Purification and Properties of Eukaryotic Initiation Factor 2 and Its Ancillary Protein Factor (Co-eIF-2A) from Yeast *Saccharomyces cerevisiae*

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Two peptide chain initiation factor activities, eIF-2, and Co-eIF-2A, were purified from the high speed supernatant fraction of the yeast *Saccharomyces cerevisiae* and their properties were studied. 1) In sodium dodecyl sulfate-polyacrylamide gels, purified eIF-2, showed two major polypeptide bands corresponding to molecular weights of 54,000 and 36,000. The molecular weight of eIF-2, determined using a density gradient centrifugation method, was approximately 140,000. 2) In sodium dodecyl sulfate-polyacrylamide gel, purified Co-eIF-2A showed a single polypeptide band corresponding to a molecular weight of 20,000. A similar molecular weight for Co-eIF-2A was also found using a density gradient centrifugation method. 3) In peptide reactions eIF-2, bound Met-tRNA, in the presence of Mg++, The reaction required GTP. Co-eIF-2A stimulated Met-tRNA, binding to eIF-2, (2-3-fold) and also rendered the complex stable to 3 x 10^{-2} M aurintricarboxylic acid. 4) This Co-eIF-2A activity was heat-labile and N-ethylmaleimide-insensitive. 5) Antibodies were prepared by injecting rabbits with homogeneous Co-eIF-2A. Such anti-Co-eIF-2A inhibited (60%) protein synthesis in a yeast cell-free protein synthesizing system and completely blocked Co-eIF-2A stimulation of Met-tRNA, 40 S initiation complex formation. Protein synthesis inhibition by anti-Co-eIF-2A was almost completely reversed by preincubation of the antibodies specifically with homogeneous Co-eIF-2A.

The presence of eukaryotic initiation factor 2 (eIF-2) and one or more eIF-2-ancillary protein factor activities (Co-eIF-2) has been reported in widely divergent eukaryotic organisms (for recent reviews, see Refs. 1-4). However, the relationships of different Co-eIF-2 activities present in different eukaryotic organisms and their precise roles in peptide chain initiation are not clear. In reticulocyte protein synthesis, several eIF-2-ancillary protein factor activities have been reported to be involved in a complex regulation mechanism which also included phosphorylation of the a-subunit of eIF-2 by protein synthesis inhibitors HRI (heme-regulated inhibitor) and dsI (double-stranded RNA activated inhibitor) (1-4). In our laboratory, we have isolated two low molecular weight polypeptides (M, 80,000 and 25,000) which we term Co-eIF-2A and Co-eIF-2B (5-9) and two high molecular weight protein complexes, Co-eIF-2 (10, 11) and RF (12, 13). The characteristics of these eIF-2 ancillary factors have been described in earlier papers (2, 5-13).

The characteristics of eIF-2 and Co-eIF-2 activities in lower eukaryotic organisms are less clear. Most of the work has been done using wheat germ (14-17), *Artemia salina* (18-22), and yeast (23). As with reticulocyte eIF-2, the ternary complex (Met-tRNA, eIF-2-GTP) formation by wheat germ eIF-2 is inhibited by Mg++ (15, 16), and preformed eIF-2-GDP complex is more stable in the presence of Mg++ than in its absence (16). On the other hand, *Artemia* eIF-2 activity is not inhibited by Mg++ (21, 22) and yeast eIF-2 requires Mg++ for maximum activity (23). Two different eIF-2-ancillary protein factor activities have been isolated from both wheat germ, Co-eIF-2A (M, ~ 19-21,000) and Co-eIF-2B (M, ~ 83,000) (15, 16), and *Artemia*, Co-eIF-2A (M, ~ 65,000) and Co-eIF-2B (M, ~ 105,000 and 112,000) (20). All four Co-eIF-2 activities resemble reticulocyte Co-eIF-2A activities (Co-eIF-2A and Co-eIF-2B) in that all the Co-eIF-2 activities stimulated Met-tRNA, binding to the homologous eIF-2 and formed aurintricarboxylic acid-resistant complexes (5, 8, 15, 16, 19, 20). None of the activities enhanced the rate of exchange between GDP bound to eIF-2 and free GDP (15, 16, 22), although one of the factors, wheat germ Co-eIF-2A, stimulated the binding of GDP to eIF-2 (16).

It is clear from the above that the characteristics of eIF-2 and Co-eIF-2 activities in different eukaryotic organisms differ considerably, and further studies will be necessary to establish which features in the mechanisms on the regulation of peptide chain initiation are the general and which are specific to particular organisms. With this objective, we have initiated a comparative study of the mechanism of peptide chain initiation in two widely divergent eukaryotic organisms, namely rabbit reticulocytes and yeast. We selected yeast as a second eukaryotic organism because of the recent development of an efficient *in vitro* yeast protein synthesizing system (24) and the availability of several temperature-sensitive yeast
peptide chain initiation mutants (25) which may be defective in one or more eIF-2-ancillary factor activities. In this paper, and in the accompanying paper (26), we present the results of our recent studies on purification and characterization of eIF-2 and Co-eIF-2A activities from yeast Saccharomyces cerevisiae and a comparison of the activities with similar activities in rabbit reticulocytes.

**EXPERIMENTAL PROCEDURES AND RESULTS**

In this paper, we have described purification of eIF-2, and Co-eIF-2A activities from yeast S. cerevisiae to near homogeneity. In SDS gels, purified eIF-2, shows two major polypeptide bands corresponding to molecular weights of 54,000 and 36,000. The M, 54,000 band is significantly more intense than the M, 36,000 band, suggesting the possible presence of two polypeptides of equal molecular weights in this band. Further studies using two-dimensional gel electrophoresis would be helpful to establish the subunit composition of eIF-2. The molecular weight of purified eIF-2, determined using a density gradient centrifugation is approximately 140,000.

The properties of purified eIF-2, preparation described here resemble in many ways the properties of purified eIF-2 preparation isolated from the same organism as reported previously by Bann et al. (24). However, whereas the previous authors reported a requirement for GDP for stabilization of eIF-2, activity during purification and storage, we have purified GDP at any stage of purification and storage did not have any significant effect on eIF-2, activity. Also, our purified Co-eIF-2A;O activities from yeast resemble in many ways the properties of purified eIF-2 preparations in rabbit reticulocytes.

The specific activities in rabbit reticulocytes (Co-eIF-2A;O, Co-eIF-2AS0, and Co-eIF-2AZ5) in rabbit reticulocytes (5), the characteristics of Co-eIF-2A;O activity reported here appear similar to the lower molecular weight Co-eIF-2A activities (Co-eIF-2A;O, Co-eIF-2A;O, and Co-eIF-2A;O) in rabbit reticulocytes (5, 7, 9), for maximum effect on the ternary complex it is necessary to add a severalfold molar excess of Co-eIF-2A;O relative to Co-eIF-2. We have previously offered several reasons for such requirements for reticulocyte Co-eIF-2A;O (9). We believe that similar reasons are also applicable for yeast Co-eIF-2A;O. The studies using anti-Co-eIF-2A;O reported in this paper clearly indicate that this activity, like the reticulocyte Co-eIF-2A;O activity reported earlier (5, 9), is required for protein synthesis in yeast. Anti-Co-eIF-2A;O inhibited (60%) protein synthesis in a yeast cell-free protein synthesizing system and such inhibition was almost completely reversed by preincubation of the antibodies specifically with homogeneous Co-eIF-2A;O.

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**REFERENCES**

Yeast eIF-2 and Co-eIF-2A

PURIFICATION AND PROPERTIES OF KAPRAVY'S INITIATION FACTOR 2 (eIF-2) AND ITS ANTAGONIST PROTEIN FACTOR (Co-eIF-2A) FROM YEAST

EXPERIMENTAL PROCEDURES

Materials

(eIF-2) bovine heart eIF-2 was prepared and purified by the procedure of Kapralov et al. (21). Purified bovine heart eIF-2 was stored in 50 mM TRIS-HCl, pH 7.5, 0.2 M NaCl containing 0.01% NaN3, 0.1 mM DTT, 0.1% sulfhydryl oxidase, and 0.1% phenylmethylsulfonyl fluoride (Sigma). Yeast proteins were separated using 10% SDS gel electrophoresis. Yeast protein mixtures were electrophoresed to separate yeast proteins from other sources of materials used in these studies as were previously described (21).

Growth of Yeast Cells and Preparation of Lysate and Postmitochondrial Supernatant

Yeast cells were grown in minimal synthetic media, washed, and resuspended in buffer A containing 20 mM TRIS-Cl, pH 7.5, 0.05 M KCl, and 0.1% NaN3. Yeast cells were disrupted by sonication as previously described (21). The supernatant was collected by centrifugation at 50,000 g for 30 min and stored at -20°C. The supernatant was then used as the source of eIF-2.

Purification of eIF-2 and Co-eIF-2A

These two activities were separated using DEAE-cellulose chromatography. The DEAE-cellulose was separated by anion exchange using a linear gradient of sodium phosphate (pH 7.5) by elution with buffer A containing 0.5 M KCl. The final preparation was dialyzed overnight against the same buffer with one change of the dialysis buffer (Fraction I).

Results

TABLE I

| Fraction | Total Protein (mg) | Total Activity (pmol) | Specific Activity (pmol/mg) | Purification Yield (fold)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>600.0</td>
<td>6,000</td>
<td>10</td>
<td>1.6</td>
</tr>
<tr>
<td>Fraction II</td>
<td>150.0</td>
<td>5,750</td>
<td>25</td>
<td>0.9</td>
</tr>
<tr>
<td>Fraction III</td>
<td>165.0</td>
<td>2,850</td>
<td>17</td>
<td>1.7</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>45.0</td>
<td>1,350</td>
<td>30</td>
<td>3.1</td>
</tr>
<tr>
<td>Glycerol gradient</td>
<td>0.65</td>
<td>501</td>
<td>870</td>
<td>0.9</td>
</tr>
</tbody>
</table>

One unit of activity is defined as that amount of protein required to bind 1 pmol of [35S]Met-tRNAf in the presence of GTP under the assay conditions.
Yeast eIF-2 and Co-eIF-2A

Upon SDS-polyacrylamide gel electrophoresis, the final eIF-2 preparation showed two polypeptide bands corresponding to molecular weights of 54K and 36K (Fig. 1). However, the 36K polypeptide band was significantly more intense than the 54K polypeptide band indicating the possible presence of two proteins. The molecular weight of Fraction V eIF-2, determined using a glycerol density gradient centrifugation method, was found to be 36K (Fig. S). This result also suggests that eIF-2 is possibly composed of one 36K subunit and two other subunits of approximate molecular weight, 54K.

Figure 1: SDS-polyacrylamide gel electrophoresis of eIF-2 and Co-eIF-2A. Electrophoresis of protein on SDS-polyacrylamide gel was performed according to the method of Laemmli (13) as described previously (9). Approximately 5 μg of eIF-2 (lane 2) and 2 μg of Co-eIF-2A (lane 1) were used in this experiment. The molecular weight of the 54K polypeptide band was calculated by comparison with the mobility of several known molecular weight markers.

Figure 2: Determination of the approximate molecular weights of eIF-2 and Co-eIF-2A using a glycerol density gradient centrifugation method. Samples (0.5 ml) of eIF-2 (125 μg) and Co-eIF-2A (100 μg) were applied to the top of linear glycerol gradients (10 - 30%, total volume 3 ml) containing 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.1 mM EDTA and 1 mM dithiothreitol. Samples were centrifuged at 45,000 rpm for 14 h (Exp. 2A; eIF-2) or 18 h (Exp. 2B; Co-eIF-2A) in a Beckman SW27.1 rotor at 4°C. The gradients were then fractionated and fractions collected. SDS-polyacrylamide gel electrophoresis of fractions 9.3 from Exp. 2A and 2B were plotted against the log of their molecular weights in this band. The molecular weight of the Co-eIF-2A preparation showed two polypeptide bands corresponding to molecular weights of 54K and 36K. The properties of purified yeast eIF-2, preparation (Fraction V) described here appear similar to those previously described for purified yeast eIF-2 (14). Our preparations are comparable to the results of Laemmli (13) and Periany et al. (23), who have also reported comparable specific activities.

The properties of purified yeast eIF-2, preparation (Fraction V) described here appear similar to those previously described for purified yeast eIF-2. Preparation (24), which are comparable to the results of Laemmli (13) and Periany et al. (23), who have also reported comparable specific activities. According to David et al. (25), yeast eIF-2 is composed of three polypeptides (M, 31K, 46K, 48K) and the molecular weight of the complex was determined using a glycerol density gradient centrifugation method in 127K. An interesting difference is that whereas the previous preparations have reported a requirement for GDP for stabilization of eIF-2, purification during and also during storage, we have purified eIF-2 activity in the absence of GDP, and our purified eIF-2 preparations are stored in liquid nitrogen in the absence of GDP for several months without appreciable loss in activity.

Co-eIF-2A was purified from the yeast ribosomal salt (5 M KCl) wash. This preparation was also contained eIF-2 activity. eIF-2, and Co-eIF-2A activities co-purified during Bio-Rex cellosil chromatography (Fraction II); the ternary complex formed with both Fraction I and II preparations were resistant to nucleotidized eIF-2 by the method of Laemmli (13) as described previously (9). Approximately 5 μg of eIF-2 (lane 2) and 2 μg of Co-eIF-2A (lane 1) were used in this experiment. The molecular weight of the 54K polypeptide band was calculated by comparison with the mobility of several known molecular weight markers.

Table II: Purification of Co-eIF-2A from yeast Saccharomyces cerevisiae

| Fractions | Total Protein (μg) | Total Activity (units) | Specific Activity (units/μg) | Purification (Fold) | Yield (%)
|-----------|-------------------|------------------------|-----------------------------|---------------------|--------|
| Fraction I | Hydroxyapatite | 50.0 | 7,500 | 150 | 1.0 | 100
| Fraction II | Phosphocellulose | 4.0 | 1,980 | 350 | 2.2 | 26
| Fraction III | Glycerol gradient | 0.9 | 990 | 1,100 | 7.3 | 13

*One unit of activity is defined as the amount of protein required to increase [35S]Met-tRNA to binding to purified eIF-2 from 1 pmol to 2 pmol.

Table II: Purification of Co-eIF-2A from yeast Saccharomyces cerevisiae

The results presented in Table III describe the effects of aurintricarboxylic acid, heat and NEM-treatment on Co-eIF-2A (25). We have previously reported that retinol and Co-eIF-2A in heat stable (5) whereas Co-eIF-2A in heat labile (9). Both Co-eIF-2A (retinol) and Co-eIF-2A (5,7) standards and also rendered the complex resistant to aurintricarboxylic acid. Yeast Co-eIF-2A activity is, however, heat labile. Significant loss in Co-eIF-2A activity was observed after heating the Co-eIF-2A preparation at 50°C for 4 min and the loss was more pronounced at higher temperatures. Co-eIF-2A activity was assayed both by its stimulation of eIF-2 activity and formation of aurintricarboxylic acid resistant complex. Both activities were lost in parallel upon heat treatments of Co-eIF-2A preparations. As shown above, Co-eIF-2A activity is insensitive to NEM-treatment.
Yeast eIF-2 and Co-eIF-2A

Table III

| Treatment | 5^35S|Met-18MA bound to Millipore filters (pmol) |
|-----------|-----------------------------------------|
| Experiment 1 | None | 0.60 | 0.04 | 1.25 | 0.35 | |
| Heat 50'  | 0.91 | 0.90 | 0.52 | 0.65 | 0.10 | 0.15 |
| 65'      | 0.70 | 0.70 | 0.15 | 0.70 | 0.10 | 0.15 |
| Experiment 2 | None | 0.4 | 0.95 | 0.95 | 0.95 |

Standard Millipore filtration assay conditions were used. In experiment 1, Co-eIF-2A was preincubated with 3 mM ANA at 27°C as described previously. Excess ANA was then removed by treatment with excess dithiothreitol. Where indicated 1 X 10^-5 M ATP was added to the reaction mixture.

Studies using Co-eIF-2A Antibodies

We prepared antibodies against homogeneous Co-eIF-2A preparations and studied the effects of such antibodies on in vivo protein synthesis and also in partial peptide chain initiation reactions. The in vivo yeast cell-free protein synthesis system used was prepared by the recently described procedure of Gotoh et al. (24). We found (Table IV) that this protein synthesizing system efficiently promoted amino acid incorporation and the addition of exogenous eIF-2 and Co-eIF-2A did not have any significant effect on such amino acid incorporation, indicating that this system contains saturating amounts of these peptide chain initiation factors. As shown in Table IV, addition of immune γ-globulin to Co-eIF-2A (anti-Co-eIF-2A) inhibited protein synthesis approximately 40% and the addition of control γ-globulin had no significant effect. The extent of this inhibition markedly increased at higher anti-Co-eIF-2A concentrations (data not shown). However, at higher concentrations, the control γ-globulin also inhibited protein synthesis. This protein synthesis inhibition by anti-Co-eIF-2A was mostly overcome by preincubation of anti-Co-eIF-2A with homogenous Co-eIF-2A and not with eIF-2. These results demonstrate that yeast Co-eIF-2A like recombinant Co-eIF-2A (5,6) is an integral component of protein synthesis in yeast.

Figure 1: Effects of anti-Co-eIF-2A on yeast eIF-2.

Table IV

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Addition</th>
<th>[3H]Leu incorporated/20 µl incubation mixture (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>None</td>
<td>26.4</td>
</tr>
<tr>
<td>2.</td>
<td>Control γ-globulin (3 µg)</td>
<td>23.3</td>
</tr>
<tr>
<td>3.</td>
<td>Immune γ-globulin (3 µg)</td>
<td>11.5</td>
</tr>
<tr>
<td>4.</td>
<td>None + eIF-2 (1 µg)</td>
<td>26.5</td>
</tr>
<tr>
<td>5.</td>
<td>None + Co-eIF-2A (10 µg)</td>
<td>27.0</td>
</tr>
<tr>
<td>6.</td>
<td>Control γ-globulin (3 µg) + Co-eIF-2A (10 µg)</td>
<td>24.0</td>
</tr>
<tr>
<td>7.</td>
<td>Control γ-globulin (3 µg) + eIF-2 (5 µg)</td>
<td>22.9</td>
</tr>
<tr>
<td>8.</td>
<td>Immune γ-globulin (3 µg) + Co-eIF-2A (10 µg)</td>
<td>22.8</td>
</tr>
<tr>
<td>9.</td>
<td>Immune γ-globulin (3 µg) + eIF-2 (15 µg)</td>
<td>13.7</td>
</tr>
</tbody>
</table>

[3H]Leu incorporation was determined following the procedure of Gotoh et al. (24). Reaction mixture (25 µl) contained 229 nCi/µl yeast lysate, 20 µM HEPES-KOH (pH 7.4), 0.1 M potassium acetate, 2 µM magnesium acetate, 0.1 µM ATP, 0.5 M UTP, 0.5 M CTP, 0.5 M GTP, 1 mM creatine phosphate, 20 µM of each of the 20 natural amino acids including radiolabeled [3H]leucine (sp. act. 10,000 cpm/µg), 100 µg of crude yeast HAM, and where indicated, control or immune γ-globulin at the indicated amounts. 10 µg Co-eIF-2A and 5 µg eIF-2 were used. In experiments 4 to 9, the peptide chain initiation factors were preincubated with γ-globulin (control and immune) at 37°C for 30 min. The reaction mixtures were then mixed with other components of the protein synthesis system and were incubated for 15 min. [3H]Leu incorporation in protein was determined on 20 µl aliquotes.

The results presented in Fig. 4 show the effects of addition of control and immune γ-globulin to Co-eIF-2A on Met-18MA 40S AUG initiation complex formation. As shown here, eIF-2, promoted Met-tRNAi binding to 40S ribosomes in the presence of AUG codon and such binding was significantly enhanced by the addition of homogenous Co-eIF-2A. Addition of control γ-globulin had no effect on such Met-tRNAi 40S AUG complex formation but addition of anti-Co-eIF-2A almost completely inhibited Co-eIF-2A stimulated formation of such complexes. These results show that Co-eIF-2A activity is required for optimum Met-tRNAi 40S complex formation in yeast.