CHAPTER 13

Methods for Studying Iron Metabolism in Yeast Mitochondria

Sabine Molik, Roland Lill, and Ulrich Mühlenhoff
Institut für Zytobiologie und Zytopathologie
Philipps-Universität Marburg, 35033 Marburg, Germany

I. Introduction

Iron–sulfur (Fe/S) clusters are versatile, ancient cofactors of proteins that are involved in electron transport, enzyme catalysis, and regulation of gene expression (Beinert et al., 1997). Recent years have shown that the synthesis of these cofactors and their insertion into apoproteins involve the function of complex cellular machineries in all kingdoms of life (reviewed by Balk and Lill, 2004; Johnson and Dean, 2004; Lill and Mühlenhoff, 2005, 2006). The budding yeast *Saccharomyces cerevisiae* serves
as a key model organism for the study of this novel biochemical pathway in eukaryotes. Mitochondria play a central role in this pathway, as they are essential for the maturation of all cellular Fe/S proteins. The so-called mitochondrial iron-sulfur cluster (ISC) assembly machinery is responsible for the de novo synthesis of Fe/S clusters and for the insertion of these cofactors into mitochondrial Fe/S apoproteins (Fig. 1). This system is also involved in the maturation of Fe/S proteins that are located outside the mitochondria, in the cytosol or in the nucleus. A mitochondrial export system and a recently discovered cytosolic Fe/S protein assembly (CIA) system specifically participate in the maturation of cytosolic and nuclear Fe/S proteins (Fig. 1). Of the ~20 assembly components known to date, many are encoded by essential genes, including several components of mitochondria. This indicates that the process is indispensable for life.

In fact, the maturation of cellular Fe/S proteins is the only mitochondrial function known so far that is essential for viability of eukaryotes (Lill and Muhlenhoff, 2005). In *S. cerevisiae*, defects in Fe/S protein maturation result in respiratory deficiency due to a collapse of the respiratory chain and/or the citric acid cycle, and auxotrophies for certain amino acids and vitamins, which require Fe/S proteins for their biosynthesis (Jensen and Culotta, 2000; Kispa* et al.*, 1999). Severe defects result in cell death. The first essential Fe/S protein that was identified in budding yeast (and is not a member of the ISC or CIA machineries) is Rli1p, which plays a key role in ribosome biogenesis (Kispa* et al.*, 2005; Yarunin* et al.*, 2005). The finding that this protein is essential for cell viability indicates that Fe/S biogenesis is also an essential process.

![Fig. 1](image)

**Fig. 1** Fe/S protein biogenesis and Aft1/2p-dependent gene regulation in *S. cerevisiae*. In yeast and other eukaryotes, the mitochondrial ISC assembly system is required for the maturation of mitochondrial Fe/S protein. An unknown component (X), which is produced and exported by the ISC assembly and ISC export systems (export), is required for the maturation of cytosolic and nuclear Fe/S proteins by the cytosolic Fe/S protein assembly system (CIA). In addition, this component is required for attenuation of the iron regulon by the transcriptional activators Aft1p/Aft2p. Defects in the ISC assembly and export systems induce the import of Aft1p into the nucleus and a constitutive transcriptional activation of many genes involved in iron uptake and storage. The connection between Fe/S protein biogenesis, the mechanism for iron sensing, and the transcriptional regulation of iron-responsive genes are not well understood.
In *S. cerevisiae*, defects in the mitochondrial ISC assembly and export apparatus are associated with low levels of cellular heme (the second most abundant iron-containing cofactor of the cell), mitochondrial iron overload, and a massive constitutive transcriptional deregulation of iron-responsive genes that is similar to the transcriptional response of yeast to iron deprivation (Shakoury-Elizeh *et al.*, 2004). These phenotypes strongly suggest that the mitochondrial Fe/S protein assembly system plays a crucial role in the sensing of iron, in the transcriptional regulation of cellular iron homeostasis, and in the control of cellular heme levels (Lange *et al.*, 2004). However, the underlying regulatory mechanisms for these processes are unknown. In yeast, defects in the mitochondrial ISC assembly and export apparatus elicit the induction of iron-uptake genes, the so-called “iron regulon.” The iron regulon comprises a set of ~30 genes with products that function in ionic iron uptake at the cell surface (*FET3*, *FTR1*, *FRE1*, and *FRE2*), siderophore uptake (*ARN1–4*, *FIT1–3*), iron transport across the vacuolar and mitochondrial membranes (*FET5*, *FTH1*, and *MRS4*), and the modulation of the mRNA stability of iron-dependent genes (Rutherford and Bird, 2004; Rutherford *et al.*, 2003; Shakoury-Elizeh *et al.*, 2004). The induction of these genes is a physiological response by yeast to iron deprivation that contributes significantly to the coordinated adaptation of cellular iron usage and uptake to low iron conditions. The expression of the iron regulon is controlled by the iron-sensing transcriptional activators Aft1p and Aft2p (Rutherford and Bird, 2004). Aft1p resides in the cytoplasm under iron-replete conditions, but shuttles to the nucleus on iron deprivation (Yamaguchi-Iwai *et al.*, 2002). The precise mechanism of the iron-responsive activation of Aft1p is unclear, since it is unknown what this transcription factor is actually sensing. Analyses have shown that Aft1 (and possibly Aft2p) are regulated by an unidentified key molecule that is produced and sequestered by the mitochondrial ISC systems, and which functions as a regulatory signal for the sensing of iron and for the expression of iron-regulated genes in the nucleus (Fig. 1) (Rutherford *et al.*, 2005). The constitutive activation of Aft1/2p in ISC assembly- or ISC export-deficient cells results in an increased iron uptake linked with mitochondrial iron accumulation (Kispal *et al.*, 1997; Lill and Muhlenhoff, 2005; Rutherford *et al.*, 2005).

The present compendium includes assays for the analysis of *de novo* synthesis of Fe/S clusters and heme formation in *S. cerevisiae* under *in vivo* and *in vitro* conditions in Section II. Methods to determine the mitochondrial iron content and reporter assays to analyze iron-dependent gene expression are described in Sections III and IV. These approaches are crucial to elucidate the mechanisms underlying the maturation of Fe/S proteins and may aid in the identification of new members of this evolutionary ancient process.

### II. Determination of Cellular Fe/S Protein Formation and Heme Synthesis

The phenotypes outlined above show that cellular Fe/S protein maturation is tightly connected to mitochondrial respiration, heme synthesis, and cellular iron homeostasis in *S. cerevisiae*. A full understanding of Fe/S cluster maturation thus
requires an integrative approach that takes all of these processes into account. This chapter provides a comprehensive compilation of the most important routine methods used for the analysis of these processes in S. cerevisiae in our laboratory.

We first describe standard methods for analyzing the de novo maturation of Fe/S proteins by radiolabeling iron-starved yeast cells with $^{55}$Fe in vivo (Fig. 2). Following the radiolabeling, cells are lysed, and the $^{55}$Fe/S proteins are immunoprecipitated with specific antibodies. The amount of radioactive iron that is copurified with the protein is quantified by liquid scintillation counting. The uptake of $^{55}$Fe by the cells during the labeling reaction may serve as an indication of cell viability. This semiquantitative method can be used for the analysis of endogenously synthesized proteins (e.g., aconitase and Leu1p) and of overproduced proteins (e.g., Bio2p, Yah1p, Isu1, Nbp35p, Nar1p). If suitable antibodies are not available, appropriate tags can be used. In this context, we have good experience with the use of hemagglutinin (HA)- or tandem affinity purification (TAP)-tagged proteins for analysis of both mitochondrial and cytosolic Fe/S proteins. Finally, the formation of heme can be analyzed by this in vivo labeling assay. Due to its high solubility in organic solvents, radiolabeled heme can be extracted quantitatively from a cell extract into the organic phase. The degree of incorporation of $^{55}$Fe into heme is quantified by liquid scintillation counting of the organic phase.

In addition, Section II includes two assays for the analysis of Fe/S cluster synthesis activities in mitochondrial extracts in vitro. The first assay is based on radiolabeling with radioactive $^{55}$Fe or $^{35}$S-cysteine of yeast mitochondria overproducing an endogenous Fe/S protein (Muhlenhoff et al., 2002a). Mitochondria are lysed with detergent and incubated with cysteine, iron, NADH, and DTT under anaerobic conditions. Radiolabeled Fe/S proteins are immunoprecipitated with specific antibodies and are quantified by scintillation counting. To obtain mitochondria with a large portion of Fe/S proteins in the apoform, organelles prepared from yeast cells cultivated in iron-free medium are optimal. In the second assay, a soluble apoferredoxin is added to the mitochondrial extract and the reconstituted holoferredoxin is subsequently purified either by anion exchange or by native gel electrophoresis (Lutz et al., 2001). This method can be used with or without radiolabel and is suitable for mitochondria or cell extracts from a variety of organisms with only minor adjustments. However, in the case of mammalian tissue or cell cultures or of pathogenic protists, experiments may be complicated by low sample amounts.

A. Determination of Cellular Fe/S Cluster Formation by Radiolabeling of Yeast Cells In Vivo

Materials

“Iron-free” minimal medium for growth of S. cerevisiae. This medium corresponds to regular synthetic complete (SC) medium (Sherman, 2002), lacking iron chloride. A ready-made powder is commercially available (Formedium, United Kingdom).

$^{55}$FeCl$_3$ (NEN/Perkin-Elmer)

Citrate buffer: 50-mM sodium citrate; 1-mM EDTA, pH 7.0
Fig. 2  Flow sheet for the analysis of de novo Fe/S protein formation by radiolabeling of yeast cells in vivo. (A) Yeast cells that were cultivated in iron-free medium are incubated with radioactive $^{55}$Fe for at least 1 h. During this time, the radioactive iron is taken up and incorporated into Fe/S apoproteins by the mitochondrial ISC assembly, ISC export, and CIA systems. (B) Subsequently, a cell extract is prepared and the labeled $^{55}$Fe/S reporter proteins are immunoprecipitated with specific antibodies coupled to protein-A Sepharose. (C) The radioactive $^{55}$Fe that is copurified with the immunobeads is quantified by scintillation counting. Both tagged and untagged (native) proteins can be analyzed in this assay. (D) The conditional ISC mutant Gal-ISU1/Δisu2 (Gal-ISU) was radiolabeled under permissive conditions in the presence of galactose (Gal) and under repressive conditions in the presence of glucose (Glc), and the amount of iron incorporation into the mitochondrial aconitase was determined. Wild-type cells (WT) were analyzed in parallel. Antibodies against aconitase ($\alpha$Aco) and preimmune serum (PIS) were used for immunoprecipitation.
**TNETG buffer:** 20-mM Tris–HCl, pH 7.4; 2.5-mM EDTA; 150-mM NaCl; 10% (w/v) glycerol; 0.5% (w/v) Triton X-100

20-mM HEPES-KOH, pH 7.4; saturated PMSF in ethanol; 0.5-mm glass beads; 25% trichloroacetic acid (TCA) (optional)

**Appropriate antibodies coupled to protein-A Sepharose.** Antibodies are coupled to protein-A Sepharose for immunoprecipitation as follows:

Resuspend 50 mg of dried protein-A Sepharose (GE Healthcare, Chalfont St. Giles, UK) in cold 500-μl TNETG. The beads are swollen by incubation for at least 30 min at 4°C with occasional mixing. Do not vortex immunobeads. Collect the beads by centrifugation at 850 × g for 5 min. Add 500 μl of antiserum and incubate at 4°C with gentle mixing using a rotating shaker for at least 1 h.

Collect the beads by centrifugation. Wash five times in 500-μl TNETG, spin down in between washes. The beads are resuspended in 500-μl TNETG and stored at 4°C.

**Procedure**

1. Harvest yeast cells from a 50-ml overnight culture by centrifugation (1500 × g for 5 min), wash once in 10-ml sterile water, and dilute cells in 100-ml “iron-free” SC medium supplemented with the appropriate carbon source. Continue growth until cell density reaches an OD600 of 0.2. Incubate overnight in a shaking incubator at 30°C.

2. Collect cells by centrifugation (1500 × g for 5 min), wash once in 10-ml sterile water and determine the wet weight. Resuspend 0.5-g cells in 10-ml iron-free medium in a 50-ml culture flask and incubate for 10 min at 30°C in a shaking incubator.

3. Dilute 10-μCi ⁵⁵FeCl₃ in 100 μl of 0.1-M sodium ascorbate, and add mixture to the preincubated cells. The final concentration of ascorbate in the medium is 1 mM. Incubate for 1–2 h at 30°C.

4. Transfer the radiolabeled cells to a 15-ml Falcon tube and harvest by centrifugation (1500 × g for 5 min). Wash the cells once in 10-ml citrate buffer, and once in 1 ml of 20-mM Hepes-KOH, pH 7.4. Concentrate the cells by centrifugation (1500 × g for 5 min).

5. Resuspend the cell pellet in 0.5-ml TNETG buffer. Add 10-μl saturated PMSF and 1/2 volume of glass beads. Lyse the cells by three bursts of 1 min on a vortex at maximum speed with intermediate cooling for 1 min on ice. Tubes are best vortexed upside down. All subsequent steps are carried out at 4°C.

6. Remove the coarse cell debris by centrifugation for 5 min at 1500 × g. Transfer the supernatant to a 1.5-ml reaction tube and centrifuge at 17,000 × g for 10 min. Transfer the supernatant to a fresh tube. At this point, carefully avoid carrying over any membrane debris. Remove 5 μl of the extract for scintillation counting. This sample serves as a crude measure of the cellular iron uptake. Another 25 μl of the extract are precipitated with TCA for immunoblotting (optional). About 250 μl
are used for immunoprecipitation of the proteins of interest. Usually, two immunoprecipitations can be carried out with one labeling reaction.

7. For immunoprecipitation, add 20–50 µl of IgG-coupled immunobeads or 10 µl of commercially available coupled anti-hemagglutinin A (HA) or anti-Myc beads (Santa Cruz) to 250 µl of cell extract. Cut off the end of the pipette tip when handling immunobeads. Do not vortex the beads. Incubate the reaction tubes in a rotating shaker at 4°C for 1 h.

8. Collect the beads by centrifugation at 1500 × g for 5 min. Remove the supernatant completely with a syringe. Wash the beads three times in 500-µl TNETG buffer and collect the beads by centrifugation. Virtually no labeled supernatant should remain in the reaction tubes after each washing step.

9. Add 50 µl of water and 1 ml of scintillation cocktail to the beads, vortex briefly, and determine the radioactivity associated with the beads in a scintillation counter using the counter settings for ³H (Muhlenhoff et al., 2002b).

Comments

1. In all labeling experiments involving ⁵⁵Fe, it is essential that all solutions and glassware are iron free. Standard dishwasher detergent and laboratory glassware frequently contain iron. Glassware should be acid-washed in 1-M HCl. Double-distilled water of the highest quality should be used throughout. The contaminated glass flasks used for in vivo radiolabeling of yeast are incubated with citrate buffer and washed in distilled water to remove remnant radioactivity. The flasks are rinsed with 70% ethanol for sterilization.

2. Reduction of ⁵⁵FeCl₃ is essential, as oxidized Fe³⁺ is virtually insoluble at neutral pH. Therefore, labeling reactions with ⁵⁵FeCl₃ in vivo or in vitro are always carried out in the presence of 1-mM fresh ascorbate to avoid precipitation of ferric iron. The radiation safety conditions for ⁵⁵Fe (an electron capture radiation) are similar to those for radioactive ³H. For the quantification of ⁵⁵Fe, the counter settings for ³H are appropriate.

3. For yeast, best results are obtained with cells overproducing the Fe/S protein of interest from a high-copy plasmid under the control of a strong promoter. If antibodies are not available, HA-, Myc-, or TAP-tagged versions of the Fe/S protein can frequently be used. In S. cerevisiae, the endogenous levels of aconitase, Yah1p (ferredoxin), and Leu1p are sufficient for analysis without overexpression. For other organisms, a suitable reporter protein has to be determined empirically.

B. Determination of Cellular Heme Levels by Radiolabeling of Yeast Cells In Vivo

Materials

Stop solution: 100-mM FeCl₂ in 5-M HCl
Butyl acetate
Procedure

1. Perform steps 1–4 of an in vivo $^{55}$Fe radiolabeling assay as described in Section II.A.
2. Resuspend the washed, radiolabeled cells in 0.5-ml water and split the suspension in two halves.
3. To each half add 25-µl Stop solution, 800-µl butyl acetate, and 1/2 volume of glass beads at 0°C. Lyse the cells by three bursts of 1 min each on a vortex at high speed with intermediate cooling for 1 min on ice. The tubes should be vortexed upside down.
4. Separate the organic phase by centrifugation at 10,500 × g for 10 min at 20°C. Transfer two 250-µl aliquots (for duplicate values) of the upper organic phase into two reaction tubes. Carefully avoid touching the membrane debris or parts of the lower aqueous phase.
5. Add 1 ml of scintillation cocktail to the beads, mix, and determine the $^{55}$Fe radioactivity in the organic phase by scintillation counting, using the standard settings for $^3$H.

Comments

1. The two assays described in Sections II.A and II.B can be performed in parallel on the same sample.
2. The heme biosynthesis assay takes advantage of the high solubility of protonated heme in organic solvents at low pH. Butyl acetate is the preferred solvent as it does not interfere with scintillation counting.
3. Due to the short incubation times (usually 60 min), this in vivo heme formation assay gives a measure mainly for the activity of ferrochelatase. The steady-state levels of heme in yeast cells in vivo can be determined using cells cultivated in medium supplemented with $^{55}$FeCl$_2$ for longer periods. To this end, cells from a preculture are diluted in 50-ml “iron-free” SC medium supplemented with the appropriate carbon source, 1-mM ascorbate, and 10-µCi $^{55}$Fe, at a density of OD$_{600} = 0.1$. The cultures are incubated overnight at 30°C in a shaking incubator and the amount of radioactive heme is determined as described above. For details see Lange et al. (2004). This assay is highly sensitive and works well with mammalian cell cultures.

C. Determination of Fe/S Cluster Formation in Isolated Mitochondria In Vitro

Materials

In vitro experiments for the de novo formation of Fe/S proteins essentially require anoxic conditions and oxygen-free buffers. We work in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) filled with 95% (v/v) nitrogen and 5% (v/v) hydrogen gas. Stock solutions are introduced to anaerobic conditions at least 1 week in advance. They can be stored under anaerobic conditions indefinitely.
Anaerobic solutions of the following chemicals:

2 × Mitobuffer

- 40-mM HEPES, pH 7.4
- 100-mM KCl
- 2-mM MgSO₄
- 1.2-M Sorbitol

Other reagents:

- 10% (w/v) Triton X-100
- 2-Mercaptoethanol
- 25% (v/v) HCl
- 1-M Tris–HCl, pH 8.3
- 0.5-M EDTA

The following stock solutions are prepared freshly with oxygen-free water:

- 0.1-M Sodium ascorbate
- 0.1-M DTT
- 10-mM Cysteine
- 1-mM Pyridoxal phosphate
- 0.1-M NADH
- 30-mM Ferric ammonium citrate
- 30-mM FeCl₂
- ⁵⁵FeCl₃ (NEN/Perkin-Elmer) or ³⁵S-cysteine (GE Healthcare, Chalfont St. Giles, UK)

Procedure

Steps 1–4 are carried out under anaerobic conditions.

1. Store isolated mitochondria and all other solutions in an anaerobic chamber with open caps for 1 h on ice to remove oxygen.

2. In a standard reaction tube, add 125 μl of 2× mitobuffer, 2.5 μl of 100-mM sodium ascorbate, 2.5 μl of 0.1-M DTT, 2.5 μl of 0.1-M NADH (1-mM final), 2.5 μl of 1-mM pyridoxal phosphate (10-μM final), 5 μl of 10-mM cysteine (0.2-mM final), 2.5 μl of 10% (w/v) Triton X-100, and bring volume to 250 μl (minus the volume of mitochondria) with anaerobic water.

3. Add mitochondria (100-μg protein) isolated from iron-starved cells, 5-μCi ⁵⁵FeCl₃ (reduced in 10-mM ascorbate), and incubate with gentle shaking for 2.5 h at 25°C under anaerobic conditions. [For labeling with ³⁵S, add 10-μCi ³⁵S cysteine and FeCl₂ (0.3-mM final concentration) instead of non-radioactive cysteine and FeCl₃.]

4. Terminate the labeling reaction by addition of 2.5 μl of 0.5-M EDTA on ice. All further steps are carried out aerobically at 4°C.

5. Remove membranes by centrifugation at 17,000 × g for 10 min at 4°C. Transfer the supernatant to a fresh tube. Using a Pipetman with the ends of the
pipette tips cut off, add 20–40 µl of immunobeads. Incubate the tubes in a rotating shaker for 1 h.

6. Collect the beads by centrifugation at 3000 × g for 5 min at 4°C. Carefully remove all of the supernatant with a syringe. Wash the beads three times in 500 µl of ice-cold TNETG buffer. In between the washing steps, collect the beads by centrifugation. It is essential that virtually no supernatant remains in the reaction tubes after each wash.

7. Add 50 µl of water and 1-ml scintillation cocktail, vortex, and count the radioactivity associated with the beads in a scintillation counter.

Comments
Mitochondria are isolated from iron-starved yeast cells grown in iron-free medium (Diekert et al., 2001). We obtained the best results with mitochondria isolated from cells that overproduce biotin synthase (Bio2p) for this type of experiment (Muhlenhoff et al., 2002a).

D. Analysis of Fe/S Cluster Formation In Vitro Using Recombinant Ferredoxins

Materials

Materials as in Section II.C.

An acidic, low molecular weight [2Fe-2S] ferredoxin. These can be obtained in recombinant form from E. coli or are commercially available.

Additional reagents requiring no deoxygenation.

20-mM HEPES-KOH, pH 7.4; saturated PMSF in ethanol; 25% TCA (optional); appropriate antibodies coupled to protein-A Sepharose; Bromophenol blue (0.2% w/v).

Procedure

Preparation of apoferrodoxin

1. Incubate a recombinant ferredoxin (4–10 mg/ml concentration) under anaerobic conditions for at least 1 h on ice to remove oxygen.

2. In a standard reaction tube, add 250-µl ferredoxin, 10-µl 2-mercaptoethanol, and bring volume up to 1 ml with anaerobic water. Cool the sample on ice, add 25% HCl to a final concentration of 0.5 M, mix gently, and incubate on ice for 5 min. A white precipitate forms immediately.

3. Collect the precipitated protein by centrifugation at 12,000 × g for 10 min at 4°C. Remove the supernatant completely and rinse the pellet briefly with 500 µl of ice-cold water containing 0.1% 2-mercaptoethanol. Remove water completely.

4. Add 250 µl of 50-mM Tris–HCl, pH 8.3. Resuspend the pellet carefully with a pipette and store the sample on ice. Do not vortex. The protein should dissolve within 10–15 min. If necessary, add 1 µl drops of unbuffered 1-M Tris until the solution is completely clear.

5. Repeat steps 1–4. For details see comment 2 and Meyer et al. (1986).
**Reconstitution assay**

1. Under anaerobic conditions, combine 125 µl of 2× mitobuffer, 2.5 µl of 100-mM sodium ascorbate, 2.5 µl of 0.1-M DTT, 2.5 µl of 0.1-M NADH (1-mM final), 2.5 µl of 1-mM pyridoxal phosphate (10-µM final), 2.5 µl of 10% Triton X-100, and bring volume up to 250 µl with anaerobic water. In cases where holoferredoxin formation is detected by native PAGE, smaller reaction volumes (50–100 µl) should be used.

2. Add isolated mitochondria or cell extracts corresponding to at least 100 µg of protein and 20 µg of apoferredoxin. For radioassays, add either 5 µl of 10-mM cysteine (0.2-mM final) and 5-µCi 55FeCl3 (reduced in 10-mM ascorbate) or 2.5 µl of 30-mM FeCl3 (0.3-mM final) and 10-µCi 35S-cysteine. Control reactions without mitochondria and added apoferredoxin should be analyzed in parallel. For nonradioactive assays, add ferric ammonium citrate to a final concentration of 0.3 mM and cysteine to 4 mM. Higher amounts of ferredoxin (50 µg) may be used. Incubate with gentle shaking for 2 h at 25°C under anaerobic conditions.

3. Terminate the reconstitution reaction by addition of 2.5 µl of 0.5-M EDTA on ice.

4. Remove membrane debris and aggregates by centrifugation at 17,000 × g for 10 min at 20°C. Transfer the supernatant to a fresh tube. For analysis of holoferredoxin formation continue with one of the two following protocols. All further steps are carried out aerobically at 4°C.

**Isolation of radiolabeled holoferredoxin by binding to ion exchange resins**

1. Add 25 µl of a 1:1 slurry of anion exchanger beads (Q-Sepharose, GE Healthcare, Chalfont St. Giles, UK) in TNETG-buffer containing 200-mM NaCl (TNETG-200) and incubate in a rotating shaker for 10 min at 4°C.

2. Collect the beads by centrifugation at 3000 × g for 5 min. Carefully remove all of the supernatant with a syringe. Wash the beads three times in 500-µl TNETG-200 buffer containing 1-mM ascorbate. Between the washing steps, collect the beads by centrifugation. Remove the supernatant completely.

3. Add 50 µl of water and 1-ml scintillation cocktail, vortex, and count the radioactivity associated with the beads in a scintillation counter.

**Separation by native PAGE**

1. Prepare a 17.5% polyacrylamide gel with a 6% stacking gel and cold standard electrophoresis buffer according to the standard Laemmli procedure, with the exception that SDS is omitted.

2. Add 1 µl of 0.2% bromophenol blue to each 100 µl of reaction mix, load 50 µl of the samples onto the chilled native polyacrylamide gel. Electrophoresis is carried out at 4°C at 30 mA and 200 V until the dye front reaches the bottom of the gel.

3. For detection of radiolabeled ferredoxin, the gel is fixed by gentle shaking in cold 20% ethanol at 4°C for 30 min, dried under vacuum, and analyzed by
autoradiography. Since Fe/S cofactors are acid-labile, the gels should not be stained or fixed with acetic acid. In case of nonradioactive assays, the red color of the reconstituted holoferredoxin may be visible by eye. The gel is stained with Coomassie Brilliant Blue. Holoferredoxin forms a sharp band slightly above the dye front that is well separated from the majority of proteins.

Comments

1. The preparation of apoferredoxin and Fe/S cluster reconstitution necessitate anaerobic conditions and oxygen-free solutions.

2. These protocols take advantage of the fact that most low molecular mass [2Fe-2S] ferredoxins are soluble in their apoform, which can be generated by acid precipitation. Care should be taken that the Fe/S cluster is removed completely and no oxidation occurs. In an optimal case, the UV/Vis spectrum of the apoferredoxin lacks any absorption above 300 nm. If this is not the case, the procedure should be repeated.

3. Biochemical reconstitution of mitochondrial Fe/S protein assembly requires ATP. For mitochondria from S. cerevisiae, the endogenous ATP levels are sufficient and the addition of ATP is not recommended since ATP is an effective chelator of iron. For mitochondria from other sources, however, the addition of low amounts of ATP (0.2–0.5 mM) may improve the reconstitution. In any case, control reactions without added ATP, apoferredoxins or mitochondria should be analyzed in parallel.

4. The quantitative estimation of holoferredoxin formation takes advantage of the acidic character of this type of ferredoxin, which allows its binding to either an anion exchange resin at relatively high ionic strength or a rapid mobility in an electric field. It is therefore essential that a model protein with a low pI is used. We use either yeast ferredoxin Yah1p or plant-type ferredoxins.


III. Determination of Mitochondrial Iron Contents

Defects in mitochondrial Fe/S cluster formation or in the ISC export machinery result in an increased uptake of iron that is eventually accumulated within the mitochondria. The latter is an unphysiological event, as the vacuole usually serves as the physiological storage organelle for iron. Section III includes two methods for the determination of the iron content of isolated mitochondria or of cell lysates, which are based on the formation of colored iron complexes with the chelators bathophenantroline and nitro-PAPS, respectively. The bathophenantroline assay is a rapid assay useful for the determination of artificially increased mitochondrial iron levels. The nitro-PAPS assay is more sensitive but more time consuming, as labile iron is quantitatively released from proteins by acid prior to quantification. This assay may be used to determine physiological iron levels of cell lysates.
A. Bathophenantroline Assay

**Materials**

- 100-mM Bathophenantroline (prepare freshly)
- 1-M Sodium dithionite (prepare freshly)
- 10% (w/v) SDS
- 1-M Tris–HCl, pH 7.4

**Procedure**

1. Mix 100 μl of 1-M Tris–HCl, pH 7.4 with 60 μl of 10% SDS (0.6% final), 20 μl of 1-M dithionite, 100-μl bathophenantroline (10-mM final) and isolated mitochondria (at least 200-μg protein), and bring to a final volume of 1 ml with distilled water. Incubate at 20°C for 5 min.

2. Remove membrane debris by centrifugation for 5 min at 10,000 × g. Record the absorption spectrum of the sample between 500 and 700 nm using a reference sample lacking mitochondria. Determine the difference between OD_{540} minus OD_{700} (to account for light scattering). The absorption coefficient $\varepsilon_{540\text{ nm}}$ is $\sim23,500 \text{ M}^{-1} \text{ cm}^{-1}$ (Li *et al*., 1999).

B. Nitro-PAPS Assay

**Materials**

- 2.5% (w/v) SDS
- 1% HCl
- 7.5% (w/v) Ammonium acetate solution
- 4% (w/v) Ascorbic acid (prepare fresh)
- 1.3 mM 2-(5-nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)phenol (Nitro-PAPS)

Recommended iron standard: 0.2-mM (NH$_4$)$_2$Fe(SO$_4$)$_2$·6H$_2$O; FW = 392.14 [Mohr’s Salt, 0.008% (w/v), ~39 mg/500 ml, prepare fresh]

**Procedure**

1. Dilute samples and a blank to 100 μl with water in standard reaction tubes.

2. Add 100 μl of 1% HCl, mix by gentle shaking, and incubate at 80°C for 10 min. Allow the tubes to cool down (keep closed) and centrifuge for 1 min at 9000 × g.

3. In the following order: add 500 μl of 7.5% ammonium acetate, 100 μl of 4% ascorbic acid, and 100 μl of 2.5% SDS. Vortex after each addition.

4. Centrifuge for 5 min at 9000 × g, transfer supernatant (855 μl) into a fresh tube, and add 95 μl of the iron chelator nitro-PAPS (final concentration 130 μM).
Determine the absorbance at 585 nm against the blank sample in quartz cuvettes. The absorption coefficient $\varepsilon_{585\,\text{nm}}$ is $\sim 94,000 \, \text{M}^{-1} \text{cm}^{-1}$ (Makino et al., 1988).

Comments
The same protocol may also be used for the iron chelator Ferene (3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine). The absorption coefficient $\varepsilon_{593\,\text{nm}}$ is 35,000 $\, \text{M}^{-1} \text{cm}^{-1}$.

IV. Reporter Assays for Analysis of Iron-Dependent Gene Expression in S. cerevisiae

Defects in mitochondrial Fe/S cluster formation or in ISC export systems induce the constitutive transcriptional deregulation of iron-dependent genes, a response that resembles the physiological reaction of yeast to iron deprivation (Shakoury-Elizeh et al., 2004). The transcriptional (de)regulation of iron-responsive genes can be studied conveniently by determining promoter activities in reporter assays. In S. cerevisiae, reporter assays based on the $\beta$-galactosidase (LacZ) are most frequently used. In Section IV, we describe alternative reporter assays that take advantage of green fluorescent protein (GFP) or luciferase for monitoring gene expression in S. cerevisiae. Both GFP and luciferase from Renilla reniformis can be measured directly in whole yeast cells and do not require cell lysis. The use of these whole cell assays, however, is limited to the analysis of strong promoters. Weak promoters can be routinely analyzed in cell extracts using firefly or Renilla luciferase as reporters. Both luciferase systems are highly sensitive and can be analyzed in parallel in a single cell (Harger and Dinman, 2003; McNabb et al., 2005; Szittner et al., 2003). Although we use these reporter assays in the context of iron-responsive transcriptional regulation, these assays can be used generally for monitoring gene expression in S. cerevisiae.

Our GFP reporter constructs are based on the CEN/ARS plasmid pPS1372 that carries two GFP open reading frames in tandem, under the control of the ADH2 promoter (Taura et al., 1998). The ADH2 promoter can be excised by double digests with SacI and SmaI or SacI and EcoRI, and replaced by the promoter of interest. Our luciferase reporter constructs are based on the CEN/ARS plasmids p414-MET25 and p416-MET25 (Funk et al., 2002) (Fig. 3). Coding regions for luciferase from firefly or Renilla reniformis were taken from the original vectors pGL3 and pGL4.70 (Promega Corporation, Madison, WI), respectively, and inserted into the SmaI and Sali sites downstream of the MET25 promoter. The latter can be replaced by the promoter of interest by directional cloning. For details see Fig. 3. For an example of iron-responsive genes, we choose the promoter of CYC1, encoding cytochrome c, and FET3, a member of the iron regulon that is frequently used for the analysis of iron-responsive transcription in S. cerevisiae. These genes are constitutively deregulated in cells with defects in mitochondrial Fe/S protein biogenesis (Fig. 3).
Fig. 3 Reporter assays based on luciferase and GFP in *S. cerevisiae*. Physical map of the luciferase reporter plasmids p414-MET25-Luc1 (A) and p416-MET25-hRLuc (B) carrying the firefly (luc1) or *Renilla* luciferase (hRluc) gene. The sequence and restriction sites of the poly-linker regions between the MET25 promoter and the start codons of the luciferase open reading frames are shown below the vector maps. (C) The conditional ISC export mutant Gal-ATM1 harboring the reporter plasmid p414-CYC1-Luc1 was grown under permissive conditions in the presence of galactose (Gal) and under repressive conditions in the presence of glucose (Glc), and the transcriptional activity of the CYC1 promoter was determined by recording the luciferase-specific luminescence of cell extracts. Five microliters of cell extracts at a protein concentration of 0.1 μg/μl were analyzed over a 5-min interval. The maximal activity was observed ~3 min after the addition of substrate. (D) Regular and iron-starved (-Fe) wild-type cells (WT) and the ISC mutants Δyfh1 and Gal-ATM1 harboring plasmid pFET3-GFP were grown in the presence of glucose, and the transcriptional activity of the FET3 promoter was determined by recording the GFP-specific fluorescence emission of whole cells.
A. Promoter Assay Based on Green Fluorescent Protein

Procedure

1. Grow the yeast cells carrying the GFP reporter plasmid in a small volume of minimal medium supplemented with the required amino acids and carbon source overnight at 30°C. Collect the cells by centrifugation (1500 g for 5 min), dilute in 25 ml of the desired growth medium to a final cell density of OD₆₀₀ = 0.2, and incubate at 30°C in a shaking incubator. For a single end point measurement, cells are grown up to density of OD₆₀₀ of ~0.5. Otherwise, aliquots are withdrawn at the desired time points.

2. Harvest the cells by centrifugation (1500 g for 5 min), resuspend the cell pellet in 3 ml of distilled water, and determine the optical density at 600 nm.

3. For the determination of the GFP-specific fluorescence, cells are diluted in a 3-ml fluorescence cuvette to an OD₆₀₀ of 1 with distilled water. The fluorescence emission of the cells is set to zero at 600 nm and the emission spectrum is recorded between 500 and 550 nm. The maximum of the emission is at ~512 nm. For an accurate readout, the spectrum of a sample lacking the reporter plasmid is subtracted. We use the following fluorescence spectrometer settings. Excitation wavelength: 480 nm with 10 nm bandwidth; emission: 5 nm or 10 nm bandwidth; detector at medium amplification. We use a FP6300 fluorescence spectrometer (Jasco Ltd., Tokyo, Japan).

Comments

1. Since yeast cells settle quickly, the use of a built-in magnetic stirrer during fluorescence measurements is recommended.

2. Since the GFP fluorescence is partially quenched by the growth medium, medium must be removed completely prior to the measurement. The fluorescence is also reduced in cells at stationary phase.

3. The assay is much simpler, but also less sensitive, than reporter assays based on β-galactosidase or luciferase. This assay is useful for the analysis of strong promoters only.

4. The assay can be carried out in microplate formats and quantified in microplate readers equipped with fluorescence detection.

5. FET3 and promoters of other genes of the yeast iron regulon are induced on iron starvation, which can be mimicked by growth of yeast in the presence of up to 50-μM bathophenantroline, an iron chelator. The FET3 promoter is constitutively induced in cells with defects in the mitochondrial ISC assembly or export systems and may be used for identification of these types of mutations in yeast (Fig. 3).

B. Promoter Assays Based on Luciferase Reporters

1. Analysis in Cell Extracts

Procedure

Sample preparation

1. Grow yeast cells carrying the luciferase reporter plasmids in a small volume of minimal media with the required amino acids and carbon sources overnight at
30°C in a shaking incubator. Collect the cells by centrifugation (1500 × g), dilute in 5 ml of the desired growth medium until the cell density reaches an OD_{600} of 0.2, and incubate at 30°C in a shaking incubator until the cell density reaches ~0.5.

2. Collect the cells by centrifugation (1500 × g for 5 min), resuspend in 1-ml water, and transfer the cells to a 2.2-ml reaction tube. Collect the cells by centrifugation (1500 × g for 5 min). At this point the cells may be shock frozen.

3. Resuspend the cells in 300-μl cell culture lysis reagent (provided by the manufacturer with the luciferase substrates). Add 50–100 μl of 0.5-mm glass beads and 2.5-μl saturated PMSF and cool the cells on ice. Vortex two times for 3 min with intermediate cooling on ice.

4. Sediment the glass beads and membrane debris by centrifugation at 17,000 × g for 10 min at 4°C. Transfer the supernatant to a fresh tube and determine the protein concentration (typical yields are 0.2–0.5 μg/μl). At this point the extracts may be shock frozen.

**Luminescence measurements**

**Firefly luciferase:**

1. Add 10-ml Assay Reagent buffer to the lyophilized luciferase substrate (Luciferase Assay System, Promega). Store the working solution in aliquots at −20°C.

2. Mix 5-μl yeast extract with 20-μl substrate working solution in a microplate, mix well, and incubate for 1 min. Determine the luminescence in a microplate reader equipped with luminescence detection over 2–5 min with an integration time of 1000 μsec. Dilution of the cell extracts may be required.

**Renilla luciferase:**


2. In a microplate, mix 10-μl yeast extract with 50 μl of substrate working solution in Renilla Assay buffer. Mix well and determine the luminescence in a microplate reader equipped with luminescence detection over 1 min with an integration time of 1000 μsec. Serial dilutions of the extracts may be required.

**Comments**

1. To minimize plasmid loss, cells must be grown with selection for the auxotrophic marker of the plasmid throughout the analysis.

2. Both luciferase systems give comparable results and can be measured in parallel in the same sample (McNabb et al., 2005). In general, the firefly system is more sensitive. Extracts are stable on ice for extended times. Specialized luminometers are not necessary. The luminescence can be quantified in microplate readers equipped with luminescence detection or in scintillation counters, provided the coincidence mode is switched off. We routinely use a microplate reader (Infinite M200, Tecan AG, Männedorf, Switzerland) or a scintillation counter. The protocols described above are for microplate readers, but they can be easily adjusted for scintillation counters. Since the latter are far more sensitive, dilutions of the extracts up to 1:100,000 may be required.
3. The luminescence of luciferase is time dependent. In yeast extracts, the luminescence of firefly luciferase peaks after ~2–3 min, while that of the *Rhenilla* enzyme is maximal immediately after addition of the substrate. Hence, it is necessary to monitor the time course of the luminescence. As a result, only a restricted number of samples can be analyzed in parallel in a microplate reader. Luciferase assays are thus difficult to adjust for high through-put analysis.

**2. Promoter Assay with Whole Cells Using *Renilla* Luciferase**

*Procedure*

1. Grow the yeast cells carrying the luciferase reporter plasmid in a small volume of the desired growth medium overnight at 30°C in a shaking incubator. Collect the cells by centrifugation (1500 \( \times \) g for 5 min), dilute in 5 ml of the desired growth medium until cells reach a density of \( \text{OD}_{600} = 0.2 \), and incubate at 30°C in a shaking incubator until the cell density reaches ~0.5. Dilute the cells to an \( \text{OD}_{600} \) of 0.1 in 1-ml water.

2. Dilute the *Renilla* luciferase substrate (*Viviren*, Promega) 1:20 in DMSO.

3. Transfer 2 \( \mu l \) of diluted substrate to a fresh tube and bring final volume to 100 \( \mu l \), accounting for the volume of cells that will be assayed. Mix gently.

4. Record the luminescence of the sample for 1 min. This is necessary for preequilibration and to get a background reading.

5. Add the cells, mix gently, and monitor the luminescence of the sample for 2–3 min.

6. Alternatively, cells and substrate are mixed in 100-\( \mu l \) water and the luminescence is recorded directly for 2–4 min. A control lacking cells expressing luciferase is analyzed separately.

*Comments*

1. The luminescence of *Renilla* luciferase in whole yeast cells is much weaker than in cell extracts. The analysis in luminometers or microplate readers frequently gives unsatisfactory results. We routinely quantify the luminescence in a standard scintillation counter with the coincidence detection mode switched off. Scintillation counters are much more sensitive than luminescence readers and are generally recommended for highly sensitive luminescence assays or simply when a luminescence device is not available.

2. Store the *Viviren* substrate in small aliquots at ~80°C. Avoid repetitive freeze thawing. The working solution is best prepared a day or two in advance. For reasons that are unclear, preparation of the substrate in advance reduces the autofluorescence of the substrate and increases the luminescence signal. The working solution can be stored in the refrigerator for at least 1 week.

3. All samples and controls have to be prepared freshly and analyzed with the same substrate-working solution. Cells should not be frozen.
This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB593 and Gottfried-Wilhelm-Leibniz program), Fonds der Chemischen Industrie, Deutsches Humangenomprojekt, and the Fritz-Thyssen-Stiftung.

References


