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The α and γ subunits of initiation factor eIF-2 can be cross-linked to 18S ribosomal RNA within the quaternary initiation complex, eIF-2·Met-tRNA$_f$·GDPCP·small ribosomal subunit

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ABSTRACT

Initiation factor eIF-2 from rat liver was reacted with the hetero-bifunctional cross-linking reagents ABAI or APTPI without diminishing its ability to form the quaternary initiation complex with Met-tRNA$_f$, GDPCP and the small ribosomal subunit. Upon irradiation with UV light, subunits α and γ of eIF-2 became covalently linked to 18S ribosomal RNA. The subunits were identified electrophoretically after isolation of the covalent protein-rRNA complexes and subsequent degradation of the rRNA by nuclease and alkali treatments. The close proximity of the two factor subunits to sequences of ribosomal RNA within the quaternary complex could be confirmed in a second set of experiments using unmodified, ^{125}I-labeled factor and diepoxybutane as cross-linking reagent.

INTRODUCTION

Initiation factor eIF-2 forms a ternary complex with Met-tRNA$_f$ and GTP (1-5), which binds to the small ribosomal subunit forming a quaternary complex (3-6). Within this complex, ribosomal proteins S3, S3a, S6, S13/16, S15 and S15a could be cross-linked to the factor with dimethyl-5,6-dihydroxy-4,7-dioxo-3,8-diazadecanbisimidate (7). These proteins have therefore been suggested as candidates for functional interactions between eIF-2 and the ribosomal subunit. From the binding of eIF-2 to rRNA, which was used for factor purification by affinity chromatography (8), it could be supposed that interactions between rRNA and eIF-2 might also occur during the initiation complex formation. In the present study a close proximity of factor subunits α and γ, and 18S rRNA within the quaternary initiation complex, eIF-2·Met-tRNA$_f$·GDPCP·40S ribosomal subunit, is demonstrated by cross-linking with different reagents.

MATERIALS AND METHODS

Initiation factor eIF-2 from rat liver (8), [^{35}S]Met-tRNA$_f$ (9) and 40S

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rat liver ribosomal subunits (10) were prepared as described. GDPCP was purchased from Serva International (Heidelberg, GFR) and diepoxybutane (DEB) from Merck-Schuchardt (Munich, GFR).

Preparation of reagents

Methyl-*p*-azidobenzoylaminoacetimidate hydrochloride (ABAI): *p*-azidobenzoic acid, 1 g, prepared from *p*-aminobenzoic acid (11) was heated with 0.73 ml $SOCl_2$ in a water bath until complete solubilisation. Excess $SOCl_2$ was removed by evaporation and the residue solubilized in 12 ml acetone. The chloride was added in portions to a 12 ml aqueous solution of 1 g $NaHCO_3$ and 0.83 g aminoacetonitrile hydrochloride (Aldrich-Europe, Beerse, Belgium). After evaporation of the acetone, the nitrile was collected on a filter and recrystallized from ethanol (m.p. 118-120^oC). Of the nitrile, 0.1 g was suspended in 0.5 ml dry methanol and saturated with dry HCl at 0^oC. After 2 h at 0^oC, ether was added to the solution until slight turbidity. After 16 h at 0^oC, more ether was added. The crystallized imidate was collected rapidly by filtration and recrystallized from methanol by the addition of ether in small portions (m.p. 173-175^oC; decomposition).

Methyl-5-(*p*-azidophenyl)-4,5-dithiapentanimidate hydrochloride (APTPI): In 50 ml benzene 4.5 g 4,4'-dithiobisphenylazide prepared from 4,4'-dithiodianiline (Aldrich, Milwaukee, Wis.) (11) was reacted for 16 h at 20^oC with 1.66 g 3-mercaptopropionitrile (12). The solution was then heated for 30 min at 50^oC, extracted with 0.2 N NaOH, dried with anhydrous Na_2SO_4, and chromatographed on an aluminium oxide 90 (Merck, Darmstadt, GFR) column in benzene. Of the resulting yellow oil, 0.5 g was solubilized in 3 ml dry methanol and the imidate synthesized as described for ABAI (m.p. 180-183^oC; decomposition).

Reaction of eIF-2 with ABAI and APTPI

To 0.15 mg eIF-2 in 100 µl buffer containing 100 mM KCl, 20 mM triethanolamine•HCl (TEA•HCl), pH 8.0, 5 mM thiodiethanol and 0.1 mM EDTA, 11 µl of a freshly prepared solution of 5 mM ABAI or APTPI in 0.5 M TEA•HCl, pH 8.0 were added. After incubation for 30 min at 0^oC in the dark, the factor was labeled by the addition of *N*-succinimidyl-3-(4-hydroxy,5-[^{125}I]iodophenyl)-propionate prepared from 0.1 mCi sodium [^{125}I]iodide (13). After 30 min at 0^oC, Tris•HCl, pH 7.8, was added to a final concentration of 10 mM. The specific activity of the factor was approximately 0.1 µCi per µg.

Formation of quaternary initiation complex and cross-linking procedure

The reaction mixtures contained, in final volumes of 0.2 ml, 0.15 mg ABAI- or APTPI-modified [^{125}I]eIF-2, 0.5 mg [^{35}S]methionine-charged rat liver tRNA, 100 mM KCl, 0.2 mM GDPCP and 35 mM TEA•HCl, pH 7.8. After 5 min incu-

bation at 37°C, 2 mg rat liver, 40S ribosomal subunits in 50 mM KCl, 50 mM TEA•HCl, pH 7.8, 5 mM $MgCl_2$ and 5 mM thiodiethanol were added and the magnesium concentration was increased to 7 mM. The incubation was continued for 3 min at 37°C and the complex separated from unbound factor and tRNA by chromatography on a 6 x 50 mm Sepharose 4B (Pharmacia, Uppsala, Sweden) column in 50 mM KCl, 50 mM TEA•HCl, pH 7.8, 7 mM $MgCl_2$ and 0.1 mM GDPCP. Fractions of 3 droplets were collected and pooled (cf. Fig. 1). An aliquot of the complex was used for [^{35}S]methionine determination, and the remaining material was irradiated for 60 min using a UV lamp (140 W, Solimed, GDR), emitting light between 300 and 400 nm. The sample beaker was kept in ice and protected from heat irradiation by a quartz filter cell through which ice water was circulated. Initiation complex containing unmodified, ^{125}I-labeled eIF-2 was cross-linked by twice repeated incubations for 30 min at 37°C with fresh aliquots of 1 mM DEB.

Isolation of covalently linked complexes between eIF-2 subunits and rRNA

To the irradiated samples, 0.7 volumes of ethanol was added and the precipitate was collected by centrifugation for 5 min at 10 000 x *g*. The precipitate was solubilized in 0.3 ml buffer containing 0.2 M Tris•HCl, pH 7.8, 1% (w/v) lithium dodecylsulfate, 1 mM iodoacetamide and 0.5 M LiCl by incubation for 5 min at 56°C. The material was layered onto a 15-30% (w/v) sucrose gradient in 20 mM Tris•HCl, pH 7.8, 1% (w/v) lithium dodecylsulfate and 50 mM LiCl, and the 18S rRNA separated from non covalently linked proteins by centrifugation for 14 h at 40 000 rpm (0°C) in a SW 40 rotor (Beckman Instruments, Palo Alto, Cal.). Ribonucleoproteins and RNA in the pooled fractions (see Fig. 2) were precipitated by the addition of 2.5 volumes of ethanol at -20°C. Precipitated material was recentrifuged in a second sucrose gradient as described above.

Identification of cross-linked eIF-2 subunits

The precipitated mixture of 18S RNA and RNP complexes was solubilized in 25 µl buffer containing 0.1 M Tris•HCl, pH 7.8, and 1 mM iodoacetamide. After incubation with 10 µg RNase A (Serva, Heidelberg, GFR) and 5 units of ribonuclease T1 (Calbiochem, San Diego, Cal.) for 30 min at 37°C, 10 µg of protein from small ribosomal subunits were added. Thereafter 20% SDS and 4 N NaOH were given to final concentrations of 1%, and 0.2 N, respectively. After incubation for 2 h at 37°C the proteins were precipitated with 10% trichloroacetic acid, redissolved in 50 µl 2% SDS, 0.1 M Tris base, 1 mM iodoacetamide and separated by polyacrylamide gel electrophoresis (14). Autoradiography was performed for 7 days at -78°C using Kodak X-Omat film and Cronex High Plus intensifying

Screen (Du Pont de Nemours & Co., Wilmington, Del.).

Estimation of [^{35}S]methionyl-tRNA$_f$

An aliquot of the quaternary initiation complex [^{35}S]Met-tRNA$_f$·^{125}I--labeled eIF-2·GDPCP·40S ribosomal subunit was incubated with 0.1 N NaOH and 10 mM thiodiethanol for 10 min at 37°C and dried by lyophilization. The residue was dissolved in 10 µl water and spotted onto a TLC aluminium sheet Silica Gel (Merck, Darmstadt, GFR), which was developed in butanol/acetic acid/water (4:1:1). The area between Rf 0.25 and 0.35 containing the [^{35}S]methionine was scraped off and counted in a toluene based scintillation mixture.

RESULTS AND DISCUSSION

The use of gel chromatography on Sepharose 4B for separating the quaternary initiation complex, [^{125}I]eIF-2·[^{35}S]Met-tRNA$_f$·GDPCP·40S ribosomal subunit, from uncomplexed components of the incubation mixture is illustrated in Fig. 1. Assuming a molecular weight of eIF-2 of 125 000 (15,16), 0.2 pmol Met-tRNA$_f$ per pmol added factor was recovered in the isolated complex. The factor could be reacted with ABAI or APTPI at concentrations up to 0.5 mM without considerable loss of activity. Thus, after modification with ABAI or

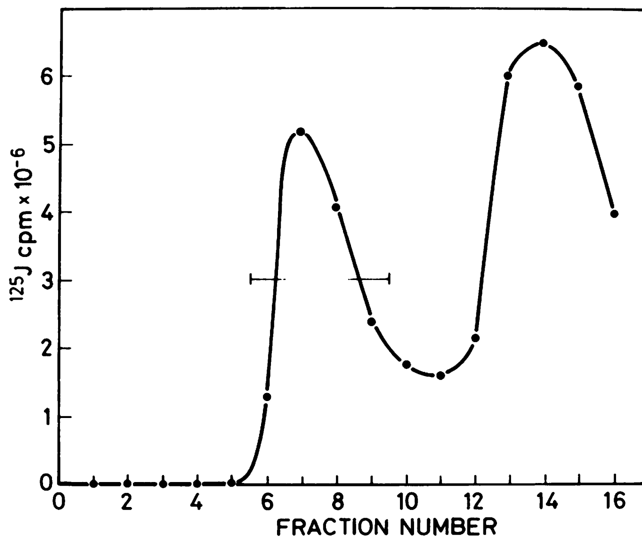

Fig. 1. Separation of the quaternary initiation complex, [^{125}I]eIF-2·[^{35}S]-Met-tRNA$_f$·GDPCP·40S ribosomal subunit, from hydrolyzed iodination reagent and uncomplexed components by gel chromatography on Sepharose 4B. The fractions marked by a bar were pooled and used for cross-linking.

APTPI under standard conditions, and subsequent labeling with ^{125}I, one pmol factor bound 0.13-0.18 pmol [^{35}S]Met-tRNA$_f$ into quaternary complex isolated as shown in Fig. 1.

When the isolated quaternary complex with modified eIF-2 was irradiated with UV light for 1 h, the subunits of the factor became covalently bound to 18S rRNA. Following sucrose gradient centrifugation in the presence of lithium dodecylsulfate they were found in the 18S rRNA fraction (Fig. 2). After degradation of the rRNA with ribonuclease and alkali treatments, the α and γ subunits of eIF-2 could be identified by electrophoresis (Fig. 3a,b).

The same result was obtained in experiments using DEB (17) for the cross--linking of unmodified ^{125}I-labeled factor to 18S rRNA within the complex (Fig. 3c). The proximity of the terminal C-atoms of this reagent (about 4 Å) suggest that subunits α and γ of initiation factor eIF-2 are very close to the 18S rRNA within the complex.

The β subunit of eIF-2 is involved in initiation complex formation (18) by interacting with Met-tRNA$_f$ (19). Subunit β as such was not recovered after the degradation of the 18S rRNA, and was therefore not directly cross-linked to RNA. However, its presence in covalently linked protein-rRNA complexes was

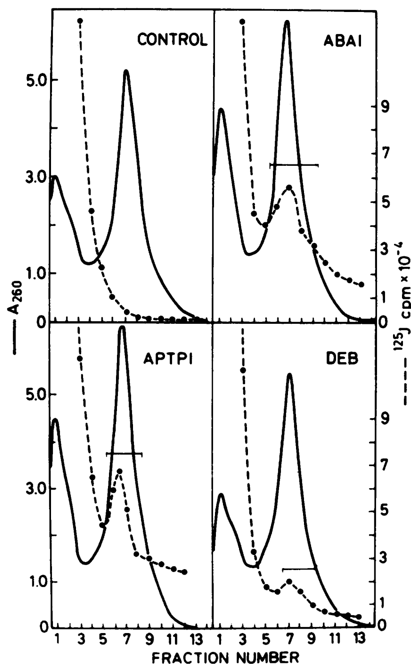

Fig. 2. Sucrose gradient centrifugation of covalently linked complexes between subunits of [^{125}I]eIF-2 and 18S rRNA in the presence of lithium dodecylsulfate. Marked fractions were pooled, precipitated and recentrifuged once. Experimental details are described under "Materials and Methods".

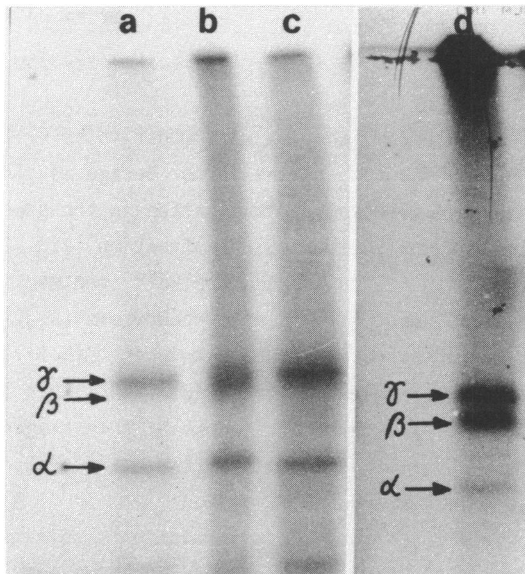

Fig. 3. Identification of ^{125}I-labeled polypeptides cross-linked to 18S rRNA with ABAI (a), APTPI (b and d) or DEB (c). The mixture of RNA and covalent ribonucleoprotein complexes was degraded by nuclease and alkali treatments (a-c), or incubated with 0.1 M β-mercaptoethanol (d), and analyzed by SDS gel electrophoresis. The gels were exposed for 7 days at -70°C using an intensifying screen.

demonstrated after the reductive cleavage of all cross-links introduced with APTPI (Fig. 3d). This suggests that cross-links occurred between the factor subunit themselves.

Since subunits α and γ of eIF-2 can also be cross-linked to ribosomal proteins (P. Westermann, unpublished results), it is concluded that these subunits are involved in the contact between the ternary complex, eIF-2•Met--$tRNA_f$•GDPCP, and the small ribosomal subunit.

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