Purification of the Cytochrome c Reductase/Cytochrome c Oxidase Super Complex of Yeast Mitochondria

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Abstract
The protein complexes of the respiratory chain interact by forming large protein particles called respiratory supercomplexes or “respirasomes”. Biochemical characterization of these particles proved to be difficult because of their instability. Here we describe a strategy to isolate and characterize the cytochrome c reductase/cytochrome c oxidase supercomplex of yeast, also termed the III + IV supercomplex, which is based on lactate cultivation of yeast, gentle isolation of mitochondria, membrane solubilization by digitonin, sucrose gradient ultracentrifugation, and native gel electrophoresis. The procedure yields pure forms of two varieties of the III + IV supercomplex composed of dimeric complex III and one or two copies of monomeric complex IV. Supercomplex preparations can be used for physiological or structural investigations.
1. INTRODUCTION

The cytochrome $c$ reductase and cytochrome $c$ oxidase complexes can be separately purified from mitochondrial fractions of many organisms. This led to the view that the two complexes should be considered as independent structures (reviewed in Hackenbrock et al., 1986). However, early investigations already indicated the occurrence of ordered supramolecular structures that consist of defined interactions of respiratory proteins complexes (Hatefi et al., 1962). These findings were considerably supported by recent investigations on the basis of gentle biochemical preparations, native gel electrophoresis procedures, and electron microscopy (EM) (reviewed in Boekema and Braun, 2007). As a result, several so-called respiratory supercomplexes could be defined. One of the best-described respiratory supercomplexes is the cytochrome $c$ reductase/cytochrome $c$ oxidase supercomplex, which also is termed the III + IV supercomplex. It initially was described for the bacteria *Paracoccus denitrificans*, PS3, and *Sulfolobus sp.* (Berry and Trumpower, 1985; Iwasaki et al., 1985; Sone et al., 1987). In yeast mitochondria, its existence first was described physiologically by inhibitor titrations (Boumans et al., 1998) and later by biochemical procedures (Cruciat et al., 2000; Schägger and Pfeiffer, 2000). Its stability was found to depend on the cardiolipin concentration of the inner mitochondrial membrane (Pfeiffer et al., 2003; Zhang et al., 2002; 2005). Recently, a pseudo-atomic structure was presented for the III + IV supercomplex of yeast by comparison of medium-resolution 2-D maps from EM and the crystal structures of the two individual respiratory complexes, which are available for higher eukaryotes (Heinemeyer et al., 2007; Nelson and Cox, 2008). According to the proposed structure, the cytochrome $c$ binding pockets of cytochrome $c$ reductase and cytochrome $c$ oxidase are in close proximity, allowing cytochrome $c$ to efficiently transfer electrons from one complex to the other by a simple ping-pong mechanism. Here we describe procedures to gently purify the III + IV supercomplex of yeast for its physiological and structural characterization.

2. ISOLATION OF THE III + IV SUPERCOMPLEX

2.1. Cultivation of yeast cells in lactate medium

Biosynthesis and assembly of respiratory complexes in yeast depends on growth conditions during cultivation (Heinemeyer et al., 2007; Schägger and Pfeiffer, 2000). Lactate media lead to induced biosynthesis of complex IV and also induce formation of the III + IV supercomplex. For preparing
the III + IV supercomplex, yeast cells are cultivated in 2.5 L lactate medium (five 2-L Erlenmeyer vessels with 500 ml medium each composed of 0.3 % [w/v] yeast extract, 5 mM glucose, 7 mM KH$_2$PO$_4$, 20 mM NH$_4$Cl, 4 mM CaCl$_2$, 9 mM NaCl, 3 mM MgCl$_2$, 2.2 % [v/v] lactate, 0.2 M NaOH, pH 5.5) for 24 h at 30°C and 120 rpm. Cells are harvested at an optical density (OD) of 1.2 to 1.3 and directly used for the preparation of mitochondria.

2.2. Isolation of mitochondria

Yeast mitochondria are prepared according to standard procedures. Preparations should be carried out as fast and gentle as possible. A very suitable protocol is outlined in Meisinger et al. (2006). For this procedure, the yeast cell wall is digested by zymolyase, cells are disrupted mechanically, and finally organelles are purified by differential centrifugations and a sucrose gradient ultracentrifugation. Purified mitochondria are resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) at a protein concentration of 20 mg/ml and can be stored at −80°C. For reproducibility of experiments, it is recommended to store stock solutions of mitochondria at one specific concentration.

2.3. Solubilization of the mitochondrial membranes

The solubilization step is most crucial for supercomplex characterization. Digitonin (1–8 g detergent/g protein) nicely allows stabilization of the III + IV supercomplex from yeast (Cruciat et al., 2000; Heinemeyer et al., 2007; Schägger and Pfeiffer, 2000). Routinely, 1 ml resuspended organelles (corresponding to 20 mg mitochondrial protein) are sedimented by centrifugation for 10 min at 14,000g. The pellet is directly resolved in 1 ml solubilization buffer (5% [w/v] digitonin, 30 mM HEPES, 150 mM potassium acetate, pH 7.4) and incubated for 20 min on ice. Insoluble material is removed by centrifugation for 10 min at 18,000g.

2.4. Separation of mitochondrial protein complexes by sucrose gradient ultracentrifugation

The solubilized mitochondrial protein complexes are directly transferred onto a linear sucrose gradient (volume: 12 ml; 0.3 to 1.5 M sucrose in 15 mM TRIS base, pH 7.0, 20 mM KCl, 0.2% digitonin) and centrifuged for 20 h at 150,000g and 4°C. Afterwards, the gradient is fractionated into twenty-six 0.5-ml fractions from bottom to top, which most easily is achieved by use of a peristaltic pump linked to a needle, which gently is transferred into the tube.
2.5. Blue-native PAGE

Small aliquots of the sucrose gradient fractions (~50 μl) can be used for analysis of the protein complex composition of the fractions by blue-native PAGE (Wittig et al., 2006). Because 5% digitonin specifically destabilizes the dimeric ATP synthase supercomplex from yeast, the two largest bands visible on the gels represent III + IV supercomplexes (Fig. 10.1). As revealed by densitometric measurements, the largest band represents a supercomplex composed of dimeric complex III and two copies of monomeric complex IV, the second largest band a supercomplex of dimeric complex III and one copy of complex IV (Heinemeyer et al., 2007; Schägger and Pfeiffer, 2000). Overall purity of the two supercomplexes is >90% in fraction 4 and >80% in fraction 5 of the gradient (Fig. 10.1) as estimated by silver staining of blue-native gels (not shown). These fractions can be directly taken for physiological measurements or structural

**Figure 10.1** Purification of III + IV supercomplexes from yeast mitochondria. Mitochondrial membranes are solubilized by digitonin, and protein complexes are separated by sucrose gradient ultracentrifugation (horizontal separation dimension). Aliquots of 13 fractions of the gradient are subsequently separated by 1-D blue-native PAGE (vertical separation dimension). Identities of the four largest protein complexes are given to the left of the gel: III$_2$ + IV$_1$ and III$_2$ + IV$_2$, supercomplexes composed of dimeric complex III and one or two copies of monomeric complex IV; V, ATP synthase complex; III$_2$, dimeric complex III.
analysis with EM (see below). Activity of the purified supercomplexes also can be directly monitored by in-gel activity assays for complex IV and complex III (Wittig et al., 2007; Zerbetto et al., 1997). For an even higher purification of the yeast III + IV supercomplexes, which might be necessary for cryo-EM or x-ray crystallography, the bands representing these supercomplexes can be directly electroeluted from a 1-D Blue-native gel according to standard procedures (Wittig et al., 2006).

3. Characterization of the III + IV Supercomplex by EM

Purified supercomplex fractions can be used for structural analysis with negative stain single particle EM (Heinemeyer et al., 2007). EM can record the signal of single molecules, and EM images can typically contain the projection maps of dozens to hundreds of proteins or other macromolecules. These images, however, are very noisy, and to retrieve the signal of molecules thousands of single particle projections need to be summed. Because molecules may have freedom to attach to the support film of the EM specimens, extensive image processing is necessary. Single particle image analysis is a well-developed method to analyze EM projections. In short, projections are compared by statistical methods and subsequently sorted into homogeneous groups (“classes”), which represent specific angular projections of studied proteins. Before they can be summed into final 2-D maps, the projections also need to be aligned, which means that rotational and translational shifts are calculated and imposed to bring them in optimal equivalent positions. The 2-D maps disclose many features of the studied macromolecules. In favorable cases, where the sample is homogeneous and randomly oriented molecules can be recorded, projections can become merged into 3-D structures. Recent reviews give an overview of the methods of single particle EM (Frank, 2002; van Heel et al., 2000).

On single particle analysis, the obtained 2-D maps of the yeast III2IV2 supercomplex show that it has a symmetric shape with dimeric complex III in the center and two laterally attached complex IV monomers at opposite positions (Fig. 10.2).

4. Perspectives

With lactate medium for yeast cultivation, a gentle method for the preparation of yeast mitochondria, membrane solubilization by digitonin and sucrose gradient ultracentrifugation, supercomplexes composed of cytochrome c reductase and cytochrome c oxidase can be efficiently
puriﬁed. Blue-native PAGE is a helpful tool to monitor the puriﬁcation steps. Furthermore, blue-native PAGE can be used as the starting point for another puriﬁcation step, which is based on electroelution and which leads to highly pure supercomplexes suitable for x-ray crystallography. However, despite considerable success in supercomplex puriﬁcation, procedures still have to be further developed to characterize even larger protein structures, which are assumed to occur under in vivo conditions. Laboratory strains of *Saccharomyces cerevisiae* lack the NADH dehydrogenase complex (complex I), which in other organisms additionally is associated to complexes III and IV. Further protein complexes of the inner mitochondrial membrane possibly associated with III + IV supercomplexes are the ADP/ATP translocase or the preprotein translocase of the inner mitochondrial membrane, the so-called TIM complex (Dienhart and Stuart, 2008; Saddar et al., 2008). Development of novel procedures might allow unraveling the megacomplex organization of the inner mitochondrial membrane.

**Figure 10.2** Structure of the III$_2$ IV$_2$ supercomplex from yeast as obtained by single particle electron microscopy. Upper part, 2-D map of 832 aligned projections in a position vertical to the membrane plane. Lower part, pseudo-atomic structure of the III$_2$ + IV$_2$ supercomplex derived by ﬁtting the crystal structures for the III$_2$ complex (in red) of yeast (Lange and Hunte, 2002) and complex IV (in green) of beef (Tsukihara et al., 1996) into the projection map. Modiﬁed from Heinemeyer et al. (2007).
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