PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: EVIDENCE FOR TWO FORMS OF THE Met-tRNA$_f^{Met}$ BINDING FACTORS

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Received April 16,1974

Summary

Two forms of Met-tRNA$_f^{Met}$ binding factors (IF1A and IF1B) were separated by DEAE-cellulose chromatography. These two forms can be distinguished by the stabilities of their respective Met-tRNA$_f^{Met}$:IF1:GTP complexes to Mg^{++}. The Met-tRNA$_f^{Met}$:IF1B:GTP complex dissociates extensively in the presence of Mg^{++} where as the Met-tRNA$_f^{Met}$:IF1A:GTP complex is distinctly more stable under similar conditions. The molecular weight of Met-tRNA$_f^{Met}$:IF1A:GTP complex as determined by sucrose density gradient centrifugation is 160,000 and that of the Met-tRNA$_f^{Met}$:IF1B:GTP complex is 65,000.

Reports from several laboratories indicate that the first step in peptide chain initiation in rabbit reticulocytes (1-4) and also in mouse L-cells (5) is the formation of a ternary complex, Met-tRNA$_f^{Met}$:IF1:GTP. This complex formation does not require Mg^{++}, ribosomes and AUG codon and the complex formation is markedly inhibited by addition of Mg^{++}. The reason for such inhibition by Mg^{++} is not obvious and it is not clear how the Met-tRNA$_f^{Met}$:IF1:GTP complex interacts with ribosomes and AUG codon.

We recently reported (6) that the Met-tRNA$_f^{Met}$:IF1:GTP complex formed with a partially purified mixture of peptide chain initiation factors, dissociates extensively into free Met-tRNA$_f^{Met}$ upon addition of Mg^{++}.

In this paper, we will describe the separation of two forms of Met-tRNA$_f^{Met}$ binding activities (IF1A and IF1B) by DEAE-cellulose chromatography of a partially purified mixture of peptide chain initiation factors. These two

forms can be clearly distinguished by the relative stabilities of their Met-$tRNA_f^{Met}$ complexes towards Mg^{++}; Met-$tRNA_f^{Met}$:IF1A:GTP complex is distinctly more stable to Mg^{++} than Met-$tRNA_f^{Met}$:IF1B:GTP complex. The properties of these two forms of Met-$tRNA_f^{Met}$ binding factors and their roles in peptide chain initiation will be discussed.

MATERIALS AND METHODS

The preparation of preincubated reticulocyte ribosomes and ribosomal 0.5 M KCl wash (I fraction) were the same as previously described (2,7). The crude I fraction was further purified by passage through a DEAE-cellulose column previously equilibrated with Buffer D (5 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol and 50 μM EDTA, 10 percent glycerol). A partially purified mixture of the peptide chain initiation factors was obtained by eluting the column with Buffer D containing 0.3 M KCl (Fraction II). The Fraction II was concentrated by ammonium sulfate precipitation (0-80 percent saturation). The precipitate was dissolved in Buffer D and dialyzed against Buffer D (Fraction III).

The dialyzed Fraction III preparation (approximately 15 ml, 5 mg per ml) was applied to a second DEAE-cellulose column (1 cm x 15 cm) previously equilibrated with Buffer D. The column was then washed with 10 ml Buffer D and was then eluted with 100 ml of a linear KCl gradient (0.1 → 0.3 M) in Buffer D. The fractions were concentrated by dialysis against Buffer D containing 20 percent polyethylene glycol. The dialyzed and concentrated fractions were stored in ice before use.

Poly r(U-G) directed methionine transfer reaction and Millipore filtration assay for [^{35}S]Met-$tRNA_f^{Met}$ binding to IF1 have been described (2,6,7). Other materials and methods were the same as described previously (2,6,7).

RESULTS

Fig. 1 describes a typical DEAE-cellulose chromatography of the initiation factor preparation (Fraction III). Fig. 1A represents the optical density pro-

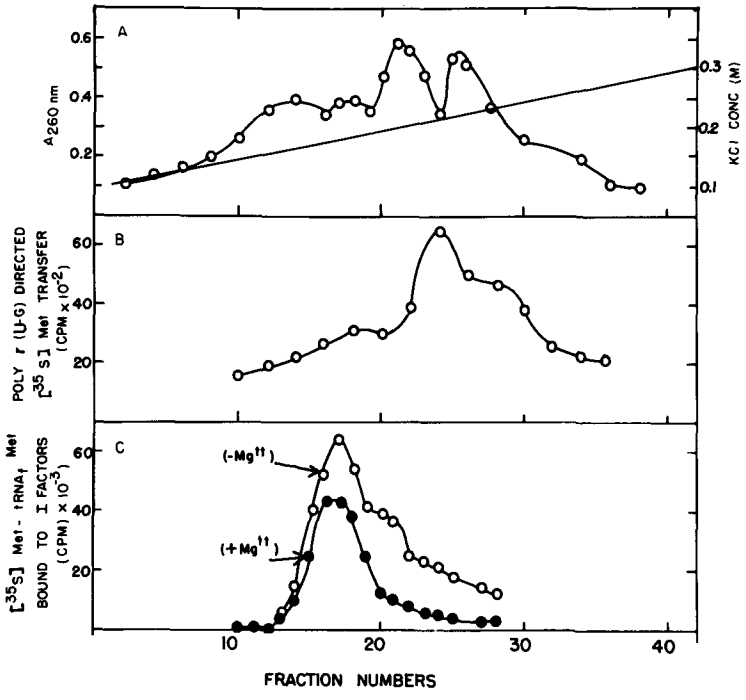

Figure 1. DEAE-cellulose chromatography of initiation factor(s) (Fraction III). The preparation of Fraction III and DEAE-cellulose chromatography have been described under Materials and Methods. Figure A represents the optical density profile of different column fractions. Figure B represents the activities of the fractions for poly r (U-G) directed methionine transfer reaction. Methionine transfer assay using 0.01 ml of the fractions, was performed as described previously (2,7). Figure C represents the activities of the factors to bind Met-tRNA$_f^{Met}$. The Met-tRNA$_f^{Met}$ binding assay was first performed using the standard assay method without Mg^{++} (Curve o-o-o). A 0.01 ml aliquot of each fraction was used in the assay. In another set of identical experiments (Curve ●-●-●-) Met-tRNA$_f^{Met}$ was first bound to the initiation factor in the absence of Mg^{++}, then Mg^{++} was added to the reaction mixture to make the final Mg^{++} concentration 3 mM. The reaction mixtures were then incubated in ice for 5 minutes. The reactions were then terminated by addition of wash buffer containing 3 mM Mg^{++}. [^{35}S]Met-tRNA$_f^{Met}$ bound radioactivity was then analyzed by Millipore filtration.

file of the eluted proteins. Fig. 1B describes the activities of different fractions as assayed by their abilities to catalyze the methionine transfer reaction in response to poly r(U-G) messenger. As before (1-2), three distinct activity peaks corresponding to IF1, IF2 and IF3 were observed. Fig. 1C describes the Met-tRNA$_f^{Met}$ binding activity of the different fractions. The

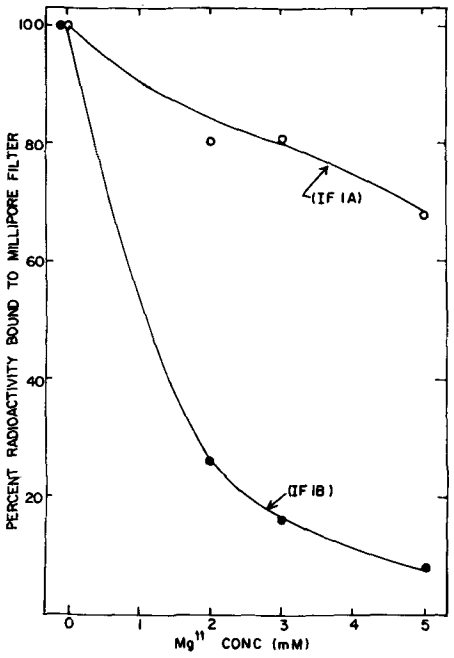

Figure 2. Effects of addition of Mg^{++} on preformed Met-$tRNA_f^{Met}$:IF1:GTP complexes with IF1A and IF1B. Met-$tRNA_f^{Met}$:IF1:GTP complex was preformed using either IF1A (8 μg) (110,000 cpm) or IF1B (10 μg) (80,000 cpm). The standand Met-$tRNA_f^{Met}$ binding assay was used. The incubation mixtures were then mixed with Mg^{++} as indicated and put in an ice bath for 5 minutes. The reactions were then terminated by addition of wash buffer containing Mg^{++} at the same concentration as the incubation mixture. The solutions were then assayed for Met-$tRNA_f^{Met}$ binding by the usual Millipore filtration assay.

Met-$tRNA_f^{Met}$ binding assay was first performed using the standard assay method without Mg^{++} (curve o-o-o). Met-$tRNA_f^{Met}$ binding activities separated into at least two peaks. In another set of identical experiments, Met-$tRNA_f^{Met}$ was first bound to the initiation factors in the absence of Mg^{++}, then Mg^{++} was added to the reaction mixture to make the final concentration 3 mM and incubation was continued for 5 minutes in an ice bath. The reactions were then terminated by addition of wash buffer containing 3 mM Mg^{++} and filtered through Millipore filters. The results of this binding reaction are also shown in Fig. 1C (curve ●-●-●). A Met-$tRNA_f^{Met}$ binding peak, coincident with the main Met-

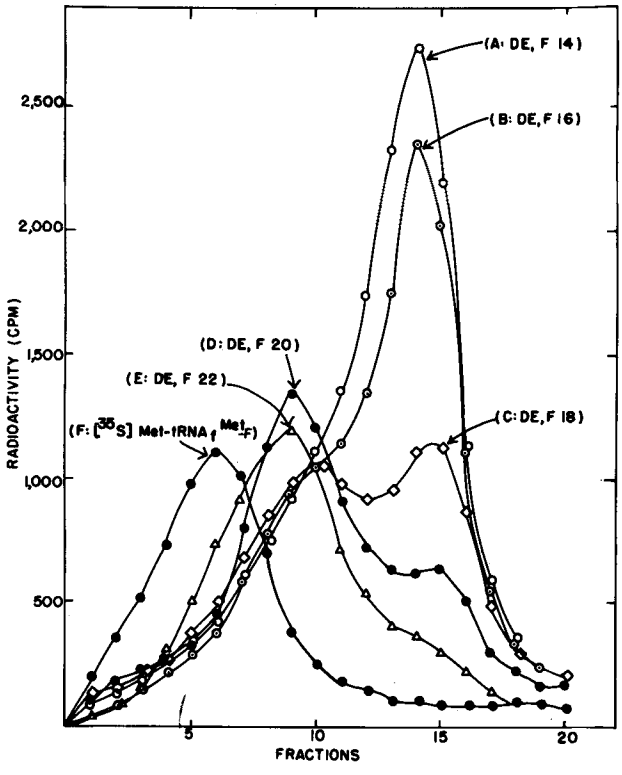

Figure 3. Sucrose density gradient analysis of Met-$tRNA_f^{Met}$:IF1:GTP complexes formed with different DEAE-cellulose fractions. The Met-$tRNA_f^{Met}$:IF1: GTP complexes were formed with different DEAE-cellulose fractions (Fraction 14 to 22; fraction 14 of this preparation represented IF1A peak and fraction 22, the IF2 peak). 0.05 ml aliquots of the reaction mixtures were then applied on sucrose gradients (5 - 15 per cent linear) containing 20 mM Tris-HCl, pH 7.5; 100 mM KCl; 5 mM mercapto-ethanol. The gradients were run in SW 50.1 rotors (Spinco Division, Beckmann) for 12 hours. Fractions (0.25 ml) were collected and aliquots (0.1 ml) were spotted on filter paper discs (Whatman No. 3). The filter paper discs were washed three times with cold 5 per cent trichloracetic acid, once with ethanol:ether (1:1) and once with ether. The paper discs were dried and counted for radioactivity.

$tRNA_f^{Met}$ binding peak was observed and the Met-$tRNA_f^{Met}$ binding activity associated with the second peak was greatly reduced. These results together with the results reported previously (6), indicate that the Met-$tRNA_f^{Met}$ binding activity in the partially purified initiation factor preparation exists in two forms and one form of the Met-$tRNA_f^{Met}$:Initiation factor:GTP complex dissociates in the presence of Mg^{++}.

The results shown in Fig. 2 describe the effects of addition of different concentrations of Mg^{++} to preformed [^{35}S]Met-tRNA$_f^{Met}$:IF1(A or B) complexes. The IF1A complex is distinctly more stable than the IF1B complex at all Mg^{++} concentrations tested. It is not clear if some dissociation of the Met-tRNA$_f^{Met}$: IF1A:GTP complex observed in these experiments is not due to contamination of the IF1A fraction with the IF1B species.

The Met-tRNA$_f^{Met}$:IF1:GTP complexes formed with different DEAE-cellulose fractions were further analyzed by sucrose density gradient centrifugation. The results of such an experiment are shown in Fig. 3. Different DEAE-cellulose fractions of an initiation factor preparation, starting from the IF1A peak fraction (curve A, Fraction 14) through fractions corresponding to the IF2 region (curve E, Fraction 22) were used in these studies. The Met-tRNA$_f^{Met}$: IF1:GTP complexes were formed using standard binding conditions in the absence of Mg^{++} and the complexes were analyzed by sucrose density gradient centriugation. It is clear from the results that the Met-tRNA$_f^{Met}$ complexes formed with the IF1A fractions sediment as the higher molecular weight form (approximate mol. wt. 160,000) and the complexes formed with the IF1B fractions (approximate mol. wt. 65,000) sediment as the lower molecular species. The Met-tRNA$_f^{Met}$ complex formed with fraction 22, a fraction enriched with IF2 activity, also sedimented similarly as the lower molecular form suggesting lack of any interaction of Met-tRNA$_f^{Met}$:IF1B:GTP complex with IF2.

DISCUSSION

The results presented in this paper clearly indicate the presence of two forms of Met-tRNA$_f^{Met}$ binding factors, IF1A and IF1B. The molecular weight of the Met-tRNA$_f^{Met}$:IF1A:GTP complex as determined by sucrose density gradient centrifugation is approximatley 160,000 and that of Met-tRNA$_f^{Met}$:IF1B:GTP complex is 65,000. After subtraction of the molecular wieght of Met-tRNA$_f^{Met}$ (25,000) the approximate molecular weight of IF1A is 135,000 and that of IF1B is 40,000. The lower molecular weight form, Met-tRNA$_f^{Met}$:IF1B:GTP dissociates

rapidly in the presence of Mg^{++} while the higher molecular weight form, Met-$tRNA_f^{Met}$:IF1A:GTP is distinctly more stable under these conditions.

Further work will be necessary to establish the relationship between these two forms of Met-$tRNA_f^{Met}$ binding factors and if IF1A is an oligomeric (possibly tetrameric) form of IF1B. The results of preliminary experiments indicate that these two forms, at least in the crude factor preparation (Fraction III) are interconvertible. For example, most of the Met-$tRNA_f^{Met}$ activity present in the crude preparation is in the IF1B form; the Met-$tRNA_f^{Met}$ complex formed using the crude initiation factor preparation dissociates extensively at 5 mM Mg^{++} and low temperature (6). However, when the same incubation mixture was held for a longer period at 37°, a substantial part of the Met-$tRNA_f^{Met}$ complex becomes stable to Mg^{++} and low temperature. Further work is in progress to determine if these two factors are interconvertible and how this interconversion regulates this first step in protein synthesis.

We have not yet been able to demonstrate any clear difference in biological activities of these two binding factors. Both factors bound to Met-$tRNA_f^{Met}$ and in each case such binding was GTP dependent and in a similar manner sensitive to aurintricarboxylic acid (6). Recently, we described a Millipore filtration assay for studies of Met-$tRNA_f^{Met}$ binding to ribosomes (6). Using this assay method, we have studied the requirements for Met-$tRNA_f^{Met}$ binding. We have observed (6) that both factors catalzyed the binding of Met-$tRNA_f^{Met}$ to ribosomes in the absence of AUG codon and addition of AUG codon does not have any significant effect on such binding. When IF2 and IF3 were added, both factors (IF1A and IF1B) stimulated AUG directed Met-$tRNA_f^{Met}$ binding to ribosomes in a similar manner. However, we can not, at present, draw any definite conclusion regarding the specific roles of these factors as we are not sure about the purity of the factor preparations and as mentioned above, there are indications that these factors interconvert under the assay condition.

It is not clear, at present, how our present observation is related to different peptide chain initiation activities in reticulocyte protein synthesis

as have been reported by other workers. Schreier and Staehelin mentioned the purification of four (4) or possibly five (8) peptide chain initiation factors from reticulocyte ribosomal salt wash and Cashion and Stanley described (9) the requirements of only two protein factors for formation of initiation complex with Met-$tRNA_f^{Met}$, 80 S ribosomes and AUG codon. Our work demonstrates that at least one of the peptide chain initiation factors, the Met-$tRNA_f^{Met}$ binding activity (IF1), can exist in two forms separable by DEAE-cellulose column chromatography. Both forms can stimulate protein synthesis initiation. The Met-$tRNA_f^{Met}$ binding activities of these two forms are dependent on assay conditions. Both forms bind to Met-$tRNA_f^{Met}$ in the absence of Mg^{++}. In the presence of Mg^{++} (3-5 mM), only the Met-$tRNA_f^{Met}$ binding activity of IF1A is apparent.

ACKNOWLEDGEMENTS

This work was supported by a NIH Research Grant (GM-18796), an American Cancer Society Grant NP-113 and a U.S. Public Health Service Career Development Award (1-K4-GM-46,240) to N.K.G.

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