Cytoplasmic-type 80 S Ribosomes Associated with Yeast Mitochondria

I. EVIDENCE FOR RIBOSOME BINDING SITES ON YEAST MITOCHONDRIA*

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SUMMARY

A class of 80 S ribosomes has been isolated from purified yeast mitochondria. These ribosomes are distinguished from cytoplasmic ribosomes of the postmitochondrial supernatant fraction by having a greater stability against the dissociating effect of 0.4 M KCl. These results confirm observations of Schmitt (1969) Fed. Europ. Biochem. Soc. Lett. 4, 234-238. Ribosome dissociability has been examined as a function of the stage of cell growth. Free and membrane-bound cytoplasmic ribosomes of the postmitochondrial supernatant fraction and the 80 S ribosomes associated with mitochondria are all more resistant to the dissociating effect of 0.4 M KCl when isolated from log phase as compared with stationary phase cells. In all cases, however, 80 S ribosomes associated with mitochondria show resistance to KCl dissociation to a significantly larger degree than do the other classes of ribosomes.

Three lines of evidence support the conclusion that the 80 S ribosomes (bound 80 S) we isolate from purified mitochondria are cytoplasmic-type ribosomes: (a) Incorporation of 32P into rRNA of these 80 S ribosomes is insensitive to 15 \mu g/ml of ethidium bromide, (b) this class of 80 S ribosomes is found in the mitochondrial fraction from a cytoplasmic petite which lacks mtDNA, and, (c) poly(U)-directed phenylalanine incorporation catalyzed by purified preparations of 80 S ribosomes associated with mitochondria is sensitive to inhibition by cycloheximide but not by chloramphenicol.

Evidence is presented that purified mitochondria have binding sites for the bound 80 S ribosomes; additional binding sites can be exposed by washing mitochondria with 2 mM EDTA. We observe at least two classes of binding sites with EDTA-washed mitochondria. “Low affinity” sites bind 260 \mu g of ribosomes per mg of mitochondrial protein and “high affinity” sites bind 116 \mu g of ribosomes per mg of mitochondrial protein. Ribosome binding sites on EDTA-washed mitochondria are labile; storage of mitochondria at 0°C reduces their ability to bind exogenously added bound 80 S ribosomes in an apparent first order process with a half-life of 33 hours.

It is now well established that mitochondria possess an intrinsic system for synthesizing protein that differs in many respects from the cytoplasmic system. Among these differences include the generally smaller size of mitochondrial ribosomes (1-4), differential sensitivity of the cytoplasmic and mitochondrial systems to inhibitors of protein synthesis (5-8), system-specific elongation factors (9, 10), tRNAs, and amino acyl synthetases (11-14), and the participation of formyl methionine tRNA in the initiation of mitochondrial but not cytoplasmic protein synthesis (15-18).

Although mitochondria possess an active intrinsic protein-synthesizing machinery, it is not possible to account for more than about 10 to 15% of the total mitochondrial protein as translation products of this intrinsic system (19-21). While clearly, the remaining 55 to 90% of the total mitochondrial protein must therefore be derived from the extramitochondrial translation apparatus, little information is available on the coordination of the two translation systems or on the mechanisms by which particular cytoplasmic translation products are directed toward the developing mitochondrion. Additional problems are also apparent when one considers that specific mitochondrial proteins, for example, those located within the matrix space, must somehow be transported across a double membrane barrier. Although a number of models have been presented to account for the transport of products of cytoplasmic protein synthesis into the mitochondrion (22-27), conclusive experimental support for these is not yet available.

One consideration for the transfer of particular translation products across membrane barriers which has received some experimental support is that of vectorial translation as carried out by polysomes bound to the endoplasmic reticulum of some secretory cells (for a recent review, see Campbell (28)). There are, in addition, considerable data to show that membranes of the endoplasmic reticulum have ribosome binding sites (29-36).

In this connection, recent demonstrations (37, 38) of 80 S ribosomes associated with the mitochondrial fraction of yeast, in addition to the presence of smaller 72 to 75 S ribosomes (39-42), have prompted us to investigate the possibility that (a) 80 S ribosomes associated with yeast mitochondria are cytoplasmic-type ribosomes, and (b) mitochondria possess binding sites for these ribosomes.

In this paper, we confirm the finding of 80 S ribosomes associated with yeast mitochondria. Evidence is presented that these are not intrinsic mitochondrial ribosomes and, in addition,
that yeast mitochondria possess binding sites for these 80 S ribosomes. We discuss possible functions for mitochondrial bound ribosomes in light of known functions of membrane-bound ribosomes in other systems.

**EXPERIMENTAL PROCEDURE**

**Strains and Growth Conditions**

A diploid strain of Saccharomyces cerevisiae cloned from commercial baker's yeast (Standard Brands Incorporated) was used for most of this study. Strain 183 is a haploid cytoplasmic petite (a, ur-, p-) that lacks mtDNA (43). Except where otherwise noted, cells were grown at 28°C to stationary phase on liquid media containing 1% Difco Yeast extract, 1% Difco Bacto-peptone, 0.1% KH₂PO₄, 0.12% (NH₄)₂SO₄, and either 2% galactose (YPGal) or 2% glucose (YPG).

**Preparation of Mitochondria**

Mitochondria were prepared by the following three procedures. 

**Method A**—Cells were harvested by centrifugation, washed once with distilled water, once with standard buffer containing 10 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, 50 mM NH₄Cl, and 1 mM mercaptoethanol, and resuspended in 1 ml of standard mannitol buffer (standard buffer made 0.25 M in mannitol, designated Buffer A) per g of cells, wet weight. The cell suspension was subjected to 40-s homogenization in a CO₂-cooled Braun MSK Homogenizer at 4,000 rpm with 2 g of glass beads (0.5 mm diameter) per g of cells, wet weight. The suspension was decanted and the beads rinsed with 3 × 5 ml of Buffer A, per g of cells, wet weight. Mitochondria were pelleted from the supernatant suspension by centrifugation at 12,000 × gmax for 20 min. The crude mitochondrial pellet was resuspended in a small volume of Buffer A and layered onto a 30 to 70% linear sucrose gradient prepared in standard buffer. After centrifugation in a Beckman SW 25.1 rotor for 2½ hours at 90,200 × gmax, the mitochondrial band was removed with a syringe, diluted 2-fold with Standard Buffer, and pelleted. The purified mitochondria were resuspended in Buffer A at a protein concentration of about 10 mg per ml.

**Method B**—For large scale preparation of mitochondria, the method described by Tzagoloff (44) was used to reduce 2 pounds of commercial pressed yeast (standard brands) to a frozen powder. The powder was thawed in 1.5 liters of Buffer A and the pH adjusted to 7.4 with 5 N KOH. Crude mitochondria were isolated from this homogenate as described in Method A above. Mitochondria were purified by isopycnic banding on a 30 to 60% linear sucrose gradient prepared in standard buffer. Centrifugation was carried out with a Sorvall SS-34 reorienting density gradient zonal rotor for 4 hours at 40,500 × gmax. By this procedure, up to 2.0 g of mitochondrial protein could be purified in one run. The gradient was fractionated and those fractions containing the bulk of the cytochrome oxidase activity were pooled, diluted 2-fold with standard buffer, and the mitochondria pelleted. The purified mitochondria were resuspended in Buffer A at a protein concentration of about 25 mg per ml.

**Method C**—Yeast spheroplasts were prepared according to the method of Kovâ et al. (45) except that EDTA was omitted from the buffers and disruption of the spheroplasts was carried out in Buffer A. Mitochondria were isolated and purified as described in Method A.

In all three procedures, 1 g of cells, wet weight, yielded about 2 mg of mitochondrial protein.

**Isolation and Purification of Ribosomes**

From purified mitochondria (prepared by Method A or B, see "Results"), 80 S ribosomes were extracted by resuspension and homogenization of mitochondria in lysing buffer (standard buffer containing 2% Triton X-100) to give a final protein concentration of 2 to 3 mg per ml. Insoluble material was removed by centrifugation at 12,000 × gmax for 10 min. Ribosomes were pelleted from the supernatant solution by centrifugation for 3 hours at 105,700 × gmax and resuspended in Buffer A.

Total cytoplasmic ribosomes were obtained by making the postmitochondrial supernatant solution 2% in Triton X-100 and proceeding as described above for isolation of 80 S ribosomes from the mitochondrial fraction. In some instances (see "Results") cytoplasmic ribosomes in the postmitochondrial supernatant fraction were separated into membrane-bound and free ribosomes according to the procedure of Blobel and Potter (46). Ribosomes bound to the endoplasmic reticulum were extracted and isolated exactly as described for the 80 S ribosomes obtained from purified mitochondria.

For in vitro studies, 80 S ribosomes from mitochondria (prepared by Method B), were isolated as described above and were purified by sedimentation through a 15 to 30% linear sucrose gradient prepared in standard buffer. Centrifugation was carried out for 9 hours at 76,300 × gmax in a Beckman SW 25.1 rotor. The gradients were simultaneously fractionated and scanned for absorbance at 254 nm with a lscio model 183 density gradient fractionator and model 224 ultraviolet flow analyzer. The fractions containing monosomes were pooled and the monosomes pelleted. Ribosomes obtained in this way had absorbance ratios A₂₆₀:A₂₃₅ and A₂₆₀:A₂₈₀ of greater than 1.7 and 1.9, respectively. About 3 mg of ribosomal protein were routinely obtained from 1 g of mitochondrial protein.

All ribosome preparations described above were resuspended in Buffer A, divided into small aliquots, quickly-frozen in liquid nitrogen, and stored at −70°C.

**Sucrose Density Gradient Analysis of Ribosomes**

Ribosomes were layered on a 15 to 30% linear sucrose gradient in standard buffer with or without 0.4 M KCl and centrifuged for 2 hours in a Beckman SW 50.1 rotor at 221,800 × gmax. Gradients were simultaneously fractionated and scanned for absorbance as described above.

Sedimentation coefficients of ribosome subunits were estimated by the procedure of Martin and Ames (47) with yeast cytoplasmic ribosomes as an 80 S marker (48).

**Analytical Methods**

Cytochrome oxidase activity was measured by the procedure of Wharton and Tzagoloff (49). Protein was determined by the method of Lowry et al. (50). Radioactivity of aqueous samples was determined in the toluene-Triton X-100 scintillant of Paterson and Greene (51). Growth of cells was measured as optical density with a Klett photometer with a No. 66 filter.

**RESULTS**

**Membrane-bound Ribosomes Isolated from Purified Mitochondria**

—A class of membrane-bound ribosomes was isolated from purified mitochondria by extraction with buffer containing 2% Triton X-100. When these ribosomes are sedimented together with cytoplasmic 80 S ribosomes on sucrose gradients prepared in the absence of KCl (Fig. 1A), we observe no resolution of the monosome peaks (hereafter, the ribosomes obtained from mitochondria...
some species. Since the intercalating dye ethidium bromide was added to a final concentration of 15 \(\mu g\) per ml in order to avoid possible interference from intrinsic mitochondrial ribosomes (see Fig. 2 and text). After 30-min incubation at 28\(^\circ\)C, 1.0 mCi per ml of \(^{32}\)P\(_{i}\) (carrier-free, New England Nuclear) was added and the cells were incubated for an additional 3 hours. Labeled cells were then combined with 10 g of cold carrier cells, wet weight, and bound 80 S ribosomes were isolated from purified mitochondria prepared by Method A (see Experimental Procedure). \(^{32}\)P\(_{i}\)-Labeled cytoplasmic ribosomes were prepared as described under "Experimental Procedure" and were referred to as "bound 80 S". On the other hand, bound 80 S ribosomes are clearly distinguished from bulk cytoplasmic ribosomes by having a greater stability against the dissociating effect of KCl. As shown in Fig. 1B, bound 80 S monosomes sedimented through a sucrose gradient containing 0.4 M KCl only partially dissociate into 60 and 40 S subunits; cytoplasmic 80 S monosomes completely dissociate into subunits under these conditions. These observations are in agreement with those previously reported by Schmitt (37).

Bound 80 S Ribosomes Are Not Intrinsic Mitochondrial Ribosomes—In a number of reports, the sedimentation coefficient for the monosome of the intrinsic mitochondrial ribosome from yeast has been estimated and these estimates vary between 72 to 80 S (37–42). In view of this ambiguity regarding the identification of a unique intrinsic mitochondrial ribosome species, we felt it necessary, therefore, to establish unambiguously the origin of the bound 80 S ribosome reported here; that is, whether it is an intrinsic mitochondrial ribosome or a cytoplasmic-type ribosome.

It is now well established that the rRNA of intrinsically mitochondrial ribosomes in fungi and other eukaryotic organisms is a gene product of mtDNA (52–55). Since the intercalating dye ethidium bromide selectivity inhibits the transcription of mtDNA in yeast (56), as well as sufficiently induces cytoplasmic pettis even under nongrowing conditions (57, 58), it should be possible to distinguish between intrinsic mitochondrial ribosomes and cytoplasmic ribosomes on the basis of incorporation of label in the presence of ethidium bromide into the rRNA of the various ribosome species.

Toward this end, cells were incubated with 15 \(\mu g\) per ml of ethidium bromide. Under these conditions nearly 70% of the cell population was converted to petites after 30 min (Fig. 2). At this time \(^{32}\)P\(_{i}\) was added to the culture and the cells were incubated for an additional 3 hours after which time bound 80 S and cytoplasmic 80 S ribosomes were isolated and examined on sucrose gradients. Inspection of the data presented in Fig. 3 shows that bound 80 S and cytoplasmic ribosomes were labeled nearly the same specific radioactivity even after a large percentage of the cell population had been converted to petites. The optical density and labeling profiles again show the resistance of bound 80 S monosomes to the dissociating effect of 0.4 M KCl.

Additional support for the view that bound 80 S ribosomes isolated from mitochondria are not intrinsic mitochondrial ribosomes comes from the fact that it is possible to isolate bound 80 S ribosomes from the "mitochondrial" fraction of a cytoplasmic petite which lacks mtDNA. Fig. 4 shows a sucrose density gradient profile of bound 80 S ribosomes obtained from such a petite (strain 183) which was shown previously to lack mtDNA (43) and illustrates the resistance of these ribosomes to KCl dissociation. Cytoplasmic ribosomes from this strain are completely dissociated in 0.4 M KCl (data not shown).

Taken together, the insensitivity of the incorporation of label into the RNA of bound 80 S ribosomes in the presence of ethidium bromide and the presence of this class of ribosomes in a cytoplasmic petite which lacks mtDNA leave little doubt that bound 80 S ribosomes are not the intrinsic mitochondrial ribosomes in yeast.

We have also demonstrated the presence of bound 80 S ribosomes in anaerobically grown cells and cells grown in the presence of chloramphenicol (3 mg per ml) (data not shown). The resistance of these ribosomes to dissociation by KCl is similar to that of bound 80 S ribosomes obtained from cells grown aerobically under nonrepressing conditions.

Comparison of Poly(U) directed Phenylalanine Incorporation with Bound 80 S and Cytoplasmic Ribosomes—The data presented in Table I show that purified preparations of bound 80 S and cytoplasmic ribosomes are active in poly(U)-directed phenylalanine incorporation. As expected, both classes of ribosomes are inhibited by 125 \(\mu g\) per ml of cycloheximide and insensitive to 100 \(\mu g\) per ml of chloramphenicol. However, these two classes of ribosomes are distinguished further by the fact that cytoplasmic ribosomes are about 13 times more active than bound 80 S ribosomes under the assay conditions of Table I. It is of interest that differences have been observed by others between membrane-bound and free ribosomes used in cell-free protein synthesis (59).

Comparison of Ribosome Dissociability as a Function of Cell Growth—In view of recent demonstrations that ribosomes isolated from rapidly growing cells are more difficult to dissociate than ribosomes from nongrowing cells (60, 61), an observation possibly related to the extent of engagement of ribosomes with mRNA (80), we decided to examine the dissociability of bound 80 S ribosomes as a function of the growth phase of the culture; in addition, we wished to compare these results with the dissociability of cytoplasmic ribosomes obtained from the postmitochondrial supernatant fraction. The latter were separated into membrane-bound (presumably bound to the endoplasmic reticulum and referred to as ER-ribosomes) and free ribosomes according to the procedure described by Blobel and Potter (46).

Fig. 5 shows the results of an experiment where bound 80 S ribosomes, ER-ribosomes, and free cytoplasmic ribosomes were isolated from cells obtained from cultures in various stages of the growth phase and examined on sucrose gradients containing 0.4 M KC1.
Kinetics of petite induction by ethidium bromide.

In the experiment described in the legend to Fig. 1 for labeling of bound 80 S ribosomes with $^{32}$P, the percentage of petites in the cell population induced by ethidium bromide prior to the addition of label was determined by plating about 200 colonies at the times indicated on differential media as described by Sherman (68). Colonies were scored visually as either grandes or petites after growth for 4 days at 28°.

Fig. 2 (right). Comparison of the labeling of bound 80 S and cytoplasmic ribosomes in the presence of ethidium bromide. $^{32}$P-Labeled bound 80 S ribosomes are those described in Fig. 1. $^{32}$P-Labeled cytoplasmic ribosomes were isolated from the same cells used in that experiment for the isolation of $^{32}$P-labeled bound 80 S ribosomes; about 0.8 A$_{260}$ unit each of bound 80 S (A and B) and cytoplasmic ribosomes (C and D) were applied to sucrose gradients prepared with (A and C) and without (B and D) 0.4 M KCl and analyzed for absorbance (---) and radioactivity (O---O) as described under "Experimental Procedure."

**Table I**

Activity of bound 80 S and cytoplasmic 80 S ribosomes in poly(U) directed phenylalanine incorporation.

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Ribosome</th>
<th>Phenylalanine incorporated per mg ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>Cytoplasmic 80 S</td>
<td>514</td>
</tr>
<tr>
<td>Complete plus chloramphenicol (100 µg/ml)</td>
<td>Cytoplasmic 80 S</td>
<td>529</td>
</tr>
<tr>
<td>Complete plus cycloheximide (125 µg/ml)</td>
<td>Cytoplasmic 80 S</td>
<td>17</td>
</tr>
<tr>
<td>Complete</td>
<td>Bound 80 S</td>
<td>38</td>
</tr>
<tr>
<td>Complete plus chloramphenicol (100 µg/ml)</td>
<td>Bound 80 S</td>
<td>37</td>
</tr>
<tr>
<td>Complete plus cycloheximide (125 µg/ml)</td>
<td>Bound 80 S</td>
<td>8</td>
</tr>
</tbody>
</table>

Ribosome Binding Sites on Mitochondria—The fact that bound 80 S ribosomes were isolated from mitochondria purified by isopycnic banding on sucrose gradients suggested to us that mitochondria contain ribosome binding sites. In order to show this directly, purified mitochondria were repeatedly washed with...
buffer containing 2 mM EDTA (Buffer B: 0.25 M mannitol, 10 mM Tris-Cl, pH 7.4, 2 mM EDTA). Mitochondrial preparations treated in this manner are referred to as "stripped" mitochondria. In this connection, it has been shown that EDTA treatment of membranes of the rough endoplasmic reticulum from rat liver depletes the membrane of bound ribosomes and exposes ribosome binding sites (29-31). Fig. 6 shows the results of a series of experiments where stripped mitochondria were incubated for 1 hour with 32P-labeled bound 80 S ribosomes and the reaction mixture subsequently sedimented through 30 to 70% sucrose. Clearly, with increasing amounts of mitochondrial protein in the reaction mixture, more ribosomes enter the gradient and importantly, all the counts which do enter co sediment with cytochrome oxidase activity (Fig. 6C).

If EDTA treatment does strip mitochondria of bound 80 S ribosomes, such preparations should have the capacity to bind more exogenously added ribosomes than untreated (Buffer A-washed) preparations. That this is indeed the case, is shown by the data in Fig. 7; stripped mitochondria (EDTA-washed) bind nearly twice the amount of 32P-labeled bound 80 S ribosomes as Buffer A-washed mitochondria.

For routine measurements of ribosome binding, we have also developed a rapid assay procedure which simply involves the separation of mitochondria from unbound ribosomes by differential centrifugation. The data presented in Fig. 7 show that this method gives nearly identical results to the gradient assay procedure described above. In all subsequent experiments, the rapid assay procedure was used to measure ribosome binding.

Fig. 8 shows the results of a binding experiment where mitochondria were held constant (0.5 mg) and binding was determined as a function of increasing amounts of free ribosomes. It is seen that ribosome binding to mitochondria shows a saturation behavior. When the data of Fig. 8 are recast according to the method of Scatchard (62) (Fig. 9), at least two classes of ribosome binding sites are readily apparent. The "high affinity" binding sites have a binding capacity of 116 pg of ribosomes per mg of mitochondrial protein and the "low affinity" sites, a binding capacity of 260 pg of ribosomes per mg of mitochondrial protein. The significance of these different classes of binding sites is unclear at the present time.

Stability of Binding Activity—If stripped mitochondria are maintained at 0°, their capacity to bind ribosomes at 28° decreases as an apparent first order process with a half-life of about 33 hours (Fig. 10). A similar instability of ribosome binding to

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**Fig. 5.** Comparison of the dissociation of ribosomes prepared from cells at different stages of growth. Cells were grown on YPGal (1 liter) and harvested at the Klett values indicated in the figure. Bound 80 S ribosomes (A, B, and C), ER-ribosomes (D, E, and F), and free cytoplasmic ribosomes (G, H, and I) were isolated from the various subcellular fractions, applied to sucrose gradients containing 0.4 M KCl, and analyzed for dissociation by absorbance as described under "Experimental Procedure."

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**Fig. 6.** Bound 80 S ribosome binding to mitochondria. Mitochondria were prepared by Method A from cells grown on YPGal (30 ml) minus KHP04, containing 0.3 mCi per ml of 32P and mixed with mitochondria (2.5 g of protein) prepared by Method B. Bound 80 S ribosomes were extracted from the combined mitochondria and purified as described under "Experimental Procedure." Mitochondria prepared by Method C to be used for binding were washed twice with Buffer B, once with Buffer A, and resuspended in Buffer A at 10 mg of mitochondrial protein per ml. Increasing amounts of mitochondrial protein as indicated in the figure were incubated with 35 μg of 32P-labeled bound 80 S ribosomes for 1 hour at 28° in a reaction mixture brought to 0.2 ml with Buffer A. The incubation mixtures were then layered directly on 5-ml 30% to 70% sucrose gradients prepared in Standard Buffer and centrifuged for 1 hour at 243,000 X g in a Beckman 50.1 rotor. Gradients were fractionated and aliquots (5 μl) of each fraction were assayed for cytochrome oxidase activity (O-O-O); the remaining material was assayed for radioactivity (O-O-O).
stripped membranes of the endoplasmic reticulum has been reported by Schires et al. (31).

**DISCUSSION**

Some controversy exists regarding the size of the intrinsic mitochondrial ribosome in yeast. Morimoto and Halvorson (38) reported the isolation of an 80 S ribosome from purified yeast mitochondria, and later Scragg et al. presented evidence that in vitro protein synthesis catalyzed by these 80 S ribosomes is inhibited by chloramphenicol and not by cycloheximide (63). On the basis of hybridization data, Morimoto et al. have concluded that the rRNA of this 80 S ribosome is a gene product of mtDNA (64). While those results provide strong evidence for the designation of this ribosome as the intrinsic mitochondrial ribosome in yeast, Schmitt demonstrated that depending upon the extraction conditions, a 72 S ribosome could be isolated from yeast mitochondria in addition to an 80 S species (39). Subsequently, Grivell et al. (41) showed that a 74 S ribosome from yeast mitochondria was the “minimal structure capable of catalyzing protein synthesis,” and thus concluded 74 S to be the size of the intrinsic mitochondrial ribosome.

In view of the discrepancy regarding the size of the intrinsic mitochondrial ribosome from yeast, it was necessary to establish unambiguously if the bound 80 S ribosome extracted from purified yeast mitochondria reported here was an intrinsic mitochondrial or a cytoplasmic-type ribosome. We have presented three lines of evidence which strongly support the latter conclusion: (a) bound 80 S ribosomes are labeled to the same extent with °P, as cytoplasmic ribosomes when labeling is carried out in the presence of ethidium bromide, (b) bound 80 S ribosomes can be isolated from a cytoplasmic petite which lacks mtDNA microfuge. Aliquots (50 µl) of the supernatant solution were assayed for radioactivity. The data in both assay procedures are calculated as percentage of the total radioactivity sedimenting with the mitochondria.

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**Fig. 7 (left).** Ribosome binding to EDTA-washed (stripped) and Buffer A-washed (unstripped) mitochondria: a comparison of two assay methods. Mitochondria prepared by Method C were divided into two portions. One portion was washed twice with Buffer B and once with Buffer A (SMB) (○—○, △—△), and the other portion was washed 3 times with Buffer A (Δ—Δ). As indicated in the figure, increasing amounts of mitochondrial protein were incubated for 1 hour at 98° with 55 µg of °P, labeled bound 80 S ribosomes prepared as described in Fig. 6. The total volume of each reaction mixture was adjusted to 0.6 ml with Buffer A. At the end of the incubation period the reaction was divided into two equal portions. One portion was assayed for ribosome binding as described in Fig. 6 (gradient assay). For the rapid assay procedure aliquots (50 µl) of the reaction mixture were assayed for radioactivity. The remaining material was centrifuged at 0° for 1 hour at 13,500 rpm in a Beckman model 152 microfuge. Aliquots (50 µl) of the supernatant solution were assayed for radioactivity. The data in both assay procedures are calculated as percentage of the total radioactivity sedimenting with the mitochondria.

**Fig. 8 (center).** Dependence of free ribosome concentration on bound 80 S ribosome-binding to mitochondria. EDTA-washed mitochondria (0.5 mg) were incubated for 1 hour at 28° with increasing amounts of °P, labeled bound 80 S ribosomes (prepared as described in Fig. 6) in a reaction mixture adjusted to 0.3 ml with Buffer A. The amount of ribosomes bound to mitochondria was calculated by determining specific radioactivity assuming 4° unit = 0.060 mg of ribosomal protein.

**Fig. 9 (right).** Scatchard plot of bound 80 S ribosome-binding to mitochondria. The data obtained in Fig. 8 are replotted according to Scatchard (62).

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**Fig. 10.** Decay of mitochondrial-binding activity. EDTA-washed mitochondria were prepared as described in Fig. 6 and stored at 0°. At 24-hour intervals, aliquots (1 mg of mitochondrial protein) were removed and incubated with freshly thawed °P, labeled bound 80 S ribosomes (35 µg). Binding was determined by the rapid assay procedure described in Fig. 7.
terparts. First, bound 80 S ribosomes are more resistant to KCl dissociation than either free or membrane-bound ribosomes isolated from the postmitochondrial supernatant fraction. Second, bound 80 S ribosomes are less active in an in vitro system of poly(U)-directed phenylalanine incorporation than are bulk cytoplasmic ribosomes under the assay conditions described in Table I.

While the effect of KCl on monosome dissociation was shown to vary depending on the stage of growth of cells from which ribosomes were isolated, bound 80 S ribosomes were always the most resistant in this respect. Grivell et al. (41) suggested that the resistance of yeast 80 S ribosomes to KCl dissociation is the result of contaminating membrane fragments in the preparation. While we cannot rule out this possibility, we have not observed any effect of the degree of purity on the dissociability of preparations of bound 80 S ribosomes. Those preparations with absorbance ratios \( A_{260}/A_{232} \) and \( A_{260}/A_{380} \) greater than 1.7 and 1.9, respectively, showed the identical extent of dissociation in KCl gradients as cruder preparations.

Furthermore, even free cytoplasmic ribosomes show resistance to KCl dissociation which varies as a function of the stage of growth of the cells from which the ribosomes were obtained. A more likely explanation to account for differences in ribosome dissociability which has received some experimental support (60, 61) is that ribosomes actively engaged in protein synthesis are more difficult to dissociate, possibly due to the presence of mRNA fragments (90). With this consideration in mind, our data suggest that compared to free or membrane-bound ribosomes of the postmitochondrial supernatant fraction, bound 80 S ribosomes are more actively engaged in protein synthesis or, alternatively, that mRNA associated with bound 80 S ribosomes is more stable.

Our results further suggest that yeast mitochondria have ribosome binding sites and that these are limited in number. This is seen by the saturation behavior of binding as a function of ribosome concentration and the fact that mitochondria have a greater capacity to bind ribosomes if first washed with EDTA (conditions which would be expected to remove externally bound ribosomes). Moreover, the ability of mitochondrial preparations to bind ribosomes is a labile property, since mitochondria have a greater capacity to bind ribosomes if first washed with EDTA (conditions which would be expected to remove externally bound ribosomes).

This type of experiment has been carried out by others (67) and provides an important basis for the view that specific proteins destined for export from the cell are first released during the translation process into the internal space of the endoplasmic reticulum. We are currently testing the possibility that bound 80 S ribosomes which bind to mitochondria function in a manner similar to ribosomes of the rough endoplasmic reticulum; that is, that bound 80 S ribosomes selectively translate protein destined for release into the mitochondrion.

**Note Added in Proof**—K. Dawidowicz and H. R. Mahler have recently presented evidence (International Symposium on Protein Synthesis and Nucleic Acids, the XI Latin American Symposium, in press (1971)) showing that the mitochondrial particle active in protein synthesis in vivo is a 75 S particle and its polysomes.

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