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Toward an Understanding of the Formylation of Initiator tRNA Methionine in Prokaryotic Protein Synthesis. II. A Two-State Model for the 70S Ribosome†

Hans Uffe Petersen, Antoine Danchin,* and Marianne Grunberg-Manago

ABSTRACT: The 70S ribosomes can select the proper initiator tRNA between Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$. Experiments on binding and on formation of aminoacylpuromycin, as a function of magnesium, potassium, or initiation factors, suggest a two-state equilibrium for 70S particles, involving a minor, active conformation and a major one which is not readily active. The formyl group would act as a specific trigger to select the active conformation. Experimental results are interpreted following this simple model and equilibrium parameters, together with kinetic constants of the peptidyltransferase activity, are presented.

One of the differences between eukaryotes and prokaryotic organisms in the polypeptide initiation step is that in prokaryotes the initiation proceeds through the formylation of methionyl initiator tRNA. In order to understand the reason for this difference, we have studied the role of the formyl group in initiation by acylated initiator tRNA in an in vitro *Escherichia coli* system.

As reported in the preceding article of this series (Petersen et al., 1976), formylated and unformylated initiator tRNA bind equally well to 30S ribosomal subunits in the presence of a messenger, in particular poly(A,U,G). Furthermore, both species are recognized by initiation factors.

The situation is quite different with 70S ribosomes; only the attachment of the formylated species is stimulated by the initiation factors, which strongly inhibit the attachment of the unformylated Met-tRNA$_f^{Met}$. In the absence of initiation factors, both species are bound to the 70S ribosomes. However, only the formylated tRNA reacts completely with puromycin, as will be shown in this paper. When the unformylated tRNA is first bound to the 30S particles, and the 50S subunits are subsequently added, the inhibitory effect of initiation factors is lost and, under these conditions, most of the Met-tRNA bound reacts with puromycin.

It is generally accepted that protein synthesis starts by initiator tRNA and the initiator codon being positioned on the small ribosomal subunit. However, the finding that formylation is more important when the ribosomes are in the form of 70S couples led us to believe that fMet-tRNA might be bound directly to the 70S couples at a puromycin reactive site. Since this reaction does not readily occur with the unformylated species, we were prompted to investigate the behavior of the 70S ribosomal couples toward initiator tRNA.

In the present article we report that 70S ribosomes are able to distinguish between the formylated and unformylated initiator tRNA, even in the absence of initiation factors and an energy source. We propose a two-state model for the 70S ribosomal conformation during initiation, which leads to quantitative parameters suggesting a function in vivo for the formylation of initiator tRNA.

Materials and Methods

The materials used are the same as those described in the preceding article (Petersen et al., 1976), with the addition of elongation factor T (T_u and T_s), which was a gift from Dr. Pongs from the Max-Planck Institute for Molecular Genetics of Berlin, and of tetracyclin (hydrochloride) which was a product of Roussel-Uclaf. The degree of association of ribosomal subunits, as a function of magnesium and potassium concentrations, was measured by the light scattering technique, as described by Godefroy-Colburn et al. (1975). All the other methods used were described in the preceding article (Petersen et al., 1976).

Results

Binding of Formylated and Unformylated Initiator tRNA to 70S Ribosomes. In the absence of initiation fac-

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Table I: Effect of [K^+] on the Binding of fMet- and Met-$tRNA_f^{Met}$ to 70S Ribosomes.[b]

	Binding at 5 mM Mg^{2+} (pmol)		Binding at 20 mM Mg^{2+} (pmol)		Binding at 35 mM Mg^{2+} (pmol)	
[K^+](mM)	fMet	Met	fMet	Met	fMet	Met
0	0.22	1.29	1.16	1.33	1.29	1.11
100			0.99 (15)[a]	1.20 (10)		
200			0.81 (30)	1.23 (8)		
400	0.06 (73)	0.18 (86)	0.69 (41)	1.21 (9)	1.06 (18)	1.02 (8)

[a] The figures in parentheses indicate the percentage of inhibition. [b] Incubation for 20 min at 37 °C of the following mixture (50 μl): 15 pmol of 70S ribosomes; 0.11 A_{260} unit of poly(A,U,G); 1 mM GTP; 50 mM Tris-HCl (pH 7.4); 50 mM ammonium chloride; magnesium acetate and potassium chloride as indicated; and 3.0 pmol of fMet-$tRNA_f^{Met}$ (4100 cpm/pmol) or 3.8 pmol of Met-$tRNA_f^{Met}$ (4600 cpm/pmol). The amount of tRNA bound was analyzed by the Millipore filter assay.

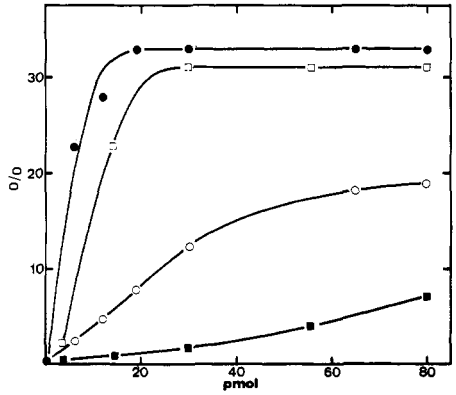

FIGURE 1: Binding of formylated and unformylated initiator tRNA to 70S ribosomes as a function of ribosome concentration. The incubation mixture contained 50 mM Tris-HCl buffer, 50 mM ammonium chloride, and 0.11 A_{260} unit of poly(A,U,G), and in addition: in the case of formylated species, 3.8 pmol of fMet-$tRNA_f^{Met}$ (4600 cpm/pmol), 1 mM GTP, and 6 mM magnesium acetate (○), and when indicated, 5 μg of crude initiation factors (IF) (●); in the case of the unformylated species, 4.0 pmol of Met-$tRNA_f^{Met}$ (4100 cpm/pmol), 5 mM magnesium acetate (□), and 50 μg of crude initiation factors (IF) (■). The ordinate shows the percentage of added fMet-tRNA which binds to the ribosomes.

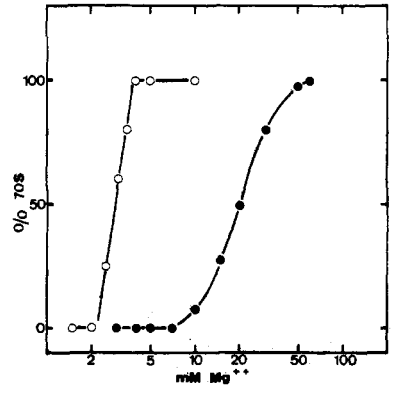

FIGURE 2: Light-scattering measurement of the equilibrium between 70S ribosomes and 30S + 50S subunits. Measurements as a function of magnesium concentration, as indicated, at 24 °C as explained under Materials and Methods, in the absence of potassium (○) and at 400 mM potassium (●) (semilogarithmic plot).

tors, both the formylated and unformylated Met-$tRNA_f^{Met}$ bind to ribosomes. In the presence of messenger [poly(A,U,G) (Petersen et al., 1976) or R17 phage RNA (data not shown)] there is considerably more binding of the unformylated tRNA; this association, however, is inhibited by the presence of initiation factors, whereas binding of the formylated tRNA is stimulated. Figure 1 shows the binding of both initiator tRNAs as a function of ribosome concentration; the amount of tRNA bound (30–32%) is the same under optimal conditions for each (i.e., in the presence of initiation factors for the formylated species and in the absence of initiation factors for Met-$tRNA_f^{Met}$).

As will be explained further in more detail, we suppose that the 70S ribosome can exist in two different conformations, both of which bind Met-$tRNA_f^{Met}$, but only one of which is active in binding the formylated species. In order to calculate the equilibrium between these two conformational forms, the experiments presented here were done at a ribosomal concentration corresponding to the initial part of the curve of Figure 1, where the attachment is linear with respect to ribosome concentration. When the equilibrium constants were calculated at high ribosome concentrations, we used a semireciprocal plot of Figure 1, i.e., the binding of aminoacyl-tRNA against $(rib)^{-1}$, where the limiting percentage of aminoacyl-tRNA bound as ribosome concentration approaches infinity is the same in the absence or presence of initiation factors.

Since increasing the ribosomal concentration beyond a certain point does not increase the amount of tRNA bound, we are led to conclude that part of the tRNA is not in a proper conformation to react at its binding site, as a result of chemical modification or tertiary structural change. We have therefore calculated the binding constants supposing that the plateau value represents total binding of the active fraction.

Effect of Magnesium and Potassium on the Binding and Puromycin Reactivity of Both Initiator tRNAs. In the absence of initiation factors, as has been found previously (Petersen et al., 1976), magnesium concentration exerts a pronounced stimulation on the binding of formylated tRNA, whereas no effect is observed on the unformylated species. As shown in Table I, potassium, at a high concentration (400 mM), inhibits the binding of both species when the Mg^{2+} concentration is low; when the Mg^{2+} concentration is higher than 20 mM, potassium has no effect. We may relate this inhibition to the ribosomal dissociation produced by potassium. Figure 2 shows the percentage of associated 70S couples as a function of Mg^{2+}, in the presence and absence of potassium, measured by light scattering. It can first be seen that the Mg dependence indicates that the ribosomes used belong to the type we called "preparation A" (Debey et al., 1975), also known as "tight couples", where the range of Mg^{2+} concentration between 0 and 100% association is narrow and where the ribosomes are fully associated at 4 mM Mg^{2+}. However, addition to the

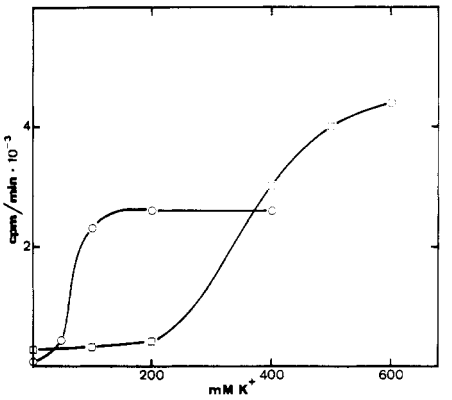

FIGURE 3: Initial rate of puromycin reaction with formylated and unformylated initiator tRNA at varying potassium concentrations. The reaction mixture (50 μl) contained: 15 pmol of 70S ribosomes; 0.11 A_{260} unit of poly(A,U,G): 50 mM Tris-HCl (pH 7.4); and 35 mM magnesium acetate; plus 1.0 pmol of fMet-tRNA$_f^{Met}$ (4600 cpm/pmol) and 1 mM GTP (○); or 4.0 pmol of Met-tRNA$_f^{Met}$ (4100 cpm/pmol) (□); potassium concentration as indicated. After 20 min at 37 °C, 25 μg of puromycin was added and the reaction continued for 0.25, 0.5, 1, 2, and 5 min. The reacted amount was plotted against the incubation time and the initial rates were calculated from the linear part of the curves obtained at different potassium concentrations.

Table II: Effect of Messenger (Poly(A,U,G)) on Binding and Puromycin Reaction of fMet-tRNA$_f^{Met}$ and Met-tRNA$_f^{Met}$.[a]

	pmol	
	−Poly (A,U,G)	+Poly (A,U,G)
fMet-tRNA$_f^{Met}$ binding	0.30	1.05
fMet-puromycin reaction	2.01	1.68
Met-tRNA$_f^{Met}$ binding	0.11	0.95
Met-puromycin reaction	1.75	1.41

[a] Same incubation mixture as in Table I in the absence of initiation factors except that GTP was omitted and that the amounts of magnesium acetate and of potassium chloride were 35 and 400 mM, respectively. Binding was measured after 20 min at 37 °C. For the puromycin reaction, 25 μg of puromycin was added after 20 min at 37 °C and the reaction was continued for 5 min. The reacted amounts were analyzed by the ethyl acetate extraction technique.

70S particles of potassium at a concentration of 400 mM has a strong dissociating effect; at 20 mM Mg^{2+} the ribosomes are associated 50% of the time at 25 °C and 25% at 37 °C (Debey et al., 1975). It is only at 35 mM Mg^{2+} that, in the presence of potassium, ribosomes are predominantly in the form of 70S couples.

It is known that a monovalent cation (ammonium or potassium) is required by both ribosomal subunits. The 30S particle needs the ion for optimal binding of aminoacyl-tRNA (Zamir et al., 1971) and the peptidyltransferase activity of the 50S particle is strongly stimulated by potassium or ammonium (Maden and Monro, 1968).

The puromycin reactivity seen when Met-tRNA$_f^{Met}$ is first bound to 30S particles and the 50S subunit is subsequently added (Petersen et al., 1976) is not found when preassociated 70S ribosomes are used at low potassium and magnesium concentrations. However, when the salt concentrations are increased, the unformylated initiator tRNA binds to a puromycin-sensitive site directly on the 70S ribosome (Figure 3 and Table II). It is remarkable that the potassium concentration required is higher for the unformylated species (Figure 3); for maximal reaction velocity, the optimum potassium concentration is 400–600 mM for the unformylated species, while it is only 100 mM for the formylated species (Figure 3). These values reflect the overall effect of K^+, namely on the 70S particles and on the peptidyltransferase. Potassium can be replaced by NH_4^+ at the same concentrations (data not shown). The noninitiator tRNA, Met-tRNA$_m$, does not react with puromycin, whatever the potassium or ammonium concentration (Figure 4).

Influence of mRNA on the Binding and Puromycin Reaction. While the presence of messenger, such as poly(A,U,G), is absolutely required in our assay conditions (slow exchange) for the binding of initiator tRNA, it is not necessary for the puromycin reaction (Table II). Both formylated and unformylated tRNAs react with puromycin in the absence of messenger even better than in its presence, and the dependence upon potassium of the peptidylpuromycin formation remains the same (data not shown).

This leads to the conclusion that it is not the binding of the initiation codon to a preexisting site on the ribosome which directs the binding of initiator tRNA, but that initiator tRNA has a structure which enables it to find its proper site on the ribosome.

Effect of Tetracyclin. It is well established that the antibiotic, tetracyclin, at low concentrations, especially inhibits binding to the A site on the ribosome (Pestka, 1971). Binding to the P site may be stimulated by the antibiotic (Bodley and Zieve, 1969).

We find that at a concentration of 0.1 mM tetracyclin abolishes EF-T_u stimulated binding of Met-tRNA$_m^{Met}$ (Table III). However, the binding of initiator tRNA (formylated or not) is not inhibited by tetracyclin at that concentration, but is somewhat stimulated, whether or not initiation factors are present (Table III). Our results, therefore, indicate that the unformylated initiator tRNA is bound at a site different from the A site.

Theoretical Interpretation: A Two-State Model for the 70S Ribosome Conformation during Initiation

The above results indicate that, although barely distinguishable in the binding test on 30S ribosomes, Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ behave quite differently on the 70S particles both in the presence of initiation factors which inhibit the binding of the unformylated species and in the absence of initiation factors. The binding of the unformylated species is much higher and does not depend on the magnesium or potassium concentration, in contrast to that of the formylated species; under the usual conditions of the assay (100 mM [NH_4 + K]Cl) Met-tRNA$_f^{Met}$ hardly reacts with puromycin, in contrast to what happens with fMet-tRNA$_f^{Met}$. However, the unformylated species can be triggered to react with puromycin in absolute contrast to Met-tRNA$_m^{Met}$. We thus observe that 70S ribosomes are able to distinguish not only between initiator tRNA$_f^{Met}$ and noninitiator tRNA$_m^{Met}$ but also between formylated and unformylated acylated initiator tRNAs, and this even in the absence of initiation factors. Since no energy is required in this recognition step (although the presence of IF-2 and GTP enhances the ability to discriminate between Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$) one may presume the existence of at least either two sites or two ribosomal conformations for the binding of initiator tRNA.

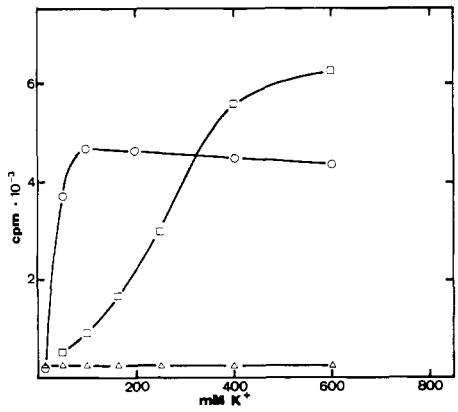

FIGURE 4: Effect of potassium concentration on the puromycin reaction with pre-bound initiator tRNA. The incubation mixture contained: 16 pmol of 70S ribosomes; 50 mM Tris-HCl (pH 7.4); 1 mM GTP; 35 mM magnesium acetate; and 4.2 pmol of fMet-tRNA$_f^{Met}$ (○) (4100 cpm/pmol), 3.8 pmol of Met-tRNA$_f^{Met}$ (□) (4600 cpm/pmol), or 4.6 pmol of Met-tRNA$_m^{Met}$ (△) (4100 cpm/pmol). Incubation and analysis of reaction products are as described in Table II.

The experiments with tetracyclin support the idea that, although bound in a different manner than formylated tRNA, Met-tRNA$_f^{Met}$ is not bound preferentially at the A site under our conditions; moreover, it is easy, using magnesium and potassium to select a ribosome-Met-tRNA$_f^{Met}$ readily reactive toward puromycin, whereas this is always impossible with Met-tRNA$_m^{Met}$. We shall therefore introduce in our model the concept of a dynamic equilibrium between two forms of 70S ribosomes, equilibrium which does not imply a conformational change of site A into site P. The model we present follows this hypothesis and we shall compare it to the experimental data before discussing its biological significance.

In the simplest two-state model, one would have the following equilibria:

$$70S \overset{L}{\rightleftharpoons} (70S)^* \qquad (1)$$

$$70S + T_i \overset{K_{1i}}{\rightleftharpoons} T_i70S \qquad (2a)$$

$$(70S)^* + T_i \overset{K_{2i}}{\rightleftharpoons} T_i(70S)^* \qquad (2b)$$

where T_i is the aminoacylated initiator tRNA where i = M for Met-tRNA$_f^{Met}$ and i = F for fMet-tRNA$_f^{Met}$.

The peptidyltransferase reaction is supposed to occur only in one of the ribosome conformations, namely (70S)*:

$$T_i(70S)^* + p \xrightarrow{k_{+i}} (70S)^* + aa_ip + t \qquad (3)$$

where p stands for the puromycin, aa_ip is the aminoacylpuromycin adduct, and t is the uncharged tRNA.

For the sake of conveniency, let us write [70S] = R and [(70S)*] = R^*; one can then derive from eq 1, 2a, 2b, and 3 that:

$$R^* = LR$$

$$T_iR/T_i{\cdot}R = K_{1i}$$

$$T_iR^*/T_i{\cdot}R^* = K_{2i}$$

$$v_i = d[aa_ip]/dt = k_{+i}pT_iR^*$$

Table III: Effect of Tetracyclin on the Binding of fMet-tRNA$_f^{Met}$, Met-tRNA$_f^{Met}$, and Met-tRNA$_m^{Met}$.[a]

	Addition	Tetracyclin	tRNA Bound (pmol)	Inhibition by Tetracyclin (%)
fMet-tRNA$_f^{Met}$	None	−	0.55	−11 (stimulation)
		+	0.61	
	Crude IF	−	1.44	−2 (stimulation)
		+	1.47	
Met-tRNA$_f^{Met}$	None	−	1.96	−12 (stimulation)
		+	2.20	
	Crude IF	−	0.39	−33(stimulation)
		+	0.52	
Met-tRNA$_m^{Met}$	None	−	0.42	0
		+	0.42	
	EFT	−	0.84	100
		+	0.40	

[a] Incubation mixtures are as in Table I, except for the amount of magnesium acetate which was 5.5 mM and, where indicated: 50 μg of crude initiation factors (IF); 50 μg of elongation factor T (EFT); 0.1 mM tetracyclin; 2.3 pmol of fMet-tRNA$_f^{Met}$; 5.2 pmol of Met-tRNA$_f^{Met}$; or 4.6 pmol of Met-tRNA$_m^{Met}$ (all at 4100 cpm/pmol).

The amount B_i, bound to ribosomes (see Appendix), $B_i = T_iR + T_iR^*$, can be written:

$$B_i = (1/2)(T_{0i} + \bar{K}_i^{-1} + R_0 - \sqrt{\Delta}) \qquad (A)$$

where

$$\Delta = (\bar{K}_i^{-1} + R_0 - T_{0i})^2 + 4\bar{K}_i^{-1}T_{0i}$$

$$\bar{K}_i = \frac{K_{1i} + LK_{2i}}{1 + L}$$

R_0 is the total ribosome concentration and T_0 is the total charged tRNA concentration.

The initial velocity of the puromycin reaction is (see Appendix):

$$v_i = \frac{k_{+i}pK_{2i}L}{K_{1i} + K_{2i}L}B_i \qquad (B)$$

K_i is readily obtained from the experimental results (see Appendix):

$$\bar{K}_i = \frac{B_i}{(R_0 - B_i)(T_{0i} - B_i)}$$

and Figure 5 summarizes the values obtained from our experiments. In these experiments the values are only accurate when the amounts T_{0i} or R_0 are different enough from B_i; however, one increases the accuracy in using plots of B_i as a function of $1/R_0$ or $1/T_{0i}$ for variable ribosome and tRNA concentrations (see Appendix). This has been done in several instances to check the accuracy of our $\bar{K}_i$ determinations. In addition this provides a means to estimate the exact T_{0i} value for each batch of charged tRNA. This appears to be important since there always remains a fraction of tRNA which is absolutely not reactive toward ribosomes (25–65%). However, the final result is only a semiquantitative evaluation which, nevertheless, gives a self-consistent picture of the behavior of 70S ribosomes, and this is enough for the purpose of this article.

Although the proposed theoretical model involves the minimum number of hypotheses, there remain uncertainties

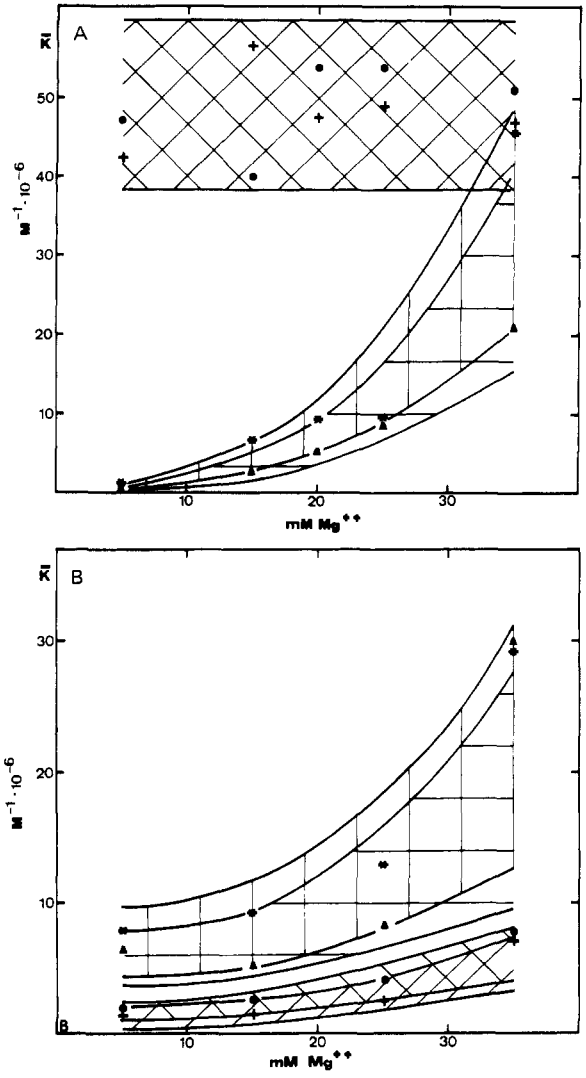

FIGURE 5: Average binding constant for the two ribosomal conformations, $\bar{K}_i$ (see text), as a function of magnesium and potassium, in the absence (A) and the presence (B) of initiation factors. The data are always very unprecise due to the difficulty in evaluating the actual proportions of native ribosomes and tRNA. We have therefore plotted a number of points which are averages of several experiments; experimental points generally fall under the crossed areas. It is only the general trend of the curves which is important for our purpose: (A) is in the absence of initiation factors and (B) under conditions of saturated initiation factors effect: (* and |||) $\bar{K}_F$ 50 mM K^+; (▲ and ≡) $\bar{K}_F$ 450 mM K^+; (● and \\\) $\bar{K}_M$ 50 mM K^+; (+ and ///) $\bar{K}_M$ 450 mM K^+.

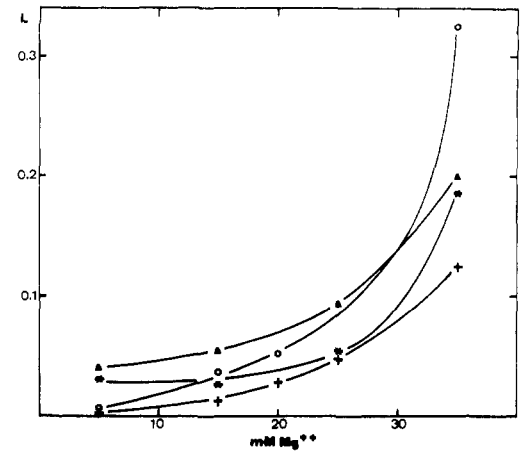

FIGURE 6: Equilibrium constant for the binding of initiator tRNA to ribosomes. The conformational equilibrium constant L (see text) of 70S ribosomes is derived from the points in Figure 5 without initiation factors at 50 mM potassium (○), or 450 mM potassium (+). In the presence of initiation factors (IF) and 50 mM potassium (△) or 450 mM potassium (*).

if one lets all the thermodynamic constants be specified as functions of magnesium, potassium, or initiation factor concentration. We must, therefore, introduce a few constraints in order to obtain a qualitative evaluation of the evolution of the dynamic equilibrium of 70S ribosomes as a function of magnesium, potassium, or initiation factors.

We note that in the absence of initiation factors, K_M does not change with potassium or magnesium; we therefore make the assumption (theoretically justified in the Appendix) that $K_{1M} = K_{2M}$, and we then assume that one of the functions of initiation factors is to prevent the binding of initiator tRNAs on the ribosome conformation which is not ready for initiation. This means that $K_{1M}(+IF)$ is set to be 0. On the other hand, one observes that, in the absence of initiation factors, fMet-tRNA$_f^{Met}$ binding decreases to a very low value as the magnesium concentration is decreased; since this means that $K_{1F}(-IF)$ is very small, we set this constant to zero. Finally, we assume that K_{2M} and K_{2F} do not change, either in the presence of magnesium, potassium, and initiation factors, or in their absence.

It is thus easy to roughly evaluate the five different parameters involved in our hypothesis. One first computes $K_{1M}(-IF) = K_{2M}(-IF) \simeq 50\ \mu M^{-1}$; taking this value in the experiments performed in the presence of initiation factors, one is able to obtain $L(+IF)$; this value is the same for both formylated and unformylated initiator tRNA binding and can therefore be used to derive $K_{2F}(+IF) \simeq 200\ \mu M^{-1}$, and since we assume that $K_{2F}(+IF) = K_{2F}(-IF)$ the $L(-IF)$ value can be obtained. The results are summarized in Figure 6.

For the puromycin reaction these L values can be used to compute k_{+M} and k_{+F}, and one finds that they are probably identical. This would mean that peptidyltransferase does not distinguish between formylated and nonformylated methionine residues. In addition these kinetic constants appear to vary in a linear fashion with respect to the potassium concentration, i.e., $k_+ = 170[K^+]\ M^{-1}\ s^{-1}$, suggesting that the cation plays a direct role in catalysis of peptidyl transfer with an apparent binding constant of 10–20 M^{-1}.

At low magnesium and in the absence of initiation factors, L appears to be small (on the order of 0.003) and increases when magnesium is added to the assay medium; potassium antagonizes this positive effect. Thus, the 70S particles appear to be mostly in a conformation which is not able to readily sustain peptidyl transfer. The action of initiation factors is certainly complex and only roughly depicted in our model, but we can nevertheless observe that L is still small (about 0.04), albeit stimulated of one order of magnitude, as compared to the case where no initiation factors are present. At higher magnesium concentration, the general trend of L evolution is similar to that in the absence of factors, and at concentrations higher than 15 mM, the factors barely have any effect.

Thus, it appears that, in the absence of factors Met-tRNA$_f^{Met}$ binds equally well to both 70S conformations, whereas fMet-tRNA$_f^{Met}$ binds only to the form sustaining peptidyl transfer, thus shifting the unfavorable equilibrium to a reactive state. With our simplifying assumptions, the binding constants range between 50 and 200 μM^{-1}. They

certainly could depend on potassium, magnesium, or initiation factors, with a correlative variation of the L values; however, the general trend of variation of L would be the same, the actual values being lower than those given here.

Inhibition of the binding of both tRNA species, at 5 mM Mg^{2+}, due to potassium (Table I) can be explained by the dissociation of ribosomes (Figure 2). However, since we have shown (Petersen et al., 1976) that 30S particles are not able to discriminate between formylated and unformylated tRNA under such conditions, we tentatively interpret part of the influence of potassium as due to an antagonizing effect against the positive action of magnesium, rather than to actual dissociation. Indeed, at higher magnesium concentrations we found that potassium had almost no effect on the binding of unformylated initiator tRNA, whereas the formylated species binding is inhibited. This can therefore only partly be caused by dissociation of the ribosomes which is only significant at 400 mM K^+, but can certainly be explained by a shift of the equilibrium toward the unactive form.

The strong activating effect of the peptidyltransferase activity by potassium, or ammonium, is shown in Figures 3 and 4. The initial rate of the puromycin reaction as a function of potassium concentration is due to the combination of two antagonistic effects of this ion: a shift to the preexisting ribosomal equilibrium toward a form which does not support peptidyl transfer and an activation of peptidyltransferase; this explains the sigmoidicity of the curves.

We shall now discuss these results in terms of what we already know about ribosomes and formylation.

Discussion

The hypothesis that, at the 70S ribosome level, a dynamic equilibrium exists between two forms, one, minor, at low magnesium concentration, yielding an active peptidyl transfer, and the other which cannot perform this transfer, is mainly founded on two sets of observations: first, the completely different behavior of Met-$tRNA_m^{Met}$ and Met-$tRNA_f^{Met}$, the former always being unable to react with puromycin, whereas the latter can be triggered (by magnesium or potassium) to react with the antibiotic; second, the difference between formylated and unformylated species of initiator tRNA, the latter being easily bound to ribosomes, albeit under conditions where only part of it is ready to react with puromycin.

This interpretation of the experimental results suggests a function for formylation. Since fMet-$tRNA_f^{Met}$ is only able to bind to the active, minor conformation of 70S ribosomes, it may have a triggering function in initiation. This assumption is further supported by the fact that peptidyltransferase does not seem to discriminate between formylated and unformylated methionine, as shown by the kinetic constants of peptidyl transfer which seem to be equal for both species. The formyl group might therefore act as a positive effector for initiation of protein synthesis on 70S ribosomes. The binding constants (of the order of 100 μM^{-1}) appear to be consistent with the assay used: isolation of the bound complex by nitrocellulose filtration. The equilibrium constant of the dynamic equilibrium of ribosomes (from 0.002 to 0.3) reflects the positive influence of the magnesium ion. This must be taken only as an order of magnitude because of the many necessary simplifications made in the model and because of the lack of precision of binding experiments. We shall not try, therefore, to obtain a quantitative picture of the influence of magnesium on the evolution of the ribosome conformation.

In the presence of initiation factors, the picture—at least at low magnesium concentrations—must be much more complex. Our model can nevertheless be used as a phenomenological description giving an overall behavior. The fine influence of initiation factors has been described elsewhere (Godefroy-Colburn et al., 1975) showing its complexity.

Potassium appears as a necessary cofactor for peptidyltransferase, and its linear contribution to the rate constant for the reaction suggests a direct action on the catalytic site. Since potassium depresses the proportion of ribosomes in the active conformation but stimulates peptidyl transfer, it exhibits a pseudocooperative action on the initial velocity for peptidyl transfer between concentrations of 50 and 200 mM. Since potassium concentration in vivo is at most 200 mM (Solomon, 1962) one may wonder whether this phenomenon cannot be used as a homeostatic regulation, coupling internal potassium concentration and protein synthesis (Lubin and Ennis, 1964).

The observation that initiator tRNA reacts with puromycin even in the absence of synthetic messenger raises the question of whether or not there is a sequential order for the binding of the different components on ribosomes. The various groups working on this subject (Vermeer et al., 1973; Jay and Kaempfer, 1974) do not agree on the order of attachment for mRNA and initiator tRNA. It seems difficult to answer this question from our present experiments since the observed puromycin reaction is probably due to a fast turnover arising from a weak binding of charged tRNA to ribosomes in the absence of the template which is not detectable on Millipore filters; however, our data show that a proper fixation site does exist on 70S particles even in the absence of any messenger.

In the preceding article, we have seen that formylated and unformylated initiator tRNAs bind almost equally well to the 30S ribosomal subunit. This is in contrast to the binding to 70S ribosomes where Met-$tRNA_f^{Met}$ binds much better than fMet-$tRNA_f^{Met}$ in the absence of initiation factors.

These results indicate that initiator tRNA has, by itself, a structure which is responsible for its proper positioning on the 30S subunit, even in the absence of formylation or initiation factors. The factors enhance this binding and have a further directing influence on 70S ribosomes; however, when Met-$tRNA_f^{Met}$ is already bound to the 30S particle, before the junction of the 50S particle, the inhibitory effect of initiation factors is lost, which suggests that a slow conformational equilibration between two states of the 70S ribosomes has to take place. This is reflected by the fact that the puromycin reaction with Met-$tRNA_f^{Met}$ is much slower than with fMet-$tRNA_f^{Met}$, although it is considerably higher than that observed on preassociated 70S ribosomes.

These results are in agreement with those given in the preceding article and suggest that 50S ribosome subunits have an essential influence on the equilibrium of the 70S particles; the ribosomes may be visualized as follows:

$$\begin{array}{ccc} 30S & \rightleftharpoons & (30S)^* \\ + & & + \\ 50S & & 50S \\ \Downarrow & & \Downarrow \\ (70S) & \rightleftharpoons & (70S)^* \end{array}$$

where the (30S) equilibrium lies predominantly on the side

of the *active* (30S)* subunit whereas the 70S equilibrium is in favor of the *inactive* (70S) ribosome. In turn this ribosome is partially dissociated which is consistent with the effect of potassium and magnesium described in the preceding article. The whole set of equilibria can therefore be easily used at the initiation step of protein synthesis.

These hypotheses are in agreement with the experiments of Ginzburg and Zamir (1975) showing that 30S ribosomal subunits associated with the 50S subunits react with *N*-ethylmaleimide similarly, although not identically, to 30S subunits in the *inactive,* rather than in the *active* form.

Experiments on initiation of protein synthesis are usually discussed in terms of peptidyl ("P") or aminoacyl ("A") binding sites for tRNA. The experiments presented here using tetracyclin and the results of binding studies on 30S particles (Petersen et al., 1976) suggest that Met-tRNA$_f^{Met}$ does not bind to a true A or P site but only to a "P-like" site: this term would encompass two different peptidyl sites, depending on the ribosomal conformation, and would correspond to an active peptidyltransferase on the active 70S ribosome and to a nonactive one on the inactive 70S particle.

Accordingly, one could tentatively describe the 70S $\rightleftharpoons$ (70S)* equilibrium as an equilibrium of the type P$_-$ $\rightleftharpoons$ P*A, where P$_-$ means a particle only able to attach Met-tRNA$_f^{Met}$ in a specific, nonactive P site, and P*A means a fully active particle ready for initiation and acceptance of the second aminoacyl-tRNA. This is consistent with the observation of Springer and Grunberg-Manago (1972).

Finally, we would like to propose a general function for the regulatory action of the formyl group. It is generally considered that initiation of polypeptide synthesis proceeds via a complex involving initiator tRNA, messenger RNA, and the 30S ribosomal subunit (Lucas-Lenard and Lipmann, 1971). Since this subunit is unable to discriminate between the formylated and unformylated tRNA species (Petersen et al., 1976) one may wonder whether, in special cases, the 70S particle might not be used, *as such,* in initiation complexes. In particular, in the case of polycistronic messengers, it may be possible that the first cistron is translated via dissociated ribosomes, but when passing to the next cistron, a nonnegligible portion of the ribosomes would remain as 70S particles requiring formylation for the initiation step.

One of the striking differences between prokaryotic and eukaryotic protein synthesis is that whereas prokaryotes initiate translation at several points on polycistronic messengers, eukaryotic messengers, as far as is known, are monocistronic and contain only one initiation site. Eukaryotic viral messengers, even when coding for more than one protein, appear to be translated as a single polypeptide which is processed subsequently by proteases (Smith, 1975). Thus, eukaryotes would not need formylation in the way that we suggest is necessary for prokaryotes.

In accordance with this idea, it has been shown that polyoma virus DNA can be transcribed and translated in an *Escherichia coli* cell-free system but that apparently only one protein is synthesized in the absence of formylation of Met-tRNA$_f^{Met}$ (Crawford and Gesteland, 1973). On the other hand, at least eight initiation sites have been shown on Poliovirus RNA when fMet-tRNA$_f^{Met}$ is present (Rekosh et al., 1970). Formylation might, therefore, be used as a regulatory device in the translation of polycistronic messengers in which the different cistrons are contiguous (such as lactose operon, galactose operon, etc.), since lowering the formylation level would increase the polarity effect. Accordingly, mutant strains in which the ribosomes are readily dissociated would more easily overcome the absence of formylation. Our hypothesis might also be supported by the fact that *E. coli* can grow (albeit very slowly) on a medium supplemented with trimethoprim where the level of formylation is drastically reduced; under such conditions the ribosomes are almost completely dissociated (Harvey, 1973).

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Appendix: Derivation of tRNA Binding Constants on Ribosomes, Equilibrium Constants of Ribosome Conformation, and Rate Constant for the Formation of Aminoacylpuromycin

The equations derived from the model (where i = F or M) are:

$$R \rightleftharpoons R^* \qquad R^* = LR \tag{1}$$

$$R + T_i \overset{K_{1i}}{\rightleftharpoons} RT_i \qquad RT_i/T_i \cdot R = K_{1i} \tag{2a}$$

$$R^* + T_i \overset{K_{2i}}{\rightleftharpoons} R^*T_i \qquad R^*T_i/T_i \cdot R^* = K_{2i} \tag{2b}$$

$$R^*T_i + p \xrightarrow{k_{+i}} aa_ip + t + R^* \tag{3}$$

$$daa_ip/dt = k_{+i}pR^*T_i$$

The amount bound, B_i, equals:

$$B_i = RT_i + R^*T_i$$

and, using the total tRNA (T_{0i}) and ribosome (R_0) concentrations, one has:

$$R_0 = R + R^* + RT_i + R^*T_i \tag{4}$$

$$T_{0i} = T_i + RT_i + R^*T_i \tag{5}$$

Let us set:

$$K_i = K_{1i} + LK_{2i}$$

$$\bar{K}_i = \frac{K_{1i} + LK_{2i}}{1 + L}$$

Then combining eq 1, 2a, 2b, 4, and 5 one obtains:

$$T_i^2 + (\bar{K}_i^{-1} + R_0 - T_{0i})T_i - \bar{K}_i^{-1}T_{0i} = 0$$

which allows the computation of free tRNA, T_i:

$$T_i = (1/2)(-\bar{K}_i^{-1} - R_0 + T_{0i} + \sqrt{\Delta})$$

where $\Delta = (\bar{K}_i^{-1} + R_0 - T_{0i})^2 + 4\bar{K}_i^{-1}T_{0i}$ and the amount bound is:

$$B_i = T_{0i} - T_i = (1/2)(T_{0i} + \bar{K}_i^{-1} + R_0 - \sqrt{\Delta}) \tag{A}$$

Thus, B_i is a function of $\bar{K}_i$ and therefore a function of L. In order to compare the variations of B_i as a function of L one must know the sign of the derivative $B_i'(\bar{K}_i^{-1})$:

$$B_i'(\bar{K}_i^{-1}) = -2R_0T_0\Delta^{-1/2}(\sqrt{\Delta} + T_{0i} + R_0 + \bar{K}_i^{-1})^{-1}$$

which is always negative.

B_i is therefore an increasing function of $\bar{K}_i$. Now, $\bar{K}_i$ is a function of L which has the sign of $K_{2i} - K_{1i}$ if L is increasing, and of $K_{1i} - K_{2i}$ if L is decreasing. This shows that when B_i is constant over a large range of conditions,

K_{1i} must be equal to K_{2i}. In addition, as a function of $\bar{K}_i$ one can show that for the small values of $\bar{K}_i$ one has:

$$\lim K_i \longrightarrow 0 \qquad B_i = R_0 T_{0i} \bar{K}_i + \sigma(\bar{K}_i)$$

Therefore, if B_i is small, $\bar{K}_i$ is small which means that L is small and K_{1i} is small.

$\bar{K}_i$ may be obtained directly from the amount bound:

$$\bar{K}_i = \frac{B_i}{(T_{0i} - B_i)(R_0 - B_i)} \tag{6}$$

and also in the limiting case obtained at high ribosomal or tRNA concentrations:

$$R_0 \longrightarrow \infty \qquad B_i = T_0 \left(1 - \frac{\bar{K}_i^{-1}}{R_0}\right) + \sigma(1/R_0) \tag{7a}$$

$$T_0 \longrightarrow \infty \qquad B_i = R_0 \left(1 - \frac{\bar{K}_i^{-1}}{T_0}\right) + \sigma(1/T_0) \tag{7b}$$

The equations for binding being solved, there remains the equation for the puromycin reaction:

$$v_i = \frac{d a a_i p}{dt} = k_{+i} p R^* T_i \tag{B}$$

$$= k_{+i} p K_{2i} L T_i R$$

$$= \frac{k_{+i} p K_{2i} L}{K_{1i} + K_{2i} L} B_i$$

Then

$$\frac{v_M}{v_F} = \frac{k_{+M}}{k_{+F}} \frac{\bar{K}_F}{\bar{K}_M} \frac{B_M}{B_F}$$

In order to obtain the different values of the constants one follows the order given in the text.

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