CROSSLINKING OF INITIATION FACTOR eIF-2 TO PROTEINS OF THE SMALL SUBUNIT OF RAT LIVER RIBOSOMES

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1. Introduction

Eukaryotic initiation factor eIF-2 binds Met-tRNA$_f$ and GTP to 40 S ribosomal subunits [1–7]. The binding site for eIF-2 on the 40 S subunit was studied with ^{14}C-labeled eIF-2 and ^{3}H-labeled ribosomal subunits, using DBI as a crosslinking reagent. High molecular weight protein conjugates were isolated which predominantly contained ribosomal proteins S2, S3 and S18 (according to [11]) covalently bound to the initiation factor.

2. Materials and methods

2.1. *Preparation of ^{14}C-labeled initiation factor eIF-2*

Initiation factor eIF-2 from rat liver was isolated as in [6,7] and methylated by a procedure modified from [4] 0.5 mg eIF-2 in 0.1 ml buffer was dialyzed for 3 h against 100 mM TEA–HCl buffer (pH 8.0) containing 100 mM KCl, 0.1 mM EDTA, 5 mM β-ME and 10% (v/v) glycerol The glycerol had been purified as a 20% (v/v) aqueous solution by adding 10 mM sodium borohydride, stirring for 1 h, and filtration through an Amberlite mixed bed ion exchanger (MB 1, Serva, Heidelberg). After dialysis, 0.54 μmol [^{14}C]formaldehyde (Isocommerz, Dresden, 42 Ci/mol) was added, followed at 5, 10, 15 and 20 min by successive 0.14 μmol portions of sodium borohydride. After 30 min (0°C) the preparation was dialyzed against buffer A (20 mM TEA–HCl (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 5 mM β-ME and 10% glycerol). The specific activities of the factor preparations were 5–6 × 10^3 dpm/μg protein.

Abbreviations DBI, dimethyl 4,7-dioxo-5,6-dihydroxy-3,8-diazadecanbisimidate; TEA, triethanolamine; β-ME, β-mercaptoethanol, SDS, sodium dodecyl sulfate; GMPPCP, β, γ-methylene-guanosine-5′-triphosphate

2.2. *Preparation of ^{3}H-labeled 40 S ribosomal subunits*

Small ribosomal subunits from rat liver [8] were dialyzed against buffer B (50 mM TEA–HCl, (pH 7.6) 50 mM KCl, 5 mM $MgCl_2$ and 5 mM β-ME). To 10 mg subunits in 0.3 ml buffer B, 0.08 ml 500 mM potassium borate (pH 9.0) and 2 μmol formaldehyde were added, followed by successive 0.5 μmol portions of sodium [^{3}H]borohydride (Institute of Isotopes, Budapest, 300 Ci/mol) at 5, 10, 15 and 20 min. After 30 min (0°C) the labeled subunits were dialyzed against buffer B. The specific activities of the subunit preparations were 3–5 × 10^4 dpm/μg.

2.3. *Formation of the quaternary complex [eIF-2 · GMPPCP · Met-tRNA · 40 S subunit]*

^{14}C-Labeled eIF-2 (0.5 mg in 0.3 ml buffer A) was incubated for 5 min at 37°C with 60 nmol GMPPCP and 0.6 mg rat liver tRNA, charged with methionine by use of *E. coli* aminoacyl-tRNA synthetases [7]. Then 10 mg ^{3}H-labeled 40 S ribosomal subunits in 0.3 ml buffer B were added, and Mg^{2+} adjusted to 4 mM. Unbound protein and tRNA were removed by filtration through a 1.5 × 20 cm column of Sepharose 4B (Pharmacia, Uppsala). Complex formation was assayed by using [^{3}H]Met-tRNA (6000 dpm/pmol) in combination with unlabeled ribosomal subunits (fig.1).

2.4. *Crosslinking of the quaternary complex*

The crosslinker DBI [9] was synthesized from tartaric acid bisazide and aminoacetonitrile (P W., in preparation). Preparations of quaternary complex from 3 mg 40 S subunits in 0.2 ml buffer B were reacted with 0.25, 0.75 or 1 25 μmol DBI solubilized in 0.05 ml 500 mM TEA–HCl (pH 9.0). After 30 min at 20°C the reaction was stopped by the addition of 12.5 μmol NH_4Cl.

2.5. *Isolation of crosslinked protein complexes*

Crosslinked quaternary complexes were sedimented through 3.0 ml 50 mM TEA–HCl buffer (pH 7.6) containing 15% (w/v) sucrose, 500 mM KCl, 5 mM $MgCl_2$ and 5 mM β-ME. Centrifugation was for 16 h at 33 000 rev./min using a Spinco SW 60 rotor. The sediments were suspended in 0.2 ml buffer B and the proteins extracted by the addition of 0.01 ml 2 M $MgCl_2$ and 2 vol. acetic acid. The extract was dialyzed for 2 h against 5 mM β-ME and then for 2 h against 1 mM β-ME in 100 mM sodium phosphate (pH 7.0). The solution was heated for 5 min at 60°C with 1% (w/v) SDS, incubated for 30 min at 37°C with 5 mM iodoacetamide, mixed with 10 mM β-ME, and applied to a 1.5 × 80 cm Sepharose 6B column equilibrated with 50 mM sodium phosphate (pH 7.0), 0.5% SDS and 1 mM β-ME [10]. Fractions of the eluate were combined as indicated in fig.2, supplemented with 0.2 mg unlabeled protein from 40 S ribosomal subunits and concentrated by use of an Amicon ultrafiltration cell (Filter PM-10).

2.6 *Identification of crosslinked proteins*

The concentrated protein solutions (0.5 ml) were divided into two portions One of these was dialyzed for 45 min against 20 mM sodium phosphate buffer (pH 6.0) containing 0.05% SDS and 15 mM sodium periodate. The control was dialyzed in the absence of periodate Proteins were precipitated with 5 vol acetic acid · acetone (3 : 500, v/v), dried in vacuum, solubilized in 0.1 ml 6.0 M urea and analyzed by two-dimensional electrophoresis [11]. The radioactivity of the ribosomal proteins was estimated as in [12]. Background values obtained with control proteins not treated with periodate were subtracted.

3. Results and discussion

The activity of eIF-2 in the binding of Met-$tRNA_f$ to 40 S ribosomal subunits was not markedly impaired by reductive methylation. During gel filtration through Sepharose 6B ^{14}C-labeled eIF-2 comigrated with the 40 S subunits and bound [^3H]Met-tRNA (fig.1, peak A).

When ^3H-labeled 40 S subunits alone were treated with 5 mM DBI no protein complexes >6–8 × 10^4 daltons were observed by gel filtration in the presence of SDS (fig.2A).

The quaternary initiation complex ^{14}C-labeled eIF-2 · ^3H-labeled 40 S subunit · Met-$tRNA_f$ · GTP was treated with 1, 3 or 5 mM DBI either directly or

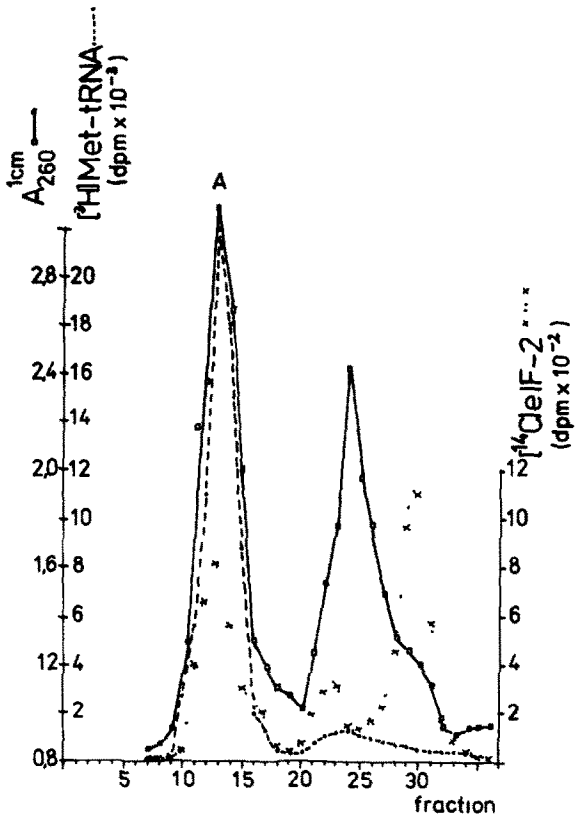

Fig.1. Gel filtration of the quaternary complex formed with 1.0 mg 40 S ribosomal subunits, 0.1 mg [^{14}C]eIF-2, 50 pmol [^{3}H]Met-tRNA and GTP through a 0.9 × 20 cm Sepharose 6B column equilibrated with 50 mM TEA · HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-ME and 0.2 mM GTP. From consecutive 15-drop fractions 0.3 ml aliquots were withdrawn, precipitated with 10% (w/v) trichloroacetic acid (0°C) and collected on Millipore filters (HA, 0.45 μm) for differential liquid scintillation counting.

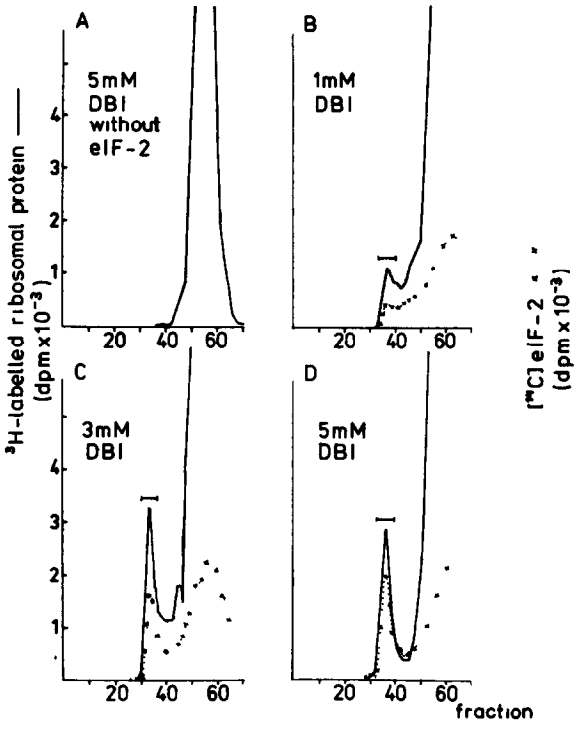

Fig.2. Gel filtration of proteins extracted from DBI-treated 40 S ribosomal subunits (A) or quaternary initiation complex (B–D). The proteins were pre-labeled by reductive methylation as indicated. The 1.5 × 80 cm Sepharose 6B column was equilibrated with 50 mM sodium phosphate, 0.5% SDS and 1 mM β-ME. Fractions marked with horizontal lines were pooled and used for further analysis

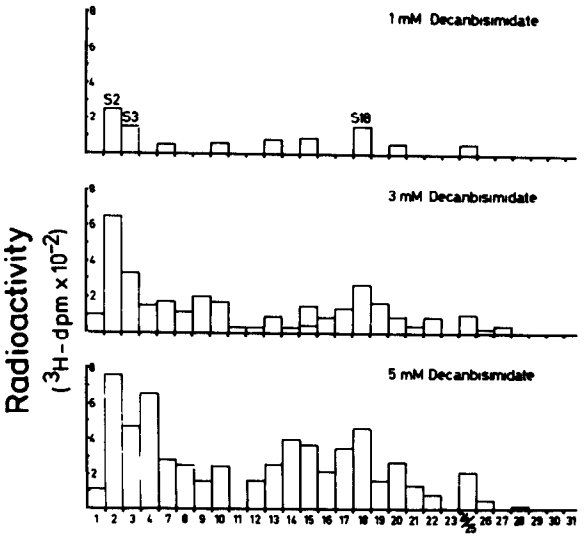

Fig.3. Ribosomal proteins obtained by periodate cleavage of high molecular weight protein conjugates as indicated in fig.2B–D. The proteins were separated by two-dimensional gel electrophoresis [13] and prepared for liquid scintillation counting [14]. Bars represent mean values of 7 independent experiments Standard deviations do not exceed 15%.

after gel filtration through Sepharose 6B. Gel filtration of the extracted proteins in the presence of SDS yielded protein fractions $>10^5$ daltons, containing initiation factor as well as ribosomal proteins (fig.2B,C,D). Protein fractions marked with horizontal lines (fig.2) were treated with periodate and submitted to two-dimensional electrophoresis together with carrier protein. No ^{14}C-activity (from labeled eIF-2) was found in areas occupied by ribosomal proteins. After crosslinking with 1 mM and 3 mM DBI ^{3}H-labeled cleavage products mainly migrate into the positions of proteins S2, S3 and S18 (fig.3). It is concluded that in the quaternary complex amino groups of these ribosomal proteins are within 1.3 nm from amino groups of the initiation factor. These proteins may therefore belong to the factor binding site of the small ribosomal subunit or to its immediate neighborhood.

The additional proteins which became crosslinked at 3 mM and 5 mM DBI may be bound to eIF-2 either directly or via other ribosomal proteins. Studies of DBI-induced crosslinking within the 40 S ribosomal subunit proper have shown that proteins S1, S4, S7, S8 and S10 can be crosslinked to proteins S2 or S3, and proteins S15 and S17 to protein S18 (P.W., in preparation). The possibility of an indirect binding mechanism is therefore obvious.

For two of the ribosomal proteins crosslinked to eIF-2 at low DBI concentrations there is additional evidence for interaction with eIF-2 or tRNA. Antibodies against protein S2 inhibit eIF-2 dependent binding of [^{3}H]Met-tRNA$_f$ to small ribosomal subunits [13]. The accessibility of protein S18 in 80 S ribosomes to reaction with iodo [^{14}C]acetamide or methyl [^{14}C]acetimidate is dimished by peptidyl-tRNA bound to the ribosomal P-site [14]. This suggests that S18 is located at or near the P-site, which also binds Met-tRNA$_f$ [15] probably still complexed with eIF-2.

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