CELL BIOLOGY

A LABORATORY HANDBOOK

Edited by

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VOLUME 1

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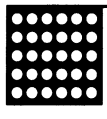

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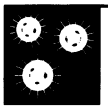

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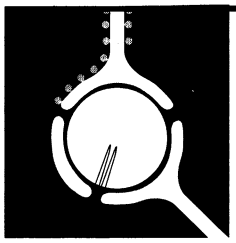

Isolation of Yeast Mitochondria and Study of Mitochondrial Protein Translation

Johannes M. Herrmann, Heike Fölsch, Walter Neupert, and Rosemary A. Stuart

I. Introduction

The formation of mitochondria is a detailed process that involves the precise cooperation of two separate genetic systems, one in the mitochondria the other in the nucleus. Studies on the biogenesis of mitochondria address the synthesis and translocation of these proteins across or into the mitochondrial membranes and finally their assembly, often into multimeric subunit complexes. Unlike most organisms, *Saccharomyces cerevisiae* can survive with defective respiratory chain and oxidative phosphorylation because it can use fermentable carbon sources for energy production. This ability to grow anaerobically, together with the ease of genetic manipulation of this yeast, has enabled the identification of many mutants defective in aerobic growth. Such mutants have led to the identification and cloning of genes that encode proteins essential for mitochondrial function (for reviews see Grivell, 1989; Tzagoloff and Dieckmann, 1990; Bolotin-Fukuhara and Grivell, 1992).

Approximately 5% of the mitochondrial proteins are encoded by the mitochondrial genome and here we describe how one can study the synthesis of these proteins in isolated mitochondria. The vast majority of mitochondrial proteins, however, are encoded by the cell nucleus and are synthesized in the cell cytosol as precursor proteins. These precursors are imported into mitochondria in a posttranslational manner. Our knowledge of mitochondrial protein import has increased over the past years due to a number of detailed *in vitro* studies using mainly *S. cerevisiae* and *Neurospora crassa* as model systems (for reviews, see Hartl and Neupert, 1990; Baker and Schatz, 1991). These *in vitro* import systems employ radiolabeled precursor proteins which have been cloned, transcribed *in vitro*, and then translated in a lysate (usually rabbit reticulocyte) in the presence of a radiolabeled amino acid (e.g., [^{35}S]methionine) and which are incubated with isolated mitochondria. Here we describe a procedure for the growth of *S. cerevisiae* and subsequent isolation of mitochondria that is an adaptation of an earlier protocol from Daum *et al.* (1982). The resulting isolated mitochondria are suitable for use in both *in organello* translation studies, a protocol for which is outlined here (as previously described by McKee and Poyton, 1984), and *in vitro* studies of import of the nuclear-encoded proteins, which has been described in detail elsewhere (Wienhues *et al.*, 1992; Glick, 1991).

II. Materials and Instrumentation

Yeast extract (Cat. No. 0127-05-3) was purchased from Difco, agar (Cat. No. 1614), glucose monohydrate (Cat. No. 4074), KH_2PO_4 (Cat. No. 4873), K_2HPO_4

(Cat. No. 5104), NH_4Cl (Cat. No. 1145), $CaCl_2 \cdot 2H_2O$ (Cat. No. 2382), NaCl (Cat. No. 6404), KCl (Cat. No. 4936), $MgSO_4 \cdot 7H_2O$ (Cat. No. 5886), $FeCl_3 \cdot 4H_2O$ (Cat. No. 5886), lactate (Cat. No. 366), trishydroxymethylaminomethane (Tris, Cat. No. 8382), sorbitol (Cat. No. 7758), sucrose (Cat. No. 7651), and EDTA (Titriplex, Cat. No. 8418) were all obtained from Merck. Fatty acid-free BSA (Cat. No. A-6003), PMSF (Cat. No. P-7626), Mops (Cat. No. M-1254), β-mercaptoethanol (Cat. No. 6250), LiDS (Cat. No. 4632), SDS (Cat. No. 20760), glycerol (Cat. No. G-7757), and bromophenol blue (Cat. No. 15375) were purchased from Serva. ATP (Cat. No. 635316), GTP (Cat. No. 414581), α-ketoglutarate (Cat. No. 127205), phosphoenolpyruvate (Cat. No. 182112), pyruvate kinase (Cat. No. 127418), and dithiothreitol (DTT, Cat. No. 708992) were all obtained from Boehringer-Mannheim. [^{35}S]Methionine (10 mCi/ml, 1142 Ci/mmole) was obtained from ICN, acrylamide (Cat. No. 10675) and *N,N'*-methylenebisacrylamide (Cat. No. 289195), *N,N,N',N'*,-tetramethylenediamine (Cat. No. 35925) were obtained from Serva, and ammonium persulfate (Cat. No. 1201) was obtained from Merck. Zymolyase (20,000 U/g) was obtained from Seikagaku (Cat. No. 120491), and the protein assay from Bio-Rad (Bio-Rad Protein Assay Kit I, Cat. No. 500-0001). *S. cerevisiae* strain, D273-10B can be obtained from the American Tissue Culture Collection (ATCC No. 24657).

III. Procedures

A. GROWTH OF *Saccharomyces cerevisiae*

Solutions

1. *YPEG agar plates:* To make 600 ml, solubilize 6 g yeast extract, 12 g Bacto-peptone, and 12 g agar in distilled water, adjust the pH to 5 with concentrated HCl, and bring to a final volume of 570 ml. Autoclave 20 min at 120°C. Prior to preparation of the agar plates mix 18 ml sterile 87% glycerol and 12 ml ethanol to the hot solution. Store the solid plates at 4°C.

2. *Lactate medium:* To make 1 liter, solubilize 3 g yeast extract, 1 g glucose monohydrate, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl, and 1.1 g $MgSO_4 \cdot 7H_2O$ in ±700 ml distilled water. Add 0.3 ml of a 1% $FeCl_3$ solution and 22 ml 90% lactate. Adjust the pH to 5.5 with 10 *M* KOH and bring to a total volume of 1 liter. Autoclave 20 min at 120°C. Store at room temperature.

Steps

1. Streak out the yeast strain D273-10B onto a YPEG agar plate and grow for 2–3 days at 30°C.

2. Inoculate 20 ml of lactate medium in a 100-ml Erlenmeyer flask with a loop full of the culture. Grow overnight at 30°C and shaking at 120 rpm.

3. Use the overnight culture to inoculate fresh lactate medium (100 ml in an 500-ml Erlenmeyer flask). The initial OD_{578} should be 0.5–1.0. Grow the culture overnight as described in step 2.

4. Repeat step 3 at three or four times.

5. For the main culture inoculate 1.5 liters of lactate medium into a 5-liter Erlenmeyer flask with the preculture to an initial OD_{578} of 0.05. Grow the culture for 14–15 hr at 30°C and 120 rpm.

6. Measure the OD_{578} of the culture, which should be 1.0–1.5.

B. ISOLATION OF YEAST MITOCHONDRIA

Solutions

1. *100 mM Tris–SO_4, pH 9.4:* To make 1 liter, solubilize 12.11 g of Tris in distilled water, adjust pH to 9.4 with H_2SO_4, and adjust to a total volume of 1 liter. Store at 4°C.

2. *100 mM Tris–HCl, pH 7.4:* To make 1 liter, solubilize 12.11 g Tris in distilled water, adjust pH to 7.4 with HCl, and adjust to a total volume of 1 liter. Store at 4°C.

3. *1 M KPi buffer, pH 7.2:* First make 100 ml of a 1 *M* K_2HPO_4 solution (17.4 g) and 100 ml of a 1 *M* KH_2PO_4 solution (13.6 g). To 50 ml of the K_2HPO_4 add the KH_2PO_4 solution until a pH of 7.2 is achieved. Store at 4°C.

4. *1 M DTT:* To make 1 ml solubilize 154.3 mg DTT in 1 ml distilled water. This solution should be prepared freshly each time.

5. *0.2 M PMSF:* To make 1 ml, solubilize 34.5 mg PMSF in 1 ml ethanol. Prepare fresh each time.

6. *2.4 M Sorbitol:* To make 500 ml, solubilize 218.6 g sorbitol in distilled water and adjust to a total volume of 500 ml. Store at 4°C.

7. Zymolyase buffer: To make 500 ml, mix 250 ml 2.4 *M* sorbitol with 10 ml 1 *M* KP_i buffer, pH 7.2, and adjust to a total volume of 500 ml. Store at 4°C.

8. Homogenization buffer: To make 500 ml, mix 125 ml 2.4 *M* sorbitol and 50 ml 100 m*M* Tris–HCl, pH 7.4, add 100 mg fatty acid-free BSA, and adjust to a total volume of 497.5 ml. Finally add 2.5 ml 0.2 *M* PMSF.

9. *SEM buffer:* To make 1 liter, solubilize 85.58 g sucrose, 2.1 g Mops, and 0.37 g EDTA in distilled water, adjust pH to 7.2 with KOH, and bring to a total volume of 1 liter. Store at 4°C.

Steps

1. Collect cells of the main culture by centrifugation at 3000 rpm (Beckman JA2-21, rotor JA10) for 5 min at 4°C.

2. Decant supernatant and resuspend the cells in a total of 100 ml of distilled H_2O.

3. Spin as described in step 1 in a preweighed centrifuge bottle.

4. Decant supernatant and determine the weight of the pellet.

5. Resuspend the cells in 100 m*M* Tris-SO_4, pH 9.4, using 2 ml/g of cells.

6. Transfer the cells with a pipette into an Erlenmeyer flask and determine the volume of the suspension which ideally should be one-tenth of the volume of the flask. Add DTT from a 1 *M* stock to a final concentration of 10 m*M*.

7. Incubate the cells for 10 min at 30°C in a shaking water bath.

8. Spin down the cells at 4000 rpm (Beckman JA2-21, rotor JA20) for 5 min at 4°C.

9. Resuspend cells in 1.2 *M* sorbitol using 2 ml/g cells.

10. Repeat step 8.

11. Resuspend the pellet in zymolyase buffer to a final concentration of 0.15 g/ml, and add 2–3 mg zymolyase per gram wet weight. Remove a small aliquot prior to the addition of zymolyase to use as a control for the spheroplast test (see step 13).

12. Incubate the cells for 20–40 min at 30°C in a shaking water bath.

13. Check for efficient spheroplast formation by adding 50 μl sample to 2 ml H_2O and measuring the $OD_{600\ nm}$. Incubation should be continued until the $OD_{600\ nm}$ is in the range 10–20% of the value measured prior to the addition of zymolyase.

NOTE

The sample should be kept cold at all times throughout the following steps.

14. Spin the spheroplasts at 4000 rpm (Beckman rotor JA20) for 5 min at 4°C.

15. Resuspend the spheroplasts in 100 ml 1.2 *M* sorbitol and spin them again at 4000 rpm (Beckman rotor JA20) for 5 min at 4°C.

16. Decant the supernatant carefully and resuspend the spheroplasts in the "homogenization buffer" at a concentration of 0.15 g/ml.

17. Transfer the spheroplast suspension to a glass douncer and dounce for 10–15 times, avoiding foaming of the sample.

18. Spin at 3000 rpm for 5 min (Beckman rotor JA20) at 4°C.

19. Decant the supernatant into fresh tubes and centrifuge again at 4000 rpm (Beckman rotor JA20) for 5 min at 4°C.

20. Decant the supernatant again into fresh tubes and spin at 10,000 rpm for 12 min at 4°C (Beckman rotor JA20).

21. Discard the supernatant and resuspend the pellet carefully in approximately 25 ml of SEM buffer.

22. Spin at 4000 rpm for 5 min (Beckman rotor JA20) at 4°C.

23. Decant the supernatant again into fresh tubes and spin at 10,000 rpm for 12 min at 4°C (Beckman rotor JA20).

24. Resuspend the mitochondrial pellet in 300 μl of SEM buffer and determine the protein concentration using the Bio-Rad assay method and then adjust the protein concentration to 10 mg protein/ml.

25. Freeze aliquots (50 μl) of the mitochondrial suspension in liquid nitrogen and store at −70°C.

C. TRANSLATION OF MITOCHONDRIA-ENCODED PROTEINS IN ISOLATED YEAST MITOCHONDRIA

Solutions

1. *1 M KCl:* To make 100 ml, solubilize 7.5 g KCl in distilled water and adjust to a total volume of 100 ml. Store at 4°C.

2. *1 M $MgSO_4$:* To make 100 ml, solubilize 24.6 g $MgSO_4 \cdot 7H_2O$ in distilled water and adjust to a total volume of 100 ml. Store at 4°C.

3. *1 M Tris–HCl, pH 7.2:* To make 100 ml, solubilize 12.1 g Tris in 70 ml distilled water, adjust the pH to 7.2 with 5 *M* HCl, and add water to a total volume of 100 ml. Store at 4°C.

4. *200 mM ATP:* Dissolve 13 mg ATP in 100 μl distilled water and adjust with 10 *M* KOH to a pH around 7. Make fresh each time.

5. *50 mM GTP:* Dissolve 2.8 mg GTP in 100 μl distilled water. Make fresh each time.

6. *Amino acid stock solution:* Solubilize 20 mg each of the amino acids alanine, arginine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, and valine in 10 ml distilled water. Aliquot in 100-μl portions and keep at −20°C.

7. *10 mM Cysteine:* Solubilize 1.2 mg of cysteine in 1 ml of distilled water. Aliquot in 20 μl and store at −20°C.

8. *1 mg/ml Tyrosine:* Solubilize 1 mg of tyrosine in 900 μl of distilled water, adjust to pH 7 with KOH, and add water to a total volume of 1 ml. Aliquot in 20 μl and keep at −20°C.

9. *200 mM Methionine:* Solubilize 30 mg of methionine in 1 ml of distilled water. Make fresh each time.

10. *BSA stock solution:* Solubilize 1 g of fatty acid-free BSA in 10 ml of distilled water. Aliquot in 100-μl portions and store at −20°C.

11. *1.5× Translation buffer:* To make 1 ml of the buffer, add 375 μl 2.4 *M* sorbitol, 225 μl 1 *M* KCl, 22.5 μl 1 *M* KP_i buffer, pH 7.2, 30 μl 1 *M* Tris–HCl, pH 7.2, 19 μl 1 *M* $MgSO_4$, 45 μl BSA stock solution, 30 μl 200 m*M* ATP, 15 μl 50 m*M* GTP, 1.7 mg α-ketoglutarate, 3.5 mg phosphoenolpyruvate, 9.1 μl amino acid stock solution, 10 μl 10 m*M* cysteine, and 18.2 μl 1 mg/ml tyrosine. Adjust to 1 ml with distilled H_2O.

12. *500 mM EDTA:* Dissolve 18.6 g EDTA in distilled water. To help dissolve EDTA, adjust pH to 7.2 with NaOH and stir at room temperature. Adjust total volume to 100 ml.

13. *Washing buffer:* Mix 1.25 ml 2.4 *M* sorbitol, 10 μl 500 m*M* EDTA, and 125 μl 200 m*M* methionine, and adjust to 5 ml with distilled water.

14. *LiDS sample buffer:* To make 50 ml of the solution solubilize 1 g LiDS, 5 ml glycerol, and 0.36 g Tris in 40 ml of distilled water. Adjust with HCl to a pH of 6.8, add 5 mg bromophenol blue 1.25 ml β-mercaptoethanol and adjust total volume to 50 ml. Store at room temperature.

Steps

1. Heat the thermoblock to 30°C.

2. Thaw mitochondria immediately before you start the experiment.

3. Mix 20 μl 1.5× buffer, 1.5 μl pyruvate kinase (0.5 mg/ml), 5.5 μl distilled water, and 2 μl mitochondria in SEM (10 mg protein/ml). Incubate the mixture for 2 min at 30°C.

4. Add 1 μl [^{35}S]methionine and incubate for 20 min at 30°C.

5. Add 30 μl 0.2 *M* methionine to the reaction mix and centrifuge at room temperature for 5 min at 14000 rpm in an Eppendorf centrifuge.

6. Remove the supernatant and wash the mitochondrial pellet carefully with 200 μl washing buffer.

7. Centrifuge again as in step 5 and remove the supernatant again.

8. Add 25 μl LiDS sample buffer and shake for 45 min in an Eppendorf mixer at 4°C to achieve good solubilization of the proteins.

9. Resolve the mitochondrial proteins by SDS–polyacrylamide gel electrophoresis and the radiolabeled proteins can be visualized by fluorography of

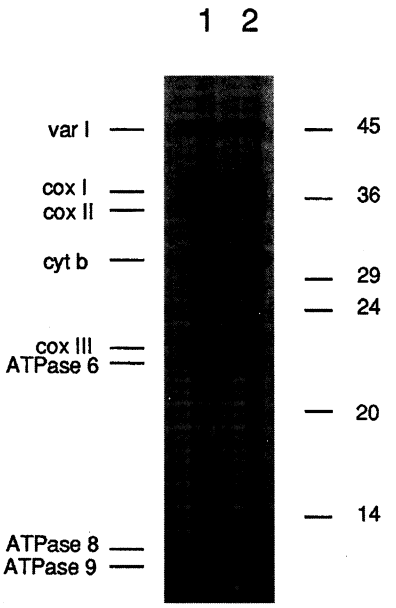

FIGURE 1 Products of translation in isolated yeast mitochondria. Proteins were translated for 10 min (lane 1) or 30 minutes (lane 2) as described, and labeling was stopped following the addition of cold methionine. After a 5-min chase, mitochondria were reisolated by centrifugation, washed, and resuspended in sample buffer. Labeled proteins were separated by SDS–PAGE and visualized by fluorography. Translation products are indicated. var I, a protein of the small ribosomal subunit; cox I–III, subunits I–III of the cytochrome c oxidase complex; cyt b, cytochrome b of the bc_1 complex; ATPase 6, 8, and 9, subunits 6, 8, and 9 of the ATP synthase. The positions marked by 45, 36, 29, 24, 20, and 14 indicate the mobility of the protein standards used and the numbers refer to their molecular weight (in kDa).

the resulting gel (Fig. 1) (Laemmli, 1970; see also article by Julio E. Celis and Eyδfinnur Olsen). For gel analysis we recommend using a gel whose final concentrations of acrylamide and bisacrylamide are 16 and 0.1% (w/v), respectively.

IV. Comments

The mitochondria prepared using the protocol described in Section IIIB are stable for several months if stored at −70°C. It is essential though that they are frozen and thawed only once as they are not suited to refreezing. The isolated mitochondria can be used for *in vitro* experiments to study the import of various mitochondrial preproteins as described by Hartl and Neupert (1990), Koll *et al.* (1992), and Glick *et al.* (1992). The mitochondria isolated according to this protocol are also suitable for *in vitro* analysis of mitochondrial protein translation, as was described in Section IIIC. In addition, one can use the isolated mitochondria for submitochondrial localization of proteins, for example, using hypotonic swelling of mitochondria to rupture specifically the outer membrane and leave the inner membrane intact (Glick, 1991). For the latter purpose we observed that best results are achieved if one isolates mitochondria from yeast cells harvested prior to reaching an OD of 1. The normal yield of mitochondria is between 2 and 5 mg per gram of yeast cells.

V. Pitfalls

1. Sometimes the zymolyase treatment does not work efficiently within a 30- to 45-min period; this usually happens if the yeast cultures were grown too long

and the cells are harvested at an OD of 2 or higher. If this occurs, the same amount of zymolyase should be added again and incubated for a further 15–30 min.

2. Take care that the zymolyase treatment does not occur too long after the spheroplast formation is complete because the zymolyase is often contaminated with other proteases, whose activities may affect the quality of your mitochondria preparation, i.e., degradation of mitochondrial surface receptors required for preprotein import.

3. The douncing step is critical: douncing with too much force will result in broken mitochondria, whereas insufficient douncing often results in a high level of intact spheroplasts, thereby decreasing the yield of mitochondria.

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