TRANSLATION INITIATION FACTOR C (f2): SELECTIVE INACTIVATION OF ITS F-MET-tRNA BINDING ACTIVITY WHICH DOES NOT AFFECT MESSENGER RNA BINDING TO THE 30 S RIBOSOME

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Received 12 January 1970

1. Introduction

Initiation factor C (f2) stimulates both the binding of messenger RNA to the 30 S ribosome [1, 3] and the attachment of formyl-methionyl tRNA (f-met-tRNA) to this subunit [2, 3]. Attempts to separate these two activities during the purification procedure [3] have remained unsuccessful. The mRNA - 30 S interaction promoted by factor C (f2) does not, however, require the presence of f-met-tRNA [4]. The two activities of the factor seem therefore to be independent from each other, suggesting that the protein might have multiple active sites. Recently, Mazumder et al. [5] reported that factor f2 (C) possesses an essential sulfhydril group whose blocking produced a complete inactivation of the effect of the factor on f-met-tRNA binding to the ribosome. The present work shows that factor C dependent f-met-tRNA binding is indeed abolished after treatment of the protein with *N*-ethyl maleimide (NEM) but the activity of the factor for messenger RNA binding to the 30 S ribosome is not impaired. These findings support the notion that different sites in the protein are responsible for f-met-tRNA and mRNA binding.

2. Materials and methods

The preparation of high salt (2 M NH_4Cl) washed ribosomes and of ribosomal subunits from *E. coli* MRE 600 was described previously [1, 4]. Initiation factor C (f2) was extensively purified from crude factor by Sephadex G200, DEAE cellulose and hydroxyl apatite according to the published procedure [3]. Initiation factors A (f1) and B (f3) were obtained as before [1]. F-met-tRNA, free of non-formylated met-tRNA was prepared [3] with ^{14}C-methionine (200 μCi/μmole) or ^{35}S-methionine (1–2 mCi/μmole) and used for the binding studies as detailed in fig. 1. Procedures for the measurement of ribosome binding to nascent T4 mRNA by the direct electron microscopy method [3, 6] and for the study by zone sedimentation of ^{3}H-T4 mRNA binding to 30 S and to 70 S ribosomes [4, 6] have been extensively described in the references indicated. *N*-ethyl maleimide from Aldrich was used to prepare factor NEM-C as in fig. 1. *N*-ethyl maleimide-1 ^{14}C (10 mCi/mmole) was purchased from Schwartz.

3. Results

3.1. *Effect of N-ethyl maleimide on initiation C (f2) activities*

Treatment of a purified preparation of factor C (f2) with *N*-ethyl maleimide produces a rapid inactivation of the effect of this protein on the binding of f-met-tRNA to the ribosome. As shown in fig. 1-I, more than 90% inactivation was seen when the protein was preincubated 10 min at 34°C with 5 mM NEM. The reagent does not affect the other components of the f-met-tRNA binding reaction, as ribosome or factor A (f1), since the addition of NEM at the same final concentration did not produce any inhibition (fig. 1-I).

Inactivation of AUG dependent binding of f-met-

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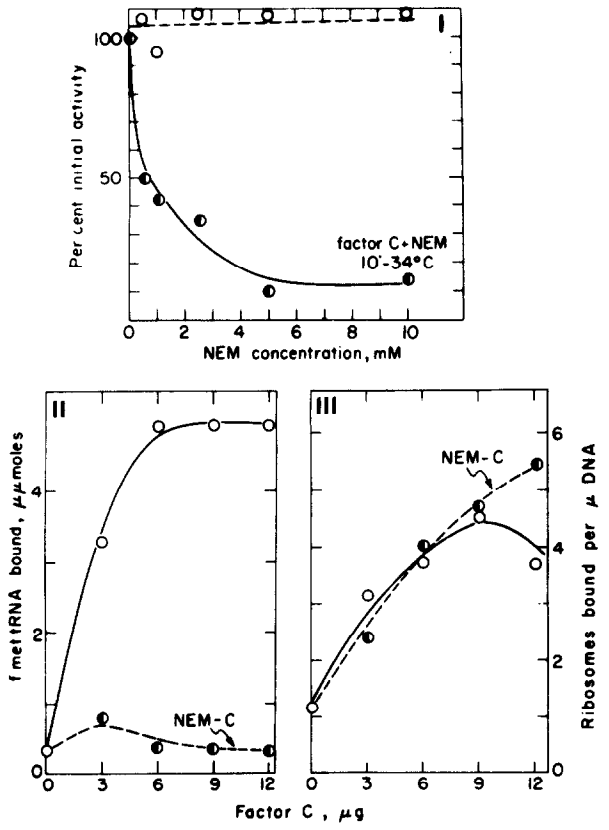

Fig. 1. (I) Purified factor C, 12 μg, was incubated with NEM at the final concentration indicated in 0.05 ml of tris-HCl pH 7.5 10 mM, $MgCl_2$ 2 mM, NH_4Cl 60 mM at 34°C for 10 min. From this 0.01 ml was used to measure the activity for f-met-tRNA binding (•) in a 0.05 ml reaction mixture containing tris-HCl, pH 7.5, 0.1 M, NH_4Cl 0.08 M, $MgCl_2$ 7.5 mM, dithiotreitol 8 mM, GTP 1 mM, 50 μg 70 S ribosomes, 2 μg factor A, ApUpG 6 μg and 15 μg ^{14}C-f-met-tRNA. A 100% activity corresponded to 5.25 pmoles bound (0.66 pmoles bound without factor C were substracted. Binding without factor A: 1.2 pmoles). As a control (○) 0.01 ml of the medium containing NEM at the concentration indicated was added to the reaction. (II) Quantitative comparison of the activity of factor C incubated 10 min at 25°C alone (○) or with 20 mM NEM (•) for the binding of ^{14}C-f-met-tRNA to 70 S ribosomes. (III) Quantitative comparison of the activity of factor C incubated 10 min at 25°C alone (○) or with 20 mM NEM (•) for the binding of ribosomes to T4 DNA bound nascent mRNA. The indicated amount of factor was added to a reaction mixture [2, 7] containing 15 μg ribosomes and 2 μg T4 DNA (previously transcribed with RNA polymerase and nucleoside triphosphates). The reaction is examined by electron microscopy and the number of ribosomes seen bound to the T4 DNA-RNA complexes is counted.

Table 1
Inactivity of factor NEM-C for f-met-tRNA binding to the 30S ribosome.

Additions	pmoles ^{35}S-f-met-tRNA bound
30 S ribosomes	0.35
30 S ribosomes + factor C, 15 μg	2.25
30 S ribosomes + factor NEM-C, 15 μg	0.28

Ribosomes and factors were those used in fig. 2. Factor C was treated with 1 mM NEM, 10 min at 30°C. Binding was measured as in fig. 1, without factor A.

Table 2
Labeling of factor C with *N*-ethyl maleimide-1-^{14}C.

Conditions	NEM-^{14}C, cpm*
1. Retained after extensive dialysis	576
2. Insoluble in 5% TCA	563
3. Insoluble in 5% TCA if protein omitted	45

* 0.05 ml of NEM-^{14}C (0.5 mM; 11 cpm/pmole) mixed with 3.75 μg factor C were incubated 40 min at 35°C in the buffer used in fig. 1-1. Same buffer used for dialysis.

tRNA to ribosomes was virtually complete after treatment of factor C with 20 mM NEM (fig. 1-II). The same treatment, however, did not impair the effect of the protein on mRNA-ribosome interaction (fig. 1-III). The electron microscopy method used, allows a quantitative study of the action of factor C on the binding of ribosomes to nascent T4 mRNA and even small changes in activity would be detected. As seen in fig. 1-III, factor NEM-C had the same specific activity as the untreated protein.

3.2. *Comparison of the mRNA-ribosome complex formed with C and NEM-C*

Formation of the DNA-RNA-ribosome complexes measured by the electron microscopy method, takes place without f-met-tRNA [1, 3]. If, however, the mRNA-ribosome binding is analyzed by zone sedimentation, only the binding of mRNA to the 30 S subunit is observed without f-met-tRNA, but the formation of a 70 S-mRNA complex stable enough to be seen by

zone sedimentation necessitates the concomitant binding of f-met-tRNA and GTP [4, 7]. Since after treatment with NEM, factor C has lost all its activity for f-met-tRNA binding it was of interest to compare its effects on the formation of the 30 S and 70 S complexes with mRNA.

The effect of factor C and NEM-C on the binding of ^{3}H-T4 mRNA to the 30 S subunit was studied as shown in fig. 2-II and both factors stimulated the attachment of mRNA more than 3 fold. No loss of activity after NEM treatment was seen, in agreement with what was observed by the electron microscopy method. To study the transfer of mRNA from 30 S to 70 S the experiment shown in fig. 2-I was performed. ^{3}H-T4 mRNA was incubated with factor C or NEM-C, factors A(f1) + B(f3), f-met-tRNA and GTP, in the presence of 30 S or a mixture of 30 S + 50 S ribosomal subunits. While NEM-C stimulated normally the attachment of mRNA to 30 S (fig. 2-I) it did not promote the formation of a 70 S-mRNA complex (fig. 2-I). Reassociation of 30 S with 50 S took place only if untreated factor C was added, that is when f-met-tRNA binding could take place.

Factor C treated with NEM is completely inactive for the binding of f-met-tRNA, even to the isolated 30 S ribosomal subunit as shown by table 1. These data therefore demonstrate that the mRNA-30 S-factor C interaction must take place before and independently from the binding of initiator tRNA.

3.3. *Labelling of initiation factor C (f2) with ^{14}C-N-ethyl maleimide*

Since NEM treatment destroys f-met-tRNA binding activity, without affecting mRNA binding, it should be possible with radioactive NEM to label selectively the site of the protein which is involved in the f-met-tRNA binding function.

When extensively purified factor C is incubated with *N*-ethyl maleimide-1-^{14}C, some of the radioactivity becomes bound to the protein (table 2). The amount of reagent bound can be measured after extensive dialysis to remove all free NEM or by measuring trichloroacetic acid insoluble material. By both methods, (table 2) about 13 μmoles of NEM are bound per gram protein. Estimates reported in the literature for the molecular weight of the factor vary from 60,000 to 80,000 daltons [8, 9]. This would give an approximate number of NEM molecules bound per molecule factor close to one (see however footnote**).

The labeled preparation of factor C was analyzed by filtration through Sephadex G200 as shown in fig. 3-I. Radioactive NEM-C is included in the gel and eluted in one major peak. Some free NEM is also visible in this preparation. The activity of NEM-C for ribosome binding to nascent T4 mRNA was measured (fig. 3-II) and the activity coincides with the peak of NEM-C radioactivity. This indicates that it is indeed the protein which binds NEM which is also responsible for the mRNA binding activity. Gel filtration of untreated factor C (fig. 3-III) shows that the activity for f-met-tRNA binding is also eluted in the same position. When compared with protein markers, this position would correspond to a molecular weight superior to 100,000 daltons**. In addition, we have observed a second peak of activity corresponding to a lower molecular weight (around 50,000)**. Both fractions have f-met-tRNA binding activities as shown in fig. 3-IV. Existence of these two forms of factor C was seen repeatedly. The ratio between them is variable; some preparations even contained only the smaller form. Treatment with NEM did not modify the elution pattern. The two fractions seen on Sephadex G200 are probably related to the two peaks of activity observed on DEAE cellulose by many authors [3, 8, 10]. The relation between these fractions is unclear and is currently under investigation.

4. Discussion

Initiation factor C (f2) reacts with *N*-ethyl maleimide to give a modified protein, NEM-C, which has lost its activity for f-met-tRNA binding to the ribosome, but is still fully active to promote the association of mRNA to the 30 S ribosomal subunit. This would indicate that the two functions of factor C are not due to the same active site on the protein and rules out that both effects result from a common change induced by factor C in the structure of the 30 S ribosome. The factor might be composed of different subunits, but although two fractions were observed by gel filtration, the two

** The molecular weight of the two fractions of factor C estimated by gel filtration does not agree with the values reported by sedimentation [9]. Such a discrepancy was already mentioned by Chae et al. [9]. If the heavy fraction is larger than 100,000 daltons, the number of NEM bound would be more compatible with two per molecule, possibly suggesting that this is a dimer form.

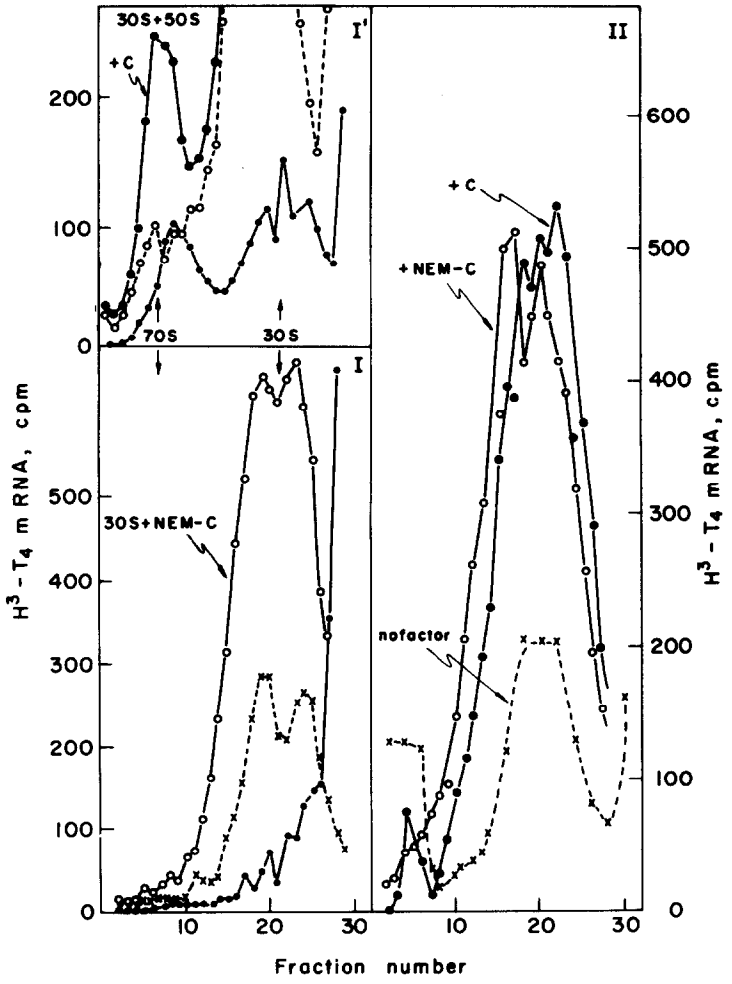

Fig. 2. Binding of T4 mRNA to 30 S and 70 S ribosomes with factor C and NEM-C.
(I) ^{3}H-T4 mRNA (*in vitro* transcription product; 6×10^{7} cpm/μmole UMP) incubated [4] with 30 μg 30 S ribosomes, 50 μg f-met-tRNA, 10 μg factor A and 15 μg factor NEM-C (as in table 1) was analyzed by centrifugation on a 5–20 percent glycerol gradient, 2 hr at 38,000 rpm. Complete (○), minus factor NEM-C (x) and minus 30 S (●).
(I') Same conditions but 35 μg reactivated 50 S ribosomes [4] were added, with factor C (●), with NEM-C (○), or without factor C (•). All six tubes were centrifuged together in Spinco Rotor 39.1.
(II) ^{3}H-T4 mRNA incubated with 30 μg 30 S ribosomes in the presence of 15 μg factor C (●), 15 μg factor NEM-C (○) or without factor (x) was analyzed as above.

activities were not separated. The identical elution profile found for the mRNA binding activity and the ^{14}C-labeled NEM-C protein (fig. 3) also rules out that the mRNA binding activity is due to contamination by another initiation factor, as B (f3), which is eluted on these columns between 17–18 ml, much later than factor C. Furthermore, we can distinguish the effects of factor B and C on mRNA-ribosome interaction [7]: the binding of mRNA observed with factor C alone can be inhibited by the addition of oligonucleotide competitors (such as ApUpG or UpUpU), while in the presence of both factor B and C these oligonucleotides do not inhibit, indicating that now natural mRNA is specifically recognized. The binding of ribosomes to T4 mRNA seen with NEM-C is inhibited by oligonucleotides, as typical for the C-dependent reaction.

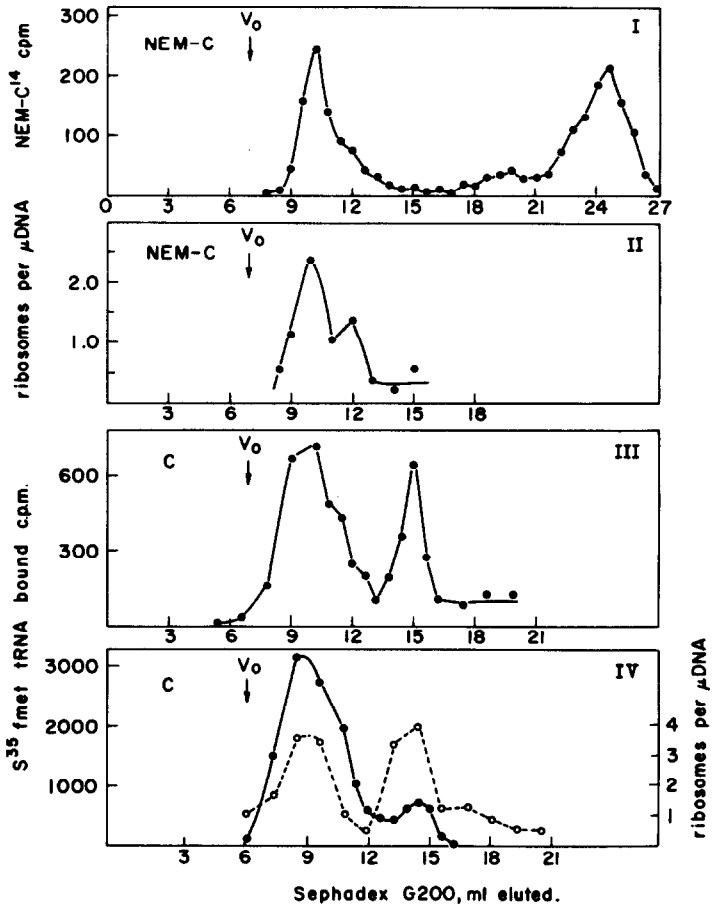

Fig. 3. (I) 50 μg ^{14}C-factor NEM-C (see table 2) were filtered through a 52 X 0.6 cm Sephadex G200 column equilibrated in tris-HCl, pH 7.5, 10 mM, $MgCl_2$ 2 mM, NH_4Cl 60 mM. Radioactivity in each fraction was measured in Bray's scintillation fluid.
(II) Same as I but activity of the fraction for ribosome binding to T4 nascent mRNA was measured.
(III) Factor C, non treated, was filtered and the activity for f-met-tRNA binding was measured. The two peaks of activity are clearly visible.
(IV) Another preparation of factor C; f-met-tRNA binding (•) and mRNA binding (o) were measured with each fraction.

Little is yet known on the mechanism by which initiation factors promote the attachment of mRNA and f-met-tRNA to the ribosome. Mazumder et al. [5] first reported that a free sulfhydryl group in factor f2 (C) is required for f-met-tRNA binding and that the cofactor GTP protects the protein against inactivation by NEM. In contrast, the effect of factor C on mRNA binding is not lost when f-met-tRNA binding activity is blocked by NEM. With this inhibitor, however, the 30 S-mRNA complex accumulates, while the junction with the 50 S subunit does not occur, in agreement with our previous results that neither f-met-tRNA nor GTP are required for the binding of T4 mRNA to the 30 S subunit [4]. Thus, 30 S-mRNA interaction must come first in the sequence of reactions leading to the formation of the 70 S initiation complex, indicating that the mechanism by which the mRNA chain is recognized and bound by the 30 S ribosome and factor C is not based on codon-anticodon interaction. The effect of factor C is most probably not specific for a given nucleotide sequence [3, 7] and it might be assumed that the recognition of the initiation signal takes place only in a subsequent

reaction involving the function of factor B (f3) as shown by the messenger competition studies [7]. It is thus of interest that with NEM-C, mRNA-ribosome interaction was not made resistant to oligonucleotide competitors upon the addition of factor B (f3) [11]. The specific recognition of messenger RNA, with factor B, would therefore, in contrast to the mere binding of the template, be dependent on some function of a free-SH group in factor C.

Acknowledgement

We thank Mr. Greenshpan for many helpful discussions. The technical assistence of Mrs. P.Federman is gratefully acknowledged. This work was supported in part by a grant from the Service d'Aide à la Recherche et au Development Français.

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