Met-tRNA$_f^{Met}$ IS LOCATED IN CLOSE PROXIMITY TO THE β SUBUNIT OF eIF-2 IN THE EUKARYOTIC INITIATION COMPLEX, eIF-2 . Met-tRNA$_f^{Met}$. GDPCP

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1. Introduction

The eukaryotic protein synthesis initiation factor eIF-2 consists of three subunits, α, β and γ, with molecular weights of 36 000, 52 000 and 57 000, respectively [1]. In the presence of Met-tRNA$_f^{Met}$ and GTP, eIF-2 forms a ternary initiation compex, eIF-2 . Met-tRNA$_f^{Met}$. GTP [2–6], in which the metabolically inert analog, GDPCP, can be stubstituted for GTP [4,5,7]. This complex binds to 40S ribosomal subunits, producing a quaternary initiation complex [4–7], in which eIF-2 is located in the neighbourhood of a limited group of ribosomal proteins [8]. In addition to the interactions with Met-tRNA$_f^{Met}$, GTP (GDPCP) and 40S ribosomal proteins in initiation complex formation, an interaction of eIF-2 with mRNA has also been reported [9,10]. The binding properties of the individual factor subunits have so far been studied only on subunits separated under denaturing conditions [11]. The present experiments were designed to investigate the functions of the subunits directly in the intact factor. By use of chemical cross-linking we have identified the β subunit of eIF-2 as the Met-tRNA$_f$ binding component in the ternary initiation complex.

Abbreviations: ABAI, methyl *p*-azidobenzoylaminoacetimidate; APTPI, 5-(*p*-azidophenyl)-4,5-dithiapentanimidate; DEB, 1,2:3,4-diepoxybutane; GDPCP, β,γ-methylene-guanosine-5′-triphosphate; GTP, guanosine-5′-triphosphate; IAA, iodoacetamide; LiDS, lithium dodecylsulfate; SDS, sodium dodecylsulfate; TDE, thiodiethanol; TEA, triethanolamine

2. Materials and methods

2.1. *Preparation of eIF-2 and covalent attachment of heterobifunctional reagents*

Initiation factor eIF-2 was isolated from rat liver (O. N., P. W. and T. H., in preparation) and dialysed for 4 h against 0.1 M KCl, 20 mM TEA–HCl, pH 7.6 and 5 mM TDE. The heterobifunctional reagents ABAI and APTPI (P. W. and O. N., in preparation) were dissolved in TEA–HCl, pH 8.0, immediately before use. The reaction mixture containing 35 μg eIF-2, 90 mM KCl, 70 mM TEA–HCl, pH 8.0, 4.5 mM TDE and 0.5 mM of either ABAI or APTPI. After 30 min at 0°C the reaction was stopped by the addition of Tris–HCl, pH 7.6, to a final concentration of 10 mM.

2.2. *Formation of the ternary initiation complex, eIF-2 . Met-tRNA$_f^{Met}$. GDPCP*

The reaction mixture contained, in a final vol. of 0.3 ml, 35 μg eIF-2 (with or without covalently bound heterobifunctional reagents), 100 mM KCl, 20 mM TEA–HCl, pH 7.6, 5 mM TDE, 0.2 mM GDPCP and 20 μg of rat liver Met-tRNA$_f^{Met}$ (charged and purified as previously described [12]). After 5 min at 37°C excess Met-tRNA$_f$ was removed by gel filtration on a 6 × 50 mm Sephadex G-100 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M KCl, 20 mM TEA–HCl, pH 7.6, 5 mM TDE and 0.2 mM GDPCP.

2.3. *Cross-linking of the ternary complex*

The gel-filtrated initiation complex formed with ABAI- or APTPI-derived eIF-2 was irradiated for

60 min (0°C) at 366 nm using an ultraviolet lamp (Desaga, Heidelberg, GFR). Control samples were kept on ice protected from irradiation.

The initiation complex formed with unsubstituted eIF-2 was cross-linked by incubation for 20 min at 37°C with 1 mM DEB [13].

2.4. *Labelling of proteins with* ^{125}I

After cross-linking, IAA was added to a final concentration of 1 mM, and the proteins were labelled by incubation for 60 min at 0°C with *N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenylpropionate) (The Radiochemical Centre, Amersham, England) [14]. The reaction was stopped by the addition of 10 mM Tris–HCl, pH 7.6. Proteins from the 40S ribosomal subunit of rat liver were added as carrier, and the material was precipitated with ethanol or 0.1 M acetic acid in acetone.

2.5. *Isolation of covalent $tRNA_f^{Met}$-protein complexes*

The precipitates were dissolved in 0.1 ml 50 mM TEA–HCl, pH 7.6, 1% LiDS and 1 mM IAA, and were placed onto 4 ml LiBr (suprapure, E. Merck, Darmstadt, FRG) density gradients containing 50 mM TEA–HCl, pH 7.6. The gradients were centrifuged for 15 h at 35 000 rev./min (20°C) in a Beckman SW60 rotor, and divided into 20 consecutive fractions, which were precipitated in the presence of 10 μg carrier protein as described above.

2.6. *Identification of polypeptides cross-linked to $tRNA_f^{Met}$*

The fractions containing the covalent $tRNA_f^{Met}$-protein complexes were dissolved in 1% SDS, 10 mM TDE and 0.1 M NaOH and incubated for 60 min at 37°C for degradation of the tRNA. The samples were neutralised with 1 M H_3PO_4 and analysed by SDS electrophoresis in 7–12% polyacrylamide slab gels [15]. Autoradiography of the stained and dried gels was carried out directly or by the use of a Cronex High Plus Intensifying Screen (Du Pont de Nemours & Co., Wilmington, DE) at −70°C.

3. Results and discussion

The activity of eIF-2 in ternary complex formation was only moderately reduced (10–20%) after coupling with the heterobifunctional reagents, ABAI and APTPI, under the conditions described. By ultraviolet irradiation of the ternary complex, the subunits of the factor became covalently linked to Met-$tRNA_f^{Met}$ and also to each other. The possibility of unspecific protein-$tRNA_f^{Met}$ interaction had been

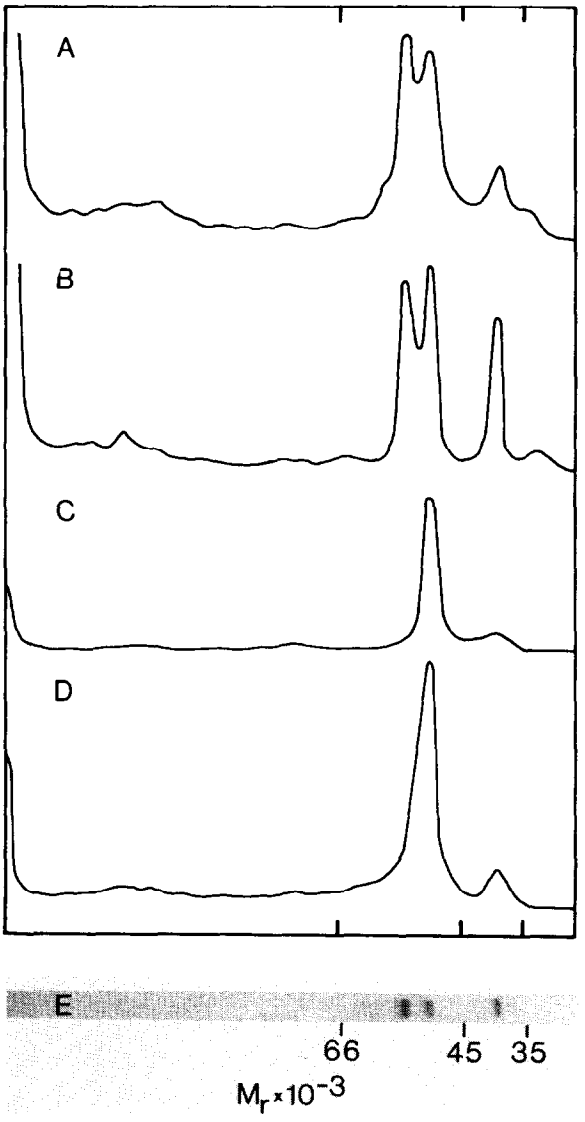

Fig.1. The use of LiBr density gradient centrifugation for isolating eIF-2 polypeptides cross-linked to $tRNA_f^{Met}$ in the ternary initiation complex, eIF-2 . Met-$tRNA_f^{Met}$. GDPCP. After cross-linking the polypeptides were labelled with ^{125}I and dissociated with LiDS. (A) Factor derivatised with ABAI or APTPI. Ternary complex not illuminated (control); (B) same as (A), but ternary complex cross-linked by illumination; (C) ternary complex cross-linked with DEB (factor unsubstituted).

reduced to a minimum by gel filtration of the ternary complex before irradiation. The presence of GDPCP in the medium during cross-linking served to increase the stability of this complex. Polypeptides not covalently bound to $tRNA_f^{Met}$ were dissociated by LiDS treatment and separated from the covalently linked polypeptide-$tRNA_f^{Met}$ complexes by density gradient centrifugation in LiBr. As shown in fig. 1B, these covalent complexes formed a distinct band at a density of 1.29–1.35 g/cm^3, while uncomplexed polypeptides accumulated at the top of the gradient. After degradation of the $tRNA_f^{Met}$, the polypeptides were identified by SDS gel electrophoresis. As can be seen from fig.2A,B, all subunits of eIF-2 were directly cross-linked to $tRNA_f^{Met}$ by both ABAI and APTPI.

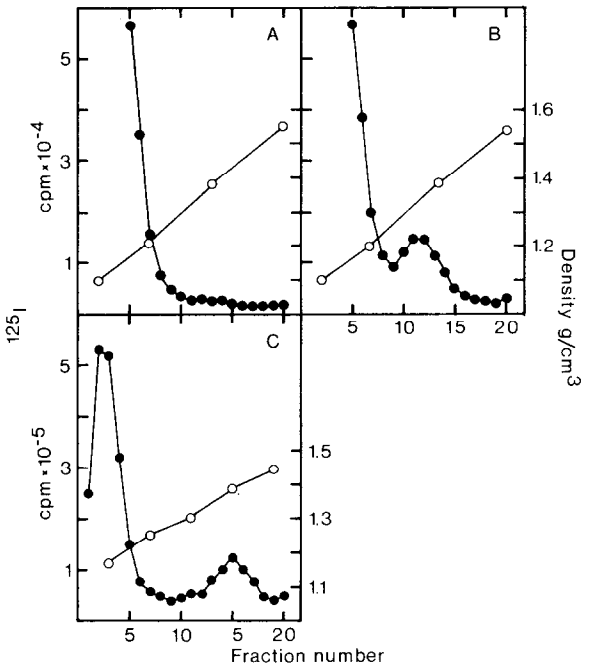

Fig.2. Identification of labelled polypeptides cross-linked to Met-$tRNA_f^{Met}$. Isolated $tRNA_f^{Met}$-linked polypeptides (fig.1) were freed from polynucleotides by alkali treatment and analysed by SDS gel electrophoresis. Autoradiographs of the stained gels were quantified by use of a microdensitometer (Joyce, Loebl and Co. Ltd, Newcastle, England). As cross-linking reagents ABAI (A), APTPI (B) and DEB (C,D) were used. In A and B the gel was exposed for 14 d (−70°C) using an intensifying screen. In C and D the gel was exposed for 1 and 3 d respectively. A sample of the original eIF-2 preparation was analysed in the same gel (shown at the bottom of the figure). The molecular weight designations refer to bovin serum albumin, ovalbumin and pepsin (Sigma, St-Louis. MO).

It is concluded that in the ternary initiation complex the distance between each of the three eIF-2 subunits and the $tRNA_f^{Met}$ was within the effective range of the two heterobifunctional reagents (i.e. 10 Å and 12 Å respectively).

A discrimination between the individual subunits was obtained by the short bifunctional reagent, DEB [14]. In these experiments unmodified eIF-2 was used for ternary complex formation. As is shown in fig.1C, the covalent $tRNA_f^{Met}$-protein complex obtained with DEB had a density in LiBr of about 1.39 g/cm^3 i.e. significantly higher than that obtained with the heterobifunctional reagents. After tRNA hydrolysis and SDS electrophoresis the β subunit of eIF-2 could be identified as the predominant polypeptide (fig.2C,D). Minor amounts of the α subunit were observed after prolonged autoradiographic exposure. In comparison with the previous experiments (fig.2A,B) much less of the labelled material accumulated at the top of the gel.

It is concluded that in the functional initiation complex the β subunit of eIF-2 is located in close proximity (4 Å or less) to Met-$tRNA_f^{Met}$. The results strongly suggest that the β subunit is the Met-$tRNA_f^{Met}$ binding component of eIF-2 in the ternary initiation complex. This is consistent with recent results of Barrieux and Rosenfeld [11] using isolated factor subunits in binding experiments.

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