A STUDY OF THE INTERACTION OF *ESCHERICHIA COLI* INITIATION FACTOR IF2 WITH FORMYLMETHIONYL-tRNA$_f^{Met}$ BY PARTIAL DIGESTION WITH COBRA VENOM RIBONUCLEASE

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Received 27 March 1981

1. Introduction

The translation initiation factor IF-2 of prokaryotic organisms functions in the correct formation of the initiation complex fMet-tRNA$_f^{Met}$:mRNA:30 S ribosomal subunit [1].

Similar to the role of the elongation factor EF-Tu in the elongation step, IF-2 can be regarded as an aminoacyl-tRNA carrier protein which ensures the correct binding of the first amino acid, formylmethionine, in the ribosomal peptidyl transferase centre.

However, the binding constant of IF-2 to fMet-tRNA is too low to allow isolation of the complex. We have studied the interaction of IF-2 with the initiator tRNA by measuring the effect of the protein on the spontaneous hydrolysis of the aminoacyl ester bond [2]. We showed that IF-2 specifically protected the formylated form of the initiator tRNA, indicating that IF-2 interacts with the amino acid acceptor region of the initiator tRNA. Furthermore, we showed that this interaction was independent of GTP.

To obtain more detailed information about the structural regions of the initiator tRNA involved in the binding to IF-2, we have employed the method of specific protection by IF-2 against ribonuclease digestion of fMet-tRNA.

Here, we have used the double strand-specific ribonuclease from the venom of the cobra *Naja oxiana*. This enzyme hydrolyses 3′-phosphoester bonds in the stems of fMet-tRNA$_f^{Met}$. In the presence of IF-2, a significant protection of these positions is observed. This effect of IF-2 appears to be specific for the formylated form of the initiator Met-tRNA$_f^{Met}$.

2. Materials and methods

Partially purified tRNA$_f^{Met}$ was obtained from the Microbiological Research Establishment, Porton Down and further purified by Sephadex A-50 column chromatography [3].

The tRNA was labelled at the 3′-end with ^{32}P and purified as in [4].

The specific radioactivity of the labelled tRNA preparations used in our experiments was ~6×10^4 cpm/μg tRNA (measured by liquid scintillation in PPO–POPOP–toluene). The tRNA was aminoacylated with [^{3}H]methionine [5] and α-amino formylated enzymatically [6] using a partially purified S-100 fraction [7].

Cobra venom ribonuclease was isolated from the venom of *Naja oxiana* according to [8]. IF-2 was isolated from *Escherichia coli* MRE 600 and purified as in [9].

Before enzymatic digestion, 13 pmol ^{32}P-labelled

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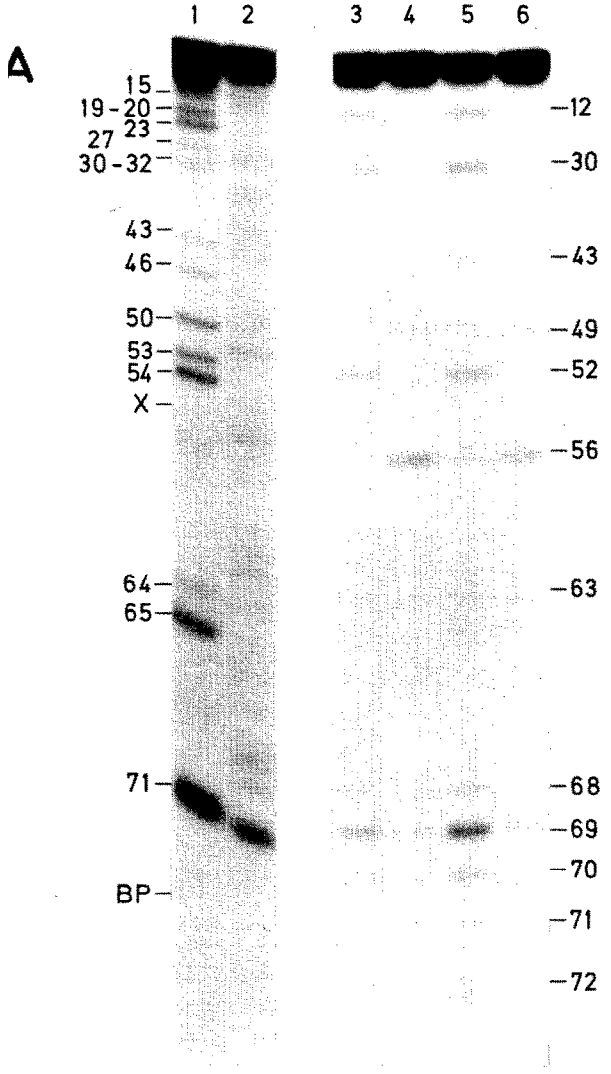
f[^{3}H]Met-tRNA$_f^{Met}$ + 4.5 μg unlabelled tRNA$_f^{Met}$ was incubated for 5 min at 37°C with 65 pmol IF-2 in a total volume of 30 μl of the following buffer: 50 mM Tris–HCl (pH 7.4), 50 mM NaCl, 100 mM KCl and 10 mM $MgCl_2$. Cobra venom ribonuclease (0.24 units) was added and the mixture incubated at 0°C for the times indicated on the figures. The reaction was stopped by phenol-extraction and the digested tRNA precipitated by 3 vol. ethanol after addition of 0.2 A_{260} units carrier tRNA. The precipitate was dissolved in 10 μl loading buffer 0.02 M Na-citrate (pH 5.0), 7 M urea, 0.001 M EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and analysed by high-voltage polyacrylamide gel electrophoresis [4]. In control experiments, IF-2 was omitted or GTP added to 0.5 mM final concentration. Similar experiments were performed using unformylated ^{32}P-labelled [^{3}H]Met-tRNA$_f^{Met}$.



3. Results and discussion

The finding in [2] and studies with small angle neutron scattering (Z. G. Li, B. Jacrot, M. G.-M., H. U. P., unpublished) indicate that the binding constant of IF-2 to fMet-tRNA$_f^{Met}$ at high [KCl] (500–700 mM) is $<10^4$ M^{-1}. Therefore, we have chosen here to form the complex at 150 mM monovalent cation.

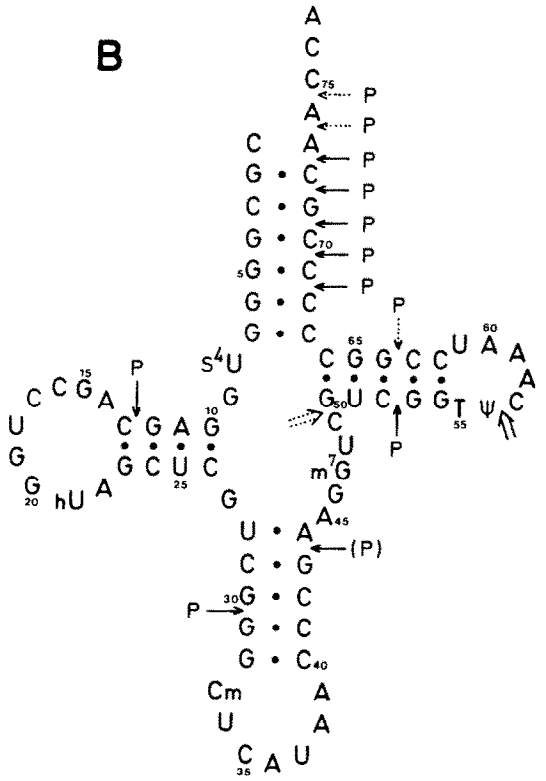
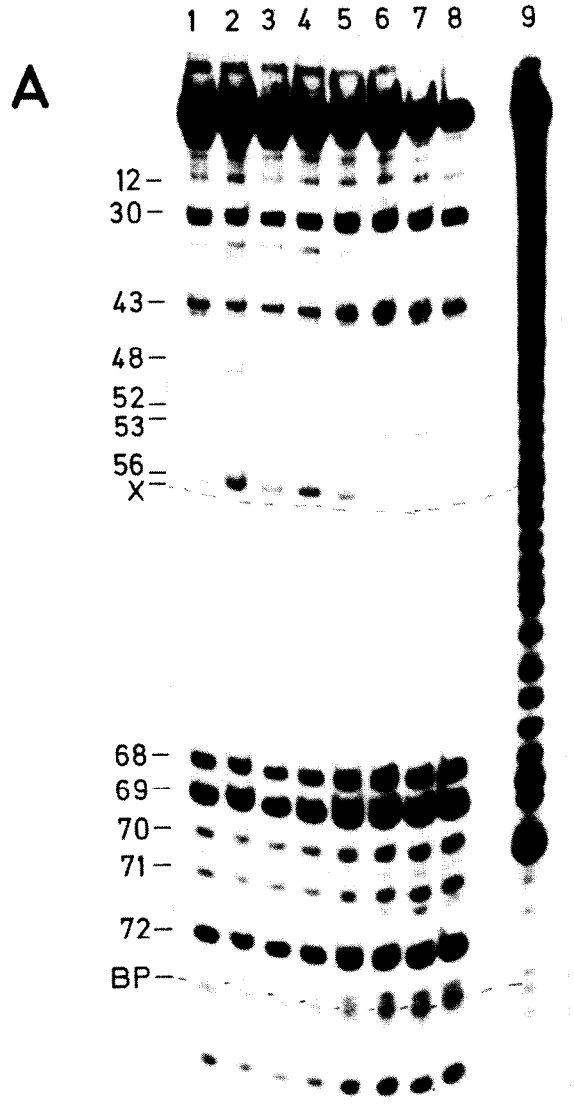


Fig.1. (A) Electrophoresis of 3′-^{32}P-labelled *E. coli* fMet-tRNA$_f^{Met}$ digested by cobra venom ribonuclease: (1) ribonuclease T_1 digestion of fMet-tRNA$_f^{Met}$; (2) random digestion of fMet-tRNA$_f^{Met}$ in formamide at 95°C for 30 min; (3,5) cobra venom ribonuclease digestion of fMet-tRNA$_f^{Met}$; (4,6) cobra venom ribonuclease digestion of fMet-tRNA$_f^{Met}$ in the presence of IF-2; (3,4) digested for 1.5 min; (5,6) digested for 3 min; (X) and (BP) indicate positions of the dyes xylene cyanol and bromophenol blue, respectively. (B) Cloverleaf structure of tRNA$_f^{Met}$. The arrows indicate cleavage positions of fMet-tRNA$_f^{Met}$ by cobra venom ribonuclease. Dotted arrows indicate positions cleaved slowly. Positions strongly protected by IF-2 are marked P and weakly protected positions (P). Cleavage positions intensified in the presence of IF-2 are indicated by double arrows.

After a short preincubation at 37°C to allow formation of a complex between IF-2 and fMet-tRNA, the incubation mixture was cooled on ice and the cobra venom ribonuclease was added. The resulting RNA fragments were separated by high-voltage polyacrylamide gel electrophoresis. An autoradiogram of such an experiment is shown in fig.1A. Denatured $tRNA_f^{Met}$ was hydrolysed by T_1 ribonuclease and by formamide to obtain reference ladders with bands of known fragments. By comparison to these, we have numbered the cobra venom ribonuclease cuts in the $fMet\text{-}tRNA_f^{Met}$. The main cuts in the absence of IF-2 are at the 3'-side of nucleotides at positions 72–68, 63, 52, (43), 30 and 12, all in double helical regions of the tRNA molecule.

When IF-2 is present, we observe a protection of these positions against nuclease digestion. In addition, new cuts appear in the extra- and TΨC-loops, as shown in fig.1. The addition of GTP did not change the digestion pattern significantly (not shown).

Similar experiments were carried out using unformylated $Met\text{-}tRNA_f^{Met}$. Digestion of $Met\text{-}tRNA_f^{Met}$ gives rise to a pattern (fig.2A) very similar to the one



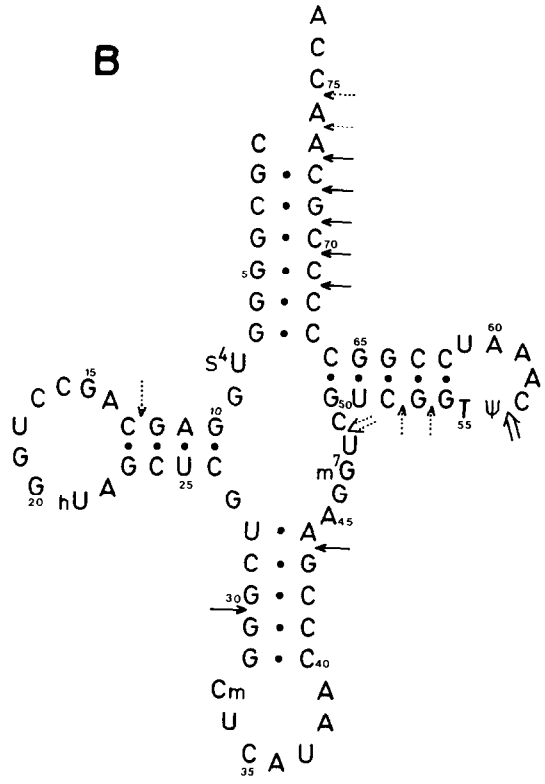


Fig.2. (A) Electrophoresis of 3'-^{32}P-labelled *E. coli* $Met\text{-}tRNA_f^{Met}$ digested by cobra venom ribonuclease: (1,5) $Met\text{-}tRNA_f^{Met}$ alone; (2,6) $Met\text{-}tRNA_f^{Met}$ + GTP; (3,7) $Met\text{-}tRNA_f^{Met}$ + IF-2; (4,8) $Met\text{-}tRNA_f^{Met}$ + IF-2 + GTP; (1–4) digested for 5 min; (5–8) digested for 20 min; (9) random digestion of $Met\text{-}tRNA_f^{Met}$ in formamide at 95°C for 30 min; (X) and (BP) indicate the position of the dyes xylene cyanol and bromophenol blue, respectively. (B) Cloverleaf structure of *E. coli* $tRNA_f^{Met}$. The arrows indicate cleavage positions of $Met\text{-}tRNA_f^{Met}$ by cobra venom ribonuclease. Dotted arrows indicated positions cleaved slowly. Cleavage positions intensified in the presence of GTP are indicated by double arrows.

obtained with fMet-tRNA$_f^{Met}$ (fig.1A), indicating no large conformational differences between the formylated and the unformylated form. However, in this case, the addition of IF-2 does not protect the tRNA, and GTP stimulates slightly the hydrolysis at residue 56 (fig.2).

It has been reported earlier that IF-2 does not protect Met-tRNA$_f^{Met}$ against chemical hydrolysis [2]. This observation in combination with the lack of protection against nuclease digestion shown in fig.2 indicates that Met-tRNA$_f^{Met}$ does not bind to IF-2, or more precisely, that the binding constant is $<5 \times 10^4$ M^{-1}. Yet unformylated Met-tRNA$_f^{Met}$ can be bound to 30 S ribosomal subunits stimulated by the 3 initiation factors [5]. Together with the results reported here, this suggests to us that binding of the initiation factors to the 30 S ribosomal subunit activates a ribosomal binding site for Met-tRNA$_f^{Met}$.

Although other explanations such as allosteric effects cannot be ruled out, the most likely explanation of the strong protective effect exerted by IF-2 as seen in fig.1, is that IF-2 sterically prevents the attack by the ribonuclease.

The intensification of the cuts in the extra- and TΨC-loops is not a result of a decreased level of other accessible sites due to the binding of IF-2 since we have a 10–15-times excess of unlabelled (uncharged) tRNA present during the digestion. Therefore, it seems more probable that the absolute affinity of the cobra venom RNase to the extra- and TΨC-loops increases by the binding of IF-2 to fMet-tRNA$_f^{Met}$, possibly due to a conformational change in this region of the tRNA leading to a more ordered structure.

Using the same technique, we have recently shown that EF-Tu:GTP protects the amino acid acceptor stem of different aminoacyl-tRNAs against cobra venom ribonuclease digestion [4]. In that case, we observed an increased intensity of the cuts in the anticodon stem when aminoacyl-tRNA bound EF-Tu:GTP.

It therefore seems likely that binding of protein factors to aminoacyl-tRNA alters the native structure of the tRNA molecule.

Based on our results we show in fig.3 a model of the interaction between IF-2 and fMet-tRNA.

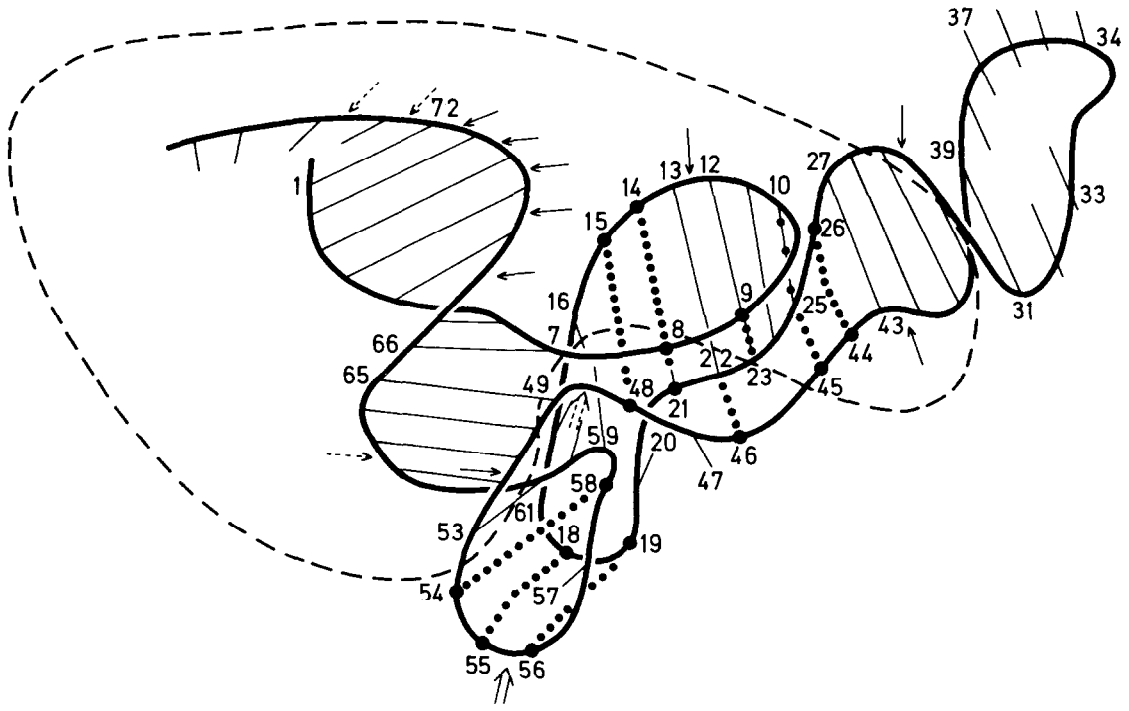


Fig.3. The structure of yeast tRNA^{Phe} [10]. The arrows indicate cleavage positions in the *E. coli* fMet-tRNA$_f^{Met}$ molecule by cobra venom ribonuclease. Cleavage positions intensified in the presence of IF-2 are indicated by double arrows. The part of the molecule protected by IF-2 is enclosed by a dashed line.

Acknowledgements

This work was supported financially by the Danish Natural Science Research Council, the Carlsberg Fund and Centre National de la Recherche Scientifique. A. S. B. held a short-term fellowship from the Federation of European Biochemical Societies.

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