THE USE OF GLUTARALDEHYDE FIXATION TO ANALYZE THE MECHANISM OF FACTOR CATALYZED REASSOCIATION OF EUKARYOTIC RIBOSOMAL SUBUNITS.

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SUMMARY

Glutaraldehyde fixation was used to analyze the mechanism of reassociation of ribosomal subunits catalyzed by a factor in rat liver cytosol. Unstable 40S-60S couples formed spontaneously in buffer alone; the couples were dissociated by hydrostatic pressure during centrifugation unless they were fixed with glutaraldehyde. Increased numbers of stable 80S ribosomes were formed in the presence of poly (U), Phe-tRNA and G-25 fraction (which contains the initiation factor EIF-1). The factor would seem then to both increase formation of 80S ribosomes and stabilize the monomer. An additional effect of the factor is to inhibit the formation of the unstable 40S-60S couples which form in the presence of Phe-tRNA alone.

The reassociation of eukaryotic ribosomal subunits is catalyzed by a factor present in rat liver cytosol (G-25 fraction) and by a purified preparation of the initiation factor EIF-1 (1, 2). Reassociation was assessed from density gradient profiles: the assumption was that the pattern reflected the distribution of subunits and 80S ribosomes at the conclusion of the reassociation reaction. However, Infante and Krauss (3), Infante and Baierlein (4), and others (5, 6) have demonstrated that ribosomes can be dissociated by hydrostatic pressure during the course of centrifugation. Moreover, Spirin et al. (7) have shown that reversible interactions can occur between subunits and ribosomes in the centrifugal field. To prevent

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such an occurance Spirin et al. (8) fixed the particles with formaldehyde before centrifugation. Subramanian (9) adopted glutaraldehyde for the same purpose. Glutaraldehyde has the advantage that it can be used with Tris buffers.

We have now utilized glutaraldehyde fixation to re-examine the reassociation of ribosomal subunits catalyzed by cytosol G-25 fraction. We find that the initiation factor (EIF-1) present in the G-25 fraction catalyzes the formation of 80S ribosomes, stabilizes the monomer, and decreases formation of unstable 40S-60S couples.

MATERIALS AND METHODS

The following preparations have been described before: of rat liver ribosomal subunits (2, 10); of a factor from rat liver cytosol that catalyzes binding of Phe-tRNA to 40S subunits and reassociation--referred to as G-25 fraction (11); of rat liver tRNA (12) which was aminoacylated with 20 different amino acids (13)--because the preparation was used with poly (U) we refer to it as Phe-tRNA.

Prior to the assay of reassociation (1, 2), to reduce non-specific aggregation, the 60S subunits (21.4 μg) were incubated for 5 min at 30° in 180 μl of medium B containing 60 μg of Phe-tRNA, 20 μg of poly (U), 0.045 μmole of GTP, and 0.57 mg of G-25 fraction. In some experiments one or another component of the mixture was omitted. The exact conditions are given in the legends to the figures. After the preincubation, 8.6 μg of 40S subunits in 20 μl of medium B were added and incubation was continued for 15 min at 30°.

Ribosomal particles were fixed with glutaraldehyde by a modification of the method of Subramanian (9). An aqueous solution of glutaraldehyde (50%)

was mixed with an equal volume of 1 M Tris in an ice bath and the pH was adjusted to 7.2 to 7.6 with a few drops of 0.5 N KOH. The solution was diluted five-fold with medium B and kept on ice. The reassociation reaction was terminated by cooling the sample on ice, and 50 µl of freshly prepared glutaraldehyde solution (5%) in medium B was added (the final glutaraldehyde concentration was 1%). When G-25 was omitted from the reassociation mixture, the same amount of bovine serum albumin (0.57 mg) was added to prevent loss of ribosomal particles by aggregation during glutaraldehyde fixation (9). The sample was kept at 0° for 10 min before a 100 µl aliquot was layered onto a 5.2 ml linear 10 to 30% sucrose gradient in medium B. Centrifugation was in a SW 50.1 rotor at 45,000 rpm for 100 min at 4°. In some experiments no glutaraldehyde was added and the samples were analyzed after the reaction was stopped by cooling. The distribution of ribosomal particles in the gradient was determined with an ISCO density gradient fractionator and ultraviolet analyzer as described before (1, 2).

The media used were: 10 mM Tris-HCl, pH 7.6, 80 mM KCl, 5 mM $MgCl_2$ (medium A); 10 mM Tris-HCl, pH 7.6, 120 mM KCl, 3.5 mM $MgCl_2$ (medium B); 10 mM Tris-HCl, pH 7.6, 880 mM KCl, 5 mM $MgCl_2$ (medium C).

RESULTS AND DISCUSSION

Rat liver ribosomes dissociate to 40S and 60S subparticles when treated with puromycin and high concentrations (0.8 M) of potassium (10); the subunits will reassociate if the potassium concentration is lowered (to 80 mM). We wished to test whether glutaraldehyde might not be helpful in the analysis of the distribution of ribosomes and ribosomal subparticles by zonal sedimentation, especially if it would prevent pressure induced dissociation during centrifugation. For that purpose a mixture of 40S and 60S subunits in medium

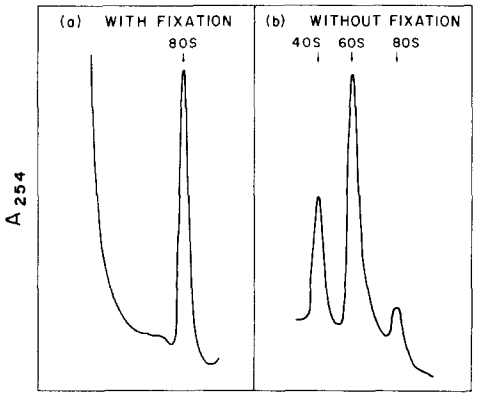

Fig. 1. Effect of glutaraldehyde fixation on the distribution of ribosomal particles in sucrose gradients. In (a) a mixture of 40S (8.6 μg of rRNA) and 60S (21.4 μg of rRNA) ribosomal subunits in medium A were mixed with 1% glutaraldehyde and analyzed on a 10 to 30% linear sucrose gradient in medium C; centrifugation was in an SW 50.1 rotor at 45,000 rpm for 80 min at 4°. In (b) the conditions were the same except that glutaraldehyde fixation was omitted.

A (the concentration of KCl in medium A is 80 mM, thus the particles would be expected to reassociate) was fixed with 1% glutaraldehyde and analyzed on linear sucrose gradients in medium C (the concentration of KCl in medium C is 880 mM, therefore unfixed ribosomes would dissociate during centrifugation). The results confirmed the prediction and substantiated the value of glutaraldehyde in the analysis (Fig. 1). The particles fixed with glutaraldehyde sedimented as 80S monomers despite the high concentration (0.8 M) of potassium in the gradient (Fig. 1a), whereas the unfixed ribosomes dissociated during centrifugation (Fig. 1b).

We undertook next to make use of glutaraldehyde to re-examine the reassociation reaction. The subunits were incubated in medium B (which has 120 mM KCl and 3.5 mM $MgCl_2$) since earlier experiments (1, 2) had indicated that 80S ribosomes did not form spontaneously in that buffer; rather association had required a template (poly (U)), Phe-tRNA, and an initiation factor (EIF-1) present in the G-25 fraction. Thus we were surprised to discover that an

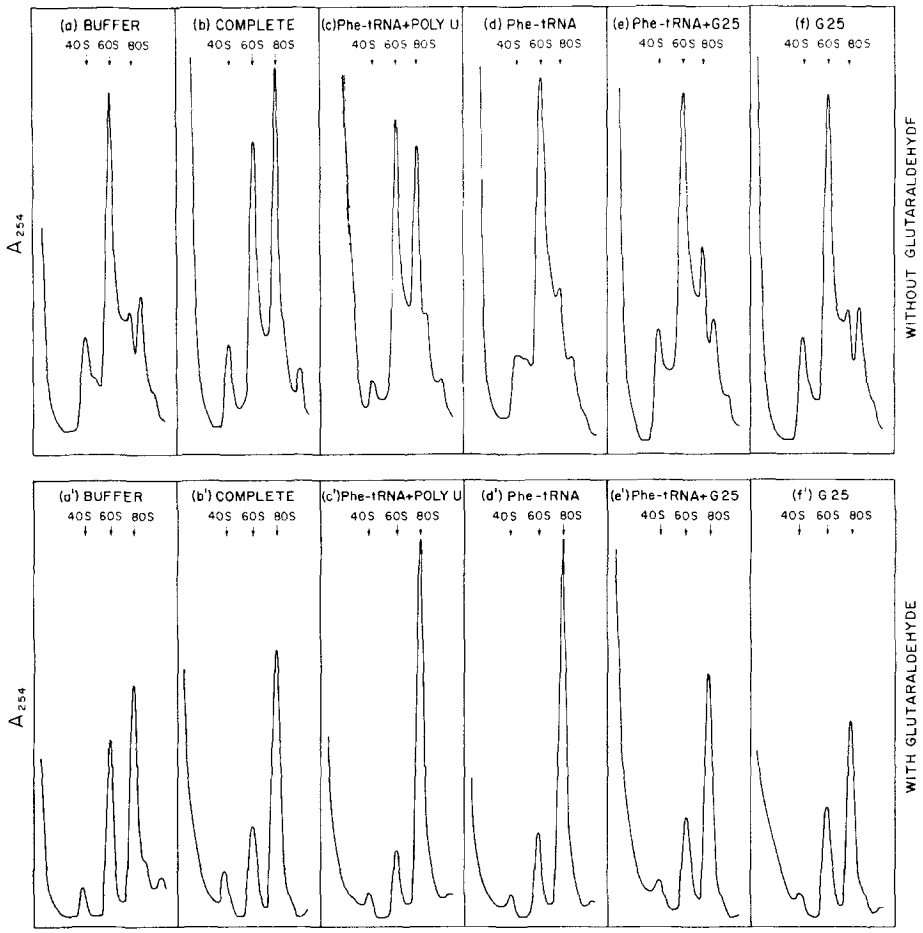

Fig. 2. Reassociation of ribosomal subunits catalyzed by G-25 fraction: analysis without and with glutaraldehyde fixation. In the complete system (b and b'), ribosomal subunits (8.6 μg rRNA of 40S and 21.4 μg rRNA of 60S) were incubated for 15 min at 30° in 0.2 ml of medium B containing: 60 μg of Phe-tRNA; 20 μg poly (U); 0.045 μmole GTP; 0.57 mg of G-25 fraction protein. In (a and a') the subunits were incubated in medium B without any of the other components and in the other experiments (c-f and c'-f') one or another of the components were omitted. In (a'-f'), after incubation, the samples were cooled and fixed with glutaraldehyde; 100 μl were layered on 10 to 30% linear sucrose gradients in medium B and centrifuged at 45,000 rpm for 100 min at 4° in a SW 50.1 rotor. In (a-f) the conditions for the analysis were the same except the particles were not fixed with glutaraldehyde before centrifugation. Since the glutaraldehyde diluted the particles in the reaction mixture by 20% (50 μl of glutaraldehyde was added to 200 μl of reaction mixture) the amount of particles on the gradients in (a-f) is greater than in (a'-f'); thus the best basis for a comparison between the two sets of experiments is of the ratio of 80S to 60S ribosomes.

appreciable number of 80S ribosomes were formed when subunits were incubated in medium B alone, i.e. without poly (U), Phe-tRNA, or G-25 frac-

tion (Fig. 2a')--the discovery required that the reassociation mixture be fixed with glutaraldehyde before zonal sedimentation. Little or no reassociation was seen in the same conditions if glutaraldehyde fixation was omitted (Fig. 2a). It would seem that the 40S-60S couples formed in the absence of template, aminoacyl-tRNA, and factors are unstable and dissociate during centrifugation. (It is important that the amount of material analyzed in the experiments without glutaraldehyde (Fig. 2a-f) and those with glutaraldehyde (Fig. 2a'-f') were different (because of the dilution resulting from addition of glutaraldehyde): the best basis for a comparison between the two sets of experiments is of the ratio of 80S to 60S ribosomes.)

In the presence of poly (U), Phe-tRNA and G-25 fraction (complete system) formation of 80S ribosomes from subunits is increased over that which occurs in buffer alone. The increase was appreciable when the reassociation mixture was fixed with glutaraldehyde (Fig. 2b'). But in conformity with previous results (1, 2) the relative increase was even greater when the particles were not fixed (Fig. 2b). Not only was there considerable formation of 80S monomers, but they were sufficiently stable to withstand dissociation by hydrostatic pressure during centrifugation. When G-25 fraction was omitted (Fig. 2c) the number of stable couples was decreased. We conclude then that a factor in the G-25 preparation (most likely EIF-1) not only catalyzes formation of 80S ribosomes (cf. Fig. 2a' and b') from subunits but stabilizes the couples as well (cf. Fig. 2a and b). It had not been possible to distinguish clearly between the two processes before.

Perhaps the greatest number of 40S-60S couples were formed with Phe-tRNA alone (Fig. 2d' - analysis with glutaraldehyde). However, those couples were not stable as was apparent from the experiments in which the particles were not fixed with glutaraldehyde (Fig. 2d). Poly (U) was not

necessary for the Phe-tRNA effect (compare Fig. 2c' and d'). It should be pointed out that only a portion of the 80S particles formed in the presence of poly (U) and Phe-tRNA (but in the absence of G-25 fraction) were stable (Fig. 2c). Addition of G-25 fraction actually decreased the amount of reassociation of subunits in the experiments with glutaraldehyde (compare Fig. 2b' and d'). It would appear that G-25 factor inhibits the formation of the unstable 40S-60S couples which form in the presence of Phe-tRNA alone: that effect is best seen by comparing Fig. 2d' and e', but also by comparing Fig. 2b' and c'.

Finally, it needs to be noted that the G-25 factor did not catalyze formation of 80S ribosomes in the absence of poly (U) and Phe-tRNA (Fig. 2f and f'). Thus each of the components, template, aminoacyl-tRNA and factor contribute to the formation and stabilization of ribosome monomers (80S initiation complexes).

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