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GTP interacts through its ribose and phosphate moieties with different subunits of the eukaryotic initiation factor eIF-2

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We have previously shown that a GTP derivative bearing *p*-azidoaniline at the γ-phosphate group specifically labels the γ-subunit of eukaryotic initiation factor eIF-2. In the present study a new GTP derivative carrying the photoreactive group at the ribose moiety of GTP was applied for affinity labeling of eIF-2 in different initiation complexes. Using this GTP analogue the β-subunit of eIF-2 was found to be specifically labeled in all complexes investigated. It is concluded that GTP interacts with both the β- and γ-subunit of eIF-2: the guanosine moiety is in contact with the β-subunit and the γ-phosphate group with the γ-subunit.

Photoaffinity labeling; Eukaryotic initiation factor eIF-2; GTP binding

1. INTRODUCTION

The eukaryotic initiation factor eIF-2 consisting of the subunits α (32 kDa), β (35 kDa), and γ (55 kDa) [1], is involved in the early steps of initiation of protein synthesis. It binds GTP and Met-tRNA$_f$ to form a ternary complex, which is then bound to the 40 S ribosomal subunit. At the end of the initiation cycle, GTP hydrolysis is catalyzed by eIF-5 and the [eIF-2·GDP] complex is released from the ribosome. Finally, GDP is replaced by GTP at eIF-2 by means of the guanosine nucleotide exchange factor eIF-2B (reviews in [2,3]).

In order to localize the GTP-binding site to one of the subunits of eIF-2 different approaches have been applied which, however, led to partially conflicting results (discussed in [4]). In previous affinity labeling studies using a GTP derivative carrying the photoreactive group at the γ-phosphate group, we could demonstrate that GTP contacts the γ-subunit of eIF-2 [4,5]. On the other hand, cDNA sequence analysis of eIF-2β revealed a guanine-binding site to be located at the β-subunit of eIF-2 [6,7]. This apparent discrepancy led us to extend the affinity labeling experiments by application of a new GTP analogue carrying the photoreactive group at the ribose moiety of GTP. With this analogue the β-subunit of eIF-2 was found to be specifically labeled indicating that GTP by its ribose moiety is in close contact to the β-subunit. Thus, the GTP-binding site seems to be formed by both the β- and γ-subunits of eIF-2.

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2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]GTP (111 TBq per mmol) and [^{3}H]methionine (555 GBq per mmol) were purchased from Amersham (England). Nonradioactive nucleotides (ATP, GTP, GMPPCP) and total tRNA from yeast were obtained from Boehringer Mannheim (FRG). 4-(3-Trifluoromethyldiazirino)benzoic acid (TDBA) synthesized according to [8] was a kind gift from Dr A.S. Girshovich (Poustchino).

2.2. *Preparations*

The preparation of initiation factor eIF-2 from rat liver and

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of polyclonal antibodies against eIF-2 subunits raised in rabbits have been described recently [9]. 40 S ribosomal subunits were prepared from rat liver as described by Stahl et al. [10]. The preparation of partially purified [^{3}H]Met-tRNA$_f$ has been briefly described in a previous paper [4].

2.3. *Synthesis of the GTP analogue, 4-(3-trifluoromethyldiazirino)benzoyl hydrazone of periodate oxidized GTP (Guo(2′,3′-TDBH)ppp)*

The analogue was synthesized as described by Girshovich et al. [11] for the preparation of 2-nitro-4-azidobenzoyl hydrazone of periodate oxidized GTP with minor modifications. The photoreactive compound 4-(3-trifluoromethyldiazirino)benzoyl hydrazide (TDBH) was obtained by hydrazinolysis of the *N*-hydroxysuccinimidoester of TDBA, which was prepared from TDBA according to [8]. To 10 mg (30 μmol) of *N*-hydroxysuccinimidoester of TDBA in 1 ml dioxane, 6 μl of 25% hydrazine hydrate (about 30 μmol) were added and the reaction was allowed to proceed for about 1 h at 0°C. The mixture was evaporated to an oil under reduced pressure, dissolved in acidified water, and the hydrazide was extracted in ether. After evaporation, the compound was crystallized from 50% ethanol (m.p. 183).

The hydrazone of periodate oxidized [α-^{32}P]GTP was obtained as follows: 9.25 MBq of [α-^{32}P]GTP was incubated with 1 mM $NaIO_4$ in a 20 μl reaction mixture for 30 min at 0°C. To avoid unspecific oxidation, the mixture was kept in the dark. The reaction was stopped by addition of 3 μl of 20 mM 2-mercaptoethanol. After 15 min incubation on ice, 22 μl of a hydrazide solution (1 mg/ml) in 50% ethanol was added, and the reaction was continued for 1 h at 30°C. Thereafter, the whole mixture was applied to thin layer electrophoresis on a cellulose sheet for 2 h (20 mM Na-citrate, pH 5.0, 300 V). The sheet was dried and exposed to autoradiography. The band carrying the radioactive product (electrophoretic mobility about 0.75 in comparison to GTP) was cut out, and the strip was washed with ethanol to elute the salts present in the electrode buffer. The cellulose was scraped off from the strip and the material was extracted twice with 1 ml water. The extract was lyophilized and dissolved in distilled water to a concentration of about 10^6 cpm per μl. The yield of the reaction was estimated from the autoradiograph to be about 70–80%. The analytical electrophoresis of the product showed a purity of about 95%. The analogue was stored at −20°C.

2.4. *Affinity labeling experiments*

2.4.1. Binary initiation complex [eIF-2·Guo(2′,3′-TDBH)ppp]

Reaction mixtures (30 μl total volume) containing 40 mM Hepes-KOH, pH 7.3, 100 mM NH_4Cl, 1 mM $MgCl_2$, 0.2 mM 2-mercaptoethanol, about 10^6 cpm [α-^{32}P]Guo(2′,3′-TDBH)-ppp (30 nM final concentration), 8 μM eIF-2 and further additions as indicated in fig.2 were incubated for 15 min at 37°C. After chilling, the samples were irradiated with a mercury lamp at a wavelength of ≥340 nm for 5 min, with a total dose of 10^5 erg/mm^2. The photoreaction was stopped by addition of sample buffer for electrophoresis to give a final concentration of 1% SDS, 2% 2-mercaptoethanol and 10% glycerol. The samples were incubated for 15 min at 37°C and subjected to SDS-gel electrophoresis in 1 mm thick slab gels. The electrophoresis system of Laemmli [12] in the modification of Anderson et al. [13] was applied using a homogeneous separation gel containing 15% acrylamide and a 0.09% methylene bisacrylamide. This system allows a good separation of the β- and γ-subunit of eIF-2, and the subunits migrate in the order α, γ, β [1]. Molecular mass marker proteins (bovine serum albumin, ovalbumin and chymotrypsinogen A) were run on a parallel slot. The gels were stained by Coomassie brilliant blue, dried and exposed to X-ray films, type HS 11 (ORWO, Wolfen GDR), for 7 h with an intensifying screen.

The experimental details of affinity labeling of the binary complex [eIF-2·γ-(*p*-azido)anilide of GTP] are described in [4]. They are essentially the same as above with the following exceptions: the concentration of the GTP photoanalogue (2.3 μM) was considerably higher because of its lower specific radioactivity and irradiation was performed at ≥310 nm, because the azido group needs a somewhat lower wavelength for activation. Furthermore, labeled protein bands were visualized by fluorography in order to detect tritium labeling.

2.4.2. Ternary initiation complex [eIF-2·Guo(2′,3′-TDBH)ppp·Met-tRNA$_f$]

This experiment was performed essentially as described for the binary initiation complex except that 10 pmol partially purified [^{3}H]Met-tRNA$_f$ were included in the reaction mixture. $MgCl_2$ was omitted.

2.4.3. Quaternary initiation complex [eIF-2·Guo(2′,3′-TDBH)ppp·Met-tRNA$_f$·40 S ribosomal subunit]

Reaction mixtures (50 μl total volume) contained 40 mM Hepes-KOH, pH 7.3, 100 mM NH_4Cl, 0.2 mM 2-mercaptoethanol, about 5×10^5 cpm [α-^{32}P]Guo(2′,3′-TDBH)ppp (30 nM final concentration), 8 μM eIF-2, and 40 pmol partially purified [^{3}H]Met-tRNA$_f$. After incubation for 15 min at 37°C, 60 μg of 40 S subunits and $MgCl_2$ to a final concentration of 7 mM were added and the incubation was continued for further 5 min. Quaternary complexes were separated from remaining eIF-2 and GTP analogue by centrifugation through a 1 ml Sephacryl S300-column in buffer containing 20 mM Hepes-KOH, pH 7.3, 100 mM NH_4Cl, 6 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol. The 40 S complexes (150 μl final volume, each) were irradiated in 30 μl portions as described above. Concentrated sample buffer for electrophoresis was added, and the samples were processed as described for the binary initiation complex.

2.5. *Immunoblotting*

In order to demonstrate the identity of β- and γ-subunit of eIF-2, 1 μg eIF-2 per cm gel width was run in two parallel slots of the slab gel. The protein was electrophoretically transferred to nitrocellulose membranes (Sartorius, Goettingen; 0.45 μm pore size) according to the method described by Kyhse-Anderson [14]. 5 mm-strips of the blot were incubated in the presence of 1 μg antibody against eIF-2β or eIF-2γ, respectively, in a total volume of 2 ml blocking solution consisting of 5% milk powder, 50 mM Tris-HCl, pH 7.3, 150 mM NaCl and 0.05% Tween 20 for 16 h at 4°C under gentle shaking. Bound antibodies were detected by subsequent incubation with anti-rabbit IgG antibodies conjugated with peroxidase. Staining was performed with 1,4-chloronaphthol.

3. RESULTS AND DISCUSSION

A new GTP analogue, 4-(3-trifluoromethyldiazirino)benzoyl hydrazone of periodate oxidized [α-^{32}P]GTP, designated here as Guo(2',3'-TDBH)ppp (GTP analogue II in fig.1) was synthesized as described in section 2. The procedure is quite simple; the photoreactive compound, TDBH, can be prepared beforehand and stored indefinitely. The reaction with periodate oxidized GTP can be performed in one tube and takes only a few hours. The product obtained is to our knowledge the first carrier-free GTP photoanalogue. The photoreactive trifluoromethyldiazirino group upon irradiation at $\geq$340 nm yields a very reactive carbene radical forming crosslinks only with immediate neighbours [8,15,16].

The GTP analogue was applied as affinity marker in order to label the eukaryotic initiation factor eIF-2. Different initiation complexes were formed using the GTP analogue instead of GTP: binary complex [eIF-2·GTP], ternary complex [eIF-2·GTP·Met-tRNA$_f$] and quaternary complex [eIF-2·GTP·Met-tRNA$_f$·40 S ribosomal subunit]. After irradiation, the labeled polypeptide was identified by SDS-gel electrophoresis followed by autoradiography. In order to prove the specificity of labeling an excess of unlabeled GTP or ATP was included in parallel reaction mixtures. In the case of the quaternary complex GMPPCP was applied instead of GTP, because it is more efficient in forming a stable quaternary complex. The results are shown in fig.2A. They clearly demonstrate an exclusive and specific labeling of eIF-2β in all three types of initiation complexes. The reaction is absolutely dependent on irradiation (not shown). The identity of β- and γ-subunits was additionally proved by immunoblotting (lanes 12 and 13).

From these results we conclude that in all complexes investigated the ribose moiety of GTP is in close contact with the β-subunit of eIF-2. On the other hand, in the affinity labeling experiments using γ-(*p*-azido)anilide of GTP (analogue I in fig.1) we observed a specific labeling of the γ-subunit of eIF-2 which, for comparison, is shown in fig.2B.

The different results obtained with the two photoanalogues can be explained by the assumption that GTP is bound to the β-subunit of eIF-2 by its guanosine moiety, and to the γ-subunit by its γ-

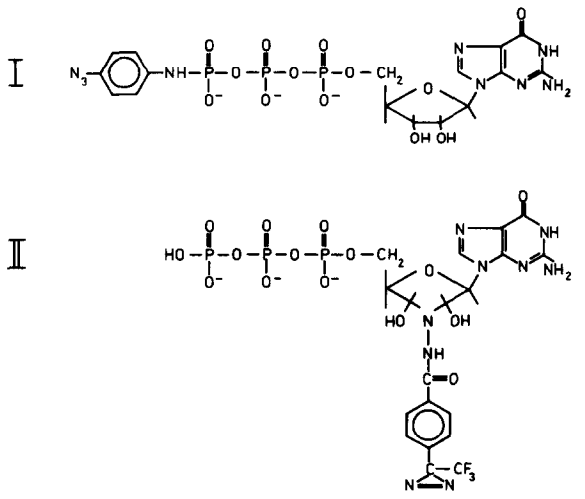

Fig.1. Structure of GTP analogues. Analogue I: γ-(*p*-azido)anilide of GTP. Analogue II: 4-(3-trifluoromethyldiazirino)benzoyl hydrazone of periodate oxidized GTP (Guo(2',3'-TDBH)ppp).

phosphate group. This hypothesis would also explain the failure to observe any labeling of the quaternary initiation complex by γ-(*p*-azido)anilide of GTP [4]. One could assume a conformational change of eIF-2 to occur upon binding to the 40 S ribosomal subunit leading to an exposure of the γ-phosphate group of GTP, whereas the guanosine part of the molecule is still bound to the β-subunit (fig.2A, lane 9). The exposure of the γ-phosphate group possibly enables its interaction with initiation factor eIF-5 later on.

After completion of the experiments, our attention was drawn to preliminary results on the use of GTP analogues carrying an azido group at the ribose or guanine moieties [17]. Although all protein bands of the eIF-2 preparation were labeled, there was some indication of specificity with respect to the β-subunit.

cDNA sequence analysis of eIF-2β also indicated the presence of a guanine-binding domain in the β-subunit [6,7]. However, a portion of the consensus sequence of the phosphoryl-binding sequence was not found in the eIF-2β sequence [7]. Thus, the phosphate-binding site could be located in the γ-subunit which is not yet sequenced.

The only result arguing against an interaction of GTP with the β-subunit is the observation that preparations of β-deficient eIF-2 are still able to fulfil all functions of the factor in the GTP-

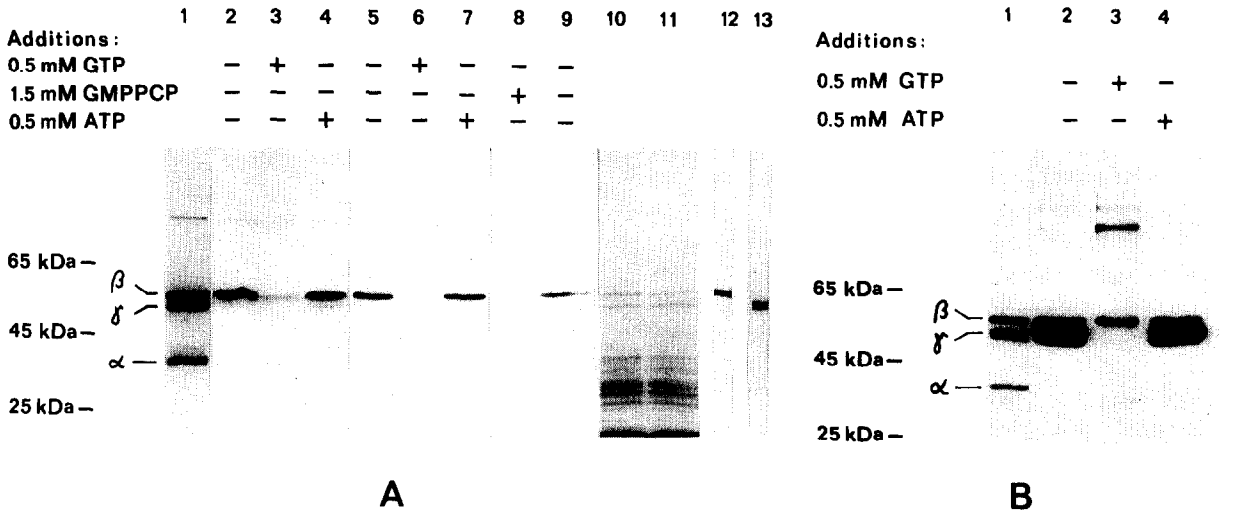

Fig.2. (A) Affinity labeling of eIF-2 in different initiation complexes by Guo(2',3'-TDBH)ppp. Lanes: 1, Coomassie-stained gel showing the positions of the eIF-2 subunits; 2–9, autoradiographs of the affinity labeling experiments (lanes 2–4, binary complex; lanes 5–7, ternary complex; lanes 8,9, quaternary complex); 10,11, Coomassie-stained gel of the autoradiographs shown in lanes 8,9; 12,13, immunoblots incubated with antibodies against eIF-2β (lane 12) and eIF-2γ (lane 13). (B) Affinity labeling of eIF-2 in the complex with γ-(*p*-azido)anilide of GTP. Lanes: 1, Coomassie-stained gel showing the positions of the eIF-2 subunits; 2–4, autoradiographs of the affinity labeling experiments.

dependent binding of Met-tRNA$_f$ to 40 S subunits [18–22]. This discrepancy cannot be explained at present.

The assumption that eIF-2α is the GTP-binding subunit [23] can be excluded in the light of the affinity labeling experiments ([4,5] and this paper) as well as on the basis of the sequence data of eIF-2α [24].

Summarizing the results, the conclusion can be drawn that the GTP-binding site is constituted by both the β- and γ-subunits of eIF-2. The guanosine moiety of the molecule appears to be bound to the β-subunit, whereas the γ-phosphate group appears to be in contact with the γ-subunit at least during those stages of the initiation process, in which eIF-2 is not bound to the ribosome.

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