Partial Reconstitution of Active Eukaryotic Ribosomes Following Dissociation with Dimethylmaleic Anhydride*

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Modification of yeast ribosomes with dimethylmaleic anhydride, a reagent for protein amino groups, is accompanied by loss of polypeptide-synthesizing activity. This activity can be recovered by incubation at pH 6, which produces regeneration of the modified amino groups. Dimethylmaleic anhydride modification also causes the dissociation of proteins from the ribosomes. Protein-deficient ribosomal particles are prepared from 80 S ribosomes or 60 S subunits by treatment with a molar excess of reagent relative to ribosomal particles equal to 6300 or 4800, respectively. The core particles from 80 S ribosomes lack 18% and those from 60 S subunits 40% of the total protein in the corresponding untreated control. In both cases, there is a selective release of proteins, the protein-deficient particles being able to reconstitute active 60 S subunits upon addition of the corresponding split proteins. The reconstituted ribosomal particles, when assayed in the presence of native 40 S subunits, are active in poly(U)-directed polyphenylalanine synthesis (30-90% of the activity of a dimethylmaleic anhydride-untreated control, as compared to 0-15% when the split proteins are excluded). This procedure could prove useful in the study of the structure and function of the eukaryotic ribosome.

Active prokaryotic ribosomes and their subunits have been reconstituted from either protein-deficient core particles and the corresponding released proteins or from free rRNA and ribosomal proteins (1-3). These reconstitution systems have been very useful in establishing structure-function relationships. In eukaryotic ribosomes, attempts to produce well-defined core particles which could be used to reconstitute active ribosomes have been accompanied by limited success. As with prokaryotic ribosomes, high salt concentrations have been used to disassemble eukaryotic ribosomes, with the hope of preparing homogeneous ribosomal "cores" from which active particles could be obtained upon addition of the released proteins. A certain specificity in the proteins released at different salt concentrations has been described as well as the preparation and characterization of more or less homogeneous core particles (4-14). Although there are reports of reconstitution of active particles from ribosomal cores and split proteins (4, 10, 11, 13, 14), a dependable method for the dissociation and assembly of eukaryotic ribosomes is still lacking.

The chemical modification of protein amino groups with organic acid anhydrides is a potential procedure to dissociate protein-containing structures (15). Using dimethylmaleic anhydride, after separation of the dissociated components, the reagent moieties can be easily removed by incubation at moderate acid pH, thus allowing the recovery of the regenerated components (16). Reversible modification with this reagent has been used to dissociate proteins from Escherichia coli ribosomes and to prepare protein-deficient ribosomal cores which reconstitute active particles upon addition of the corresponding released proteins (17-19). The usefulness of this procedure to reconstitute active prokaryotic ribosomes together with its apparent mildness and little relation with the methods so far used prompted us to investigate its applicability to the reconstitution of the more fragile eukaryotic ribosomes.

In this work, we have studied the potential value of reversible modification with dimethylmaleic anhydride in the preparation of eukaryotic protein-deficient ribosomal particles that could reconstitute active ribosomes upon incubation with the corresponding released proteins.

EXPERIMENTAL PROCEDURES

Materials and Assay of Activity—Yeast ribosomes were obtained from Saccharomyces cerevisiae haploid strain Y165 (a, his4, trp5, MA1) by grinding the cells with sea sand, differential centrifugation, and one-time washing with 0.5 M NH₄Cl (20). Ribosomal subunits were prepared by zonal centrifugation (20). Ribosomes and ribosomal subunits were suspended in 100 mM Tris-HCl (pH 7.4), 80 mM KCl, 12.5 mM MgCl₂, and 1 mM dithiothreitol at a ribosomal particle concentration of 50-100 mg/ml and kept at ~70 °C. Dimethylmaleic anhydride was purchased from Sigma. Poly(U)-directed polyphenylalanine synthesis was performed in 50 mM Tris-HCl (pH 7.4), 80 mM KCl, and 12.5 mM MgCl₂ using a crude system containing S. cerevisiae S-100 extract (100,000 x g supernatant) (21).

Modification with Dimethylmaleic Anhydride and Separation of Ribosomal Particles from Split Proteins—Ribosomes or 60 S subunits (1.5-3.0 μm) in 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (K⁺) (pH 8.2), 80 mM KCl, 12.5 mM MgCl₂, and 1 mM dithiothreitol (0.5-2.0 mM) were treated at room temperature with the desired amount of dimethylmaleic anhydride in dioxane (300 mg/ml), the pH being maintained at 8.2 by addition of base (18). This reaction was complete in 1 h. To separate the ribosomal particles from the released proteins, the preparations were centrifuged under two different sets of conditions. (a) Centrifugation took place at 2 °C and 63,000 rpm in a Beckman SW 65 rotor for 2.5 h in the buffer solution used in modification. Under these conditions there is inactivation of the ribosomal preparations including the dimethylmaleic anhydride-untreated controls. (b) The preparations were diluted with a suitable solution to the following ionic conditions: 50 mM Tris-HCl (pH 7.4), 500 mM ammonium acetate, 100 mM MgCl₂, and 5 mM 2-mercaptoethanol. Centrifugation took place at 5 °C and 30,000 rpm in a Beckman SW 50 rotor for 12-15 h. The pellet, containing the ribosomal particles, was suspended in 100-400 μl of 50 mM Tris-HCl (pH 7.4), 300 mM NH₄Cl, 15 mM MgCl₂, 250 mM sucrose, 1% glycerol, and 1 mM dithiothreitol. Only small inactivation of the controls is observed under these new conditions, which are, therefore, employed in the reconstitution experiments.

Regeneration of the Modified Amino Groups and Reconstitution

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of Active Particles—To regenerate the modified amino groups, the pH was lowered to a value close to 6.0 by dialysis against a buffer solution at this pH or by addition to the modified preparation of a low pH buffer. Dialysis took place at 0–4 °C for 24 h against 20 mM cacodylate (pH 6.0), 300 mM NH₄Cl, 15 mM MgCl₂, 250 mM sucrose, 15% glycerol, and 1 mM dithiothreitol. In the other case, a suitable solution (1-3 volumes) was added to the modified preparation to reach a final composition close to that of the above dialysis buffer, except for MgCl₂ and cacodylate which had final concentrations of 15–58 mM and 50 mM, respectively. Incubation took place at 0–4 °C for 24 h or at 37 °C for 2 h.

Reconstitution was conducted in three different ways. (a) To the modified ribosomal cores the corresponding modified split proteins were added, and the amino groups were regenerated by addition of a low pH buffer as indicated before, with incubation at 37 °C for 2 h. (b) The modified split proteins were regenerated by addition of a low pH buffer and incubation at 0–4 °C for 24 h. At the end of this incubation the corresponding modified ribosomal cores were added, and incubation at low pH was continued for 2 h at 37 °C. (c) The modified split proteins and the modified ribosomal cores were generated independently by addition of a low pH buffer and incubation at 0–4 °C for 24 h. After regeneration the split proteins and the ribosomal cores were mixed together and incubated at 37 °C for 1 h.

Sedimentation Analysis—Sedimentation was studied by centrifugation in linear 5–30% sucrose gradients containing 20 mM Tris-HCl (pH 7.4), 100 mM KCl, and 30 mM MgCl₂. The ribosomal preparations (30 pmol) were centrifuged in a Beckman SW 50 rotor at 45,000 rpm and 4 °C for 1.25 h. The distribution of particles along the gradient was determined with an ISCO density fractionator.

Electrophoresis and Quantitative Determination of Split and Core Proteins—The proteins obtained from ribosomal particles by extraction with 67% (v/v) acetic acid, as well as the split protein fraction, were dialyzed against 0.5% acetic acid and lyophilized prior to protein determination or gel electrophoresis (17). Protein concentration was determined as described by Lowry et al. (22). Two-dimensional polyacrylamide gel electrophoresis was performed by the procedure of Kaltenschmidt and Wittmann (23) as described by Howard and Traut (24).

RESULTS AND DISCUSSION

Treatment of yeast ribosomes with dimethylmaleic anhydride is accompanied by inactivation of polypeptide synthesis and dissociation of the 80 S ribosome into its large and small subunits (Fig. 1). A reagent molar excess of 800 is sufficient to cause complete inactivation of polyphenylalanine synthesis. Regeneration at pH 6.0 of the modified amino groups is followed by recovery of the polyphenylalanine synthetic capacity (Fig. 1A) and the original sedimentation pattern (Fig. 1B). After 10 h of incubation at pH 6.0 and 0 °C, the preparation that had been modified with a molar excess of reagent over ribosomes equal to 3200 recovered 65% of the original activity, showing a sedimentation pattern entirely similar to that of the control not treated with dimethylmaleic anhydride.

Modification is also accompanied by dissociation of proteins from the ribosomal particles. Attempts to reconstitute active ribosomes after separation of the split proteins from the ribosomal cores were initially unsuccessful, since ribosomal preparations, including the dimethylmaleic anhydride-treated, were inactivated during centrifugation. This inactivation could be prevented by decreasing the centrifugal force while increasing the centrifugation time and by decreasing the pH and increasing the concentrations of magnesium and monovalent cations (see centrifugation conditions (b) under "Experimental Procedures”). Fig. 2 shows the protein released from ribosomes by treatment with different amounts of dimethylmaleic anhydride when separation from the ribosomal cores is achieved by centrifugation under the two sets of conditions just mentioned. The inactivating conditions produce a higher release of proteins at all levels of dimethylmaleic anhydride used. At a molar excess of reagent over ribosomes of 30,000, more than 90% of the total ribosomal protein is liberated, while under the noninactivating conditions only 65% is released. The shapes of the liberation curves are also different. The noninactivating conditions produce a sigmoidal curve of release, which reaches a plateau at a molar excess of reagent relative to ribosomes around 1500. A sigmoidal curve of release indicates that there is cooperativity in the dissociation. When treating E. coli ribosomes with dimethylmaleic anhydride a sigmoidal release is also obtained (17). In contrast, by centrifuging the modified yeast ribosomes under the inactivating conditions, a hyperbolic shaped curve is produced. Apparently, upon modification with dimethylmaleic anhydride the ribosomal particles become more susceptible to centrifugation, and additional proteins are released.

Using the noninactivating conditions for the separation of split proteins from ribosomal cores, partial reconstitution of active ribosomes can be obtained upon addition of the isolated split proteins to the ribosomal cores and regeneration of the modified amino groups (Table I). 80 S ribosomes were treated
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Reconstitution was assayed as recovery of polyphenylalanine synthesis. 60 S subunits were treated with dimethylmaleic anhydride using a molar excess of reagent relative to subunits equal to 4800. Regeneration and reconstitution were conducted in three different ways as indicated under Experimental Procedures (experiment 1, procedure (a); experiment 2, procedure (b); experiment 3, procedure (c)). The amount of split proteins added was in all cases 3 times that dissociated from the ribosomal cores used in the mixture. The control preparations were subjected to the same treatments as the corresponding ribosomal cores but in the absence of dimethylmaleic anhydride. Polyphenylalanine synthesis was always assayed in the presence of native 40 S subunits (molar ratio of 40 S subunits to 60 S subunits or 60 S cores equal to 2).

Table I
Reconstitution of active 60 S subunits from protein-deficient particles obtained by dimethylmaleic anhydride treatment

Preparation Addition Poly[Phɛ] synthesized pmol Phe incorporated/pmol ribosomal particle in 20 min

Experiment 1 Initial 60 S subunits 6.1
60 S cores 0.3
60 S cores Split proteins 2.7

Experiment 2 Initial 60 S subunits 21.4
Control 60 S subunits 15.0
60 S cores 0.5
60 S cores Split proteins 4.9

Experiment 3 Initial 60 S subunits 10.2
Control 60 S subunits 4.7
60 S cores 0.2
60 S cores Split proteins 4.2

with a molar excess of dimethylmaleic anhydride equal to 6300, which caused the release of 18% of the total ribosomal protein. Polyacrylamide gel electrophoresis of the split and core proteins showed that some ribosomal proteins were specifically released while others remained bound in the core particles. The control ribosomes, subjected to the same treatments as the ribosomal cores but in the absence of dimethylmaleic anhydride, had 85% of the original activity in poly-peptide synthesis, while the regenerated core particles retained only 6%. The particles obtained by reconstitution of the ribosomal cores with the split proteins did not show any substantial increase in activity over the regenerated core particles alone. However, when native 40 S subunits were included in the assay mixture 76% of the activity of the control was recovered, indicating that functional 60 S subunits had been formed. Under the employed conditions assembly of active 40 S subunits was not achieved, which might be due to degradation of rRNA by a potassium-activated RNase associated with 40 S ribosomal subunits (25). Initial difficulties in the reconstitution of active 30 S subunits of E. coli ribosomes following dimethylmaleic anhydride treatment were caused by degradation of rRNA (18).

Active 60 S subunits can be reconstituted from the ribosomal cores and split proteins obtained by modification of isolated 60 S subunits with dimethylmaleic anhydride at a molar ratio of reagent to subunits equal to 4800 (Table II). This treatment causes the release of 40% of the total protein present in 60 S subunits (the released protein was separated from the ribosomal cores by centrifugation under the non-inactivating conditions), while the 5 S RNA remains bound to the ribosomal core. Polyacrylamide gel electrophoresis of the proteins released and of those that remain bound to the particles shows specificity of dissociation (Fig. 3). Some proteins appear only in the released fraction or are present in it in much higher amounts than in the cores. These proteins are L15, L17/L18, L19, L24, L26, L34, L35, L36/L37, L38, L39, L40/L42, and L44/L45, as indicated by the nomenclature of Kruiswijk and Planta (26) (Fig. 3A). In contrast, the following proteins are entirely or mainly present in the core fraction: L2/L3, L4, L5/L10/L12, L8, L11/L16, L13, L20, L21, L22/L23, L25, L28/L29, and L30 (Fig. 3B). After separation of the split proteins from the ribosomal cores by centrifugation under the non-inactivating conditions, reconstitution was performed in three different ways: (a) by regeneration of the modified groups of split proteins and ribosomal cores in the presence of each other during reconstitution (Table II, experiment 1); (b) by previous regeneration of the split protein fraction.
The reversible modification with dimethylmaleic anhydride used to dissociate the yeast ribosomes is a mild procedure and, although dissociation is also produced by a change in the electrostatic interactions of the particle, it differs significantly from treatment with high salt concentration. The results presented in this paper indicate the potential usefulness of the dimethylmaleic anhydride treatment to disassemble eukaryotic ribosomes and to reconstitute active particles from their components.

REFERENCES

Fig. 3. Electrophoresis patterns of the proteins released (A) and of those present in the ribosomal particles (B) after dimethylmaleic anhydride treatment of 60 S subunits. The treatment was performed at a molar ratio of dimethylmaleic anhydride to 60 S subunits equal to 4800. Centrifugation to separate the split proteins from the core particles was conducted under the nonactivating conditions.

The modified ribosomal cores were regenerated during reconstitution, in the presence of already regenerated split proteins (Table II, experiment 2); and (c) by independent regeneration of split proteins and ribosomal cores prior to reconstitution (Table II, experiment 3). In all cases ribosomal particles active in polyphenylalanine synthesis were obtained. The regenerated ribosomal cores retained less than 5% of the activity of the untreated control, while the activity of the reconstituted particles ranged from 30 to 90%. This reconstitution is fairly reproducible within the range indicated, no clear differences being observed between the results obtained by the three variants of the procedure.
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