CHARACTERISTICS OF N-Ac-Phe-tRNA BINDING AND ITS CORRELATION WITH INTERNAL AMINOACYL-tRNA RECOGNITION

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Evidence is given that exact accomodation of initiation and internal aminoacyl-tRNAs to their cognate decoding site is chiefly mediated by protein factors. Only the factor-mediated reaction placed N-Ac-Phe-tRNA correctly at the P site, as demonstrated by lack of sensitivity towards tetracyclin, fast reaction with puromycin, streptomycin-induced release, and clear saturation of ribosomes. Furthermore, correct N-Ac-Phe-tRNA binding to the P site (in the presence of IF1 and IF2) proved to be necessary for an EFT-dependent opening of the A site to Phe-tRNA.

When the initiation complex is complete, initiator-tRNA ($fMet\text{-}tRNA^{Met}_f$, or its analog N-Ac-Phe-tRNA) is entirely bound on the ribosome at the peptidyl site (P) leaving the aminoacyl site unoccupied; the codon following the initiation codon is then ready to recognize its specific anticodon. In the present communication we provinde information showing that initiator tRNA is correctly accomodated at the P site only in the presence of initiation factors. Furthermore, we show that the attachment of internal aminoacyl tRNA at the A site depends strictly on how the initiator tRNA was attached at the P site.

Materials and Methods

Ribosomes were prepared from E. coli MRE 600, as described previously[1], except that they were washed three times and not purified on DEAE-cellulose.

Elongation factor T (EFT) was prepared according to Ravel et al.[2] up to the DEAE-Sephadex chromatography step included. The EFT_u obtained was contaminated with EFT_s, and used as EFT in the experiments. Under our conditions EFG activity was not found at 0°C, but was slightly present at 30°C.

Initiation factors, IF1 and IF2, were prepared as previously described[1]. Aminoacyl-tRNAs were prepared as described by McLaughlin et al.[3] except that the crude enzyme fraction came

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from E. coli MRE 600, and was further purified on DEAE-cellulose. N-Ac-Phe-tRNA was prepared as already described [4].

The products used originated as follows: poly U, Miles (USA); GTP, Sigma (USA); (^{14}C)Phe (specific activity 235 mCi/mmole), and (^{3}H)Phe (specific activity 1000 mCi/mmole), C.E.A. (France); tetracyclin hydrochloride, Roussel UCLAF (France); streptomycin sulfate, Specia (France); puromycin dihydrochloride, NBC (USA).

Millipore filtration was done according to Nirenberg and Leder [5]. For all the results, except where indicated, binding values without poly U (always inferior to one tenth of the value in the presence of poly U) were substracted.

In every experiment, action of initiation and elongation factors was proved to be GTP-dependent, and under our conditions EFT did not interfere with pre-bound N-Ac-Phe-tRNA.

Results

I - Characteristics of N-Ac-Phe-tRNA binding

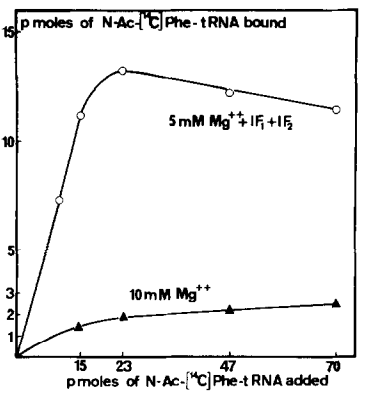

Fig. 1

Binding of N-Ac-(^{14}C)Phe-tRNA in the presence and absence of initiation factors, at 5 mM and 10 mM Mg^{++}

The incubation mixture contained in 50 µl: Tris-HCl, pH 7.50, 50 mM; NH_4Cl, 80 mM; Mg acetate, as indicated; GTP, 1 mM; poly U, 0.2 A_{260} units; ribosomes, 0.8 A_{260} units; IF1, 1.35 µg, and IF2, 2.25 µg (when indicated); N-Ac-(^{14}C)Phe-tRNA, as indicated. Incubation 15 min at 25°C.

Fig. 1 shows the binding of N-Ac-Phe-tRNA to ribosomes, stimulated by poly U, under two different sets of conditions : at 10 mM Mg^{++} without factors, and at 5 mM Mg^{++} in the presence of initiation factors IF1 and IF2. In the first experiment, only a slight part of the N-Ac-Phe-tRNA binds to ribosomes, and this binding does not reach saturation, even when as much as 90 pmoles

of N-Ac-Phe-tRNA are added for 2.9 pmoles bound. When IF1 and IF2 are present, at 5 mM Mg^{++}, the binding is strongly enhanced, and with 12.3 pmoles of N-Ac-Phe-tRNA, more than 90% binds to ribosomes; furthermore, a saturation point is very clearly reached, corresponding roughly to 0.9 pmole N-Ac-Phe-tRNA per 1 pmole ribosome. Non-enzymatic binding at 10 mM Mg^{++} was not stimulated by pre-incubation of the ribosomes under conditions reported to activate them[3,6] (pH 7.5 with 20 mM Mg in 160 mM NaCl for 10 minutes at 37°C); we believe, therefore,

TABLE I
Effect of streptomycin and puromycin on the N-Ac-Phe-tRNA - poly U - ribosome complex

Expt No	Conditions	N-Ac-Phe-tRNA bound (pmoles) - Sm	+ Sm	puromycin reaction (binding %)
1	5 mM Mg (25°C)*	1.8	-	15
2	10 mM Mg (30°C)*	2	2.8	34
3	5 mM Mg + (IF1 + IF2) (25°C)*	13.5	10.7	83
4	5 mM Mg + (IF1 + IF2) (30°C)*	13.5	4.14	-

* Temperature of second incubation.

Same conditions as in fig. 1; N-Ac-(^{14}C)Phe-tRNA, 23.5 pmoles; Streptomycin sulfate: Expts. 2 and 4, 0.75 µg; expt 3, 1.5 µg. For binding determination: in expts 2 and 4, after 15 min incubation at 25°C the temperature was shifted to 30°C and incubation resumed for another 30 min; in expts 1 and 3, incubation was 30 min at 25°C. For puromycin reaction: after 15 min incubation at 25°C, 100 µl of 0.7 mg/ml puromycin dihydrochloride solution in the respective buffers were added to the samples prior to a further 5 min incubation at 25°C.
Amount of N-Ac-(^{14}C)Phe - puromycin formed was determined by the method of Leder and Bursztyn (BBRC, 25, 233, 1966). Amount of N-Ac-(^{14}C)Phe - puromycin formed without poly U (always < 0.3 pmoles) was substracted. Streptomycin has no effect during the first incubation. Radioactive products determined after the second incubation, in the absence of puromycin, were negligible.

that the lower binding is not explained by mere inactivation of the ribosomes.

Moreover, as shown in Table I, only the factor-mediated reaction accomodated N-Ac-Phe-tRNA at the precise puromycin reactive site. In the absence of factor only one third of the N-Ac-Phe-tRNA rapidly reacted with puromycin, as compared to 83% under conditions of enzymatic attachment. Reaction with puromycin of non-enzymatically bound N-Ac-Phe-tRNA can be increased by longer incubation with puromycin, or by raising the temperature.

Streptomycin is known to displace initiatior tRNA from the

complete initiation complex on the 70S ribosomes [7,8], but only when this tRNA is correctly accomodated at the P site. As shown in Table I, 70% of N-Ac-Phe-tRNA bound enzymatically (at a temperature above 30°C) is released by streptomycin, as compared to 30% released when non-enzymatically bound. As already noted with fMet-tRNA [9] temperature has an important effect on the release of N-Ac-Phe-tRNA by streptomycin : at temperatures lower than 30°C no release occurred.

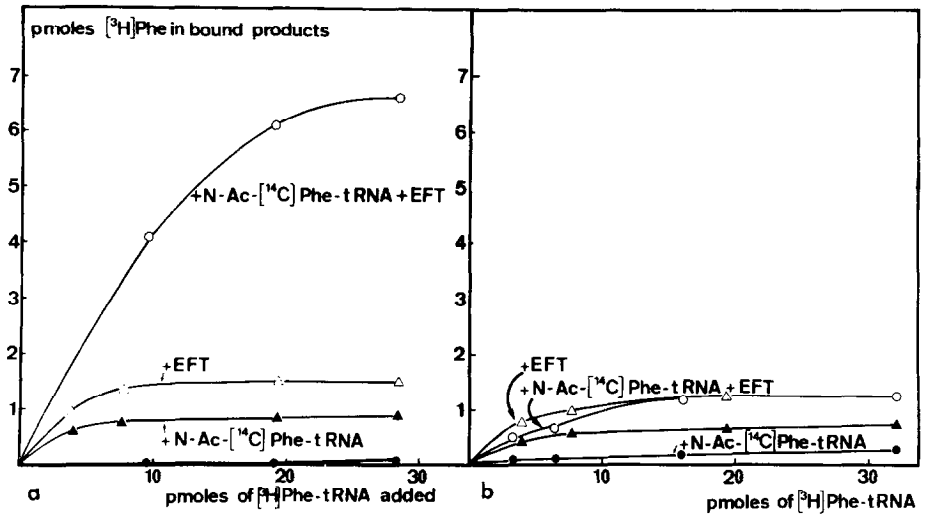

Fig.2a : 5 mM Mg^{++} Fig.2b : 10 mM Mg^{++}

(^{3}H)Phe in ribosome bound products in the presence and absence of N-Ac-(^{14}C)Phe-tRNA at 5 mM and 10 mM Mg^{++}

A first incubation was carried out under the same conditions as in Fig. 1 with 23.5 pmoles of N-Ac-(^{14}C)Phe-tRNA where indicated. The samples were then chilled to 0°C and (^{3}H)Phe-tRNA was added in the indicated amounts and, where indicated, 8 µg of factor EFT. A second incubation (final volume 55 µl) was carried out 15 min at 0°C. Binding without poly U was not substracted.

II - Correlation between A and P site occupancy by tRNAs

Fig.2 shows the binding of (^{3}H)Phe-tRNA at 0° (to avoid any enzymatic translocation) in the presence and the absence of elongation factor and under conditions where N-Ac-(^{14}C)Phe-tRNA was first bound, enzymatically or not, to the ribosomes. In fig.2a N-Ac-(^{14}C)Phe-tRNA, when present, was enzymatically pre-bound at 5 mM Mg^{++} and in fig.2b it was pre-bound at 10 mM Mg^{++} without IF1 and IF2.

Under these conditions, the only strong binding of Phe-tRNA occurs in the presence of elongation factor EFT and when N-Ac-Phe-tRNA has been enzymatically pre-bound. Thus, strong

binding at the P site very highly favors the EFT-dependent binding of Phe-tRNA. The specificity is striking since it was only when N-Ac-Phe-tRNA was enzymatically bound that the total EFT dependence appeared.

Tetracyclin, at appropriate concentrations, is known to specifically act on the A site of the ribosomes[9]. Table II

TABLE II
Effect of tetracyclin on binding of (^{3}H)Phe-tRNA to ribosomes prebound with N-Ac-(^{14}C)Phe-tRNA

Tetracyclin	N-Ac-(^{14}C)Phe (pmoles of products bound)	(^{3}H)Phe (pmoles of products bound)
0	12.45	4.61
0.05 mM	11.29	0.51
0.1 mM	10.39	0.46
0.5 mM	6.93	0.25
1 mM	1.46	0.0

Same conditions as for Table I, Expt 3. After 15 min at 25°C, the samples were further incubated for 20 min at 0°C with 19 pmoles of (^{3}H)Phe-tRNA and 18 μg of elongation factor EFT. Tetracylcin was added, as indicated, in the first incubation mixture.

TABLE III
Effect of streptomycin on the binding of (^{3}H)Phe-tRNA on ribosomes prebound with N-Ac-(^{14}C)Phe-tRNA

	N-Ac-(^{14}C)Phe (pmoles of products bound)		(^{3}H)Phe (pmoles of products bound)	
	- Sm	+ Sm	- Sm	+ Sm
	13.5	4.14	-	-
+ (^{3}H)Phe-tRNA	11.85	5.80	0.47	0.50
+ (^{3}H)Phe-tRNA + EFT	16.1	13.52	10.12	5.8

The 25°C incubation was carried out as in Table II, except that 0.75 μg of streptomycin were added (where indicated) instead of tetracyclin. After 15 min incubation, the samples are chilled to 0°C. Where indicated 32 pmoles of (^{3}H)Phe-tRNA, and 8 μg of elongation factor EFT were added prior to further incubation at 30°C for 15 min.

shows that tetracyclin at 0.05 mM, inhibits the enzymatic binding of N-Ac-Phe-tRNA by less than 10%, and the binding of Phe-tRNA by 90%, thus confirming their respective decoding sites. As previously shown [9] with fMet-tRNA, this inhibition specifically decreases with tetracyclin concentration : at over 0.1 mM, the

specificity is lost, and at 0.5 mM the binding of N-Ac-Phe-tRNA is inhibited by almost 50%.

Table III shows that addition of (^{3}H)Phe-tRNA to reaction mixtures containing ribosomes to which N-Ac-Phe-tRNA has been enzymatically pre-bound inhibits the ability of N-Ac-Phe-tRNA to be released from the P site by streptomycin. Binding of (^{3}H)Phe-tRNA, and further steps leading to the formation of N-Ac-(^{14}C)Phe-poly(^{3}H)Phe, are inhibited by streptomycin to an extent of approximately 40%.

Discussion

A possible approach to evaluating the role of factors in the matching of decoding sites by aminoacyl-tRNAs is to compare the properties of a factor-mediated and of a non-enzymatic aminoacyl-tRNA - ribosome complex. Using N-Ac-Phe-tRNA as a model for initiator tRNA, we were able to show that only the factor-mediated reaction placed N-Ac-Phe-tRNA in the P site, defined by the following characteristics : fast reaction with puromycin, chasing by streptomycin, and lack of sensitivity towards tetracyclin. Moreover, only in the presence of factors could ribosomes be clearly saturated with N-Ac-Phe-tRNA. Nevertheless, most of the non-enzymatically bound N-Ac-Phe-tRNA does react with puromycin when incubated with the antibiotic for longer periods or at higher temperatures at an Mg^{++} concentration of 0.01 M or higher. The fact that chasing by streptomycin, clear saturation point, and insensitivity towards tetracyclin, are accompanied by a fast puromycin reaction indicates that definition of the P site occupancy solely by the puromycin reaction is only correct when the reaction rate is fast. When longer incubation with puromycin is carried out, the results are difficult to interpet : a transconformation of the ribosome - tRNA - codon complex may be observed.

It can, therefore, be concluded that N-Ac-Phe-tRNA is correctly accodomated at the P site on the ribosome only in the presence of initiation factors. This does not invalidate the experiments where N-Ac-Phe-tRNA, non-enzymatically bound at 10 mM Mg^{++} was used as analog of initiator tRNA provided peptide analysis was performed [4], since a percentage of this aminoacyl tRNA is correctly bound, but it does throw some doubt on the conclusion of binding experiments under non enzymatic conditions where no peptide analysis was done.

The effect of temperature on the chasing of N-Ac-Phe-tRNA

from the P site by streptomycin is quite interesting. At a temperature below 30°C, a fast puromycin reaction occurs with N-Ac-Phe-tRNA, but no release by streptomycin is observed. This recalls the observation that fMet-tRNA reacts with puromycin as soon as it is bound to the ribosome, but will not be displaced by streptomycin at low temperature (13°), nor when its binding is under 30% completion [9]. This was interpreted [10] as reflecting a necessary conformation change between the puromycin-reactivity step and the step at which streptomycin -induced release is possible. This can be accounted for (as was previously discussed [10, 11]) by Bretscher's model [12] for ribosome structure in which the two subunits can readily slide or rotate with respect to each other, and which is such that tRNA decoding sites can adopt a transient, hybrid state where part of the site is made of the P site on the 50 S plus the A site on the 30 S, or vice-versa.

Since it is known that puromycin reacts with the P site on the 50 S subunit, while streptomycin reacts with the 30 S subunit, reported experiments could be interpreted as fMet-tRNA (or N-Ac-Phe-tRNA) entering a hybrid site consisting of the A site on the 30 S subunit and the P site on the 50 S counterpart. The release of aminoacylated initiator tRNA by streptomycin would occur only when it occupies the P site on both subunits.

The inhibition of streptomycin release of N-Ac-Phe-tRNA by Phe-tRNA indicates that growth of the N-Ac-Phe-poly-phenylalanine chain inhibits this release. This could be interpreted as an inhibition of N-Ac-Phe-tRNA release when the A site is occupied by an internal aminoacyl-tRNA, or as an impossibility for N-Ac-Phe-Phe-tRNA to be released from the ribosomes. The experiments described here do not differentiate between those two possible mechanisms, but the results are consistent with the belief that streptomycin mainly acts at the initiation step by releasing fMet-tRNA from the complete initiation complex [7, 8]. Our results also bring clear evidence that unless the P site is occupied, the A site cannot bind any internal aminoacyl-tRNA and that this A site does not recognize Phe-tRNA in the absence of elongation factor EFT. Moreover, only the enzymatic binding of N-Ac-Phe-tRNA promotes the opening of the A site.

In summary, several situations have been discussed which provide evidence that protein factors play an active role in the site selection process and do not merely act by increasing the

affinity of aminoacyl-tRNAs for their pre-existing attachment regions on the ribosome. Although initiation factors act on the 30 S and elongation factors on the 50 S ribosome subunits, several instances have been reported which suggest that each class of factor-mediated reaction has a long range effect on the subunit complementary to the one where it binds and functions.

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