA_m^{Met}$ + $tRNA^{Phe}$	4.6×10^5	1.2

The K_a values were derived from a Scatchard plot as shown in fig.1. The st- error ±5%

Table 2
Evidence that the presence of both cognate tRNAs stabilizes the 70 S · $AUGU_3$ complex

Complex	tRNA present	K_a (M^{-1})	Number of binding sites
70 S · $AUGU_3$	–	6.8×10^5	1.3
70 S · $AUGU_6$	–	8.7×10^5	1.3
70 S · $AUGU_3$	$tRNA_f^{Met}$	4.6×10^7	1.0
70 S · $AUGU_3$	$tRNA_m^{Met}$	3.9×10^6	1.0
70 S · $AUGU_3$	$tRNA^{Phe}$	4.1×10^6	1.2
70 S · $AUGU_3$	$tRNA_f^{Met}$, $tRNA^{Phe}$	2.2×10^8	1.1
70 S · $AUGU_6$	$tRNA_f^{Met}$, $tRNA^{Phe}$	2.1×10^8	1.0
70 S · $AUGU_3$	$tRNA_m^{Met}$, $tRNA^{Phe}$	0.8×10^7	1.0

The reaction conditions were the same as outlined in table 1 with the exception that 20 pmol 70 S were used. The standard error of the K_a values is ±5%

In contrast to its lack of effect on the 30 S · $AUGU_3$ · $tRNA_f^{Met}$ complex, the addition of $tRNA^{Phe}$ to the 70 S · $AUGU_3$ · $tRNA_f^{Met}$ complex increases the K_a by a factor of 4.8 (table 2). This should indicate that in this state the ribosome · mRNA complex is locked, a status which might be required for peptidyl transfer in the in vivo system. The stimulatory effect of an elongator tRNA is not observed solely with complexes which have the initiator $tRNA_f^{Met}$ at the peptidyl site. A similar albeit weaker effect can be observed with complexes having $tRNA_m^{Met}$ at the P site. With only one cognate elongator tRNA present the K_a is decreased by a factor of 53 as can be derived from the K_a of the 70 S · $AUGU_3$ · $tRNA^{Phe}$ complex (table 2). This leads to the hypothesis, that in this state the tRNA · mRNA complex should be mobile with respect to the 70 S ribosome. This would mean in vivo that the EF-G · GTP catalyzed expulsion of the deacylated tRNA will lead to a 'loosening' or increased mobility of the remaining PP-tRNA · mRNA complex with respect to the ribosome, and that this might be sufficient to allow transfer from the aminoacyl to the peptidyl site [19,20].

The next question to ask is what causes the PP-tRNA · mRNA complex to move from aminoacyl site to the peptidyl site? One plausible explanation would be a high affinity of the P site for the peptidyl moiety of the PP-tRNA [11]. To explore this possibility we compared the binding constants for deacylated and peptidyl-tRNA which, with no other tRNA present, should be bound exclusively to the peptidyl site of the ribosome (table 3). We find that

Table 3
The peptidyl moiety of a tRNA increases the affinity of the $AUGU_3$ towards the ribosome

Complex	tRNA present	K_a (M^{-1})	Number of binding sites
70 S · $AUGU_3$	$tRNA_f^{Met}$	4.6×10^7	1.0
70 S · $AUGU_3$	f-Met-$tRNA_f^{Met}$	1.1×10^8	1.1
70 S · $AUGU_3$	$tRNA_m^{Met}$	3.9×10^6	1.0
70 S · $AUGU_3$	N-Ac-Met-$tRNA_m^{Met}$	5.3×10^7	1.1

About 50% of the respective tRNA contained a blocked aminoacyl residue, 10% of which was hydrolyzed during the 12 h dialysis. Thus the true differences between the N-blocked aminoacylated and free tRNA should be even larger than indicated by the experimental numbers. The standard error of the K_a values is ±5%

the presence of a 'peptidyl' group on an elongator tRNA increases the K_a of the 70 S · $AUGU_3$ complex by a factor of >13. This supports the hypothesis that the affinity of the peptidyl moiety of the tRNA for the P site, as shown by the increase in the 70 S · $AUGU_3$ K_a, could favour movement of the PP-tRNA · mRNA complex from the aminoacyl to peptidyl site.

4. Discussion

We have tried to mimic the different states of mRNA attachment to the ribosome during the ribosomal cycle and measure the K_a of the 70 S · $AUGU_3$ complex in each state (fig.2). The replacement of a mRNA by a short oligonucleotide may be justified by the high K_a of the hexanucleotide in the presence of two cognate tRNAs and the only small increase in binding when $AUGU_3$ is replaced by $AUGU_6$. With the 30 S ribosomal subunit interactions which occur upstream, the AUG in the precistronic region are neglected with our type of mRNA model. However, the simplicity of our experimental approach, in which the sliding movement of the PP-tRNA · mRNA complex versus the ribosome is mimicked by the association–dissociation equilibrium of $AUGU_3$ · ribosome, gives to our understanding the justification for the use of the hexanucleotide as a mRNA substitute. Furthermore it should be noted that all experiments were done in the absence of the respective initiation or elongation factors at 20 mM $MgCl_2$.

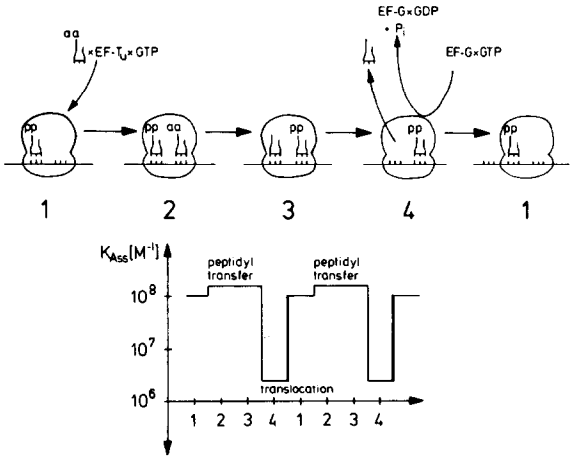

Fig.2. Relation of the different association constants of the $AUGU_3$ · 70 S · ribosome complex to the ribosomal elongation cycle. The following experiments in special support the individual steps as depicted in the above model. Step 1, complex 2, table 3; step 2,3, complex 6, table 2; step 4, complex 4 and 5, table 2.

From our data it is evident that it is the presence of the tRNAs which causes the firm and specific attachment of an oligonucleotide or the mRNA to the ribosome and that the switch from the two tRNA state to the one tRNA state renders the mRNA mobile. It is also clear, that when the PP-tRNA · mRNA becomes mobile the affinity of the P site for the peptidyl moiety may pull the PP-tRNA · mRNA complex towards the P site and thus translocate the mRNA with respect to the ribosome.

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