Preparation of yeast mitochondria and *in vitro* assay of respiratory chain complex activities

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Method Article

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Abstract

Introduction

The aim of the protocol is to obtain an enriched fraction of intact mitochondria from the yeast _Saccharomyces cerevisiae_ to perform quantitative determination of the activity of respiratory chain enzymes. All the strains used to perform this protocol are derivatives of W303 \(K699: _MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52_).

Reagents

Cell culture - Selective medium \(as appropriate) supplemented with 2% \(w/v) D-glucose - YPGAL medium: YEP medium \(1% yeast extract, 2% Bactopeptone) supplemented with 2% \(w/v) D-galactose and 0.1% \(w/v) D-glucose **Preparation of mitochondria by differential centrifugations** - 10 mM ethylenediaminetetraacetic acid \(EDTA) - Sorbitol Buffer A: 1.2 M sorbitol, 50 mM Tris-HCl, pH7.5, 10 mM EDTA \(stored at 4 °C), 0.3% \(v/v) 2-mercaptoethanol added before use - Sorbitol Buffer B: 0.7 M sorbitol, 50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA \(stored at 4 °C) - Zymolyase-100T \(Seikagaku Biobusiness Corporation) 4 mg/ml \(stored at -20 °C) - Complete^{TM} Protease Inhibitor Cocktail EDTA-free Tablets \(Roche) **Sample preparation** - 10 mM potassium phosphate \(PK) buffer, pH 7.4 \(stored at -20 °C) - Bradford microplate microassay \(Bio-Rad) **Ubiquinol:cytochrome _c_ oxidoreductase assay** - 250 mM potassium phosphate \(PK) buffer, pH 7.4 \(stored at -20 °C) - 50 mM sodium azide \(NaN_3) \(stored at -20 °C) - 1 mM cytochrome _c_ \(freshly prepared) - 1 mg/ml antimycin A in 50% ethanol \(stored at -20 °C) - sodium borohydride \($NaBH_4$) - decylubiquinone \(DB) - decylubiquinol \(DBH_2) \(stored at 4 °C and freshly diluted 1:1 with H_2O) **Cytochrome _c_ oxidase assay** - 100 mM potassium phosphate \(PK) buffer, pH 7.0 \(stored at -20 °C) - 0.8 mM cytochrome _c_ \(freshly prepared). Dissolve cytochrome _c_ in H_2O containing 10% of 100 mM potassium phosphate buffer, pH 7.0, and reduce it with sodium dithionite) - 40 mM potassium cyanide \(KCN) \(stored at -20 °C) **ATP synthase assay** - Buffer H-Mg: 10 mM $MgSO_4$ in 100 mM Hepes-KOH, pH 8.0 \(stored at -20 °C) - 30 mM NADH \(freshly prepared) - 50 mM phosphoenolpyruvic acid \(freshly prepared) - 10 mg/ml pyruvate kinase - 5 mg/ml lactate dehydrogenase \(stored at 4 °C) - 0.2 mg/ml antimycin A in 50% ethanol \(stored at -20 °C) - 25 mM ATP buffered at pH 7.0 with 3M $KHCO_3$ \(freshly prepared) - 0.2 mg/ml oligomycin in 50% ethanol \(stored at -20 °C)

Equipment

- Shaking incubator at 28 °C - Water bath at 37 °C - Refrigerated centrifuges - Spectrophotometer

Procedure

Cell culture 1. Pre-inoculate yeast cells in 5 ml of liquid selective medium supplemented with 2% \(w/v) glucose with shaking at 28 °C for 8h. 2. Harvest cells and inoculate in 200 ml of YPGAL medium. Incubate overnight with shaking at 28 °C. 3. Harvest cells by centrifuging when OD_{600nm} of the culture reaches a value of 2. 4. Wash the pellet in 20 ml of sterile H_2O. The resulting pellet can be stored at -80 °C. **Preparation of mitochondria by differential centrifugations** Yeast cell mitochondria are prepared by differential centrifugations. This protocol is adapted from Ref. 1. 1. Rinse the pellet with 5ml of 10 mM EDTA. 2. Centrifuge at 400g for 3 min. 3. Discard the supernatant and resuspend the pellet in 4.5 ml of ice-cold Sorbitol Buffer A supplemented with 0.3% \(v/v) 2-mercaptoethanol. 4. Digest the cell wall with approx. 250 µl of 4 mg/ml Zymolyase-100T \(1 mg per g of cells) at 37 °C for 45 min. 5. Verify wall digestion under the microscope. 6. Harvest spheroplasts by centrifugation at 1,800g for 15 min at 4 °C. 7. Discard the supernatant and resuspend the pellet gently in 7 ml of ice-cold Sorbitol Buffer B. 8. Centrifuge at 2,500g for 15 min at 4 °C. 9. Transfer the supernatant in a new tube and centrifuge at 20,000g for 15 min at 4 °C. 10. Discard the supernatant and resuspend the pellet by pipetting in 4 ml of ice-cold Sorbitol Buffer B supplemented with EDTA-free Complete™ Protease Inhibitor Cocktail \(Roche). 11. Centrifuge at 800g for 5 min at 4°C. 12. Transfer the supernatant in a new tube and centrifuge at 15,000g for 15 min at 4 °C. 13. Discard the supernatant and resuspend the pellet by pipetting in 4 ml of ice-cold Sorbitol Buffer B. 14. Centrifuge at 800g for 5 min at 4°C. 15. Transfer the supernatant in a new tube and centrifuge at 15,000g for 15 min at 4°C. 16. Discard the supernatant and resuspend the pellet by pipetting in 1.5 ml of ice-cold Sorbitol Buffer B. 17. Centrifuge at 800g for 5 min at 4°C. 18. Decant the supernatant in a new 2-ml-tube and centrifuge at 15,000g for 15 min at 4°C. 19. Store the pellet at -80°C. **Sample preparation** 1. Resuspend the resulting mitochondrial pellet in 300-500 µl of 10 mM PK buffer, pH 7.4. 2. Freeze and thaw three times. 3. Determine protein concentration with Bradford microplate microassay \(Bio-Rad). 4. Normalize samples for protein concentration \(recommended concentration: between 0.3 and 0.6 µg/µl). **Ubiquinol:cytochrome _c_ oxidoreductase \(Complex III) activity assay** The protocol is adapted from Refs. 2 and 3. A) Chemically reduce decylubiquinone \(DB) to decylubiquinol \(DBH_2) 1. Prepare a 10-mM solution of DB in HCl-acidified ethanol \(pH 4 or lower). 2. Add a few milligrams of sodium borohydride to reduce quinone. Reaction is completed when solution's color changes from yellow to colorless. 3. Add 2 ml of cyclohexane to the reduced solution and stir it. 4. Transfer the upper \(organic) phase to a clean tube. 5. Add 2 ml of cyclohexane to the remaining lower phase and stir it. Transfer the upper phase adding it to the previous one. 6. Repeat point 5 another time. 7. Wash the organic fraction with 2 M NaCl. 8. Dry the pellet under nitrogen and resuspend it with a volume of HCl-acidified ethanol \(pH 4 or lower) corresponding to the initial one. B) Determine ubiquinol:cytochrome _c_ oxidoreductase activity Activity is determined by measuring spectrophotometrically, at 550 nm at 30 °C, the rate of reduction of cytochrome _c_ by ubiquinol. Measure complex III activity at several protein concentrations paying attention to the linearity of the reaction. To determine the reduction of cytochrome _c_ due to the specific activity of complex III, for each sample, perform parallel measurements of activity in the presence and in the absence of antimycin A, an inhibitor of complex III. 1. Prepare two cuvettes containing 200 µl of 250 mM PK buffer, pH 7.4, 40 µl of 50 mM NaN_3, and 50 µl of 1 mM cytochrome _c_. Add 10 µl of 1 mg/ml antimycin A in one of them and an equal volume of H_2O in the other one. 2. Add 2-10 µg of mitochondrial

proteins. 3. Adjust the volume to 990 µl with H_2O. 4. Record the baseline for 2 min. 5. Start the reaction by adding 10 µl of DBH_2. 6. Measure absorbance of the sample at 550 nm for 2 min. 7. Calculate complex III specific activity using the Beer-Lambert law equation \(Fig.1). Express the activity as nanomoles of cytochrome _c_ reduced per minute per milligram of protein. **Cytochrome _c_ oxidase \(Complex IV) activity assay** Determine cytochrome _c_ oxidase activity by measuring spectrophotometrically at 30 °C for 2 minutes the oxidation of cytochrome $_c_^{red}$ as indicated by the decrease of absorbance at 550 nm \(Refs. 2-3). For each sample, measure complex IV activity twice at at least two protein concentrations that ensure the linearity of the reaction. 1. Reduce cytochrome _c_ by adding tiny amounts of sodium dithionite until the absorbance at 550 nm of 100 µl of cytochrome _c_ in 1 ml of H_2O is between 1.8 and 1.9. 2. In a cuvette, add 100 µl of 100 mM PK buffer, pH 7.0, 100 µl of reduced cytochrome _c_ and adjust the volume with H_2O \(800 µl minus sample volume). 3. Incubate the cuvette at 30 °C for 2 min. 4. Start the reaction by adding 2-10 µg of mitochondrial proteins. 5. Measure absorbance of the sample at 550 nm for 2 min. 6. Verify the specificity of cytochrome _c_ reduction by inhibiting cytochrome _c_ oxidase activity with 50 µl of 40 mM KCN. 7. Calculate complex IV specific activity using the Beer-Lambert law equation \(Fig. 1). Express the activity as nanomoles of cytochrome _c_ oxidised per minute per milligram of protein. **ATP synthase \(Complex V) activity assay** Determine complex V activity by measuring spectrophotometrically at 30 °C for 2 minutes the oxidation of NADH as indicated by the decrease of absorbance at 340 nm \(Refs. 2-3). Repeat each measurement twice at at least two protein concentrations that ensure the linearity of the reaction. 1. In a cuvette add 500 µl of Buffer H-Mg, 10 µl of 30 mM NADH, 50 µl of 50 mM phosphoenolpyruvic acid, 5 µl of 10 mg/ml of pyruvate kinase, 10 µl of 5 mg/ml of lactate dehydrogenase, and 10 µl of antimycin A. 2. Add 2-10 µg of mitochondrial proteins. 3. Adjust the volume to 900 µl with H_2O. 4. Incubate the cuvette at 30 °C for 2 minutes 5. Record the baseline for 2 min. 6. Start the reaction by adding 100 µl of 25 mM ATP. 7. Measure absorbance of the sample for 2 min \(?$Abs_{340nm}^{\text{without oligomycin}}$) 8. Add 10 µl of oligomycin. 9. Measure absorbance for 2min \(?$Abs_{340nm}^{\text{with oligomycin}}$). 10. Calculate complex V specific activity using the Beer-Lambert law equation \(Fig. 1). Express complex V activity as nanomoles of NADH oxidised per minute per milligram of protein.

Timing

3 days: - Cell culture: 8h \(pre-culture in selective medium) + 20h \(overnight culture in YPGAL) - Preparation of mitochondria by differential centrifugations: 4-6h - Sample preparation: 1h - Respiratory chain activity assay: variable, depending on the number of samples

Critical Steps

Preparation of mitochondria by differential centrifugations Keep samples on ice during the entire procedure. Following wall digestion, resuspend the pellets by gentle pipetting \(not vortexing) to avoid mitochondria fragmentation.

References

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Figures

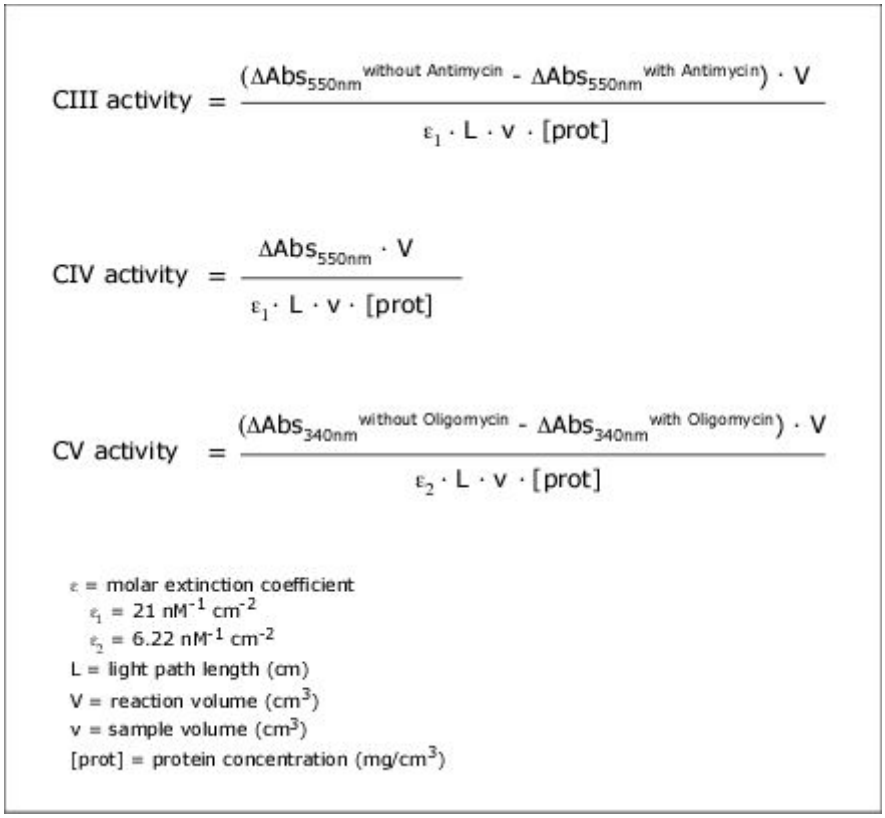

Figure 1

Formulae to calculate Complex III, Complex IV, and Complex V activity Formulae are derived from the Beer-Lambert law equation. Activities are expressed as nanomoles per minute per milligram of protein.