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Function of tRNA in Initiation of Prokaryotic Translation

ABSTRACT. *This paper is a review of studies on structural and functional aspects of the interactions between the methionine accepting tRNAs and other macromolecules (enzymes, protein factors and ribosomes) in the cellular reactions preceding the formation of the first peptide bond during protein biosynthesis. Before discussing the recent research results, the problem will be introduced in a chronological order according to the discovery of the required molecular components and their functions. This introduction also aims at giving the reader an impression of the importance of the chosen subject in the field of molecular biology.*

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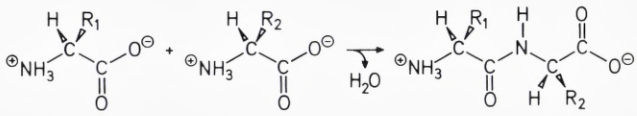
Introduction

All living cells contain a large number of different protein molecules. The exact number is not known, but estimates from the size of the genomes and two-dimensional polyacrylamide gel electrophoretic analysis of crude cell-lysates suggest the order of 2000 to 10000 different natural proteins.

These molecules consist of chains of amino acids linked together by peptide bonds as shown in Figure 1, where R_1 and R_2 are specific side-chains. All known proteins contain up to 20 different amino acids in the polypeptide chain – the primary sequence, which determines the three-dimensional structure and the function of each individual protein. The mechanism by which amino acids are linked together in the sequence specified by the nucleotide sequence of the gene is the biological translation. A four-letter "nucleotide alphabet" in DNA (and in messenger RNA) is translated into a 20-letter "amino acid alphabet" in protein molecules – a sequence of three nucleotides (codon) specifies each amino acid. The biological translation is a complicated process consisting of

Fig. 1. The formation of a peptide bond.



many steps and involving a number of specific macromolecular interactions. One of the most important components of this process is the tRNA molecule, which started its scientific career as a purely intellectual creation.

The suggestion and discovery of transfer RNA

After the elucidation of the DNA structure and the suggestion of the principles in the relation between DNA and protein in the early and mid-1950s, no one could envisage how nucleic acids could programme the synthesis of protein by direct structural interactions with amino acids. The first clue to this problem was the introduction by Francis Crick during the years 1955-57 of the "Adaptor Hypothesis" by which a small adaptor molecule would mediate between amino acids and a piece of nucleic acid which carried the genetic information for specifying the amino acid sequence during polymerisation into proteins. The Adaptor Hypothesis was almost simultaneously confirmed by the discovery of soluble RNA molecules carrying amino acids (Hoagland et al., 1959). The molecules were larger than expected by Crick, and turned out to be nucleic acids containing approximately 80 ribonucleotides and a molecular ratio of 25,000.

The first soluble RNA molecule (called sRNA – and since 1967 transfer RNA or tRNA) was sequenced by Holley et al. (1965) during the years 1958-65. The structure is shown in Figure 2A in the so-called cloverleaf representation. Many other tRNA molecules were soon sequenced, and a similar cloverleaf structure could be drawn for each molecule. The tRNA molecule can form intramolecular Watson-Crick base-pairs in four regions. One of these contains the terminals of the nucleotide chain, and the three others end in singlestranded loops. It is shown in Figure 2B where the amino acid attaches to the molecule and the "anti-codon" nucleotide sequence which "reads" the amino acid-specifying nucleotide sequence on messenger RNA is indicated at the bottom of the molecule.

The first three-dimensional structure of a tRNA molecule was obtained independently by the groups of Aaron Klug in Cambridge and Alexander Rich at M.I.T. in 1975. The folded structure (of phenyl-

alanine specific tRNA from yeast) is shown in Figure 3. Although minor and probably important differences may exist, the overall spatial structure is believed to be general in tRNA molecules.

The knowledge of the three-dimensional structure of the tRNA molecules is one of the prerequisites for the study of structural interactions between tRNA and proteins, which is one of the main subjects of this paper.

Protein biosynthesis starts at the amino terminal

At the time the tRNA molecules were discovered, only very limited knowledge existed about the molecular reactions in which it is involved during its functional cycle. The study of the components and mechanism of protein biosynthesis was just starting. However, it was becoming clear that the process took place on sub-cellular particles – first called microsomes and in 1958 named ribosomes by Richard Roberts.

Then it was a crucial question in which direction the reaction of amino acid polymerisation proceeded. If we look at Figure 1, would R_1 or R_2 be

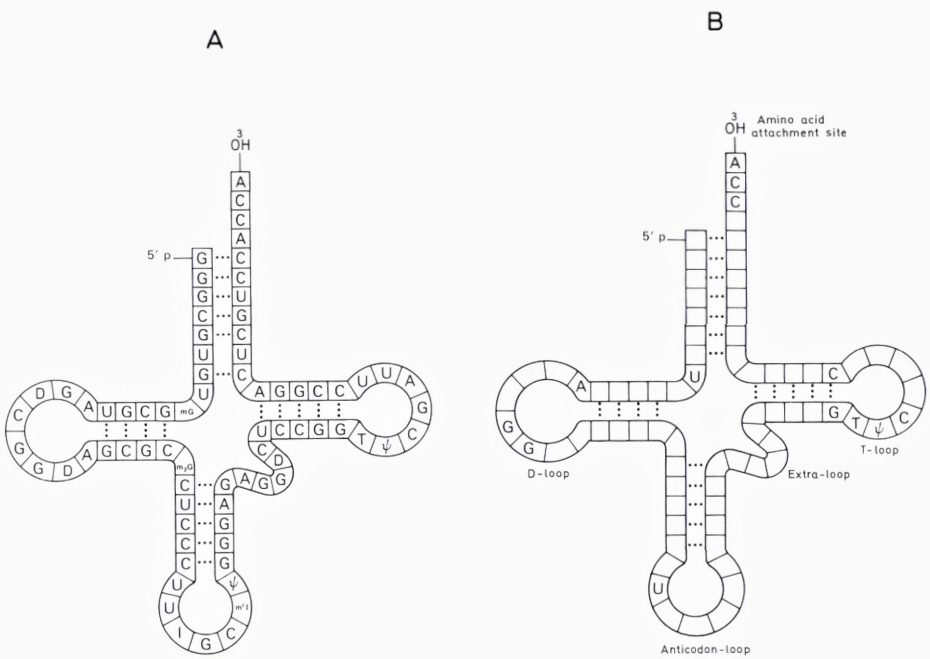


Fig. 2A. Cloverleaf representation of the nucleotide sequence of tRNA^{Ala} from yeast.
B: Common features of tRNA molecules.

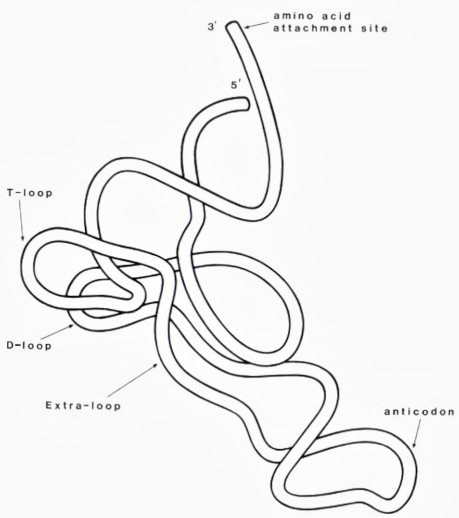


Fig. 3. Schematic diagram of the three-dimensional structure of tRNA^{Phe} from yeast. Based on the X-ray crystallographic data of Ladner et al., *1975.*

the first amino acid in the polypeptide – in other words would the initiating amino acid become the N- or C-terminal of the molecule?

The first answer to this question came from a study of the biosynthesis of haemoglobin by Bishop *et al.* (1960), who found that the polypeptide chain growth began with the N-terminal amino acid. This means that R_1 in Figure 1 is the terminal amino acid in a protein starting by this reaction. Shortly after, Goldstein and Brown (1961) showed that *E. coli* proteins are synthesized beginning with the N-terminal amino acid.

N-terminal amino acid = methionine

By that time, other workers were studying sequences of amino acids in proteins. From the work of Moriwitz and Spaulding (1958), it seemed that one particular amino acid appeared more than statistically in the N-terminal position of *E. coli* – namely methionine.

That methionine is found much more than randomly as the N-terminal amino acid of *E. coli* proteins was confirmed by Waller and Harris (1961) – for ribosomal proteins – and finally by Waller (1963) – for total *E. coli* proteins. This led to believe that proteins are synthesized by a mechanism which specifically incorporates methionine as the first and N-terminal amino acid. Twenty years ago, the picture of the molecular mechanism of protein biosynthesis can be presented as shown in Fig. 4 (Watson, 1964).

The genetic information in the chromosomal DNA (in the form of a fourletter nucleotide sequence) is transcribed into messenger RNA by

the enzyme RNA polymerase. The mRNA is then bound to a ribosome and by adaptor molecules – aminoacyl-tRNAs – translated into a 20-letter amino acid sequence in a polypeptide chain.

It was known that special protein factors (transfer enzymes) were needed to stimulate the overall process of translation and that energy was supplied by GTP, but the knowledge specifically concerning the mechanism by which the process started was clearly expressed by Watson: "Almost no hints exist about chain initiation".

The first important discovery of initiation specific molecules came from the Cambridge laboratory of Perutz.

Initiator tRNA

In their work with sulfur containing amino acids (easily labelled with high specific radioactivity) and their interaction with tRNAs from *E. coli,* Marcker and Sanger (1964) found that [^{35}S]-methionyl-tRNA upon mild hydrolysis (which splits off the amino acid as shown in Fig. 16) gave two radioactive spots on electrophoretic analysis – one from methionine and another which was identified as N-formyl-methionine. Shortly after Marcker (1965) showed that two different classes of methionyl-tRNA exist of which one could be N-formylated in the methionine by an *in vitro* system, whereas the other could not. The two species were named Met-tRNA$_1$ (not formylatable, now called $tRNA_m^{Met}$) and Met-tRNA$_2$ (formylatable, now called $tRNA_f^{Met}$) and the role of formyl-methionyl-tRNA as initiator tRNA in protein synthesis was the authors' immediate suggestion. The cloverleaf structures of the two tRNAs are

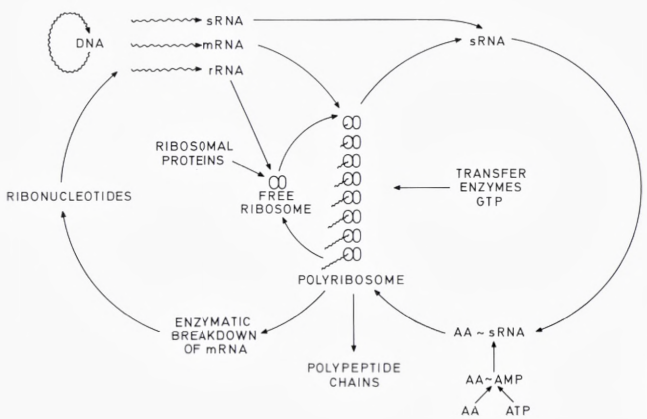


Fig. 4. Schematic view of protein synthesis as of March 1964 (Watson, 1964).

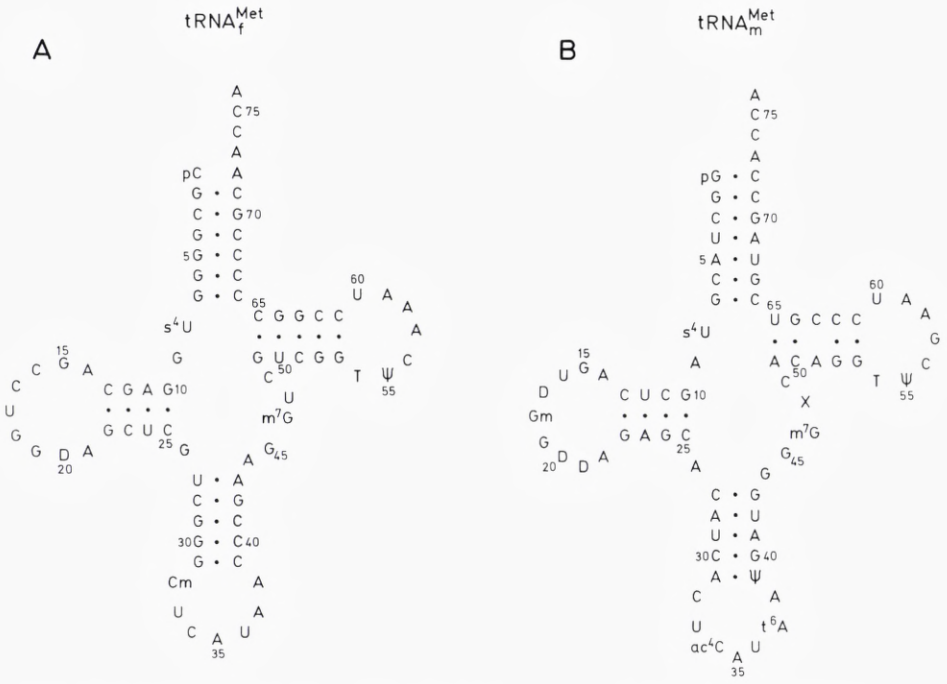


Fig. 5A. Cloverleaf representation of the nucleotide sequence of initiator tRNA$_f^{Met}$ (Dube et al., *1968).*
B. Cloverleaf representation of the nucleotide sequence of elongator tRNA$_m^{Met}$ (Cory et al., *1968).*

shown in Fig. 5. Similarities and differences in the nucleotide sequences will be discussed further in subsequent paragraphs. In an *in vitro* system similar to the one developed by Nirenberg and Matthaei (1961), Clark and Marcker (1966a) showed that N-formyl-methionyl-tRNA$_f^{Met}$ exclusively incorporated formyl-methionine into the N-terminal of a growing polypeptide chain (in response to poly(U,G)), similarly the same authors showed (1966b) that the non-formylatable Met-tRNA$_m^{Met}$ incorporated methionine into internal positions of polypeptides and not into the N-terminal. This was considered as a proof that tRNA$_f^{Met}$ is the true initiator tRNA in *E. coli*.

Aminoacylation of tRNA$_f^{Met}$

The tRNA molecules carry the amino acids from the cell cytoplasm to the ribosome – the site of protein biosynthesis. Now the question arises: How are the correct amino acids linked to the tRNA molecules? This is

an enzymatic process, which requires catalysis by an aminoacyl-tRNA synthetase specific for each amino acid.

The methionyl-tRNA synthetase aminoacylates both $tRNA_m^{Met}$ and $tRNA_f^{Met}$. The enzyme has been extensively characterized by the group of Sylvain Blanquet at the Ecole Polytechnique in Paris (Blanquet *et al.*, 1973, 1976). The native protein contains two identical polypeptide chains (type α_2) each with an M_r of 76,000.

The monomer can easily be split by trypsin, resulting in a 64 k dalton fragment, which retains the activity of aminoacylation (Dessen *et al.*, 1982). This fragment has been crystallized, and the X-ray crystallography group of Risler in Gif-sur-Yvette has determined the crystal structure at 2.5 Å resolution (Zelwer *et al.*, 1982).

The aminoacylation reaction catalyzed by Met-tRNA synthetase is shown in Fig. 8 (Reactions 1 and 2).

Formylation of initiator tRNA

Marcker (1965) showed that the formylation in the α-amino group of methionine takes place after the aminoacylation of $tRNA_f^{Met}$ and found that N^{10}-formyl-tetra-hydrofolate was a good formyl-donor in the crude *in vitro* system. The formylating enzyme was purified by Dickerman *et al.* (1967), who confirmed that N^{10}-fTHF was the formyl-donor. Therefore, the enzyme was named: N^{10}-formyl-tetrahydrofolate:L-methionyl-tRNA N-formyltransferase (E.C.2.1.2.9). This means that the formylation of Met-$tRNA_f^{Met}$ is coupled to the C-1 metabolism as shown in Fig. 6.

The transformylase has been extensively purified by Kahn *et al.* (1980) who showed that at physiological ionic conditions, the enzyme has a significantly higher affinity to charged initiator $tRNA_f^{Met}$ as compared to uncharged $tRNA_f^{Met}$ and to other tRNAs.

From the reactions of aminoacylation and formylation (and as it will be discussed later, also from the interaction with the elongation factor Tu), it seems clear that the tertiary structure of the initiator tRNA must be partially identical to that of $tRNA_m^{Met}$ but also partially different from all other tRNAs, and this specific tertiary structure seems sensitive to aminoacylation.

Not only in *E. coli* but also in cell organelles of eukaryotic organisms (chloroplasts and mitochondria) is the protein synthesis initiated by fMet-tRNA (Galper and Darnell, 1969). This led Marcker (1969) to propose the general rule that prokaryotic cells and eukaryotic cell organelles use N-formyl-methionyl-tRNA as polypeptide chain initiator

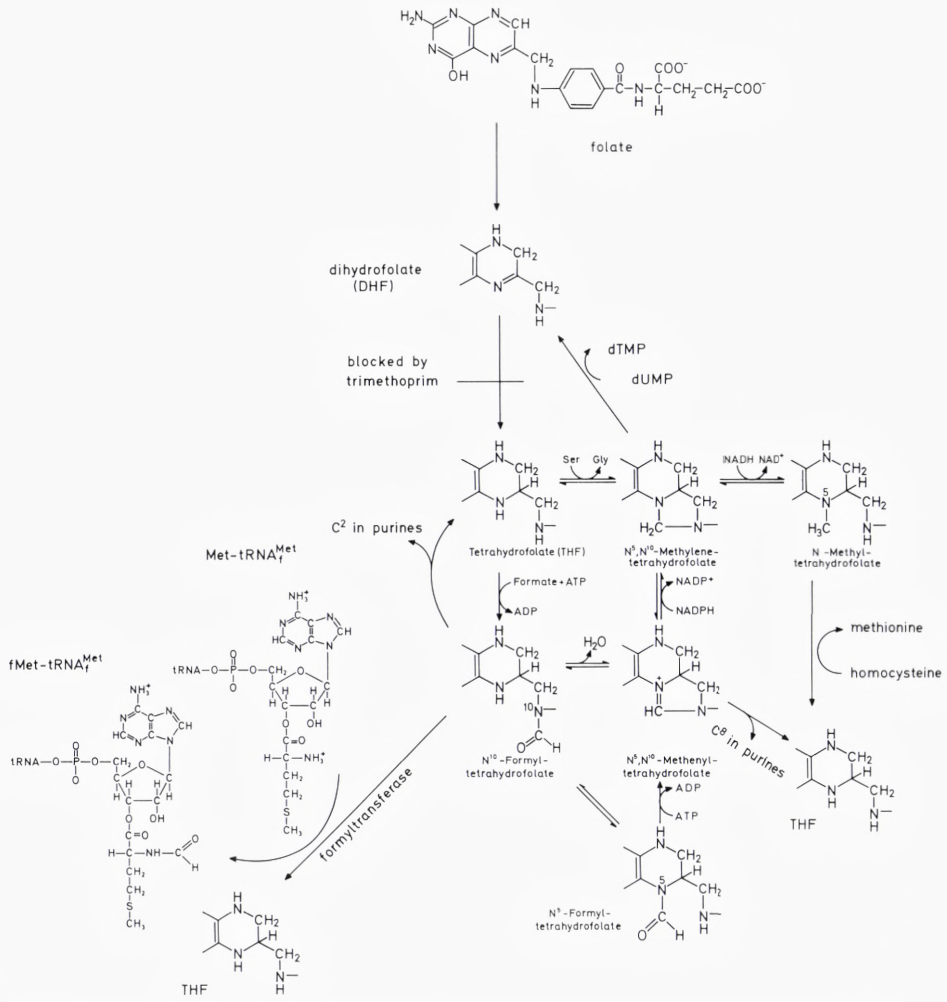


Fig. 6. The folate metabolism including the reaction of formylation of initiator Met-tRNA$_f^{Met}$.

tRNA. The initiator tRNA in eukaryotic cytoplasmic protein synthesis is also methionyl-tRNA. Although it has been shown that this tRNA (tRNA$_i^{Met}$) can be formylated by *E. coli* transformylase, no formylation takes place in the eukaryotic cytoplasm *in vivo* (Lodish, 1976).

Formylation of prokaryotic Met-tRNA_f^{Met} *is not indispensable.*

In vivo *evidence against the general rule of formylation*

However, formylation of initiator tRNA methionine in prokaryotic organisms is not general – that cells may grow without formylation has been shown in different organisms in four principal classes of cases:

1: organisms which cannot synthesize the formyl-donor *de novo* may grow in the absence of formylation
2: organisms which contain no transformylase enzyme grow without formylation
3: *E. coli* can grow when formylation is inhibited
4: mutants of *E. coli* have been isolated which grow normally in the presence of an inhibitor of formylation.

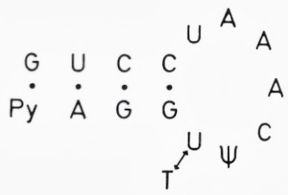
An example of each of these cases will be described briefly:

1: The organism *Streptococcus faecalis R* is (in contrast to *E. coli* and other prokaryotes) not able to synthesize folic acid *de novo,* but it was found that the cells could grow in the absence of folic acid when the substrate was supplemented with the folic acid metabolites serine, methionine, thymine and a purine base – see Fig. 6. At such conditions, the formyl-donor N^{10}-formyl-tetrahydrofolate cannot be synthesized, and formylation of the initiator tRNA must (if there is a formylation) follow other metabolic pathways (Samuel *et al.,* 1970). Indeed, no formylation or other blocking of the α-amino group of Met-tRNA methionine took place in the folate-free medium, although in the presence of folic acid, most of the Met-$tRNA_f^{Met}$ was formylated *in vivo* (Samuel *et al.,* 1972). Later, the authors showed that the $tRNA_f^{Met}$ from folate-free cells was altered as compared to the initiator tRNA isolated from cells grown in the presence of folic acid – the ribothymidylic acid normally found in the TψC loop was not found in the "folate-free" tRNA, see Figs 5A and 7. So it seemed that a structural change in the initiator tRNA could compensate for the lack of formylation (Samuel & Rabionwitz, 1974).

However, it was found that the methylase responsible for the formation of rT in the tRNA in *S. faecalis* requires a folate coenzyme as methyldonor. This means that the absence of formylation and of rT in the tRNA of "minus folate" cells are both (and possibly independently) induced by the folate deprivation (Delk & Rabinowitz, 1975).

Rabinowitz and coworkers did not study the *in vitro* binding of fMet-$tRNA_f^{Met}$ from plus and minus folate *S. faecalis* in crossed experi-

Fig. 7. The T-arm of the initiator $tRNA_f^{Met}$ from S. faecalis. *The ribothymidylic acid present in +folate cells is replaced by uridine in −folate cells (Samuel and Rabinowitz, 1974).*



ments with ribosomes (and initiation factors) isolated from the two growth conditions. It can therefore not be excluded that the ribosomes (and/or the initiation factors) play an important role for the ribosomal binding of the unformylated (and undermethylated) initiator tRNA from "minus folate" *S. faecalis* stimulated by initiation factors (and thus in the ability of *S. faecalis* cells to grow in the absence of formylation).

2: The strongly halophile *Halobacterium cutirubrum* was studied by White & Bayley (1972) who found that this organism does not synthesize the transformylase enzyme needed for the formylation of Met-$tRNA_f^{Met}$, although the methionine accepting tRNA isolated from *H. cutirubrum* could be separated into two species of which one was formylatable by *E. coli* transformylase.

As the organism requires extreme high salt concentrations, it is likely that conformational changes of the tRNA (and/or the ribosomes) may occur as compared to a hypothetical low-salt structure. Such salt induced structural changes may explain the ability of *H. cutirubrum* to grow without formylation of the initiator tRNA.

3: The antibiotic trimethoprim is an inhibitor of dihydrofolate reductase, the enzyme which reduces dihydrofolate (DHF) to tetrahydrofolate (THF) – see Fig. 6. Harvey (1973) has studied the effect of trimethoprim on the growth of *E. coli*. He found that *E. coli* wild type (B/r) cells could grow exponentially at a reduced rate in the presence of trimethoprim when the folic acid metabolites were added. Under these conditions, no formylation of Met-$tRNA_f^{Met}$ took place.

Harvey found that at the growth conditions where the initiator tRNA was unformylated, a two-fold increase in the ratio ribosomal subunits:70S ribosomes arised. As will be discussed further in the following sections, the increased level of ribosomal subunits may be involved in the explanation of the ability of the cells to grow without formylation of Met-$tRNA_f^{Met}$.

4: The isolation of *E. coli* mutants able to grow normally without for-

mylation (Danchin, 1973) is an additional support for the idea that formylation of initiator Met-tRNA is not general and vital for prokaryotic organisms.

Danchin isolated *E. coli* mutants on media containing the C-1 metabolites supplemented with trimethoprim, and found quite surprisingly that among these mutants were strains resistant to streptomycin. Such rpsL mutants are known to be point mutations in the ribosomal protein S12. Therefore, it seems clear that the ability to grow in the absence of formylation in some way is linked to alterations in the structure of the ribosomes.

Another *E. coli* mutant was isolated by Baumstark *et al.* (1977) which was able to grow without formylation of the initiator tRNA. By the same method as previously used by Danchin (1973), a K12 mutant was selected in the presence of sulfathiazole and trimethoprim. The authors showed that the initiator $tRNA_f^{Met}$ of this mutant contained a reduced (but finite) amount of ribothymidine in the TψC loop of the tRNA and concluded that this alteration in the initiator tRNA structure was possibly the reason why the mutant could grow without formylation.

However, their *in vitro* f2 directed protein synthesis did not include crossed experiments with mutant ribosomes and unformylated Met-$tRNA_f^{Met}$ isolated from the parental strain cells. This would be required to exclude that the ability to grow in the absence of formylation arises from altered ribosomes. In fact, the mutant of Baumstark is not a single mutation but derived from a parental strain which was streptomycin resistant (altered in the ribosomal protein S12).

E. coli mutants containing unmethylated uridine instead of rT in the tRNA have been isolated by Björk & Isaksson (1970). In order to test the hypothesis of Baumstark and coworkers, trimethoprim was added to the growth medium of the mutants (and the C-1 metabolites). Under such conditions no formylation of Met-$tRNA_f^{Met}$ could take place, and it would show if the lack of rT was sufficient to account for growth in the absence of formylation. The cells were not able to grow under such conditions (Danchin and Isaksson, personal communication). Thus it seems clear that the other mutations in the strain isolated and studied by Baumstark, in addition to the mutation in the methylase, must play a role in the ability of these cells to grow without formylation of the initiator Met-tRNA.

In vitro *evidence against the general rule of formylation*

Looking back at the first experiments *in vitro* with the initiator tRNA there were indications that formylation might not be indispensable. Clark & Marcker (1966a) did not find any significant difference in the activity of Met-$tRNA_f^{Met}$ whether formylated or not, and just before the discovery of the initiation factors in 1966, Bretscher & Marcker (1966) had shown that at 10-20 mM magnesium, both formylated and unformylated Met-$tRNA_f^{Met}$ could bind to ribosomes and react with puromycin. They concluded that the only role of the formyl group was to increase the rate of initiation.

This led Clark and coworkers to believe that the specificity as initiator tRNA lay in the tRNA structure and not in the presence of the formyl group on the methionine. Therefore, the two methionine accepting tRNAs were sequenced in the hope that the nucleotide primary structure could reveal some characteristic differences between the two tRNAs (on the one hand and between $tRNA_f^{Met}$ and all other tRNAs on the other).

In the two cloverleaf structures shown in Fig. 5, one observes a number of differences in nucleotides at the individual positions. However, an intensive comparison of the $tRNA_f^{Met}$ sequence with other tRNAs shows that all individual nucleotides and local sequences of $tRNA_f^{Met}$ can be found in other tRNAs. Only one difference in the primary structure seems to be characteristic for $tRNA_f^{Met}$ – namely the fact that the 5'-terminal nucleotide (cytidine) is not involved in a Watson-Crick base-pair which is the case for the 5'-terminal nucleotide of all other tRNAs.

Although this may be very important, it is unlikely that it accounts for all the specificities which are found for the initiator tRNA as compared to $tRNA_m^{Met}$ and all other tRNAs, especially as this difference is not found in eukaryotic cells which also use a formylatable (by *E. coli* tranformylase) Met-tRNA species as initiator tRNA.

Other workers then found that formylation was required for the translation *in vitro* of natural mRNA (from phage f2) at 5 mM magnesium (Eisenstadt & Lengyel, 1966), and after the discovery of the initiation factors and the establishment of their optimal salt conditions (5-10 mM magnesium), many *in vitro* experiments suggested that fMet-$tRNA_f^{Met}$ was the correct initiator tRNA as only the formylated tRNA species could bind to 70S ribosomes strongly stimulated by initiation factors (the concept of "enzymatic binding") (Grunberg-Manago *et al.*, 1969; Drews *et al.*, 1973).

In most of these experiments, 70S ribosomes were used for studying

the initiation factor stimulated binding. As the initiation mechanism was supposed to involve the formation of a 30S initiation complex with mRNA and initiator tRNA (see Fig. 8), one could ask whether 30S subunits would behave differently from the 70S ribosomes in such *in vitro* binding experiments. A first indication of such a difference can be seen from the work of Grunberg-Manago *et al.* (1969). In one experiment, 30S subunits were used, and indeed, a small stimulation (in absolute scale, but 50% relative) by the initiation factors of the binding of unformylated Met-tRNA$_f^{Met}$ at 5mM magnesium is observed.

In the work of Samuel & Rabinowitz (1974) previously described, the initiator tRNA from "minus-folate" *S. faecalis* cells was extracted and purified. In the *in vitro* ribosomal binding assay, the authors showed that initiation factors stimulated the unformylated and the formylated form of this Met-tRNA$_f^{Met}$ to the same degree. (This IF-stimulated binding was seen at Mg^{++} concentrations up to 30 mM.)

Together these results indicate that although the formylation may increase the rate of interaction of the initiator tRNA with ribosomes, the enzymatic binding of Met-tRNA$_f^{Met}$ to ribosomes or ribosomal subunits does not absolutely require formylation.

More recently, Rich and collaborators have used the single-strand specific endonuclease S1 to study structural characteristics of initiator tRNAs compared to elongator tRNAs (Wrede *et al.*, 1979). They found that S1 cleaved at two distinct sites in the anticodon loop of three different initiator tRNAs (from *E. coli,* yeast and mammalian), whereas elongator tRNAs were cut at four sites. The authors suggest that the specificity is caused by the three G-C base-pairs of the anticodon arm (see Fig. 5), which are common in the initiator tRNAs and different from the elongator tRNAs included in the experiments by Rich & Wrede. However the initiator tRNAs in Bean chloroplasts and in yeast mitochondria contain only two G-C base pairs in these positions. The nucleotide at position 29 is here A in stad of the G in *E. coli* tRNA$_f^{Met}$ (Fig. 5A). Furthermore, at least two examples are known of elongator tRNAs which contain three G-C base pairs at position 29-41, 30-40 and 31-39, namely tRNA^{Met} from Bovine liver mitochondria and tRNA^{Ser} from *Halobacterium vol.* (Sprinzl & Gauss, 1984). Therefor it seems clear that no single local nucleotide sequence in the anticodon stem of the initiator tRNA$_f^{Met}$ is determining its specific function. Indeed as it will be discussed in later paragraphs, there are indications that unformylated Met-tRNA$_f^{Met}$ can function as an elongator tRNA.

As it seems unlikely that formylation should cause a drastic change in

the structure of the anticodon stem, the results of Rich *et al.* are in agreement with our results presented in the following sections, that formylation is not the determining factor but that the tertiary structure of the initiator tRNA most probably is one of the most important features in the functional specificity of fMet-tRNA$_f^{Met}$. We have studied the accessibility of different regions of tRNA$_f^{Met}$ and tRNA$_m^{Met}$ to a number of ribonucleases and compared the results in the presence and absence of proteins. These results will be discussed in detail in subsequent paragraphs. Other important elements which will be discussed in the following is the presence *in vivo* of translation factors. The fact that unformylated Met-tRNA$_f^{Met}$ binds to the elongation factor EF-Tu (see later) indicates that at extreme growth conditions, some of the initiator tRNAs might in fact function in the elongation step of translation.

Buckingham and collaborators have found that initiation factors stimulate the binding of formylated or acetylated Trp-tRNA^{Trp} to 30S or 70S ribosomes in the presence of poly(U,G). A number of other tRNAs have been tested for similar initiator activity with negative results (Leon *et al.*, 1979). These results indicate some structural similarity between tRNA$_f^{Met}$ and tRNA^{trp}, although such similarity does not appear from a comparison of the nucleotide sequences of the two tRNAs. One point which may seem rather odd is that these two tRNAs, alone among the 21 different amino acid acceptors, do not show degeneracy in their corresponding codons, namely AUG and UGG respectively, are codons for tRNA$_f^{Met}$ and tRNA^{Trp} (with the exception that the valine codon GUG and the normal codon for isoleucine AUU in a few cases have been found as initiator codons).

Finally, the amino acid part of fMet-tRNA$_f^{Met}$ seems to be of minor importance for the functional specificity as initiator tRNA. Giegé *et al.* (1973) showed that the methionine of fMet-tRNA$_f^{Met}$ could be replaced by valine or phenylalanine without any change in the initiation factor-stimulated binding to ribosomes. The puromycin reactivity too was unchanged by the mischarged f-aa-tRNA$_f^{Met}$s.

As a conclusion, it seems that the ribosome-mRNA interaction with the initiator tRNA must be considered when determining the specificity of a tRNA as initiator. The proper selection of the initiator tRNA may at least in a final step be controlled by the initiator codon of the mRNA, and although formyl-Trp-tRNA^{Trp} can bind to the P-site of the 30S or 70S ribosome *(in vitro)*, the use as initiator tRNA *in vivo* is prevented by the lack of formylation and by the mis-matching of the tRNA^{Trp} anticodon and the initiator codon of the mRNA.

So the total recognition between the components in the initiation complex is most probably a combination of the mRNA-aa-tRNA, the mRNA-30S, the aa-tRNA-IF2 and the 30S-aa-tRNA interactions, and although one or more of these interactions might proceed by species other than (f)Met-$tRNA_f^{Met}$, the final complex will not be stable in such cases.

A summary of the reactions involving the initiator $tRNA_f^{Met}$ during the initiation of translation

A short summary of the present view of the mechanism of polypeptide chain initiation follows. Fig. 8 is a schematic representation of the steps involved in the initiation process (Petersen *et al.*, 1984b).

After the aminoacylation (reaction 1+2) and formylation (reaction 3) of $tRNA_f^{Met}$, a pre-initiation complex between fMet-$tRNA_f^{Met}$ and initiation factor IF2 is formed (reaction 4). GTP may be involved in the formation of a ternary complex fMet-$tRNA_f^{Met}$:IF2:GTP, but no direct evidence for such a complex has yet appeared; a later section discusses this subject in more detail.

The next step involves the ribosome and in particular the 30S ribosomal subunit. The 70S particle exists in equilibrium with the 30S and 50S subunits (reaction 11). *In vitro* experiments have shown that temperature, pH and especially ionic conditions are parameters of importance for this equilibrium. Thus increased concentration of potassium or decreased concentration of magnesium promotes dissociation (see also Fig. 10). However, in the cell, the equilibrium is supposed to be controlled by special proteins, the initiation factors IF1 and IF3. IF1 increases the rates of dissociation and association, and IF3 prevents reassociation by binding to the 30S subunit. IF3 is thus acting as an anti-association factor, and in the presence of IF3, IF1 acts as a dissociation factor, thus in collaboration, the two factors shift the equilibrium towards the 30S + 50S state (Grunberg-Manago, 1980).

The sequence in the following steps, the binding of mRNA and fMet-$tRNA_f^{Met}$ to the 30S subunit, is not known. There exist indications for both molecules being bound prior to the other. Therefore, in Fig. 8, the binding of both components is shown simultaneously.

In the review previously mentioned, Watson (1964) introduced the concepts of two distinct ribosomal sites for interaction with mRNA and aminoacyl-tRNA – now called the P- and A-sites. The initiator region

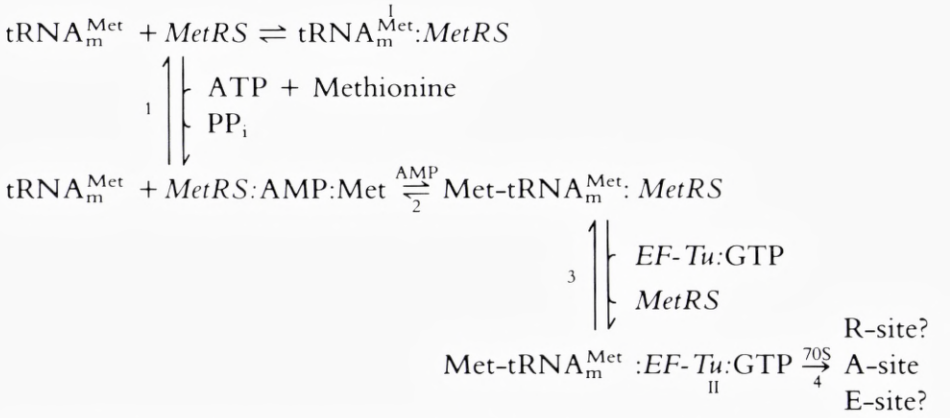


Fig. 8. Principal steps in the initiation of prokaryotic translation. Reactions involving the initiator tRNA$_f^{Met}$.

(the nucleotide sequence preceding and including the initiator AUG codon) of the mRNA is supposed to form a varying number of base-pairs with the 3'-OH terminal region of the 16S ribosomal RNA (Shine & Dalgarno, 1975), similarly a part of the fMet-tRNA$_f^{Met}$ molecule, including in particular the anticodon region, binds to the 30S ribosome. Both these interactions take place at the P-site. The P-site is thus a functional as well as a structural definition.

The implication of the P-site and the role of formylation of Met-tRNA$_f^{Met}$ in the formation of the 30S initiation complex will be discussed in more detail in the subsequent sections.

The last step in the formation of the functional initiation complex is the association of the 50S subunit with the 30S initiation complex (reaction 6). During this step, GTP is hydrolyzed, and the three initiation factors are released. The role of the GTP hydrolysis is not clear, but it seems reasonable that a final correct positioning of the four macromolecular components in the initiation complex requires a conformational adjustment which could be energy-requiring. Other molecules may play an important role in the regulation of the initiation-complex formation as for instance the ribosomal protein S1; this is currently under investigation in a number of laboratories, and is therefore not included in this short review of the initiation mechanisms.

In addition to the described classical mechanism of initiation, Fig. 8 shows reactions involving Met-tRNA$_f^{Met}$ or fMet-tRNA$_f^{Met}$, which have

Table 1: Functional differences between $tRNA_f^{Met}$ and other tRNAs

	Codon specificity	Interaction with proteins and ribosomes					
		Met-tRNA synthetase (E.C.6.1.1.10)	Transformylase* (E.C.2.1.2.9)	Initiation factor IF2	Elongation factor EF-Tu	70S ribosomal A-site	70S ribosomal P-site
$tRNA_f^{Met}$	AUG $(GUG)^{\cdot\cdot\#}$, $(AUU)^{\#}$, $(UUG)^{\cdot\cdot}$	⊕	+	⊕	⊕	±	⊕
$tRNA_m^{Met}$	AUG	⊕	–	–	⊕	+	–
other tRNAs	not AUG	–	–	–	+	+	–

* after charging with the cognate amino acid

$\cdot\cdot$ shown to stimulate Met-$tRNA_f^{Met}$ binding to ribosomes *in vitro* (Clark & Marcker, 1966b)

\# found as initiator codon in mRNA (Steitz, 1980; Sacerdot *et al.*, 1982).

⊕ complexes discussed in this paper.

only recently been investigated. Such studies are partly the subject of the following sections. The functional differences between $tRNA_f^{Met}$ and other tRNAs are shown in Table 1. The proteins and ribosomal interactions involving the different tRNAs are indicated with encircled symbols at complexes which are discussed in the subsequent sections.

In vitro studies of the 30S and 70S ribosomal Met-tRNA complex

The initiation of translation is believed to proceed via the formation of a complex between mRNA, 30S ribosomal subunit and formylmethionyl-$tRNA_f^{Met}$ as shown in Fig. 8. Because of the several exceptions to the general rule that the initiator tRNA methionine must be formylated, we wanted to study the *in vitro* formation of the 30S initiation complex. We have measured the binding of initiator Met-$tRNA_f^{Met}$, formylated and unformylated, to 30S ribosomal subunits, and we have studied the effects of different molecular components on this binding (Petersen *et al.*, 1976a).

Table 2 shows that crude initiation factor extracts stimulate the binding of both tRNA species in the presence of the synthetic messenger poly(A,G,U). In addition, it was shown that purified initiation factors

Table 2: Effect of different messengers on the binding of fMet-tRNA$_f^{Met}$, Met-tRNA$_f^{Met}$, and Met-tRNA$_m^{Met}$ to 30S ribosomal subunits in the absence and in the presence of initiation factors and GTP

				Relative molar binding to 30S ribosomal subunits		
Messenger	GTP	Crude IF	[Mg^{++}], mM	fMet-tRNA$_f^{Met}$	Met-tRNA$_f^{Met}$	Met-tRNA$_m^{Met}$
No	+	–	5	1	3	2
	+	+	5	4	2	1
ApUpG	+	–	5	3	2	2
	+	+	5	27	7	3
R17RNA	+	–	5	1	6	1
	+	+	5	74	13	1
Poly(A,G,U)	+	–	5	21	17	3
	+	+	5	183	35	8
	–	–	5	12	18	
	–	+	5	112	68	
	–	–	15	46	45	
	–	+	15	127	107	
	–	–	35	49	52	
	–	+	35	117	95	

(Petersen *et al.* 1976a).

had the same effect. This stimulation also exists in the presence of the trinucleotide codon for methionine ApUpG and in the presence of the natural messenger RNA from phage R17. No significant binding of the elongator Met-tRNA$_m^{Met}$ is found under any of these conditions.

Subsequently, preassociated 70S (tight couples) ribosomes were used in similar binding experiments, and as seen in Table 3, unformylated initiator Met-tRNA$_f^{Met}$ binds to 70S ribosomes in the absence of initiation factors. At low magnesium concentration, this binding is almost completely inhibited when initation factors are present during the incubation.

Contrastingly, formyl-Met-tRNA$_f^{Met}$ does not bind significantly to 70S ribosomes at low magnesium concentration. The addition of initiation factors shows the well-known stimulation of the binding of fMet-tRNA$_f^{Met}$.

Furthermore, Tables 2 and 3 show that GTP inhibits the binding of the unformylated initiator tRNA to both 30S and 70S ribosomes, both in the

Table 3: Effect of GTP on the binding of Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ to 70S ribosomes in the absence and in the presence of initiation factors

Addition	Crude IF	pmol	
		Met-tRNA$_f^{Met}$	fMet-tRNA$_f^{Met}$
none	–	1.89	0.15
GTP	–	1.64	0.50
none	+	0.07	0.24
GTP	+	0.04	1.85

Incubation mixtures contained: 3.8 pmol of Met-tRNA$_f^{Met}$ or 2.8 pmol of fMet-tRNA$_f^{Met}$; 15 pmol of 70S ribosomes and, where indicated, 1 mM GTP and 49 μg of crude IF. Magnesium acetate, Tris-HCl buffer, ammonium chloride, and poly(A,G,U) were in the amounts described in the legend to figure 9 (Petersen *et al.*, 1976b).

absence and in the presence of initiation factors, whereas this highenergy nucleoside triphosphate, as is well-established, stimulates the binding of fMet-tRNA$_f^{Met}$ in all cases. This may indicate that GTP binds directly to the 30S subunit and not through a complex with IF2.

To test whether the Met-tRNA$_f^{Met}$ bound to 30S ribosomal subunits is bound at the ribosomal P-site, 50S subunits were added after the binding to 30S, and subsequently puromycin was added. This antibiotic resembles the 3'-end of aminoacyl-tRNA and reacts with aminoacyl or peptidyl groups of P-site bound aa-tRNA or peptidyl-tRNA.

The results are seen in Figure 9 which shows that at least a part of the Met-tRNA$_f^{Met}$ which is bound to 30S ribosomal subunits is bound at a site which after addition of the 50S ribosomal subunit corresponds to the ribosomal P-site. About 50% of the totally bound Met-tRNA$_f^{Met}$ transfer methionine to puromycin.

The results show that formylation does not seem to be needed for the formation of a 30S initiation complex. Unformylated Met-tRNA$_f^{Met}$ is recognized by initiation factors in the presence of 30S ribosomal subunits and bound like fMet-tRNA$_f^{Met}$ at a puromycin reactive site (measured after addition of 50S ribosomal subunits).

As it will be discussed in a later section, this recognition may be caused by ribosome-bound initiation factors as free IF2 does not seem to have any significant affinity to Met-tRNA$_f^{Met}$.

This indicates that it is the structure of the initator tRNA$_f^{Met}$ – and not the formyl group – which is recognized by the 30S ribosomal subunit in the presence of initiation factors.

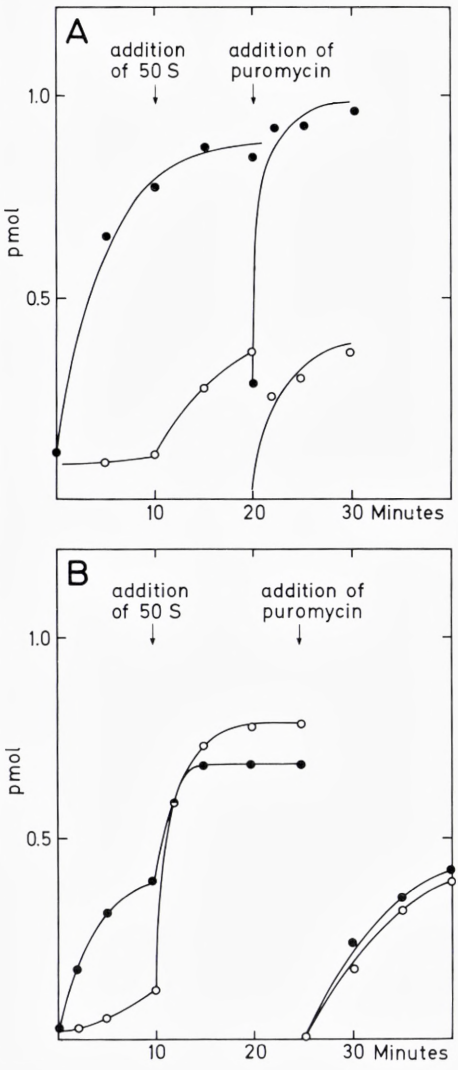


Fig. 9A. Binding of fMet-tRNA$_f^{Met}$ to 30S ribosomal subunits, effect of 50S subunits, and puromycin sensitivity in the absence (○) and presence (●) of initiation factors. Incubation mixtures (50 μl) contained, at time 0, 15 pmol of 30S subunits, 50 mM Tris-HCl (pH 7.4), 0.11 A_{260} unit of poly(A,G,U), 5 mM magnesium acetate, 50 mM ammonium chloride, 1 mM GTP and 2.1 pmol fMet-tRNA$_f^{Met}$, and where indicated, 26 μg of crude initiation factor extract. After 10 min incubation at 37°C, 15 pmol of 50S ribosomal subunits were added. After an additional 10 min incubation, 5 μl of puromycin (5 μg/μl) were added. The amount of fMet-tRNA$_f^{Met}$ bound to ribosomes was measured by nitrocellulose filtration, and the amount of formylmethionine reacted with puromycin was measured by ethyl acetate extraction.

B. Binding of Met-tRNA$_f^{Met}$ to 30S ribosomal subunits, effect of 50S subunits, and puromycin sensitivity in the absence (○) and presence (●) of initiation factors. Same incubation mixtures as in A, except 3.0 pmol of Met-tRNA$_f^{Met}$ were used instead of fMet-tRNA$_f^{Met}$, and no GTP was added. Incubation periods are indicated with arrows corresponding to the time of the various additions (Petersen et al., *1976a).*

The finding that formylation is not essential for the formation of the 30S initiation complex may explain why some *E. coli* mutants can grow in the presence of trimethoprim (which inhibits the enzyme dihydrofolate reductase and thus the formation of the formyl donor ^{10}N-formyltetrahydrofolate (see previous section)).

Nevertheless, the initiator Met-tRNA$_f^{Met}$ has been found to be entirely formylated in almost all *E. coli* cells. What then is the role of the formylation? One explanation is that the N-blocking of the initiator tRNA methionine speeds up the rate of formation of the initiation complex as

suggested by Bretscher & Marcker (1966). A second or additional possible explanation arises from our finding that only the formylated Met-tRNA$_f^{Met}$ binds to 70S ribosomes in the presence of the initiation factors which were discovered after Bretscher's and Marcker's experiments.

Thus, the formyl group is necessary in cases where initiation of translation proceeds via the (untraditional) formation of a 70S initiation complex without a previous involvement of 30S ribosomal subunits. This possibility is investigated in the following section.

A two-state model for the 70S ribosome

In the preceding section it was shown that the 30S ribosomal subunit is apparently not able to distinguish between formylated and unformylated initiator Met-tRNA$_f^{Met}$, whereas the 70S ribosome discriminates strongly in favour of the formylated species in the presence of initiation factors (which is relevant to the situation *in vivo*) but – also in the absence of initiation factors – it was found that the 70S ribosome can distinguish between the two initiator tRNA species.

The behaviour of the 70S ribosome in this binding reaction has been studied in more detail, as it seems to possess the property to select the N-formylated initiator Met-tRNA$_f^{Met}$ (Petersen *et al.*, 1976b).

The ability of the 70S ribosome to discriminate between Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ is found to be particularly strong at low

Table 4: Effect of potassium and magnesium ion concentration on the binding of fMet-tRNA$_f^{Met}$ and Met-tRNA$_f^{Met}$ to 70S ribosomes.

Potassium conc. (mM)	Magnesium conc. (mM)	fMet-tRNA$_f^{Met}$ (pmol)	Met-tRNA$_f^{Met}$ (pmol)
0	5	0.22	1.29
0	20	1.16	1.33
0	35	1.29	1.11
100	20	0.99	1.20
200	20	0.81	1.23
400	5	0.06	0.18
400	20	0.69	1.21
400	35	1.06	1.02

Incubation for 20 min at 37°C of the following mixture (50 µl): 15 pmoles of 70S ribosomes; 0.11 A_{260} unit of poly(A,G,U); 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$_f^{Met}$ or 3.8 pmol of Met-tRNA$_f^{Met}$. The amount of tRNA bound was analyzed by the Millipore filter assay (Petersen et al., 1976b).

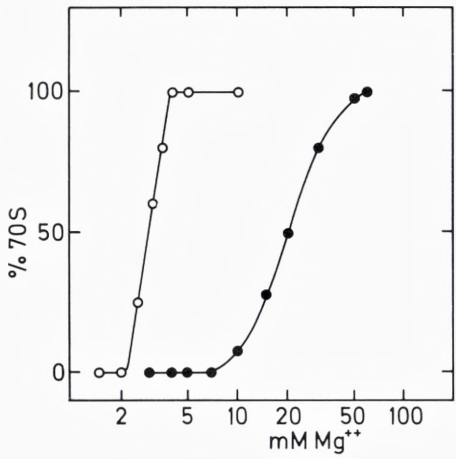


Fig. 10. The equilibrium between 70S ribosomes and 30S + 50S subunits measured by light-scattering at 24°C as a function of magnesium concentration in the absence of potassium (○) and at 400 mM (●) (Petersen et al., 1976b).

magnesium concentration (around 5 mM), whereas it disappears when the Mg^{++} concentration is increased to 20-35 mM (Table 4). It was also found (especially at low Mg^{++} concentrations) that high concentration of potassium (400 mM) strongly inhibits the binding of both initiator tRNA species to the ribosomes. The dissociation curve of ribosomes as a function of magnesium concentration (Fig. 10) shows that the ribosomes are 100% associated at 5 mM Mg^{++} in the absence of potassium (but in the presence of 50 mM ammonium chloride), whereas the ribosomes at 400 mM K^+ and 5 mM Mg^{++} are 100% dissociated. Thus the effect of potassium is in agreement with the results shown in the previous paragraph, neither of the two initiator tRNAs binds to ribosomal subunits in the absence of initiation factors.

Met-$tRNA_f^{Met}$ first bound to 30S subunits can react with puromycin after addition of 50S subunits (Fig. 9). A similar puromycin reaction is not possible for the Met-$tRNA_f^{Met}$ bound directly to the 70S ribosomes in the absence of initiation factors and at low concentrations of K^+ and Mg^{++} (50 mM and 5 mM, respectively). However, when the concentrations of these ions are increased to 400 mM and 35 mM, respectively (almost completely associated ribosomes), the Met-$tRNA_f^{Met}$ binds to a puromycin reactive site on the ribosome (Fig. 11).

The assay for the binding reaction requires filtration on nitrocellulose filters and therefore a reasonably high affinity between the compounds we want to isolate in complexed form, whereas the puromycin reaction takes place on the ribosome and liberates into solution the puromycin covalently bound to the radioactive amino acid, and the reaction product is easily extracted by ethylacetate. Thus, a much weaker binding to the

Table 5: Effect of messenger RNA (poly(A,G,U)) on the binding and puromycin reaction of fMet-tRNA$_f^{Met}$ and Met-tRNA$_f^{Met}$

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

Same incubation mixture as in Table 4 except that GTP was omitted; 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 (Petersen *et al.*, 1976b).

ribosome of the Met-tRNA$_f^{Met}$ can be detected by the puromycin reaction compared to the "binding assay". It was therefore interesting to compare the effect of mRNA (synthetic poly(A,G,U)) in the two assays. As shown in Table 2, the binding of Met- and fMet-tRNA$_f^{Met}$ to 30S subunits in the presence of initiation factors depends completely on the presence of a messenger RNA. Table 5 shows that the binding to 70S ribosomes also is messenger-dependent, whereas the puromycin reactivity is found in the total absence of messenger.

It is also shown that the binding of Met-tRNA$_f^{Met}$ as well as fMet-tRNA$_f^{Met}$ is uninfluenced by tetracyclin, an antibiotic which at low con-

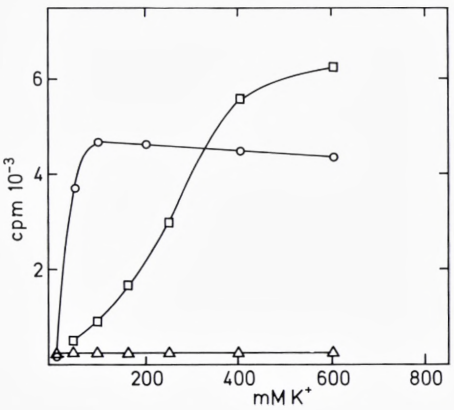


Fig. 11. Puromycin reaction of prebound initiator tRNA as a function of potassium concentration. The incubation mixture contained: 16 pmol of 70S ribosomes, 50 mM Tris-HCl (pH 7.4), 1 mM GTP, 35 mM magnesium acetate and 4.2 pmol of fMet-tRNA$_f^{Met}$ (○), 3.8 pmol of Met-tRNA$_f^{Met}$ (□) or 4.6 pmol of Met-tRNA$_m^{Met}$ (△). The reaction products were analyzed as described in the legend to Figure 9 (Petersen et al., *1976b).*

Table 6: Effect of tetracyclin on the binding of fMet-tRNA$_f^{Met}$, Met-tRNA$_f^{Met}$ and Met-tRNA$_m^{Met}$ to 70S ribosomes

	Translation Factors	tetracyclin	tRNA bound (pmol)
fMet-tRNA$_f^{Met}$	none	–	0.55
"	"	+	0.61
"	crude IF	–	1.44
"	"	+	1.47
Met-tRNA$_f^{Met}$	none	–	1.96
"	"	+	2.20
"	crude IF	–	0.39
"	"	+	0.52
Met-tRNA$_m^{Met}$	none	–	0.42
"	"	+	0.42
"	EF-Tu	–	0.84
"	"	+	0.40

Incubation mixtures were as in Table 4, 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 EF-Tu; 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$_m^{Met}$ (Petersen *et al.*, 1976b).

centrations inhibits the binding of aminoacyl-tRNA to the ribosomal A-site (Table 6).

Parallel experiments were made with the elongator Met-tRNA$_m^{Met}$. In no case was this tRNA bound in response to initiation factors or showed any reaction with puromycin.

Thus, the 70S ribosome is able to distinguish between the initiator Met-tRNA$_f^{Met}$ and the elongator Met-tRNA$_m^{Met}$. In the absence of initiation factors, the initiator Met-tRNA$_f^{Met}$ binds very well to 70S ribosomes and can be triggered to react with puromycin. Under these conditions the elongator tRNA$_m^{Met}$ does not bind to the ribosome and reacts under no conditions with puromycin. Furthermore, the results show that 70S ribosomes discriminate strongly between formylated and unformylated initiator Met-tRNA$_f^{Met}$ both in the absence and in the presence of initiation factors. Contrary to the unformylated initiator tRNA, fMet-tRNA$_f^{Met}$ does not bind to 70S ribosomes in the absence of initiation factors. The amount of Met-tRNA$_f^{Met}$ bound in the absence of initiation factors is unaffected by tetracyclin.

This raises the following question:

1: Do two different 70S ribosomal sites exist for binding of fMet-$tRNA_f^{Met}$ and Met-$tRNA_f^{Met}$, respectively, or

2: Do two different conformations exist of the ribosomal P-site – the initiator tRNA binding site?

The latter is most likely because: The Met-$tRNA_f^{Met}$ does not bind to the A-site (no effect of tetracyclin) but can be triggered to react with puromycin (the definition of P-site bound aa-tRNA).

Therefore, we propose the existence of the equilibrium:

$$70S \rightleftharpoons 70S^{\star}$$

between a 70S ribosome containing a P-site with inactive peptidyl transferase and a 70S★ ribosome with an active peptidyl transferase (or a P★-site).

This also suggests a function of the formylation of the initiator tRNA as fMet-$tRNA_f^{Met}$ only binds to the active P★-site and thus pulls the equilibrium towards the active conformation, whereas Met-$tRNA_f^{Met}$ binds equally well to both conformations and thus does not change the equilibrium. Fig. 12 shows how Met-$tRNA_f^{Met}$ (M) and fMet-$tRNA_f^{Met}$ (FM) are supposed to bind to the two conformations at different conditions.

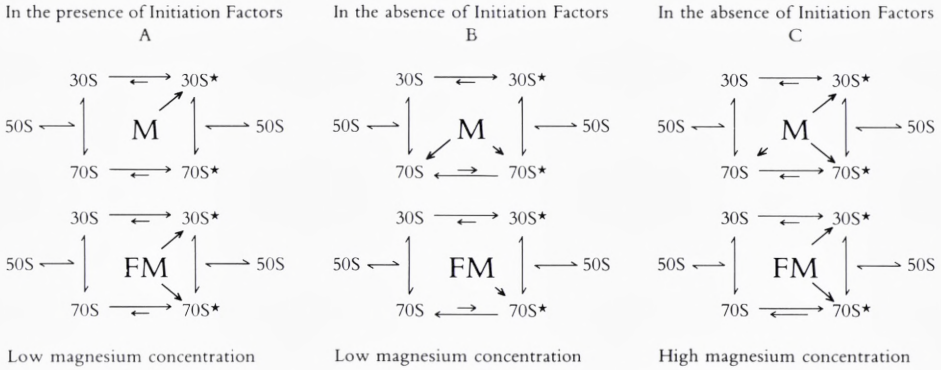


Fig. 12. A schematical presentation of how the position of the 30S and 70S ribosomal initiator tRNA binding site conformational equilibrium is supposed to be at low magnesium concentration (5 mM) (A and B), and high magnesium concentration (35 mM) (C) in the presence (A) and absence (B and C) of initiation factors. The length of the arrows from each initiator tRNA species indicates the relative affinity for the different ribosomal conformations, and the sum of arrows from one tRNA indicates the total binding at the respective conditions. M: Met-$tRNA_f^{Met}$, FM: fMet-$tRNA_f^{Met}$ (Petersen, 1980).

TP9 – a puromycin resistant E. coli *mutant*

As a consequence of the proposed hypothesis that the ribosomal P-site exists in two conformations, one would expect the equilibrium between these two conformations to be sensitive to mutations in the ribosomal components involved in the tRNA binding area. We know that puromycin binds to the ribosome in the region between the A-site and the P-site (Cooperman, 1980), and it would therefore be expected that bacterial strains permeable for but resistant to puromycin would be affected in the $70S \rightleftharpoons 70S^{\star}$ equilibrium.

From the parental strain DL1 (a puromycin sensitive strain of *E. coli* K12), Dr. Antoine Danchin, Institut Pasteur, Paris, isolated a mutant (TP9) which was able to grow in broth medium supplied with the C-1 metabolites plus puromycin and trimethoprim.

In collaboration with Dr. Leif Isaksson at the Wallenberg laboratory, Uppsala, ribosomes isolated from TP9 were analyzed by two-dimensional polyacrylamide gel electrophoresis, and we found displaced positions of the ribosomal proteins S7, S20 (= L26) and L27. S7 is located at the tetracyclin binding site (A-site), and L27 is part of the peptidyl transferase centre and thus both the ribosomal A- and P-sites (Ofengand *et al.*, 1984). S20 is situated at the "head" of the 30S subunit and most probably at the interface between the two subunits (Stöffler *et al.*, 1979).

Ribosomes isolated from TP9 and DL1 were used in *in vitro* experiments where the kinetics of binding and puromycin reaction of Met-

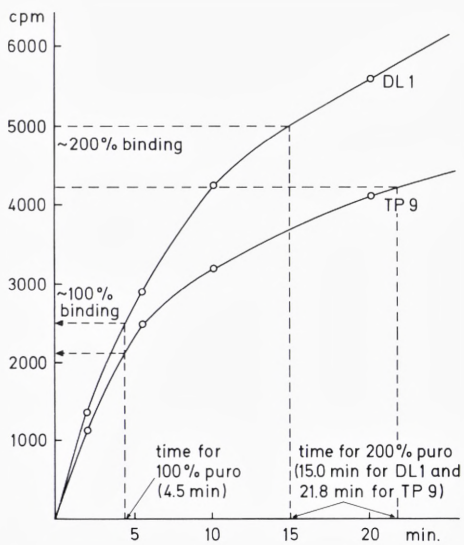


Fig. 13. Kinetics of the puromycin reaction of Met-tRNA$_f^{Met}$ bound to 70S ribosomes from wild type (DL1) and puromycin resistent (TP9) E. coli *cells. The curves show the amount of Met-puromycin formed as a function of time after 20 min binding incubation at 37°C in 50 mM Tris-HCl (pH 7.5), 35 mM magnesium chloride, 150 mM potassium chloride and 64 pmol of Met-tRNA$_f^{Met}$. The radioactivity corresponding to the amount of Met-tRNA$_f^{Met}$ bound to the ribosomes after 20 min is indicated (100%). Dotted lines indicate the time required for a puromycin reaction of twice the amount of Met-tRNA bound after 20 min (15.0 min for wild type and 21.8 min for TP9 ribosomes) (Petersen, 1980).*

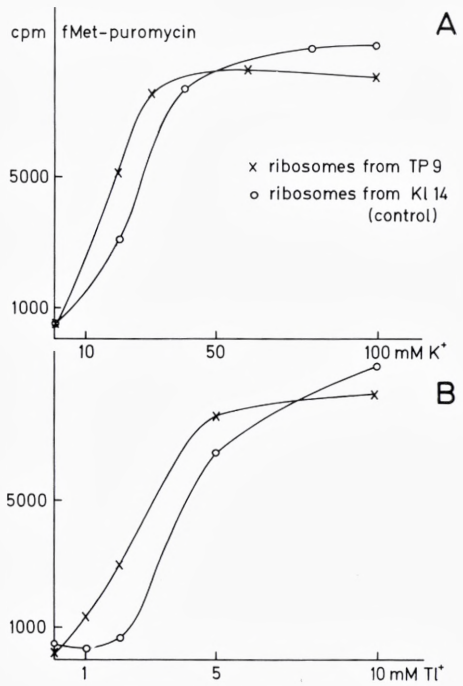


Fig. 14. Puromycin reaction of fMet-tRNA$_f^{Met}$ bound to wild type (Kl14) and TP9 ribosomes as a function of (A) potassium and (B) thallium concentration. Incubation conditions as described in the legend to Figure 13. Ethyl acetate extraction after 5 min puromycin reaction (Petersen, 1980).

tRNA$_f^{Met}$ were measured. No difference was found in the kinetics of the binding reaction, whereas a difference was observed in the puromycin reaction at prolonged time of incubation. As shown in Figure 13, the time needed to obtain a puromycin reaction corresponding to the amount of Met-tRNA$_f^{Met}$ bound is the same for the two types of ribosomes. A higher amount of puromycin reaction can be obtained if the incubation is continued, but in that case, the reaction proceeds much more slowly with the mutant ribosomes as compared to the control ribosomes.

This result may indicate that the mutation leading to puromycin resistance affects the ribosomal site of translocation in some so far unknown way.

The binding of fMet-tRNA$_f^{Met}$ to the wild type and mutant ribosomes was studied as a function of K^+ concentration (Figure 14.A). One sees the previously found sigmoidal shaped curve for the control ribosomes – indicating the double function of potassium ions in the 70S $\rightleftharpoons$ 70S* equilibrium and in the puromycin reaction, whereas the corresponding experiment with TP9 ribosomes gives a hyperbolic shaped binding curve. This indicates that with TP9 ribosomes, one of the two functions

of K^+ is suppressed – possibly the effect on the 70S $\rightleftharpoons$ 70S* equilibrium – as this may be strongly displaced toward the active form in the mutant.

As potassium ions may have a specific catalytic site at the peptidyl transferase center and not merely be required to obtain a certain ionic environment for the protein biosynthesis, we did similar experiments of binding and puromycin reaction replacing the potassium with varying concentrations of thallium, which is similar to K^+ with respect to stereochemical properties. Fig. 14B shows the results. To obtain similar amounts of fMet-puromycin, 10 times lower concentrations of Tl^+ as compared to K^+ is needed. This is a further indication of a specific ribosomal site for K^+ (Petersen, 1980).

Hypothesis on the role of the formyl group of fMet-tRNA$_f^{Met}$ in the translation of polycistronic messenger RNAs

Based on the *in vitro* experiments, a general function of the formyl group of fMet-tRNA$_f^{Met}$ in initiation of translation has been proposed (Petersen *el al.,* 1976b). In prokaryotes, the traditional view of the mechanism of initiation involved the formation of a 30S initiation complex. As shown in Table 2, the 30S ribosomal subunit (even in the presence of initiation factors) cannot discriminate between formylated and unformylated initiator tRNA, whereas in the presence of initiation factors, only the formylated species binds to 70S ribosomes (Table 3). Now, recall the situation in eukaryotic cells: 1) no formylation of the initiator Met-tRNA exists, and 2) no polycistronic mRNAs are known. Therefore, no initiation is possible at internal initiation sites in eukaryotic mRNAs.

In prokaryotes, the mRNAs commonly are polycistronic and one can imagine that the 5'-end proximal initation site always (as in eukaryotes) forms an initiation complex with 30S ribosomal subunits. This would not require formylation of initiator tRNA (although the rate of initiation is increased by the formylation which is in agreement with the fact that prokaryotic translation is 9-12 times faster than eukaryotic translation).

If the ribosome translating a polycistronic messenger does not dissociate into subunits after terminating the translation of the first cistron but as a 70S particle migrates through the intercistronic region of the mRNA (which varies considerably in length in different mRNAs), this 70S ribosome will be directly involved in the initiation complex formation at the next initiator codon. As only the formylated initiator tRNA binds to the

Table 7: Effect of trimethoprim, chloramphenicol and kasugamycin on the coordinate synthesis of β-galactosidase (Gz) and thiogalactoside acetyltransferase (Ac)

Strain	Addition (μg/ml)		Ratio (Gz/Ac)
CP78	None		1
	Trimethoprim	0.75	1.5
	Trimethoprim	1.5	2.4
	Chloramphenicol	1.5	1.06
	Chloramphenicol	2	1.14
	Kasugamycin	40	0.92
CP781a	None		1
	Trimethoprim	0.75	1.15
	Trimethoprim	1.5	1.2
	Chloramphenicol	1.5	1.1
	Chloramphenicol	2	1.2
	Kasugamycin	20	0.96

(Petersen *et al.*, 1978).

70S ribosome in the presence of initiation factors, the formylation is an absolute requirement in this situation.

From this hypothesis, one can predict that formylation of initiator tRNA methionine can be related to the polarity in the translation of polycistronic mRNAs in such a way that a decrease in the level of formylation under otherwise normal conditions will result in an increased polarity, 5'-end proximal cistrons being translated relatively more efficiently compared to 5'-end distal cistrons.

In vivo *polarity in lactose operon expression, role of formylation of initiator tRNA methionine*

It has been observed that *E. coli* mutants which were able to grow in the presence of low levels of trimethioprim – an inhibitor of formylation, see Figure 6 – contained an increased proportion of ribosomal subunits to 70S ribosomes as compared to wild type cells (Harvey, 1973). This is an indication that the cell can overcome the lack of formylation by increasing the proportion of 30S to 70S ribosomes, which is in agreement with the results that 30S and not 70S ribosomes are able to use unformylated Met-$tRNA_f^{Met}$ as initiator tRNA.

It is also expected from our hypothesis that depression of formylation

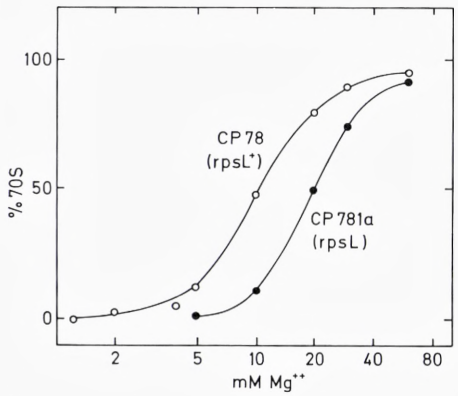


Fig. 15. Light-scattering measurement of the association of ribosomal subunits as a function of magnesium concentration at 150 mM potassium chloride and at 37°C. Ribosomes from strain CP78 (rpsL$^+$) (○) and from strain CP781a (rpsL) (●) of E. coli *(Petersen* et al., *1978).*

in normal growing cells will affect the ratio between the efficiency of translation of the 5'-end proximal and distal cistrons of polycistronic mRNAs in such a way that lack of formylation would favour the synthesis of the first protein rather than later proteins from the operon.

This is tested by measuring the *in vivo* translation of the first and the last cistron of the lactose operon in *E. coli* – β-galactosidase and thiogalactoside acetyltransferase, respectively (Petersen *et al.*, 1978). We have studied the effect of inhibition of formylation of initiator tRNA methionine on the relative synthesis of these two proteins in wild type cells and in *E. coli* mutants resistant to streptomycin and trimetroprim.

The presence of trimethoprim in the growth medium has a significant effect on the differential translation of the first and last cistrons of the lactose operon in *E. coli* wild type cells. This inhibitor of formylation results in a two-fold increase in the ratio: β-galactosidase/thiogalactoside acetyltransferase synthesized in wild type cells, whereas no significant effect was found in streptomycin resistant strains (Table 7). Control experiments with other antibiotic inhibitors (kasugamycin and chloramphenicol – which are inhibitors of initiation of translation without affecting the formylation of initiator tRNA methionine) showed that these compounds had no effect on the relative expression of the different cistrons in any of the tested strains.

Ribosomes were extracted from all tested strains and dissociation curves were measured. This showed that at conditions of pH, temperature and concentrations of mono- and divalent cations comparable to those found *in vivo,* the ribosomes from the mutant cells were significantly more dissociated than those from wild type cells (Fig. 15).

These results are consistent with the hypothesis that a significant part

of the ribosomes may pass through the intercistronic region from the previous termination step to the following initiation step without dissociating off the messenger. This could be the case when an unlimited amount of fMet-tRNA$_f^{Met}$ is available for the initiation, whereas the ribosome must dissociate and use a 30S subunit for the following initiation at low levels of formylation.

Our suggested mechanism also explains why, in eukaryotic cytoplasm, no formylation of initiator tRNA methionine is needed – as no cellular polycistronic mRNAs apparently exist.

Structural elements in initiator tRNA involved in specific interactions with proteins

As a consequence of the results discussed above, we have been interested in further investigation of the question: What are the specific structural elements in tRNA$_f^{Met}$ which are involved in and thus responsible for the different specific interactions with other macromolecules during the initiation process as shown schematically in Fig. 8? Recent developments in the methodology of nucleic acids research has made such investigations of molecular details possible.

In the following paragraphs, we will look a little more closely at the regions within the initiator tRNA$_f^{Met}$ which are implicated in these interactions. The effect of formylation is studied, and the results are compared to experiments with the elongator tRNA$_m^{Met}$ in the cases where the two tRNAs are involved in similar macromolecular interactions.

Two methods have been employed in these investigations: The footprinting method, in which ribonucleases are used for cutting the tRNA molecule at specific sites. The resulting RNA fragments are analyzed by polyacrylamide gel electrophoresis, which makes it possible to determine the exact cutting positions in the nucleotide sequence. Results from the treatment of free tRNA are compared to those from tRNA complexed to a protein molecule. In the case where different ribonucleases cut a particular position less efficiently in complexed tRNA as compared to free tRNA, it is concluded that this position is protected by the protein. The technical details of this method has been described recently (Petersen *et al.*, 1984c).

The second method is based on the lability of the aminoacyl-ester bond in aminoacyl-tRNAs. The reaction of hydrolysis of this linkage is

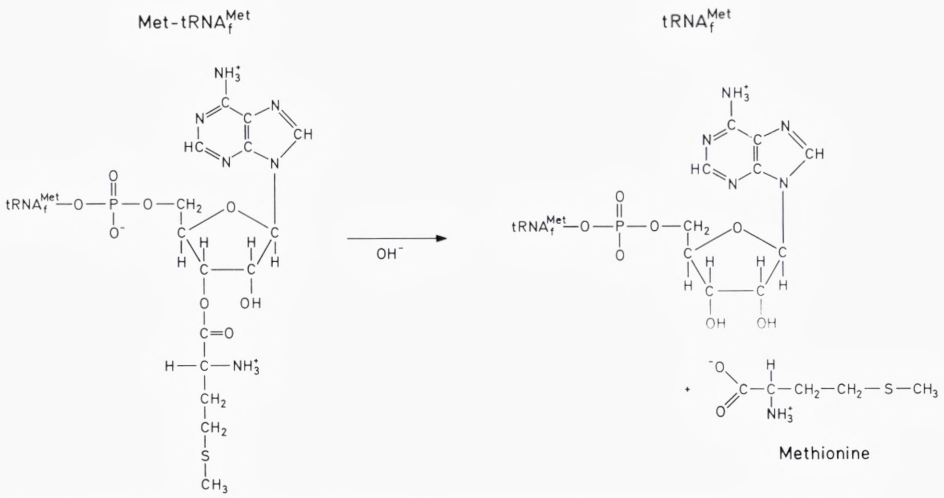


Fig. 16. Reaction of spontaneous hydrolysis of the aminoacyl ester bond in Met-tRNA$_f^{Met}$.

shown in Fig. 16. At neutral pH, the ester bond is hydrolyzed spontaneously at a rate which among other factors depends on the temperature. This method has been applied successfully in the study of interactions between elongation factor EF-Tu, GTP and different aminoacyl-tRNAs (Pingoud *et al.*, 1977). The rate of hydrolysis of fMet-tRNA$_f^{Met}$ or Met-tRNA$_f^{Met}$ is measured in the absence or presence of proteins. In the case where the halflife of the ester bond is increased by the addition of a protein, it is concluded that an interaction takes place between the two macromolecules and in particular that a closer contact must take place at the amino acid attachment site.

In the following, results are described from such studies on interactions with methionyl-tRNA synthetase, initiation factor IF2, elongation factor EF-Tu and the 70S ribosome.

Interaction with methionyl-tRNA synthetase (MetRS)

Some characteristic features of the structure of the initiator tRNA$_f^{Met}$ as compared to that of the elongator tRNA$_m^{Met}$ can be studied by comparing the footprinting results from complexes between methionyl-tRNA synthetase and the two tRNAs. As described in an earlier paragraph, the enzyme aminoacylates both tRNAs with methionine in the reactions 1 and 2 shown in Figs. 8 and 23. Although, as shown, the tRNA enzyme interaction *in vivo* takes place after the binding of a methionyl-adenylate

to the enzyme (which may change the conformation of the enzyme), a stable complex between $tRNA_f^{Met}$ or $tRNA_m^{Met}$ and MetRS can be formed and isolated *in vitro* in the absence of other ligands (complex I in Fig. 8 and complex I in Fig. 23).

Results from experiments using T1, T2 and cobra venom RNases on the binary complex enzyme:tRNA with each of the two tRNAs are shown in Figs. 17 and 18 (Petersen *et al.*, 1984a). Regions which are protected by MetRS are shown in black, whereas locations which seem more accessible for nucleases in the complexed tRNAs are shown hatched.

The 3'-side of the anticodon loop is markedly protected by MetRS in both tRNAs. Differences are seen in the 5'-side of the anticodon stem, which is protected in $tRNA_f^{Met}$, but cut more intensely in $tRNA_m^{Met}$ when complexed to MetRS. Alternatively, the D-loop and the extra-loop are

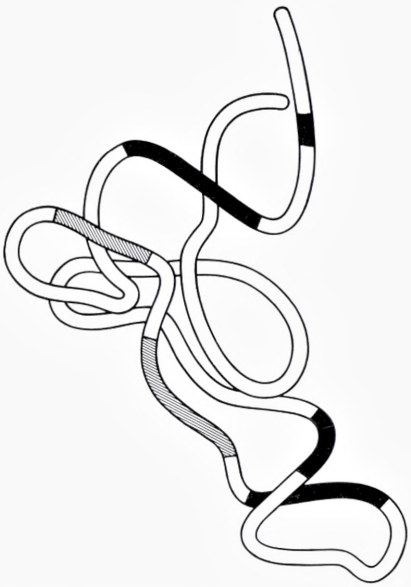


Fig. 17. Footprinting results from $tRNA_f^{Met}$ complexed to dimeric methionyl-tRNA synthetase are transferred to a tertiary structure model of $tRNA^{Phe}$ (see Fig. 3). Regions within $tRNA_f^{Met}$ which are protected (■) or cut more intensely by ribonucleases (▨) when complexed to MetRS are indicated (Petersen et al., *1984a).*

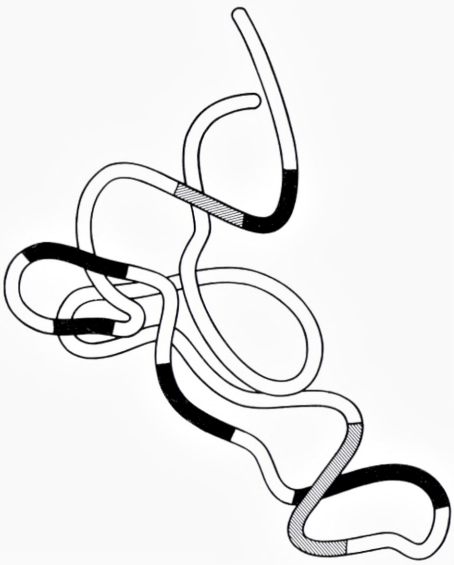


Fig. 18. Footprinting results from $tRNA_m^{Met}$ complexed to dimeric methionyl-tRNA synthetase are transferred to a tertiary structure model of $tRNA^{Phe}$ as described in the legend to Fig. 17 (Petersen et al., *1984a).*

protected in complexed $tRNA_m^{Met}$, whereas both these regions are cut more intensely by RNases in $tRNA_f^{Met}$ as a result of complex formation.

It is known that cobra venom ribonuclease cuts tRNA at many positions in the acceptor region (Boutorin *et al.,* 1981). MetRS shows only weak protection in the amino acid region. This supports the idea that (at least in the absence of the other substrates of the aminoacylation reaction), the acceptor region of tRNA is not strongly bound at the surface of the enzyme (Jacques & Blanquet, 1977).

Recent data indicate that also in the presence of methionine and ATP, no strong protection against cobra venom RNase digestion in the acceptor region takes place (H. U. Petersen, G. E. Siboska & S. Blanquet, unpublished). This result is in good agreement with the effect of MetRS on the spontaneous hydrolysis of the aminoacyl ester bond in fMet-$tRNA_f^{Met}$ in the presence of the initiation factor IF2, which is discussed in the next paragraph.

The interactions with the synthetase seem to be similar in the extreme parts (amino acid attachment site and anticodon region) of the tRNAs, whereas only $tRNA_m^{Met}$ seems to bind to the protein in the central part of the molecule. Thus, it can be concluded that the three-dimensional structures of the two molecules have differences in the region around the extra loop. In later paragraphs, it will be seen that the extra loop probably plays an important role for the specificity of the initiator tRNA.

Interaction with the initiation factor IF2

The initiation factor IF2 exists in 2 forms: IF2α (M_r 97300) and IF2β (M_r 79700), which are coded for by the same gene (Plumbridge *et al.,* 1985). The DNA sequence of this gene has recently been determined in the laboratory of Dr. M. Grunberg-Manago (Sacerdot *et al.,* 1984). As the available amounts of pure initiation factor protein has been used for functional studies and no attempt to crystallize the protein has been done, no information has been obtained on the higher order structure of these proteins. However, using small angle neutron scattering (at the equipment D11 of the Institut Laue-Langevin in Grenoble, France) we have determined the radius of gyration, R_g, of IF2 to be 45-48Å in solution, which indicates that the protein has a rather elongated shape (H. U. Petersen, M. Grunberg-Manago and B. Jacrot, unpublished).

Whilst it is well established that the protein chain elongation factor EF-Tu functions as an aminoacyl-tRNA carrier protein in a tertiary complex: EF-Tu:GTP:aa-tRNA during the elongation step of the prokaryotic translation, the question whether a similar complex is formed be-

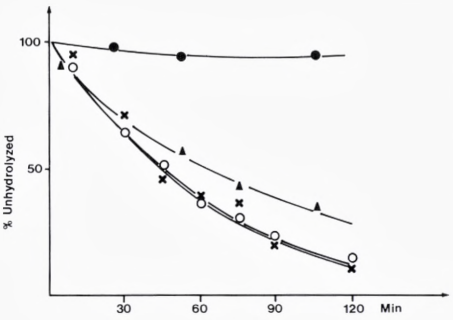


Fig. 19. Kinetics of the non-enzymatic hydrolysis of the aminoacyl ester bond of fMet-tRNA$_f^{Met}$ at 37°C in the absence (○) and presence (●) of initiation factor IF2 at 8 times molar excess, MetRS at 16 times molar excess (x) or both IF2 and MetRS at 8 and 16 times molar excess, respectively, (▲) (Petersen et al., *1984c).*

tween the initiation factor IF2, GTP and fMet-tRNA$_f^{Met}$ during chain initiation (complex II in Fig. 8) has been the subject of intense debate for more than a decade. Although many different chemical and physical methods have been applied in such investigations, a complex of native IF2, fMet-tRNA$_f^{Met}$ and GTP has never been isolated (Petersen *et al.*, 1979).

Previous studies have shown that IF2 invariably interacts with fMet-tRNA$_f^{Met}$. However, the extent of interaction with unformylated Met-tRNA$_f^{Met}$ varied from no detectable interaction to almost the same level as for the formylated species. Although it is clear that GTP hydrolysis is necessary for the formation of the functional 70S ribosomal initiation complex, no experiments have shown that GTP is required for IF2 to interact with the initiator tRNA. In addition, the ionic requirements for the formation of a binary complex IF2:fMet-tRNA$_f^{Met}$ has varied considerably. A general feature of all earlier experiments is the attempt to isolate a macromolecular complex, and such a complex may dissociate during the preparation, whereas the methods employed here do not require the isolation of a complex.

Fig. 19 shows the rate of hydrolysis of the aminoacyl-esterbond of fMet-tRNA$_f^{Met}$, free and in the presence of MetRS, IF2 or both proteins (Petersen *et al.*, 1984c). This shows that MetRS has no effect itself on the rate of hydrolysis. The lack of protective effect is an indication that MetRS does not bind strongly at the aminoacyl-linkage. It also shows that MetRS (in the absence of AMP) does not catalyze the de-aminoacylation. In the presence of IF2, complete protection is observed. When increasing amounts of MetRS are added in the presence of sufficient amount of IF2 to obtain such complete protection, the effect of IF2 disappears. This is taken as an indication that MetRS and IF2 have overlapping binding sites on the tRNA molecule and, thus, in this experi-

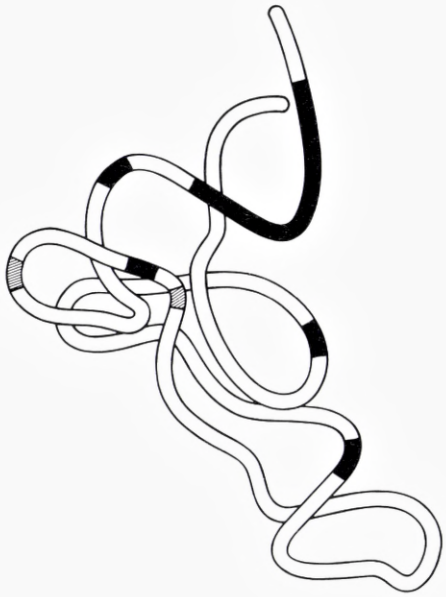


Fig. 20. Footprinting results from fMet-tRNA$_f^{Met}$ in the presence of initiation factor IF2 are transferred to a tertiary structure model of tRNA^{Phe} as described in the legend to Fig. 17 (Petersen et al., *1984c).*

ment, compete for the binding to this site. However, it is also clear that the binding site of MetRS is not the 3'-terminal part of fMet-tRNA$_f^{Met}$, which, on the other hand, seems to be one of the important binding sites for IF2.

Similar experiments were done using unformylated initiator Met-tRNA$_f^{Met}$ or elongator Met-tRNA$_m^{Met}$ (Petersen *et al.,* 1979). In both cases, no effect was observed on the rate of hydrolysis when increasing amounts of IF2 were added. Although this method does not exclude the possibility that the protein binds to the tRNA, it is clear that an interaction similar to the one found with fMet-tRNA$_f^{Met}$ does not take place.

The binding site of IF2 on fMet-tRNA$_f^{Met}$ has been further investigated by footprinting experiments (Petersen *et al.,* 1981). Fig. 20 shows the regions which are protected or cut more intensely in fMet-tRNA$_f^{Met}$ complexed to IF2. In this case, only the double strand specific ribonuclease isolated from the venom of *Naja oxiana* has been used to digest the tRNA.

The protected regions include the 3'-end, both sides of the T-stem, the anticodon stem (in particular the 5'-side) and the D-stem. It seems that the protected regions are mainly located at the extreme parts of the L-shaped tRNA molecule, and no protection is found in the extra loop. This result is very similar to the one found with MetRS except that IF2 seems to be in closer contact with the 3'-end of the tRNA.

Footprinting experiments were also done with unformylated initiator tRNA. However, IF2 had no effect on the results obtained with free Met-tRNA$_f^{Met}$. This is a further indication that no binding takes place between IF2 and Met-tRNA$_f^{Met}$ and supports the idea that formylation adds a signal to the initiator tRNA for the specific interaction with IF2 in the absence of ribosomes.

Interaction with the 70S ribosome

As shown in reaction 7 of Fig. 8 and as discussed in detail in previous paragraphs, fMet-tRNA$_f^{Met}$ can bind non-enzymatically to the ribosomal P-site at 15-20 mM magnesium ion concentration. The complex described here was formed in this way in the absence of initiation factors, using a poly(A,G,U) RNA chain as a messenger (Petersen *et al.*, 1984c). In order to ensure that no unbound tRNA is present in the footprinting study, the complex was isolated on a Sepharose 6B column prior to enzymic digestion. Fig. 21 shows the regions in the tRNA$_f^{Met}$, which are protected against ribonuclease (RNases T1 and T2) digestion in the initiation complex in the absence of initiation factors. These are seen to be located in the aminoacyl-stem, the variable loop and in the part of the anticodon stem close to the extra loop. When compared to the footprinting results with MetRS and IF2, this is the first time we observe a strong protection of tRNA$_f^{Met}$ in the extra loop. The involvement of the extra

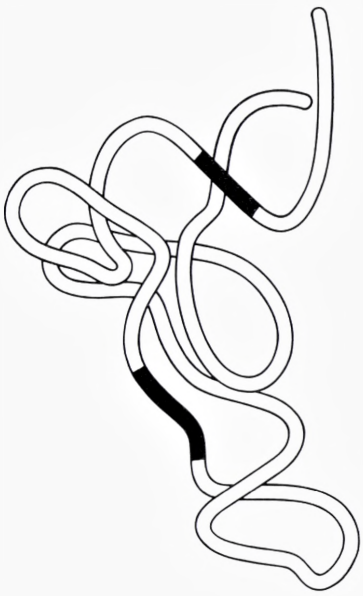


Fig. 21. Footprinting results from fMet-tRNA$_f^{Met}$ bound non-enzymatically to 70S ribosomes are transferred to a tertiary structure model of tRNA^{Phe} as described in the legend to Fig. 17 (Petersen et al., *1984c).*

loop in tRNA interaction with the 70S ribosome has also been observed for the A-site binding of $tRNA^{Phe}$ (Petersen *et al.*, 1984a), whereas other regions within the two tRNA molecules seem to have different degrees of involvement in A-site and P-site interactions (T. Jørgensen, personal communication). It must be noted that all the protected regions are found located at one side of the $tRNA_f^{Met}$ molecule, the side exposing the extra loop. Although it is too early to make a firm conclusion, this indicates the contact-face of the initiator tRNA when bound at the 70S ribosomal P-site.

Interaction with elongation factor EF-Tu

During the elongation steps of protein biosynthesis, aminoacyl-tRNAs form a ternary complex with the elongation factor EF-Tu and GTP. Elongation factor EF-Tu is an acidic protein consisting of 393 amino acids. The amino acid sequence has been determined in Aarhus (Jones *et al.*, 1980), where also a three-dimensional structure model of the molecule at 2.9 Å resolution has been obtained by X-ray crystallography (Morikawa *et al.*, 1978; Rubin *et al.*, 1981). Using the footprinting method, we have been investigating the interaction between this protein and different aminoacyl-tRNAs – in particular the elongator $tRNA_m^{Met}$ (Boutorin *et al.*, 1981; Wikman *et al.*, 1982). A summary of these results is seen in Fig. 22. In this case as well, all protected sites are found at one side of the $tRNA_m^{Met}$ molecule. Again, the extra loop seems involved in the interaction together with parts of the T-arm and the region near the amino acid attachment site.

As a comparison with $tRNA_f^{Met}$ (Fig. 8), the interactions involving $tRNA_m^{Met}$ prior to the binding at the ribosomal A-site in protein biosynthesis are shown schematically in Fig. 23. The aminoacylation is catalyzed by MetRS (Reactions 1 and 2), and the synthetase is probably displaced by EF-Tu:GTP during the formation of the ternary complex (Reaction 3). Subsequently, the Met-$tRNA_m^{Met}$ is bound at the 70S ribosomal A-site carried by EF-Tu (Reaction 4).

Until recently, it was believed that only $tRNA_m^{Met}$ and not $tRNA_f^{Met}$ could take part in these two last reactions (reactions 3 and 4 in Fig. 23) (Ofengand, 1977). However, new experiments based on the protection by EF-Tu against pancreatic RNase digestion of the (unformylated) initiator Met-$tRNA_f^{Met}$ revealed that a ternary complex Met-$tRNA_f^{Met}$:EF-Tu:GTP can actually be formed (Tanada *et al.*, 1982). This is shown as reaction 8 in Fig. 8. We have confirmed this observation by isolating a ternary complex Met-$tRNA_f^{Met}$: EF-Tu:GTP. In experiments similar to

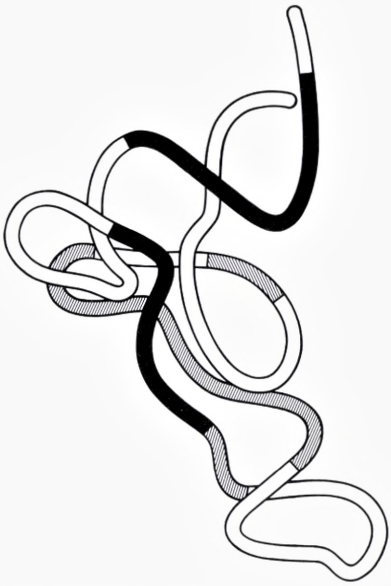


Fig. 22. Footprinting results from Met-tRNA$_m^{Met}$ complexed to the elongation factor EF-Tu and GTP are transferred to a tertiary structure model of tRNA^{Phe} as described in the legend to Fig. 17 (Wikman et al., *1982).*

those shown in Fig. 19, we obtained a strong protection against hydrolysis of Met-tRNA$_f^{Met}$ by addition of EF-Tu:GTP, whereas no effect is found on the rate of hydrolysis of fMet-tRNA$_f^{Met}$ (P. Kamp Hansen, H. U. Petersen, J. W. B. Hershey & B. F. C. Clark, unpublished).

Thus, EF-Tu:GTP in bacteria may well discriminate against only the species fMet-tRNA$_f^{Met}$. The formylation could help play a role as security against the Met-tRNA$_f^{Met}$ acting as an elongator and translating incor-

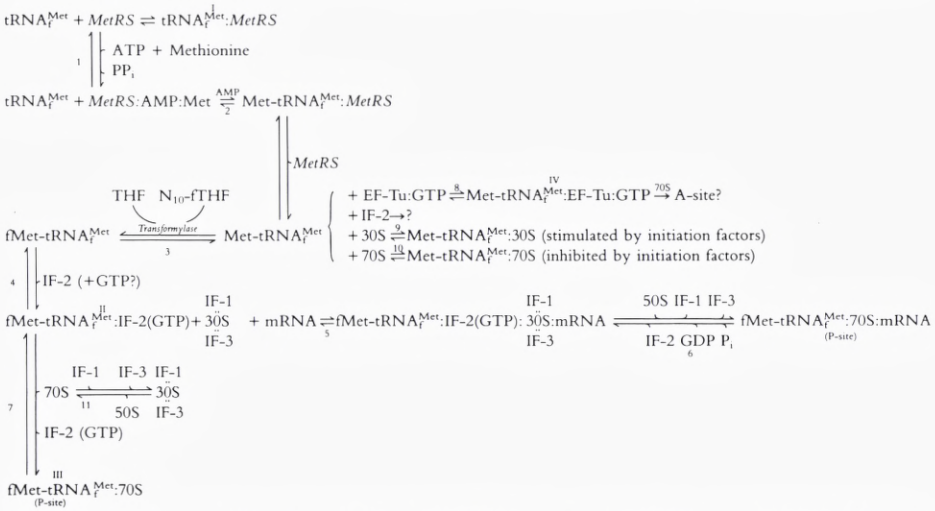


Fig. 23. Reactions involving the elongator tRNA$_m^{Met}$ prior to the binding at the 70S ribosomal A-site.

rectly at GUG and UUG, which the initiator species theoretically can do during initiation (Clark & Marcker, 1966b). What happens during eukaryotic protein biosynthesis in this connection is rather unclear, because the initiator species itself is not formylated and exists as Met-$tRNA_i^{Met}$. However, the whole selection of the initiator tRNA is more restricted in eukaryotes in the sense that more protein components are involved (Hershey *et al.*, 1984). Of course, during bacterial protein initiation, as discussed in previous sections, initiation factors play a significant role in placing the initiator tRNA in the correct P-site for initiation. More detailed information about this interaction of the initiator tRNA were obtained by footprinting studies on the complex similar to those described above for elongator tRNA. Our results (Clark *et al.*, 1984) are shown in Fig. 24. Although the studies have not been so extensive, we observed a similar general pattern of protection by EF-Tu:GTP against nuclease cutting of the initiator tRNA as for the elongator $tRNA_m^{Met}$ (Fig. 22) but with some small differences.

Again, we see protection in the aa-stem, T-stem and extra loop. In addition, the protection runs into the T-loop on both sides where we saw no changes for the elongator tRNA.

We are not able to decide at this time whether this should be interpreted in terms of extra covering by EF-Tu:GTP of the tRNA or steric hindrance due to local conformational changes.

What appears to be a reasonable conclusion is that bacterial Met-

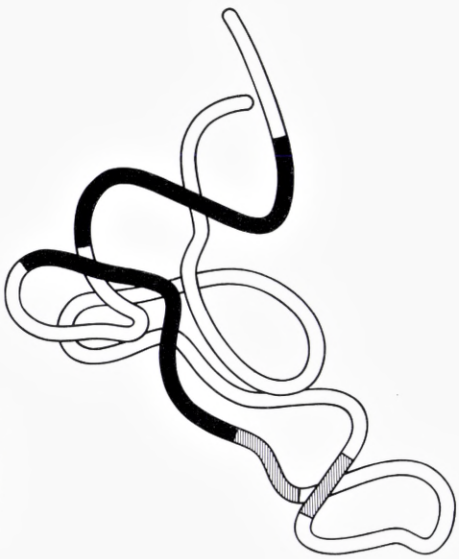


Fig. 24. Footprinting results from Met-$tRNA_f^{Met}$ complexed to the elongation factor EF-Tu and GTP are transferred to a tertiary structure model of $tRNA^{Phe}$ as described in the legend to Fig. 17 (Clark et al., *1984).*

$tRNA_f^{Met}$, the initiator tRNA in the unformylated state, can indeed form a ternary complex with EF-Tu:GTP. This ternary complex also appears almost normal in the disposition of the aa-tRNA on the EF-Tu:GTP, so it should be able to bind to the ribosomal A-site if its formation is possible *in vivo*. We are presently investigating whether the small differences in the T-loop binding are sufficient for making a fit poor enough in the A-site to be selected against.

Conclusion

In summary this paper has described work on the function of initiator tRNA during the steps of protein biosynthesis initiation. We have suggested a role for the specific formylation of prokaryotic initiator tRNA methionine in the translation of polycistronic messenger RNAs. The

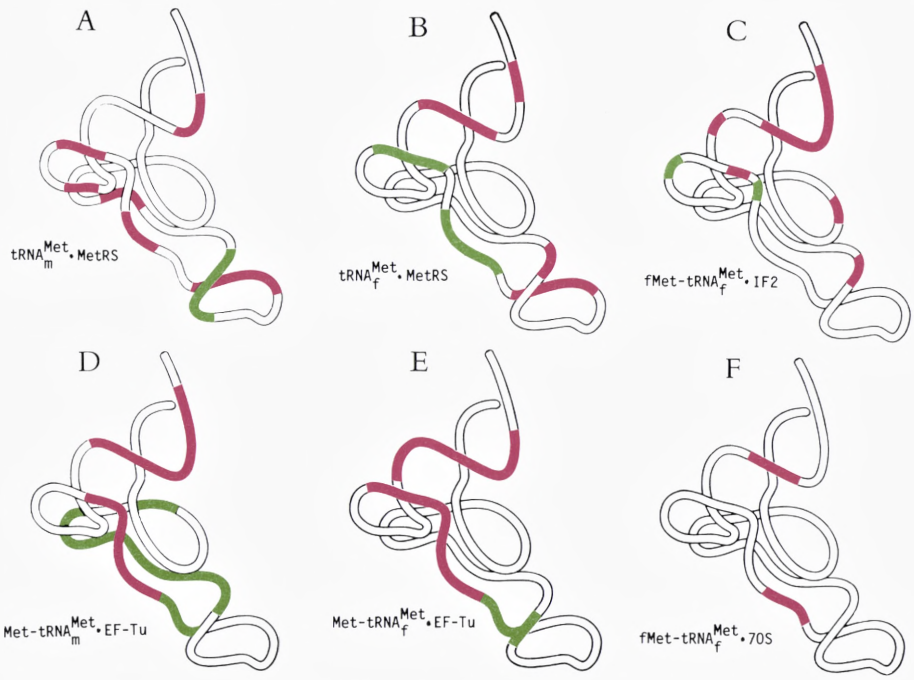


Fig. 25. A summary of the footprinting results shown in Figures 17, 18, 20-22, and 24. Regions protected in complexed tRNA are shown in red and regions cut more intesely are green. (A): $tRNA_m^{Met}$: Met-RS; (B): $tRNA_f^{Met}$: Met-RS; (C): $fMet\text{-}tRNA_f^{Met}$: IF2 (D): $Met\text{-}tRNA_f^{Met}$: EF-Tu; (E): $Met\text{-}tRNA_f^{Met}$: EF-Tu; (F): $fMet\text{-}tRNA_f^{Met}$: 70S.

regions within this tRNA which are involved in the interactions with proteins during the initiation process have been studied and the results are compared in figure 25. Although a firm conclusion about the exact sites of interactions with proteins requires further investigations – and ultimately the crystallization and X-ray diffraction structural determination of the tRNA within the complex – our results indicate some similarities and differences between the $tRNA_f^{Met}$ interactions with MetRS, IF2, EF-Tu and 70S ribosomes and also between the interactions with MetRS or EF-Tu of $tRNA_f^{Met}$ and the elongator $tRNA_m^{Met}$. The regions within the two tRNAs which are protected by proteins are shown in red and the regions which are cut more intensely in complexed tRNA are green.

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