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Aim24 stabilizes respiratory chain supercomplexes and is required for efficient respiration

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ABSTRACT

The mitochondrial respiratory chain is essential for the conversion of energy derived from the oxidation of metabolites into the membrane potential, which drives the synthesis of ATP. The electron transporting complexes bc_1 complex and the cytochrome *c* oxidase assemble into large supercomplexes, allowing efficient energy transduction. Currently, we have only limited information about what determines the structure of the supercomplex. Here, we characterize Aim24 in baker's yeast as a protein, which is integrated in the mitochondrial inner membrane and is required for the structural integrity of the supercomplex. Deletion of *AIM24* strongly affects activity of the respiratory chain and induces a growth defect on non-fermentable medium. Our data indicate that Aim24 has a function in stabilizing the respiratory chain supercomplexes.

1. Introduction

Mitochondria are essential organelles, generate energy by oxidative phosphorylation, play a central role in ion homeostasis and are necessary for heme and Fe-S cluster biosynthesis [1–3]. Mitochondria form a reticular network in the cell and are surrounded by a double membrane. The outer membrane (OM) separates the intermembrane space (IMS) from the cytosol and allows permeation of small metabolites. The respiratory chain is localized in cristae structures formed by the inner membrane (IM) and transfers electrons from reducing equivalents to its terminal acceptor molecular oxygen [4–6]. This process is coupled to the transfer of protons across the inner membrane, generating the membrane potential. This energy store allows the synthesis of ATP via the mitochondrial F_1F_O ATPase but also drives selective transport of metabolites, like ADP and ATP via the ADP/ATP carrier AAC across the membrane [7,8]. As the mitochondrial genome encodes only for a limited set of proteins, most proteins are nuclear encoded and need to be imported from the cytosol. The import of proteins into or across the inner membrane is an energy demanding process and also highly depends on the membrane potential [9–12].

The respiratory chain couples the electron transport to the proton translocation across the membrane. In baker's yeast *Saccharomyces cerevisiae* several NADH dehydrogenases catalyze the initial transfer of electrons from NADH onto ubiquinone. In contrast to mammalian cells, yeast contains single membrane enzymes which do not form large membrane integrated complexes and are not able to transfer protons [13]. The bc_1 complex (complex III), transferring electrons from ubiquinone to cytochrome *c*, and the cytochrome *c* oxidase (complex IV), mediating the final transfer of electrons onto molecular oxygen, are large membrane complexes and contribute to the generation of the membrane potential [14,15]. A number of studies revealed that single complexes of the respiratory chain oligomerize into distinct large supercomplexes [16–18]. In yeast, the dimeric bc_1 complex associates with one or two modules of the cytochrome *c* oxidase forming two distinct supercomplexes with the stoichiometry III_2IV and III_2IV_2 [19,20]. This oligomerization limits electron carrier diffusion and thereby increases the efficiency of the respiratory chain [21]. A similar oligomerization has been also reported for the mitochondrial F_1F_O ATPase [22].

The mechanisms, which determine the architecture of supercomplexes are just beginning to emerge. Studies on the assembly of complexes III and IV are suggesting that the assembly process depends on designated assembly factors, which drive the maturation of these complexes by the stepwise integration of single

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components to a core structure of proteins encoded by the mitochondrial genome [23–25]. Concomitant to the assembly of complex IV and complex III occurs their assembly into supercomplexes. Recent studies elucidate that also this process is regulated by specific assembly factors (Rcf1 and Rcf2) [26–28]. As membrane protein complexes tightly interact with the lipid bilayer, lipids also have a strong impact on the integrity of membrane complexes. Cardiolipin is a phospholipid, which is almost exclusively located in mitochondrial membranes and is predominantly found in the inner membrane [29,30]. It is necessary for the stability of respiratory chain supercomplexes [31–33] and the structural integrity of supercomplexes is affected in cardiolipin deficient yeast mitochondria [34]. A similar destabilization of supercomplexes was described in Barth syndrome patients, carrying an inherited defect in the cardiolipin biosynthesis pathway, which leads to a severe form of cardiomyopathy and other symptoms [35–37].

In order to discover novel genes, which are required for mitochondrial biogenesis a high throughput analysis identified a set of candidates defective in mitochondrial inheritance [38]. In this study, deletion of the gene *AIM24* increases the frequency of petite colony formation and causes a decrease of growth rate on non-fermentable media. Interestingly, Aim24 was previously identified as a mitochondrial protein in a high throughput localization study of chromosomally tagged green fluorescent protein fusions and was found in mitochondrial fractions in several proteomic approaches [18,39,40]. We therefore became interested, if Aim24 plays a role in the structure or function of the respiratory chain. Here, we show that Aim24 is required for maximal activity of the respiratory chain. Aim24 is integrated in the inner mitochondrial membrane and necessary for the accurate formation of respiratory chain supercomplexes.

2. Materials and methods

2.1. Yeast strains and isolation of mitochondria

S. cerevisiae strain BY4741 *aim24Δ* was obtained from the Euroscarf collection (Euroscarf, Frankfurt, Germany). The yeast strain $Aim24^{GFP}$ expressing *AIM24-GFP* fusion was generated by chromosomal integration. For complementation assays the sequence encoding for *AIM24* was cloned into the plasmid pRS416 and transformed into *aim24Δ* cells. Yeast strains YPH499 expressing a *Cor1-ZZ* and a *COX4-ZZ* fusion were described previously [26]. Yeast cultures were grown at 30 °C unless otherwise indicated in rich medium (1% yeast extract, 2% peptone and 2% dextrose or 3% glycerol) or selective medium (0.67% yeast nitrogen base with selected amino acids and 2% dextrose). Yeast mitochondria were isolated as described previously [41].

2.2. Measurement of enzymatic activities

Malate dehydrogenase activity was determined by the spectrophotometrical measurement of NADH oxidation at 340 nm. Triton X-100-lysed mitochondria were analyzed in an assay buffer containing 100 mM potassium phosphate buffer, 0.1 mM NADH and 0.2 mM oxaloacetate as a substrate. The extinction coefficient of NADH at 340 nm was 6.3 mM^{-1} cm^{-1}. Cytochrome *c* oxidase activity was followed by measuring the oxidation of chemically reduced cytochrome *c* at 550 nm. Cytochrome *c*, was reduced by dithionite before adding it 1:50 (w/v) to the assay buffer (40 mM potassium phosphate buffer, pH 7.5). Reactions were started by addition of Triton X-100-lysed mitochondria. NADH – cytochrome *c* reductase activity was assessed by the change of absorbance at 550 nm during reduction of cytochrome *c*. Mitochondria were added to 0.02% (w/v) oxidized cytochrome *c* in assay buffer (40 mM potassium phosphate buffer, pH 7.5, 0.5 mM NADH and 0.1 mM KCN). Concentrations of reduced/oxidized cytochrome *c* were determined using the extinction coefficient at 550 nm of 21.84 mM^{-1} cm^{-1} [42].

2.3. Determination of mitochondrial respiration

The consumption of molecular oxygen over time of isolated mitochondria was measured with a XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). After calibration at 30 °C, baseline respiration was measured in MAS buffer (70 mM Sucrose, 220 mM Mannitol, 2 mM HEPES, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% BSA). Subsequent measurements of oxygen consumption were performed after administration of 10 mM succinate and 4 mM ADP and after 4 mM KCN. Each sample was assayed in triplicates.

2.4. In vitro import and assembly into isolated mitochondria

For synthesis of [^{35}S]labeled Aim24 and Cox5a precursor protein, the open reading frame was amplified from genomic DNA by primers containing the SP6 promoter sequence. The PCR product was transcribed and purified (mMESSAGE mMACHINE SP6 System and MEGAclear kit, Ambion) and subsequently used for *in vitro* translation (Flexi Rabbit Reticulocyte Lysate System, Promega) in the presence of [^{35}S]labeled Methionine. Radiolabeled precursor was imported into isolated yeast mitochondria as described previously [43]. The samples were analyzed by SDS- or BN-PAGE and [^{35}S]labeled proteins were detected using Phosphor Screens (GE-Healthcare) and subsequent digital autoradiography (Storm imaging system, GE Healthcare).

2.5. Microscopy

Cells were analyzed using a DeltaVision Spectris fluorescence microscope (Olympus IX71; Applied Precision, Issaquah, WA). For each image 10–15 Z-section images were taken at 0.5 μm intervals after focusing on the middle plane of the cell. Images were deconvoluted using softWoRx, version 3.5.1 (Great Falls, MT).

2.6. Protein isolation

For the isolation of the respiratory chain complex, mitochondria containing ZZ-tagged proteins (protein A) were solubilized (100 mM NaCl, 20 mM Tris/HCl (pH 7.4), 5% (v/v) glycerol, 0.5 mM EDTA, 1% (w/v) digitonin and 2 mM PMSF). After a clarifying-spin, samples were incubated with IgG Sepharose and extensively washed with buffer containing 0.3% (w/v) digitonin. Bound proteins were eluted by TEV protease cleavage and analyzed by SDS–PAGE and Western blotting.

2.7. Lipid extraction

Isolated mitochondria were extracted in 2/1 (v/v) chloroform/methanol and subsequently washed with water and 1/1 (v/v) methanol/water. After evaporation of the solvent the extract was solved in chloroform and spotted on TLC plates (HPTLC Silica Gel 60 F254, Merck). The lipids were separated in 50/50/3 (v/v/v) chloroform/methanol/25% ammonia and stained with 470 mM $CuSO_4$ in 8.5% o-phosphoric acid and dried at 180 °C for 10 min [44].

2.8. Miscellaneous

For SDS–PAGE and Western blotting of proteins to polyvinylidene fluorid (PVDF) membranes standard techniques were used. Primary antibodies were raised in rabbit and secondary antibodies

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