The Dissociation of Yoshida Hepatoma Ribosomes into Active Particles

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1. The sedimentation pattern of Yoshida hepatoma ribosomes shows mainly a high dimer peak and an intermediate peak between those of monomer and dimer. 2. The treatment of the postmitochondrial supernatant with EDTA or potassium chloride leads to the dissociation of ribosomes into subunits, through a decrease of the ribosomal Mg^{2+} /phosphorus ratio. Provided that the deprivation of endogenous Mg^{2+} is not complete, the subunits reassociate into active polyribosomes after addition of magnesium chloride to the medium. If the Mg^{2+} /phosphorus ratio is lowered below 0.01 ($\mu\text{mol}/\mu\text{mol}$), structural changes occur, that become evident by a loss of protein and by a decreased sedimentation rate, which render the subribosomal particles unable to reassociate. 3. In the re-formation of polyribosomes from subunits, during the progressive increase of the concentration of magnesium chloride in the medium, the formation of monomeric ribosomes seems to be intermediate. 4. A dimerization of monomers and subunits occurs at concentrations of magnesium chloride greater than those required for the re-formation of polyribosomes and a preincubation for 1 h at 0°C is necessary for the maximum dimerization, whereas the complete reconstitution of polyribosomes is immediate.

In the bacterial system for protein biosynthesis translation appears to be initiated through the sequential addition of the 30 S and 50 S ribosomal subunits to the messenger RNA (Mangiarotti & Schlessinger, 1967; Nomura & Lowry, 1967); in the mammalian system there is no good evidence for such mechanism in the polyribosomal cycle, since monomeric ribosomes appear to be an essential intermediate (Pronczuk, Baliga, Triaut & Munro, 1968; Stahl, Lawford, Williams & Campbell, 1968; Cohen, 1968). A few experimental results (Vaughan, Warner & Darnell, 1967; Hogan & Korner, 1968), however, indicate a mechanism of formation of polyribosomes in which they are cycled through subunits. Moreover, attempts to prepare biologically active particles from mammalian ribosomes have generally been abortive, and only recently has the possibility of obtaining subribosomal particles able to reassociate into active polyribosomes been reported (Stahl *et al.* 1968; Bonanou, Cox, Higginson & Kanagalingam, 1968; Nolan & Arnstein, 1969; Martin, Rolleston, Low & Wool, 1969).

One of the most peculiar differences between the size-distribution of polyribosomes in the normal liver and in some experimental hepatomas (Webb & Potter, 1966) was the appearance in the hepatomas of abnormally high monomer and dimer peaks, and the presence of the component α , which sediments at a velocity intermediate between that of the monomers and dimers and results from the

combination of monomers with ribosomal subunits. Evidence was given that monomers, component α and dimers are inactive *in vivo* and represent a reserve pool of ribosomes for the formation of polyribosomes. The presence of subunits in this pool, although partly bound to the monomers in the sedimentation patterns, suggests that the ribosomal components of the polyribosomes are cycled through the subunits in a manner similar to that observed in bacteria. However, Webb & Morris (1969) have shown that the subunit pools in hepatomas were not influenced by treatments which led to severe changes in the amount of monomers and dimers, whereas some monomers and dimers appeared to be incorporated into pre-existing polyribosomes.

In the experiments reported in this paper is shown the possibility of obtaining, from Yoshida hepatoma cells, subribosomal particles able to reassociate and to incorporate amino acids *in vitro*. Some results suggest that, during the reassociation of the subunits into polyribosomes, monomeric ribosomes are an intermediate.

MATERIALS AND METHODS

Animals. Yoshida ascites hepatoma AH130 was maintained by weekly transfer of approx. 8×10^7 cells into the peritoneum of male or female rats of the Long-Evans strain (200-250 g); cells were harvested from the rat

peritoneum 6–8 days after inoculation, and collected by centrifugation (2000g for 5 min). All the subsequent procedures were carried out rapidly at 0–4°C.

Homogenization of cells. Packed cells were weighed and transferred with homogenization medium (1 ml/5 g of packed cells) to a Potter–Elvehjem homogenizer fitted with a Teflon pestle; a clearance between the pestle and the vessel was selected to give approx. 90% cell breakage with eight complete vertical movements at 1500 rev./min. The homogenate was then diluted to 20% (w/v). Homogenization medium, and medium for ribosomes, was TK or TKM buffer [50 mM-tris-HCl buffer, pH 7.8; 0.15 M-sucrose; 25 mM-KCl; with (TKM buffer) or without (TK buffer) 5 mM-MgCl₂]. In some experiments cells were resuspended to 11% (w/v) in TKM buffer and cell breakage was obtained by adding one-ninth volume of 2% Triton X-100 in TKM buffer.

Preparation and analysis of ribosomes and subparticles. The postmitochondrial supernatant was prepared by centrifuging the homogenate at 15000g for 10 min. Total ribosomes were collected, from the supernatant treated with 1% sodium deoxycholate, in a 30 or 50 rotor of a Beckman–Spinco model 50 HV ultracentrifuge at 78000g for 95 min or at 150000g for 50 min respectively. After centrifugation the tubes were rinsed with TK or TKM buffer; the loose ribosomal layer was discarded as indicated by Robinson (1969) for liver ribosomal preparations; also, in the hepatoma ribosomal pellet the loose layer is contaminated by non-ribosomal protein and phospholipid, as observed in preliminary studies. On the other hand, higher deoxycholate concentrations cause a lower recovery of RNA and protein in the pellet. The ribosomes of the packed pellet were resuspended with manual homogenization in TK or TKM buffer, at various MgCl₂ concentrations, for sucrose-gradient analysis or for incorporation studies. The ribosomal subunits were prepared by adding EDTA or KCl to the deoxycholate-treated postmitochondrial supernatant, before the centrifugation in the 50 rotor.

For determination of Mg²⁺ pellets and centrifuge tubes were carefully rinsed four times with water, and Mg²⁺ and RNA were extracted with 5% trichloroacetic acid at 90°C. Correction for free Mg²⁺ in the pellet was made by calculating the residue water, by means of [¹⁴C]dextran remaining in the pellet under identical conditions.

For sedimentation-pattern analysis of ribosomes and subunits, samples corresponding to 4–5 E units were layered over 5.5 ml concave exponential gradients, obtained with 0.4 M- and 1.2 M-sucrose by a method similar to that described by Henderson (1969), in a mixing chamber of 2.5 ml. The sucrose in the gradients was in TK or TKM buffer; the MgCl₂ concentration was always the same as that employed in the samples. The gradients were centrifuged at 165000g for 50 min, if not otherwise indicated, in the SW 50 rotor of the ultracentrifuge, and then monitored at 254 nm in an Isco UV2 model D apparatus, with a flow cell having a 0.2 cm light-path.

The determination of the amino acid-incorporating activity was carried out in a Dubnoff apparatus at 37°C for 15 min. The incubation medium contained, in a total volume of 1 ml: 50 mM-tris-HCl buffer, pH 7.8; 25 mM-KCl; MgCl₂ at the indicated concentrations; 1 mM-ATP; 10 mM-creatine phosphate; 0.25 mM-GTP; 100 µg of creatine phosphokinase; 100 µg of poly(U), where present; 0.2 ml of

cell sap (from homogenate, 20% w/v); 0.2 ml of ribosomes (about 0.3 mg of protein); 0.2 µCi of [¹⁴C]phenylalanine (specific radioactivity 244 mCi/mmol, added at zero time). The isotope was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; the other compounds were from Sigma Chemical Co., St Louis, Mo., U.S.A.

The radioactivity of the protein was determined with a method similar to that described by Stahl *et al.* (1968), employing a mark I liquid-scintillation computer of the Nuclear Chicago Corp.

Protein was determined by the biuret reaction (Gornall, Bardawill & David, 1949), with serum albumin as standard. RNA was determined by purine ribose according to Meibbaum (1939), with Sigma s-RNA type III as standard, and by phosphorus content in acid hydrolysate by the method of Beremblum & Chain (1938). Mg²⁺ was determined in the hot-5%-trichloroacetic acid-soluble fraction by the Titan Yellow method (Orange & Rhein, 1951).

RESULTS

The size-distribution pattern of Yoshida hepatoma ribosomes isolated as described in the Materials and Methods section in a medium containing 5 mM-magnesium chloride is shown in Fig. 1(a); the most evident peculiarity is the height of the monomer and dimer peaks and the presence of a low intermediate peak, similar to that of the component *x* observed in other hepatomas (Webb & Potter, 1966). The amount of particles sedimenting more slowly than monomers is very low; the peak of the small subunit, however, appears to be disproportionately high, probably because some large particles are bound to the monomers in the intermediate peak (Webb & Morris, 1969).

The evidence that monomer, component *x* and dimer are not artifacts of the isolation procedure is

Fig. 1. Size-distribution of ribosomal components of the Yoshida hepatoma: (a) ribosomes isolated in a medium containing 5 mM-MgCl₂; (b) ribosomes isolated in a Mg²⁺-free medium, before (-----) and after (—) addition of MgCl₂ to a final concentration of 5 mM. The top of the gradient is to the left of the figure; the arrow marks the position of monomeric ribosomes. Other conditions were as described in the Materials and Methods section.

Fig. 2. Size-distribution of ribosomes from Yoshida hepatoma cells lysed with Triton X-100 (0.2%). Over the gradients were layered samples corresponding to 30mg-equiv. of cell lysate (a), postmitochondrial supernatant (b) and ribosomes (c). Other conditions were as given in Fig. 1.

Fig. 3. Size-distribution of ribosomal components of the Yoshida hepatoma: (a) ribosomes isolated in a Mg^{2+} -free medium, before (-----) and after (—) addition of $MgCl_2$ to a final concentration of 5mM; (b) as (a) but the preparation was preincubated at $0^\circ C$ for 1h before layering over the gradients; (c) control ribosomes, isolated in a medium containing 5mM- $MgCl_2$. Other conditions were as given in Fig. 1.

given by experiments in which hepatoma cells, washed and suspended in TKM buffer, were rapidly lysed with Triton X-100; equivalent amounts of cell lysate, postmitochondrial supernatant and washed ribosomes from the same preparation were layered over sucrose gradients: the sedimentation patterns (Fig. 2) are substantially identical, with the exception of a broader profile of the peaks from cell lysate.

Ribosomes isolated in a Mg^{2+} -free medium (TK buffer), resuspended in the same medium and centrifuged on a Mg^{2+} -free sucrose gradient, appear to be almost completely dissociated into the two subunits (Fig. 1b, broken line); some monomers still exist as the broadening to the right of the profile of the peaks indicates. After addition of $MgCl_2$ to the medium, to bring the final Mg^{2+} concentration to 5mM, these subunits reassociate and the sedimentation pattern (Fig. 1b, continuous line) appears to be similar to that of control ribosomes, with the exception of the transposition of the height of the monomer and dimer peaks, and of the absence of the intermediate peak; moreover, the peak of the large

Fig. 4. Sedimentation patterns of subribosomal particles isolated in a Mg^{2+} -free medium containing EDTA: 1.2mM (-----); 0.9mM (----); 0.6mM (—). The gradients were centrifuged for 6h; other conditions were as described in the Materials and Methods section.

Fig. 5. Size-distribution of Yoshida hepatoma ribosomes isolated in a Mg^{2+} -free medium containing EDTA: 0.6mM (a), 0.9mM (b) and 1.2mM (c), before (-----) and after (—) addition of $MgCl_2$ to a final concentration of 5mM. The samples in 5mM- $MgCl_2$ were preincubated at $0^\circ C$ for 1h before layering over the gradients. Other conditions were as given in Fig. 1.

subunits is somewhat higher. This difference in the size-distribution pattern between control ribosomes and Mg^{2+} -reassociated particles disappears after the incubation of the samples for 1h at $0^\circ C$ in 5mM-magnesium chloride (Fig. 3).

Ribosomes obtained in TK buffer, from post-mitochondrial supernatant treated with EDTA at various concentrations, and sedimented on a Mg^{2+} -free density gradient for 6h at $165000g$, give two peaks corresponding to the subunits; a comparison of the respective patterns (Fig. 4) shows that subunits from 1.2mM-EDTA give two sharp

peaks whereas subunits prepared with lower EDTA concentrations (0.6–0.9 mM) give two peaks having a higher rate of sedimentation and a broader profile. When magnesium chloride is added back to the suspension of these ribosomal subparticles, to bring the final concentration to 5 mM, after waiting for 1 h at 0°C the particles reassociate as shown in Fig. 5; i.e. whereas subunits formed in low concentrations of EDTA reassociate to give a sedimentation pattern very similar to that of control ribosomes, the subunits obtained in higher concentrations of EDTA do not reassociate typically, but form polydispersed aggregates. The critical concentration of EDTA added to the postmitochondrial supernatant, above which subunits did not reassociate typically, varied in different experiments from 1 to 1.1 mM; the concentration of endogenous Mg^{2+} in the postmitochondrial supernatant of 20% (w/v) homogenate is about 1 mM. Therefore it is evident that, when EDTA is added to compensate for the content of

Mg^{2+} , ribosomal particles are formed that do not seem to be able to reassociate after addition of Mg^{2+} .

The results obtained when the amino acid-incorporating activity of the hepatoma preparations was tested supported the conclusions from the sedimentation profiles. [^{14}C]Phenylalanine-incorporation studies were carried out at 3 mM-magnesium chloride without poly(U) and 5 mM-magnesium chloride with poly(U) since, under the conditions used, these appeared to be the most suitable concentrations (see Fig. 6). The incorporation rate was: subribosomal particles from 1.2 mM-EDTA, 216 (± 85); subribosomal particles from 0.8 mM-EDTA, 2290 (± 328); control ribosomes, 3150 (± 927). When poly(U) was added to the incubation mixture the rate was: 180 (± 113), 9600 (± 940) and 14300 (± 1715) respectively. All counts are c.p.m. per mg of protein, after subtraction of zero-time control counts. Therefore whereas subribosomal particles obtained in the presence of excess of EDTA are practically inactive, the particles that reassociate typically after readdition of Mg^{2+} show a high mRNA endogenous-directed incorporation, which indicates the reconstitution of active polysomes.

Experiments were carried out to determine the degree of recombination of ribosomal subunits during the progressive increase in concentration of magnesium chloride in the medium. In a typical experiment, shown in Fig. 7, at 0.125 mM-magnesium chloride a recombination occurs involving most of the subunits in the form of monomers, whereas the amount of particles sedimenting faster than

Fig. 6. Incorporation of [^{14}C]phenylalanine into protein by control ribosomes (Δ , $\blacktriangle$), and subribosomal particles obtained in a Mg^{2+} -free medium containing 0.8 mM-EDTA ($\circ$, $\bullet$), with ($\blacktriangle$, $\bullet$) and without (Δ , $\circ$) poly(U) in the presence of various concentrations of $MgCl_2$. Points show the mean values of three or four experiments, and vertical bars indicate $\pm$ S.D. Other conditions are described in the Materials and Methods section.

Fig. 7. Sedimentation patterns of ribosomes isolated in a Mg^{2+} -free medium containing 0.6 mM-EDTA and re-suspended in a medium containing $MgCl_2$ to the following concentrations (mM): (a) 0; (b) 0.125; (c) 0.5; (d) 1.5; (e) 2.5; (f) 5. The samples were preincubated at 0°C for 1 h before layering over the gradients. Other conditions were as given in Fig. 1.

monomers is very low. At 0.5mM-magnesium chloride the monomer peak is decreased, whereas the re-formation of polyribosomes seems to be complete; at higher concentrations of magnesium chloride a progressive dimerization of monomers occurs, which reaches the maximum at 5mM-magnesium chloride, when the intermediate peak appears; under these conditions the sedimentation pattern of reassociated particles is identical with that of ribosomes isolated in 5mM-magnesium chloride.

It was observed that subribosomal particles isolated in high EDTA concentrations (more than 1.2mM) show a higher E_{260}/E_{280} ratio, which suggests a loss of protein. The relationship between Mg^{2+} , RNA and protein content in active and inactive subribosomal particles isolated by adding potassium chloride to the postmitochondrial supernatant was therefore studied in greater detail. It is known (Nolan & Arnstein, 1969) that high concentrations of potassium chloride cause the dissociation of 80S ribosomes into subunits, even if magnesium chloride is present in the medium; this occurs because, in the presence of high univalent cation concentration, the content of Mg^{2+} in ribosomes become less owing to its competitive displacement (Goldberg, 1966). For Yoshida hepatoma ribosomes isolated in TKM buffer (containing 25mM-potassium chloride and 5mM-magnesium chloride) a Mg^{2+} /phosphorus ratio of about 0.2 ($\mu\text{mol}/\mu\text{mol}$) was found (Fig. 8), which is in agreement with the data of different authors for ribosomes from various sources (see Spirin & Gavrilova, 1969). The decrease of the concentration

of magnesium chloride in the medium leads to a progressive lowering of this ratio to a value of 0.055 (in a Mg^{2+} -free medium). In the presence of 0.125M- and 0.325M-potassium chloride the Mg^{2+} /phosphorus ratio (0.15 and 0.1 in 5mM-magnesium chloride) decreases to 0.03 and 0.01 respectively in a Mg^{2+} -free medium.

I observed that in a Mg^{2+} -free medium containing potassium chloride in concentrations above 0.1M ribosomes are completely dissociated into subunits. The subribosomal particles with a Mg^{2+} /phosphorus ratio of about 0.03 were able to reassociate into active polyribosomes after the addition of magnesium chloride, unlike the subunits obtained in the presence of low concentrations of EDTA, whereas particles having a lower Mg^{2+} /phosphorus ratio did not reassociate. For particles obtained in potassium chloride (0.2, 0.3, 0.6 and 0.9M, in a Mg^{2+} -free medium), the RNA/protein ratios were 1.6, 1.8, 2.9 and 4.5 respectively; therefore concentrations of potassium chloride above 0.3M, that lower the ribosomal Mg^{2+} /phosphorus ratio below 0.01, lead to a structural change with a loss of ribosomal protein.

DISCUSSION

Whereas bacterial ribosomes can be dissociated into two subunits by decreasing the concentration of Mg^{2+} in the medium, mammalian ribosomes appear to be more stable. The absence of Mg^{2+} in the medium merely leads to a marked conversion of liver polyribosomes into monomeric ribosomes; liver ribosomes may be dissociated into active subunits by addition of excess of EDTA, provided that cell sap is present (Stahl *et al.* 1968).

Yoshida hepatoma ribosomes appear to be less stable, since the complete removal of Mg^{2+} is not necessary for obtaining the dissociation into subunits. I have shown that the presence of some Mg^{2+} bound to the subribosomal particles is essential for the reconstitution of particles sedimenting like polyribosomes in the sucrose gradient after addition of magnesium chloride to the medium. The phenylalanine-incorporating activity *in vitro* indicates that these reassociated particles are polyribosomes. Their incorporation rate was somewhat lower than that of control ribosomes also in the presence of poly(U); this fact may depend on a decreased activity of the transfer enzymes, since the amount of re-formed polyribosomes, in the sedimentation pattern, seems to be similar to that present in control preparations.

The lowering of the ribosomal Mg^{2+} below a certain concentration by means of high concentrations of EDTA or potassium chloride leads to structural changes, evidenced by a loss of ribosomal protein, which account for the inability of the

Fig. 8. Mg^{2+} /phosphorus ratio ($\mu\text{mol}/\mu\text{mol}$) in ribosomes isolated in a Mg^{2+} -free medium and washed in 50mM-tris-HCl buffer, pH 7.8, containing KCl: 25mM (●); 0.125M (▲); 0.325M (Δ). $MgCl_2$ at the concentrations shown was present. Other conditions are described in the Materials and Methods section.

subribosomal particles to reconstitute active ribosomes.

The re-formation of polyribosomes from subunits, which occurs at Mg^{2+} concentrations over 0.5 mM, is preceded at lower Mg^{2+} concentrations by the reassociation of most of the subunits to form monomeric ribosomes; when all polyribosomes are reformed, the amount of the monomers appears to have decreased. This fact seems to indicate that in the re-formation of polyribosomal structures from subunits, monomers are intermediate. It is necessary to specify, however, that this observation, although in agreement with that of Webb & Morris (1969) for the direct incorporation of the monomers into pre-existing polyribosomes, does not prove that in the polyribosomal cycle *in vivo* the subunits are simply precursors of 80S ribosomes and do not react directly with messenger RNA to form polyribosomes. The deprivation of Mg^{2+} and their restoration are conditions too far from the physiological one to be able to indicate what occurs in the intact cell. The evidence is clear, however, that dimeric ribosomes show some distinctive features with respect to polyribosomes: dimers and component α are formed, mainly from monomers, and also from some subunits, at concentrations of magnesium chloride much greater than those in which a complete re-formation of polyribosomes occurs; maximum dimerization takes place after incubation in 5 mM-magnesium chloride for 1 h at 0°C, whereas the re-formation of polyribosomes is immediate. The results of this paper also indicate that dimer peaks are not an artifact of the isolation procedure, since they are present in sedimentation patterns of cell lysate soon after lysis with Triton X-100. Webb & Potter (1966) have shown that high concentration of dimer is not unique to the hepatomas, since it may be induced also in the normal liver by administration of carbon tetrachloride or actinomycin D. The results of Webb & Morris (1969) suggest that the abnormal monomer-dimer peak in hepatomas may be due to a deficiency of initiation factor(s). Gravela & Dianzani (1970) have shown that the dissociation of polyribosomes and the appearance of a high monomer-dimer peak in the sedimentation pattern, *in vitro*, observed in the rat liver very shortly after carbon tetrachloride is given, are probably induced by pro-oxidant substances appearing as a consequence of poisoning by this compound. These changes in ribosomal

pattern are prevented by cycloheximide; it is therefore possible that carbon tetrachloride acts, not when ribosomes are bound to messenger RNA, but by impairing the binding of ribosomes to messenger RNA after a normal polyribosomal cycle, that is to say, at the level of initiation of translation. In the normal liver therefore some evidence exists that a decreased activity of the initiation factor(s) leads to a storage of inactive ribosomes in the form of monomer and dimer; this might be a condition naturally occurring in hepatomas.

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