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GTP interacts with the γ-subunit of eukaryotic initiation factor eIF-2

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Eukaryotic initiation factor eIF-2 is an oligomeric protein consisting of three different subunits. During initiation of protein synthesis eIF-2 interacts with GTP, Met-tRNA$_f$ and 40 S ribosomal subunit. By affinity labeling with a photo-reactive GTP analogue it was shown that in the binary complex [eIF-2·GTP] GTP is in contact with the γ-subunit of eIF-2.

Photoaffinity labeling *GTP analog* *GTP binding* *Initiation factor-2* *(Rat liver)*

1. INTRODUCTION

Eukaryotic initiation factor eIF-2 plays an important role in the first steps of protein synthesis initiation. It forms a ternary complex with GTP and Met-tRNA$_f$, which binds to the 40 S ribosomal subunit (reviews, [1,2]). At the end of the initiation cycle eIF-2 is released from the ribosome as an [eIF-2·GDP] complex and GDP is replaced by GTP by the GDP–GTP exchange factor eIF-2B (review, [3]). Nucleotide exchange on eIF-2 seems to be very important for the initiation cycle, as it is one of the targets of protein synthesis regulation [3].

Initiation factor eIF-2 is an oligomeric protein containing three different subunits of M_r values 32000 (α), 35000 (β), and 55000 (γ), as determined by sedimentation equilibrium centrifugation [4]. It is not yet clear which of these subunits is responsible for GTP binding. The only information about GTP binding was obtained with factor subunits isolated under denaturating conditions [5]. By this approach it was found that the α-subunit partially retains the ability to bind GDP. We show by affinity labeling experiments that, in the binary complex consisting of native eIF-2 and a photoreactive analogue of GTP, the γ-subunit of eIF-2 is labeled.

2. MATERIALS AND METHODS

Initiation factor eIF-2 was prepared from rat liver as described in [6] with some modifications. The 40–50% ammonium sulfate fraction of the microsomal wash preparation was adsorbed to a DEAE(DE52)-cellulose column in a buffer containing 0.1 M KCl and the eIF-2 containing protein was eluted at 0.21 M KCl. This material was adsorbed to a rRNA-cellulose column and eluted with a linear gradient (0.1–0.5 M KCl). Fractions containing eIF-2 activity were pooled, concentrated, and stored under liquid nitrogen. The initiation factor was about 85% pure as evaluated from densitometric scans of electrophoretic analyses.

The photoreactive derivative, γ-(*p*-azido)anilide of GTP (fig.1), was prepared from [^{3}H]GTP (UVVVR, Prague, CSSR; 1400 dpm per pmol) as described in [7]. ATP and GDP were purchased from Boehringer, Mannheim, FRG.

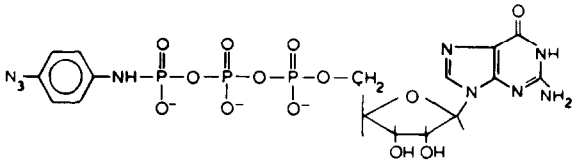

Fig.1. γ-(*p*-azido)anilide of GTP.

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Affinity labeling experiments were performed as follows: mixtures containing eIF-2 and γ-(*p*-azido)anilide of [^{3}H]GTP in the amounts indicated were incubated at 37°C for 15 min, chilled and irradiated at 313 nm with a mercury lamp with glass and water filters for 20 min with an overall dose of 10^5 erg/mm^2. For estimation of non-covalent and covalent binding, aliquots were removed after complex formation and irradiation, respectively. After irradiation sodium dodecyl sulfate (SDS) and β-mercaptoethanol up to final concentrations of 1.3 and 6%, respectively, were added, and the samples were again incubated at 37°C for 15 min. Excess of non-bound GTP analogue was removed from protein by centrifugation through a 1-ml Sephadex G 50 column in 10 mM sodium phosphate (pH 7.0), 1% SDS, 20 mM β-mercaptoethanol.

The samples were subjected to SDS–gel electrophoresis according to [8] in 6 × 180 mm rod gels containing 10% acrylamide and 0.3% bisacrylamide. Electrophoresis was performed at about 8–10 mA per gel tube for 20 h with 100 mM sodium phosphate, pH 7.0, 0.2% SDS as running buffer. After electrophoresis, gels were cut into 2-mm pieces and gel pieces were extracted at 40°C for 20 h in 0.7 ml 10 mM Tris–HCl, pH 7.5, 1% SDS each. Radioactivity was counted in 10 ml 20% Triton–toluene scintillator. Untreated eIF-2 was run on a parallel gel and stained with Coomassie G 250 to ascertain the positions of the factor subunits. The positioning of the subunits was according to [4] and with bovine serum albumin, ovalbumin and chymotrypsinogen A as molecular mass markers. For correlation to the radioactivity profiles the shrinking of the gel by staining was corrected.

3. RESULTS AND DISCUSSION

The photoreactive derivative, γ-(*p*-azido)anilide of GTP (fig.1) has been successfully used in affinity modification of bacterial elongation factor EF-T_u [7]. We use it here to identify the subunit of eukaryotic initiation factor 2 which binds GTP in the binary complex [eIF-2·GTP]. The binary complex was formed in a mixture containing 5-fold excess of eIF-2 over GTP analogue to avoid unspecific labeling of the factor. Under these conditions about 10% of GTP analogue was bound to the factor. To demonstrate the specificity of the interaction between γ-(*p*-azido)anilide of GTP and eIF-2, non-covalent binding of the analogue to eIF-2 was measured in the absence and in the presence of an excess of GDP, which binds to the nucleotide binding center of the factor with higher affinity as GTP [3] (fig.2A). For comparison, the binding was performed also in the presence of ATP, which should not interact with eIF-2. The results clearly show that the binding of the GTP analogue can be specifically inhibited by GDP but not by ATP.

The covalent linking of γ-(*p*-azido)anilide of GTP to eIF-2, however, as measured by trichloroacetic acid precipitation after irradiation of the complexes (fig.2B) is inhibited by GDP and ATP to the same extent. But as is shown by electrophoretic analyses of the irradiated complexes (fig.3), these inhibitions are due to qualitative different effects. In the absence of additional nucleotides mainly the γ-subunit of eIF-2 is labeled. In the presence of GDP the labeling of the γ-subunit of eIF-2 is completely inhibited, whereas the labeling of some contaminating proteins is not.

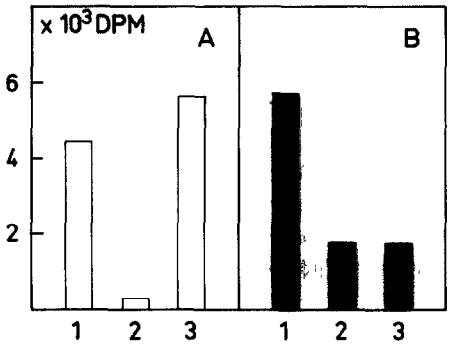

Fig.2. Non-covalent binding and covalent linking of γ-(*p*-azido)anilide of GTP to eIF-2. A. Non-covalent binding. Incubation mixtures (100 μl) contained 10 mM Hepes (pH 7.4), 100 mM NH_4Cl, 0.2 mM β-mercaptoethanol, 12.4 μM eIF-2, 2.3 μM [^{3}H]GTP analogue (1) with the following additions: 0.2 mM GDP (2) or 0.2 mM ATP (3). After incubation (37°C, 15 min) 15-μl aliquots were removed, diluted with cold buffer, filtered through membrane filters, and the radioactivity counted. B. Covalent linking. Incubation mixtures as in A were irradiated as described in section 2, 15-μl aliquots were removed, precipitated in the presence of 100 μg carrier protein with cold 5% trichloroacetic acid filtered through glass fibre filters (GF/A), and the radioactivity counted.

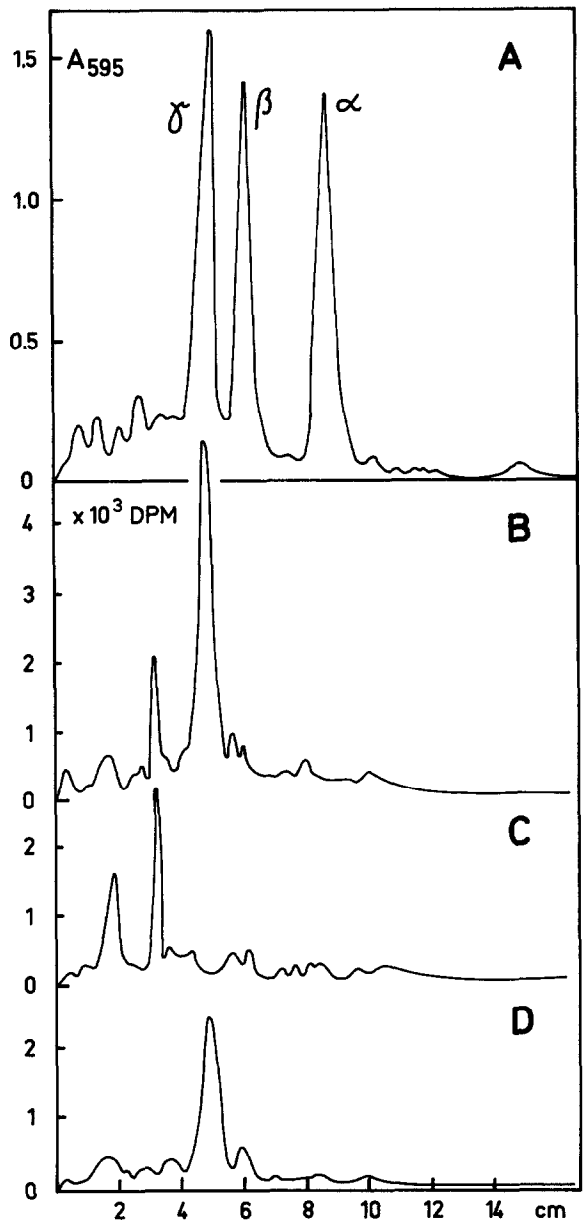

Fig.3. Analysis of affinity labeling of eIF-2 in SDS–gel electrophoresis. Incubation mixtures (100 μl each) contained 10 mM Hepes (pH 7.4), 100 mM NH_4Cl, 0.2 mM β-mercaptoethanol, 12.4 μM eIF-2, 2.3 μM [^{3}H]GTP analogue (B) with the following additions: 0.2 mM GDP (C) or 0.2 mM ATP (D). Incubation, irradiation, and processing of the samples were as described in section 2. Untreated eIF-2 (40 μg) was run on a parallel gel. The densitogram of the stained gel is shown in A.

In contrast, the labeling of the contaminating proteins can be completely inhibited by ATP but the labeling of the γ-subunit of eIF-2 only by 50%. Subunits of eIF-2 other than the γ-subunit were not found to be labeled under these conditions. We conclude from these results that in the binary complex [eIF-2·GTP] GTP is bound to the γ-subunit of eIF-2 at least with its terminal phosphate group. The partial inhibition of eIF-2 labeling by a 100-fold excess of ATP could be explained by some affinity of ATP to the factor.

To elucidate the role of the individual subunits of eIF-2 in the course of initiation complex formation two different approaches have been used so far. By crosslinking of initiation complexes it could be shown that the β-subunit is located near Met-$tRNA_f$ [9,10] and that α- and γ-subunits are in contact with the 40 S ribosome [11,12]. In the second approach, the binding activities of eIF-2 subunits isolated under denaturating conditions were measured [5]. It was found that the β-subunit is able to bind Met-$tRNA_f$ or mRNA and that the α-subunit can bind GDP. Ligand binding activity of the γ-subunit was not tested. The only indication that the γ-subunit can interact with GTP comes from phosphorylation studies with eIF-2 [13].

The fact that the α-subunit is not labeled by the GTP analogue in our experiment could be explained as follows: (i) in native eIF-2 GTP is not specifically bound to the α-subunit; (ii) the α-subunit cannot be labeled with our reagent, due to a lack of nucleophilic groups in the neighbourhood of GTP binding center. As shown in [14], the γ-(*p*-azido)anilide of ATP reacts preferentially with nucleophilic groups; (iii) the GTP binding center is located in the contact region of the α- and γ-subunits, where the terminal phosphate group is in close proximity to the γ-subunit. Further experiments with other GTP analogues should be performed to clarify these questions.

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