Extraction of Proteins from the Large Subunit of Bovine Mitochondrial Ribosomes under Nondenaturing Conditions*

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The 55 S mammalian mitochondrial ribosome (referred to hereafter as "mitoribosome") is protein-rich, containing nearly twice as much protein as the Escherichia coli ribosome. In order to produce soluble mitoribosomal proteins and protein-deficient subribosomal particles for use in functional and structural studies, the proteins of bovine mitoribosomes were extracted by washing in a series of buffers containing increasing concentrations of LiCl as the only chaotropic agent. LiCl disruption is used in order to preserve the solubilized proteins in a substantially "native" configuration. The extraction mixtures were characterized by sucrose density gradient analysis and the compositions of the stripped protein and residual pellet fractions were determined by two-dimensional polyacrylamide gel electrophoresis. In order to analyze the behavior of individual proteins, the intensity of Coomassie blue stain for each protein was normalized against the intensity of stain for the same protein in a control sample. Buffers with 1, 2, and 4 M LiCl each extract a specific subset of mitoribosomal proteins, while another group of proteins remains in the residual pellet fraction. Although very few proteins are detected in only one condition, most proteins are specifically enriched in one fraction. This LiCl procedure, therefore, produces fractionated groups of mitoribosomal proteins which can be used directly as a source for those proteins in which they are enriched or they can be used as a starting point in further purification procedures. In contrast to results with E. coli ribosomes, several mitoribosomal proteins remain core-associated, indicating a different structural organization in these ribosomes.

Mammalian mitochondrial ribosomes are the most protein-rich ribosome type. While their mass, $2.8 \times 10^9$ daltons (1), is roughly the same as that of bacterial ribosomes, they contain only about half as much rRNA, and the total protein content is correspondingly increased. This high protein content is reflected in the low buoyant density (1.44 g/cc) and low sedimentation coefficient (55 S) of the mitochondrial ribosomes (1, 2). Electrophoretic analysis of the mitoribosomal proteins has shown that the ribosome contains about 85 proteins (3), more individual proteins than either bacterial or cytoplasmic ribosomes. Since the mitoribosome functions according to the same overall protein synthesis mechanism as bacterial and cytoplasmic ribosomes, its composition leads to questions regarding the requirement for so many individual proteins.

In order to study the structure and function of the ribosome and its proteins, the particle has been disassembled by several methods. Most of these methods have relied on strong denaturants, including urea, acetic acid, or guanidinium chloride to remove the proteins from the rRNA. While the proteins produced by these techniques are suitable for some applications, their denatured state renders them less suitable for immunologic, functional, and structural studies. In recent years, extractions which use LiCl alone or in combination with other reagents, have been used to produce soluble ribosomal proteins (4-7). These preparations retain more secondary and tertiary structure (8-12); that is, their configuration is more "native," and they are more active in rRNA-protein binding experiments (10, 13).

In this paper, we report the use of LiCl to extract the proteins of the large subunit of bovine mitoribosomes in a manner which produces a series of group fractions, each specifically enriched for several proteins. Since LiCl is the only chaotropic agent used, the proteins should be preserved in a more native configuration, well suited for reconstitution, physical-chemical, and immune studies. Each of these fractions has been characterized with respect to its composition and reproducibility. In this way, a fraction produced from a small amount of material can be used directly as a source of specific, soluble, native, mitoribosomal proteins.

**Experimental Procedures**

**Buffers**

The composition of the buffers used was as follows. A: 1 M LiCl, 0.07 M KCl, 0.001 M EDTA, 0.001 M MgCl₂, 0.006 M 2-mercaptoethanol, 0.01 M Tris, pH 7.5. B: X M LiCl, 0.07 M KCl, 0.01 M EDTA, 0.001 M MgCl₂, 0.006 M 2-mercaptoethanol, 0.01 M Tris, pH 7.5. C: 9 M urea, 3 M LiCl, 0.001% butylated hydroxy-toluene, pH 3-4. D: 67% acetic acid, 0.0035 M Tris, 0.03 M MgCl₂, E: 0.1 M KCl, 0.03 M MgCl₂, 0.065 M 2-mercaptoethanol, 0.0001 M EDTA, 0.01 M Tris, pH 7.5. F: 8 M urea, 3 M LiCl, 0.02 M phosphate, pH 7.2.

**Extraction Protocol**

**Serial Extraction**—The basic extraction procedure follows that of Littlechild and Malcolm (6). This method relies on LiCl as the only chaotropic reagent. By avoiding exposure to denaturants such as urea or acetic acid, proteins are produced which retain more secondary and tertiary structure (8-12). The protocol involves extracting ribosomes by stirring in a series of buffers containing high LiCl. Core particles are separated from extracted proteins by ultracentrifugation. The recovered core particles are then used as starting material for the next extraction, performed in a buffer containing a higher concentration of LiCl.

Bovine mitochondrial ribosomal large subunits (39 S) were prepared as described previously (1, 14). 30-60 A_{260} units (1.8-3.6 mg of...
total ribosomal protein) were used for each extraction series. Before beginning the salt extractions, a control aliquot (5 A260) of the ribosomal subunit preparation was removed. This aliquot was analyzed by two-dimensional PAGE to characterize the total protein complement of the preparation.

The procedure used for the serial extractions is summarized in Fig. 1. For each step, the ribosomal subunits were suspended in a buffer at a concentration of 16 A260 units (800 pmol)/ml. The mixture was stirred at 4°C for 12-16 h. At the end of the stirring period an aliquot of 1-2.4 A260 units was removed for sucrose density gradient analysis (see below). The remainder of the extraction mixture was centrifuged in a Beckman Ty65 rotor at 50,000 rpm (approximately 200,000 × g) to pellet core particles and aggregated material. Centrifugation times were varied (5-8 h) depending on the volume and viscosity of the solution in order to maintain clearing conditions for particles of greater than 6 S (calculated clearing conditions for 2.3-5.2 S)1. After centrifugation, the supernatant was removed as quickly as possible in order to minimize resolubilization of the pellet. The supernatant was stored at -70°C until analyzed by two-dimensional PAGE.

The recovered pellet was resuspended into buffer for the next extraction. Usually the pellet was resuspended directly by homogenizing it in the appropriate buffer. In later experiments, pellets being prepared for extraction in 2 or 4 M LiCl were homogenized into buffer B containing 1 M LiCl and then buffer B containing 6 M LiCl was used to adjust to the final LiCl concentration. This procedure was designed to improve the extraction efficiency by allowing the pellets to disperse at a LiCl concentration which does not aggregate RNA.

The pellets obtained after extraction in 4 M LiCl were stored at -70°C until they were extracted for two-dimensional PAGE (see below).

One-step Extraction—Mitochondrial large subunits were adjusted directly to a concentration of 16 A260 units/ml in buffer B containing 4 M LiCl. The extraction mixture was stirred for 12-16 h, then aliquoted and centrifuged as above. The supernatant and pellet were separated and stored at -70°C until they were prepared for two-dimensional PAGE (see below).

Sucrose Density Gradients

In order to monitor the state of the extraction for core particles and aggregates, each step was analyzed by sedimentation velocity ultracentrifugation. Aliquots of 12-1.4 A260 units were removed from the extraction mixture just prior to ultracentrifugation. Samples for gradients were always analyzed immediately at the end of the extraction period. They were never frozen. The volume of the aliquots was adjusted to 0.15-0.2 ml with the appropriate buffer, buffer A with 1 M LiCl and buffer B with 2 or 4 M LiCl. The sample was centrifuged for 5 min in a Beckman Microfuge B (approximately 9000 × g) to clear any gross precipitate before being loaded onto a 10-30% sucrose density gradient. The gradients were centrifuged at 35,000 rpm for 4.25 h in a Beckman SW 50.1 rotor. Fractionation of the gradients was monitored at 290 nm.

Two-dimensional Polyacrylamide Gel Electrophoresis

The proteins present in each supernatant and pellet fraction were analyzed by two-dimensional PAGE. The first dimension was electrophoresis in 9 M urea at pH 4.3. The second dimension was electrophoresis with 0.5% sodium dodecyl sulfate and 5 M urea in phosphate buffer at pH 7.2. Conditions for electrophoresis are described elsewhere.2 An aliquot of the extraction mixture supernatant representing 4-10 A260 unit equivalents was prepared for two-dimensional PAGE. Samples were adjusted to 9 M urea, 3 M LiCl either by direct addition of solid urea and LiCl or by concentration (see below) and resuspension into buffer C. After stirring for 12-16 h at 4°C, these samples were centrifuged in a Beckman Ty65 rotor for 1.5 h, in order to remove any precipitated RNA. Samples were then dialyzed versus electrophoresis sample buffer to remove LiCl, adjust ions, and change pH before electrophoresis.

Pellets were prepared for electrophoresis by two extractions with buffer C at 10-30 A260 units/ml. Extraction mixtures were stirred for 12-16 h at 4°C and ended by centrifugation in a Ty65 rotor for 1.5 h.

1The formulas used to calculate particle clearing times during centrifugation are: \( T = K \times \frac{n}{S} \) where \( T \) = clearing time, \( n \) = relative viscosity of the solution, \( S \) = sedimentation coefficient of the particle in Svedberg units, and \( K = \) clearing factor = \( \frac{10^{12} \times 3600}{\omega^2} \times \frac{1}{\text{Int}_{\text{cum}}} \times \text{Sedimentation coefficient of particle} \)

2The control used for comparison in each experiment was a sample of the same 39 S subunits used for the salt extraction. For a given protein, the intensity of its stained spot in the control electrophrogram was normalized to "completely present" (+++++). The spot intensity for the same protein in an experimental map was then

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**FIG. 1. Schematic diagram showing method of preparation of samples for sucrose density gradient and two-dimensional PAGE analysis.** SDG, sucrose density gradient; 2D, two-dimensional.

If necessary, samples were concentrated (see below) before electrophoresis sample buffer. When the volume was excessive, samples were concentrated by a method designed to give complete recovery of proteins. Samples were dialyzed into buffer D and then concentrated by vacuum evaporation in a Savant Speed-Vac apparatus. This machine continuously centrifuges the sample during the evaporation, thereby eliminating “bumping,” and collecting the material into a tight pellet. The pellets obtained were resuspended into buffer containing 9 M urea and dialyzed versus electrophoresis sample buffer.

**Analysis**

In order to facilitate the identification of protein spots in the electrophorograms, each sample was supplemented before electrophoresis with an amount of [14C]methylated (see below) protein extracted from the control sample. The amount added (6-12 µg TP39) was too small to be visualized by Coomassie blue staining, but was analyzed by fluorography (15, 16). Thus, in the absence of a complete twodimensional PAGE pattern, spots could be positively identified by comparison to the complete pattern in the fluorogram.
assigned a comparative value (e.g. +++, +++, 100% of the control; +, 25% of the control; —, not detectable). These scaled values were allowed each protein to be considered independently from the overall appearance of the map. While not assigning an absolute quantification for each spot, these scaled values provided an objective quantitation which was easily compared among several data sets, allowing an average behavior for each protein to be deduced.

**Radiomethylation of Proteins**

In order to introduce a radiolabel onto mitoribosomal protein without affecting its two-dimensional PAGE mobility, TP39 protein was methylated at lysine residues by a gentle procedure using [3H] formaldehyde and sodium cyanoborohydride (17). Radiolabeled formaldehyde, 44 mcCi/mmol, was obtained from New England Nuclear. A mixture containing 60 μg TP39 (prepared by extraction with buffer E, as above), 50 nmol (2.2 μC) of [3H]formaldehyde, and 20 mM NaCNBH3 in a total volume of 155 μl of buffer F was stirred for 2–3 h at room temperature. At the end of the incubation period, 25 μl of 5 mM NaBH4 was added to convert the remaining formaldehyde to ethanol, and the mixture was incubated an additional 30 min at 0 °C. Aliquots were added to two-dimensional PAGE samples immediately before dialysis. Radiomethylated protein was stored at ~70 °C, when necessary.

**RESULTS**

**Sucrose Density Gradients**—The physical state of the extraction mixtures was analyzed in 10–30% sucrose density gradients. Gradient analyses indicated not only the presence or absence of core particles but also allowed evaluation of the consequences of the ultracentrifugation conditions and of the methods chosen for resuspension of the pellets. Commonly, during incomplete extraction procedures of ribosomal subunits a protein-deficient “core particle” is formed (5, 16, 18, 19). Such particles contain ribosomal RNA and a reproducible subset of ribosomal proteins. Because the particle has a characteristic sedimentation coefficient, it can be observed as a defined peak in sucrose density gradients.

Fig. 2 shows sucrose density gradient profiles of the extraction mixtures. In each case, there is a peak at the top of the gradient which includes the stripped proteins. The indicated 6 S marker shows the approximate position of the slowest S-value material cleared by the ultracentrifugation step (see below). That is, it shows a nominal dividing line for supernatant and pellet preparations. The 39 S marker shows the position occupied by an intact mitoribosomal large subunit in a preparative gradient (10–30% sucrose, buffer B).

The 1 M LiCl (Fig. 2) gradient shows well resolved “core”-type particles in addition to the peak at the top of the gradient which is formed by the stripped proteins. The major and lesser peaks in this profile may indicate two intermediate core states of differing composition or two conformations of the same particle. In this gradient, the higher S-value core runs very close to the approximate 39 S position; it may represent a ribosomal subunit missing only a few proteins. The actual composition of the individual core-like particles was not analyzed.

Gradient profiles of 2 and 4 M extraction mixtures (Fig. 2, B and C) show the expected stripped protein peak at the top of the gradient but no defined peaks entering the gradient. Instead, heterodisperse material is distributed throughout the gradient. Since these concentrations of LiCl are precipitating to ribosomal RNA, this material probably includes nonspecific aggregates of core-like particles. It might also include nonspecific protein aggregates held together by hydrophobic interactions in the high ionic strength buffer or specific structural aggregates of protein or of protein and RNA.

The soluble proteins stripped from the ribosomal subparticle by the salt treatment appear in the density gradient profiles as peaks at the top of the gradient. In order to collect these stripped proteins for analysis by two-dimensional PAGE and for use as a source of soluble, native mitoribosomal proteins, the extraction mixtures were separated into soluble supernatant and pellet material by differential centrifugation (see “Experimental Procedures”). The 6 S position indicated in each gradient profile shows the approximate position of the fastest sedimenting materials that could be included in the stripped protein fraction. In fact, the conditions of differential ultracentrifugation used will have cleared some material of less than 6 S from the stripped protein fractions. The “soluble” stripped protein fractions, therefore, contain single proteins and could contain some protein dimers or trimers.

**Two-dimensional PAGE Electropherograms**—Solubilized, extracted proteins were separated from nonextracted and pellet material by differential ultracentrifugation using conditions that would remove 6 S particles. In several cases, pellets were used as starting material for subsequent extraction with higher salt concentrations. By successive extractions with increasing concentrations of the chaotropic salt, LiCl, the mitoribosomal subunit is disassembled into specific groups of soluble proteins and a residual pellet (after 4 M LiCl containing protein and rRNA). The composition of each stripped protein fraction and of the final residual pellet fraction was analyzed by two-dimensional PAGE and the proteins present or absent in each fraction were identified. The intensity of Coomassie blue stain for each spot was normalized against its stain intensity in a control two-dimensional PAGE map (see “Experimental Procedures”). This type of analysis was used to estimate the enrichment or diminution of each protein in the sample and to allow comparison of data from several experiments.

Fig. 3 shows an electropherogram of the proteins in each fraction together with a schematic diagram. A control electropherogram of the proteins that can be extracted from 39 S mitoribosomal subunits using 9 M urea is also shown. Each of

![Fig. 2. Sucrose density gradient profiles of the extraction mixtures.](image-url)

**Table 1.** Proteins of the large subunit of bovine mitochondrial ribosomes which predominate in the stripped protein fractions

| Stripped protein fraction | Proteins |
|--------------------------|---------|
| 1 M LiCl                 | 1, 2, 16, 17, 19, 24 |
| 2 M LiCl                 | 6, 10, 11, 12, 15, 22, 23, 24, 26 |
| 4 M LiCl                 | 3, 5, 6, 10, 23, 26 |

The proteins indicated predominate in two-dimensional PAGE electrophorograms as determined by the intensity of Coomassie blue stain.
LiCl Extraction of Bovine Mitochondrial Ribosomal Proteins

Fig. 3. Two-dimensional PAGE analysis of stripped protein, pellet, and control fractions. Samples prepared as described in Fig. 1, were analyzed by two-dimensional PAGE. The first dimension is electrophoresis in 9 M urea at pH 4.3. The second dimension is electrophoresis with 0.5% sodium dodecyl sulfate and 5 M urea in phosphate buffer at pH 7.2. A–C, electropherograms of the 1, 2, and
the stripped protein fractions (Fig. 3, A–C) contain specific subgroups of the mitoribosomal proteins. The proteins which predominate in each fraction based on Coomassie stain intensity are indicated in the schematic diagrams (Fig. 3, D–F) by solid spots and are summarized in Table I. Most proteins are not removed from the particle completely by any one treatment, but are partitioning differentially among two or more conditions. For example, proteins L2 and L3 (Fig. 3, arrowheads) can be detected in all of the stripped protein fractions, but their intensity varies in a specific and reproducible manner. L2 is intense in the 1 M LiCl fraction while L3 is faint. In the 2 M LiCl fraction, both spots are obvious, although L3 is darker, and in the 4 M LiCl fraction, L3 is intense while L2 is now only a faint spot. A similar situation is observed when L23 and L24 are compared; L24 is most intense in the 1 M LiCl-stripped protein fraction and L23 is most intense in the 4 M LiCl fraction. A different result is observed with L22 and L26, which are both most intense in the 2 M LiCl-stripped protein fraction. Overall, thirteen proteins (1, 2, 3, 6, 10, 11, 12, 15, 17, 22, 23, 24, and 26) are obtained in high recovery in at least one of the soluble preparations of stripped proteins (based on the intensity of Coomassie stain). Some proteins (6, 10, 11, 14, 23, 24, and 26) which stain intensely in two-dimensional PAGE separations of complete subunits are major components of more than one stripped protein fraction.

Whereas most proteins can be obtained in useful amounts in the soluble fractions, several proteins (7, 13, 14, 21, 25, 33, 41, 42, 45, and 49) remain predominantly or exclusively with the pellet fraction (Fig. 3, G and J), even after treatment with 4 M LiCl. The most intensely stained proteins of this fraction include a few (3, 8, 9, 10, 11, and 28) which are also found in the stripped protein fractions. Several other less intense proteins (4, 5, 6, 18, 19, 32, 40, 43, and 44) appear to some extent in both the stripped protein and residual pellet fractions. A LiCl concentration of 4 M should be sufficient to disrupt any strictly ionic interactions between protein and rRNA, so it is likely that other than ionic forces, e.g. hydrophobic, are involved in maintaining the structure of the core particle.

As can be seen from Fig. 3H, all of the proteins of the mitoribosomal large subunit do not stain with the same intensity. A protein which is 80% extracted by one condition may still appear as a moderate or weak spot in the two-dimensional PAGE stained map (e.g. L31 which is stripped by 1 M LiCl). Thus, analyses based on the appearance of the two-dimensional PAGE map of a stripped protein fraction tend to be weighted in favor of darkly staining proteins. However, several of the reproducibly appearing mitoribosomal proteins routinely appear as weakly staining spots. Therefore, to analyze the behavior of each individual mitoribosomal large subunit protein during the LiCl washing procedure, the intensity of Coomassie blue stain was normalized (see "Experimental Procedures") against the intensity of stain for the same protein in a control sample (Fig. 3, H and J). This type of analysis also aided comparison for reproducibility of data from several experiments. Table II shows the compiled results of this type of analysis. Each fraction, whether stripped or core, contains detectable amounts of more than half of the different large subunit proteins, but several of these are present in small amounts. Most of the proteins are preferentially extracted into one fraction in which they are moderately to highly

| Table II |
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| Response of mitoribosomal large subunit proteins to serial extraction with LiCl |
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| Data from two-dimensional PAGE separations were analyzed by comparison against a TP39 control electrophogram as described under "Experimental Procedures." Symbols used are: ++, enriched; +, present; ±, present irreproducibly or at low levels; −, not detected. Values shown represent averages based on three (for 1 M LiCl stripped protein fraction) or two (all other) experiments. No data were available for proteins L27, L29, L30, L36, L39, and L48. |
| Large subunit proteins | Stripped protein fractions | Residual pellet |
| --- | --- | --- |
| 1 M LiCl | 2 M LiCl | 4 M LiCl |
| 1 | ++ | + | ± |
| 2 | + | + | ± |
| 3 | + | + | + |
| 4 | + | + | + |
| 5 | ± | + | ++ |
| 6 | ± | + | ++ |
| 7 | − | − | − |
| 8 | + | + | ± |
| 9 | ± | ± | ± |
| 10 | ± | ± | ± |
| 11 | ± | ± | ± |
| 12 | ± | ± | ± |
| 13 | − | − | − |
| 14 | − | − | − |
| 15 | ± | ± | ± |
| 16 | ++ | ± | − |
| 17 | ++ | ± | − |
| 18 | ± | ± | ± |
| 19 | ++ | ± | ± |
| 20 | − | − | − |
| 21 | − | − | − |
| 22 | + | + | + |
| 23 | + | + | + |
| 24 | ++ | + | + |
| 25 | ± | − | ± |
| 26 | ± | ± | ± |
| 28 | ± | ± | ± |
| 31 | ± | ± | ± |
| 32 | ± | ± | ± |
| 33 | − | − | − |
| 34 | ++ | − | − |
| 35 | ± | ± | ± |
| 37 | − | + | ± |
| 38 | + | + | + |
| 40 | + | + | + |
| 41 | − | − | − |
| 42 | ± | ± | ± |
| 43 | ± | ± | ± |
| 44 | ± | ± | ± |
| 45 | − | − | − |
| 46 | ++ | ± | − |
| 47 | ++ | ± | − |
| 49 | − | − | − |
| 50 | − | − | − |
| 51 | ± | ± | ± |
| 52 | ± | ± | ± |

*4 M LiCl-stripped protein fractions, respectively; D–F, the corresponding schematic diagrams showing the reproducibly occurring mitochondrial ribosomal large subunit proteins; G and I, electropherogram and diagram of the residual pellet fraction, H and J, electropherogram and diagram of the TP39 control. Arrowheads point to proteins mentioned in the text. Filled spots in the schematic diagrams show proteins which predominate based on Coomassie blue stain intensity.*
enriched. Enriched proteins may appear on maps with intensities which approximately equal that of the control, yet they are enriched in that fraction since other proteins will be decreased or completely absent. Highly enriched proteins will appear in a two-dimensional PAGE map as a spot which will meet or exceed the intensity of the control spot (e.g., Li6 in 1 M LiCl). The enriched proteins of each fraction are summarized in Table III. Since each condition is enriched for a specific group of proteins, the fractions produced by successive washings in LiCl are useful not only because of the soluble, native character of the proteins, but also because they allow work with a specific small group of partially purified proteins.

Interestingly, less protein is obtained in soluble form by a one-step extraction of 39 S subunits directly into 4 M LiCl (see “Experimental Procedures”) than by the serial washing procedure (data not shown). Moreover, several proteins (1, 2, 5, 10, and 23, in particular) show a greater tendency to associate with the pellet fraction under these conditions. It is noteworthy that this includes some proteins (1, 2, and 10) which are largely removed by 1 M LiCl.

**DISCUSSION**

Proteins of the large subunit of bovine mitochondrial ribosomes were extracted by washing subunits with a series of buffers containing increasing concentrations of LiCl as the only chaotropic agent. The results of each extraction condition were characterized by sucrose density gradient centrifugation, and all of the conditions show a stripped protein peak in sedimentation profiles. The material in the stripped protein and residual pellet (after 4 M LiCl) fractions was analyzed by two-dimensional PAGE to determine which proteins are present and in what amounts. Buffers containing 1, 2, and 4 M LiCl each extract a subset of mitoribosomal proteins, while another group of proteins tends to remain “core-associated,” even after 4 M LiCl. Although several of the proteins can be detected in two or more fractions, most are specifically enriched in only one fraction.

The soluble, salt-extracted proteins were separated from core material by differential centrifugation, using conditions chosen to nominally clear particles of greater than 6 S. Therefore, the soluble fractions are composed of material with a range of sedimentation coefficient values. These fractions should contain all or most material of 0-3 S, some material of 3-6 S, and a very small proportion of 6-10 S particles. Thus, the soluble protein fractions may include some small protein aggregates, dimers, and trimers, in addition to single proteins. Such aggregates may reflect specific structural associations of mitoribosomal proteins which remain bound to each other during the extraction.

Soluble proteins produced by LiCl washing exhibit different characteristics from those produced with denaturants such as urea or acetic acid. Because high salt concentrations increase the strength of hydrophobic interactions, salt-extracted proteins are expected to retain tertiary structure which sequesters hydrophobic regions to the interior, thus retaining a more native configuration. Experiments by other authors using various techniques have indicated that salt-extracted proteins retain more structure than denaturant-extracted proteins (8–12). In addition, proteins prepared in this manner retain good solubility as the salt concentration is dropped from high (1-4 M) to moderate (0.3-1 M) levels (9, 12, 20). These properties make proteins prepared by salt extraction ideally suited to certain types of experiments. In experiments requiring immune recognition, their more native structure should make them reactive with antibodies directed against conformational as well as sequence determinants. In addition, they are soluble at conditions suitable for immune precipitations without the need to add detergents. In Escherichia coli systems, proteins prepared by salt extraction have shown improved binding in rRNA-protein affinity experiments (10, 13). Because they retain some native structure, salt-extracted proteins should also be more suitable for ribosome reconstitution experiments.

In addition to the proteins solubilized by LiCl, we have also characterized those proteins which remain in the residual pellet fraction. This fraction is specifically enriched for several proteins. However, since these proteins are obtained in soluble form only by using strong denaturants, they lack the structure and solubility advantages of the stripped fraction proteins. The residual pellet fraction would include the core particles; that is, particles composed of rRNA and adhering proteins. Because core-associated proteins are determined by the operational criterion of centrifugation, aggregates of sufficient size (>6 S) containing mitoribosomal protein without rRNA could also be included in the residual pellet material. Such aggregates could include structural associations of mitoribosomal proteins which dissociated from the ribosomal subunit as a group or which reassociated after extraction. Nonspecific aggregates may also have formed during the stirring period as the effect of hydrophobic interactions was increased by the high ionic strength. Such effects may explain the apparent tendency of so many mitoribosomal proteins to remain core-associated.

In this series of experiments, group fractions of mitoribosomal proteins were characterized with respect to their composition and reproducibility. No attempt was made to purify the proteins beyond their original solubilization. There are several advantages to working with group fractions as opposed to proceeding to purified proteins. Foremost among these is the ability to generate a useful amount of protein from relatively little starting material. Less than 10 A280 units (<620 μg of protein) will produce sufficient material for some experiments. After the composition of each fraction is characterized, a particular protein of interest can be studied directly, by utilizing the fraction in which it is enriched, or that fraction can be used as a starting point in further purification procedures.

The composition of each fraction gives important clues to the structure of the mitoribosome. While ribosomes could be viewed as aggregates of basic proteins bound by largely ionic forces to negatively charged RNA, this view is inconsistent with the strong tendency of mitoribosomal proteins to remain particle-associated. The 0.1 M LiCl-stripped protein fractions will probably contain those proteins that had been held predominantly by ionic interactions. Proteins in the 0.6 M LiCl-stripped protein fraction and residual core fraction will contain proteins bound predominantly by other forces. In ribosomes, specific interactions with the rRNA are expected to stabilize the association of many proteins with the particles. Such interactions will be largely dependent on rRNA secondary and tertiary structure. Therefore, the addition of chaotropic salt, LiCl, is coupled with the removal of Mg2+ and the addition of EDTA, both of which will disrupt rRNA structure. In addition, some proteins may be tightly and specifically bound not to rRNA, but to other proteins (hydrophobically). This type of interaction may account for dimers and trimers in the stripped protein fractions as well as aggregates in the residual pellet fraction.

It is noteworthy that during a one-step extraction procedure, proteins tend to associate more with the residual pellet fraction. The tendency of some proteins to be trapped in the pellet material may result in the high salt concentration from enhanced hydrophobic interactions between proteins or between proteins and structured RNA. Interestingly, these “trapped” proteins include some which are removed by 1 M
LiCl, suggesting that the forces involved in their binding are not strictly ionic. The difficulty of extraction at concentrations of LiCl which precipitate RNA may also contribute to the low yield of soluble protein from the one-step extraction procedure.

While most of the mitoribosomal proteins are obtained preferentially in one, or occasionally two fractions, very few proteins are found exclusively in a single fraction. This result is expected for two reasons. First, with a gentle, serial extraction procedure, no one condition is stringent enough to insure complete removal of a particular protein. Other authors (4, 6, 7) have commented on this type of problem occurring when the protein concentration was too high. For this reason, the concentration of subribosomal particles used in the present experiment was purposely kept quite low (<1 µM). Second, the analysis is complicated, since both darkly and faintly staining proteins are routinely present in the mitoribosomal two-dimensional PAGE map. A darkly staining protein which is only 5% extracted into a specific fraction will still be easily detectable in that condition. Therefore, while 80% of a given protein may be in one condition, it will still be considered "definitely present" in other conditions. On the other hand, it is more difficult to judge enrichment for faintly staining proteins. If they are at all detectable in a fraction, one must assume it represents a significant proportion of that protein.

When subjected to a similar procedure (4, 6), E. coli 30 S ribosomal subunits release virtually all of their protein in 1 or 2 M LiCl washes. LiCl extraction of E. coli 50 S subunits was reported using a different procedure (4), although the results are qualitatively similar to those reported in this paper. That is, certain proteins extracted well into one condition, some extracted well into two or more conditions, and a few were never detected in reasonable quantities. The residual core after extraction of the E. coli particles is reported to contain insignificant amounts of protein (4, 6). Why then does the mitoribosomal residual pellet contain so much protein? Since the mitochondrial ribosome is the most protein-rich (RNA-poor) ribosome type it is expected that protein-protein interactions will predominate in this particle. It is reasonable to assume that the quaternary associations of these proteins involve hydrophobic interactions. In this event, the high salt concentration may serve to aggregate mitoribosomal proteins as well as RNA both specifically and nonspecifically into the residual pellet fraction.

Thus, while bacterial and mammalian mitochondrial ribosomes are similar in size and function, fundamental differences exist in the structural organization of these two ribosomes. These differences reflect an unexpected degree of freedom in the structural organization of ribosomes, allowing mammalian mitoribosomes to utilize proteins in ways that replace major amounts of rRNA. The availability of several of the mitoribosomal proteins, prepared in soluble form by the nondenaturing salt extraction, should prove to be a valuable resource for studying the structure and function of this interesting ribosome.

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