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Acyl modification and binding of mitochondrial ACP to multiprotein complexes

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ABSTRACT

The mitochondrial acyl carrier protein (ACPM/NDUFAB1) is a central element of the mitochondrial fatty acid synthesis type II machinery. Originally ACPM was detected as a subunit of respiratory complex I but the reason for the association with the large enzyme complex remained elusive. Complex I from the aerobic yeast *Yarrowia lipolytica* comprises two different ACPMs, ACPM1 and ACPM2. They are anchored to the protein complex by LYR (leucine-tyrosine-arginine) motif containing protein (LYRM) subunits LYRM3 (NDUFB9) and LYRM6 (NDUFA6). The ACPM1-LYRM6 and ACPM2-LYRM3 modules are essential for complex I activity and assembly/stability, respectively. We show that in addition to the complex I bound fraction, ACPM1 is present as a free matrix protein and in complex with the soluble LYRM4(ISD11)/NFS1 complex implicated in Fe-S cluster biogenesis. We show that the presence of a long acyl chain bound to the phosphopantetheine cofactor is important for docking ACPMs to protein complexes and we propose that association of ACPMs and LYRMs is universally based on a new protein-protein interaction motif.

1. Introduction

Acyl carrier proteins (ACPs) play a central role in fatty acid and polyketide biosynthesis pathways [13,29]. In the cytosol of animal and fungal cells ACP is an element of a multi-functional macromolecular protein complex that has been termed fatty acid synthesis type I (FAS I) system [22]. In bacteria, ACP is the central component of the fatty acid synthesis (FAS) type II machinery which operates with a set of distinct mono-functional enzymes. All enzymes of the FAS type II pathway and a bacterial-type ACP (termed ACPM, also known as NDUFAB1 or SDAP) were discovered in mitochondria [8,16,26,37,38]. Considering that mitochondria are specialized in breakdown of fatty acids to fuel energy metabolism, the presence of a highly conserved biosynthetic mitochondrial FAS type II machinery seems remarkable and was suggested to be essential to generate octanoyl-(C8)-ACPM as a precursor for lipoic acid (6,8-dithio-octanoic acid). Lipoic acid is an important cofactor for mitochondrial α-ketoacid dehydrogenases e.g. pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [9,32].

Several lines of evidence indicate that ACPM has important functions in mitochondria beyond lipoic acid synthesis. It was shown that down-regulation of human ACPM not only compromised protein lipoylation but also decreased activity of respiratory complex I [18]. Analysis of global protein-protein interaction screens in human, fruit fly and yeast model systems revealed that ACPM interacts with members of the LYR protein (LYRM) family [3,20,24,27,30]. LYR proteins are basic ~15 kDa polypeptides that carry a conserved tripeptide L-Y-R (leucine/tyrosine/arginine) sequence close to the N-terminus and a highly conserved downstream phenylalanine as well as further conserved arginine

Abbreviations: mtFASII, mitochondrial fatty acid synthesis type II; ACPM, mitochondrial acyl carrier protein; PP, phosphopantetheine cofactor; LYR protein (LYRM), leucine-tyrosine-arginine motif containing protein; NFS1, cysteine desulfurase; KAS, ketoacyl-ACPM synthase; KAR, ketoacyl-ACPM reductase; HTD2, hydroxyacyl-ACPM thioester dehydratase 2; ER (MECR), enoyl-ACPM thioester reductase; PDH, pyruvate dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; GCV3, protein H of glycine cleavage system; LIAS, lipoic acid synthase; MS, mass spectrometry; nLC, nano liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; TOF, time of flight; ISD, in-source decay; LILBID, laser induced liquid bead ion desorption

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residues (PFAM family PF05347) [3]. LYRMs are typically subunits or assembly factors of mitochondrial protein complexes and several LYRMs were associated with human diseases (reviewed in [4]). Association of ACPM with respiratory complex I was shown to be mediated by different LYRM subunits [5,48].

Complex I (NADH:ubiquinone oxidoreductase) is an L-shaped membrane protein with a hydrophilic peripheral arm and a hydrophobic membrane arm, and each arm contains one ACPM as an accessory subunit [19,44,46,49]. Complex I from the obligate aerobic yeast *Yarrowia lipolytica* harbors two similar but not identical versions of ACPM (ACPM1 and ACPM2; 63% identical, 81% similar residues) encoded by two separate genes [17] while complex I of mammals bind only one type of ACPM (NDUFAB1) [37]. ACPM1 was shown to be essential for *Y. lipolytica* while deletion of the ACPM2 gene caused a complex I assembly defect [17]. A critical function of the serine residues binding the phosphopantetheine cofactor was demonstrated for both ACPMs [17]. Two different LYRM subunits anchor the two ACPM independently to complex I forming two structurally highly similar LYRM6/ACPM and LYRM3/ACPM modules that are essential for complex I activity and assembly/stability, respectively [5,17]. While 3-hydroxy-tetradecanoate was suggested to be bound to ACPM of fungal and bovine complex I [8,12], recent cryo-EM structures of complex I from sheep and pig reported acyl chains in the range from C10 (decanoate) to C14 (tetradecanoate) [19,46].

The initial steps of the essential *de novo* Fe-S clusters biogenesis take place in the matrix of mitochondria (for a recent review see [7]). Recently, it was shown that the mitochondrial cysteine desulfurase LYRM4/NFS1 complex (*Saccharomyces cerevisiae* Isd11/Nfs1) anchors ACPM as an essential subunit [10,42]. This complex interacts with Yfh1 and Yah1 to assemble a [2Fe-2S] cluster at the Isu1 (ISCU) scaffold protein. Aided by the chaperone Ssq1 and its co-chaperone Jac1 the newly synthesized cluster is subsequently released and bound to the transfer protein Grx5. The [2Fe-2S] cluster is then either (i.) inserted into target proteins, or (ii.) transferred to the late ISC machinery for [4Fe-4S] cluster synthesis, or (iii.) used to generate a yet unknown component for export to the cytosol. The machinery for conversion of [2Fe-2S] into [4Fe-4S] clusters involves Iba57, Isa1 and Isa2. Finally, [4Fe-4S] clusters are inserted into apo-proteins by specific targeting factors.

In this study we provide evidence that the major part of ACPM is present in different mitochondrial complexes while only a minor fraction exists as a free matrix protein. ACPM in complexes but not the unbound protein was consistently found to be modified with a medium to long chain acyl group. In line with recent structural data [19,46] this suggests a new type of protein-protein interaction motif as the universal basis for ACPM/LYRM interactions that critically involves the acyl chain bound to the phosphopantetheine cofactor. Only the free ACPM in the mitochondrial matrix was found to be N-terminally acetylated. We conclude that post-translational modifications determine the role of individual ACPM molecules in mitochondrial metabolism offering potential access points for regulation.

2. Methods

2.1. Preparation of soluble fractions from Y. lipolytica and purification of complex I

We used three different *Y. lipolytica* laboratory strains: (i.) parental strain GB20 (complex I subunit NUGM-His [28]), (ii.) the deletion strain *acpm*1Δ complemented with the plasmid pUB26 carrying the *acpm1-6xalanine-StrepTagII* gene (*acpm*1Δ + pACPM1-linker-strepII, chromosomal NUGM-His, kindly provided by Martina Ding) and (iii.) the complemented wild type strain *acpm*1Δ + pACPM1-strepII (chromosomal NUGM-His) without the alanine linker [17]. Cells were grown in culture flasks or fermenter in YPD medium with or without hygromycin, respectively. For intact mitochondria preparations cells were grown in YD medium and intact mitochondria prepared with the method described in [5]. Soluble matrix proteins and mitochondrial membranes were essentially prepared following the protocol of [41]. The soluble supernatant after the ultracentrifugation step was used for purification of ACPM1-strepII by affinity chromatography. Purification of n-dodecyl-β-D-maltoside (DDM) solubilized complex I from mitochondrial membranes was achieved by Ni-NTA affinity chromatography, followed by gel filtration as detailed in [28]. The peripheral arm fragment (Iλ) of *Y. lipolytica* complex I was prepared essentially as described in [1].

2.2. Purification of NFS1 complexes and free ACPM1

Y. lipolytica cells from the plasmid complemented wild type strain (*acpm*1Δ + pACPM1-strepII) were diluted in buffer A (20 mM Tris-Cl, 500 mM NaCl, 50 mM sucrose, 1 mM EDTA, pH 8.0) and disrupted by a cell disintegrator in the presence of 2 mM PMSF and protease inhibitor cocktail. After a low speed centrifugation step (JA10 rotor, 30 min, 5000 rpm, 4 °C) the supernatant was subjected to ultracentrifugation (45 Ti rotor, 60 min, 38,000 rpm, 4 °C). The supernatant was collected and after addition of avidin, 1.5 mM PMSF and 0.05% DDM the pH adjusted to pH 8.0. The sample was sterile filtrated and subjected to a StrepTactin resin (high capacity, IBA, Germany) and washed with 20 CV buffer A supplemented with 1 mM ATP and 0.025% DDM. The sample was eluted with 10 mM desthiobiotin in buffer A + 0.025% DDM. After elution the sample was concentrated and washed with TSK buffer (20 mM Tris-Cl, 500 mM NaCl, 50 mM sucrose, 1 mM EDTA, pH 7.2 + 0.025% DDM) and subjected to TSK 3000SW gel filtration (Tosoh, Japan). Calibration was performed using 0.5 mg of immune globulin G, bovine serum albumin, superoxide dismutase and horse cytochrome *c*, respectively.

2.3. Electrophoreses

Intact mitochondria of *Y. lipolytica* were solubilized with 3 g/g digitonin/protein and separated by 4–16% blue native electrophoresis (BN-PAGE) [5,36]. Purified NFS1 complexes and free ACPM1 samples were analyzed by silver-stained 10% + 6 M urea SDS-PAGE.

2.4. Mass spectrometry

Complexome profiling was performed as described using a Thermo Scientific LTQ Orbitrap XL mass spectrometer with the exception that data were analyzed by MaxQuant (see below) [25]. Proteins from gel slices were digested as described in [6]. Fractions from chromatographically purified complexes were reduced with DTT and alkylated with chloracetamid and digested with trypsin (sequencing grade, Promega). Peptides were analysed by nano-LC ESI-MS/MS on a Thermo Scientific™ Q Exactive Plus equipped with an ultra-high performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000). Peptides were loaded on a C18 reversed-phase precolumn (Thermo Scientific) followed by separation on a 2.4 μm Reprosil C18 resin (Dr. Maisch GmbH, Germany) in-house packed picofrit emitter tip (diameter 100 μm, 15 cm long from New Objectives) using a gradient from 4% acetonitrile, 0.1% formic acid to 80% acetonitrile, 0.1% formic acid. MS data were recorded by data dependent acquisition Top10 method selecting the most abundant precursor ions in positive mode for HCD fragmentation. The Full MS scan range was 300 to 2000 *m/z* with resolution of 70,000, and an automatic gain control (AGC) value of $3 * 10^6$ total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2 +) were selected for MS/MS scans with a resolution of 35,000, an isolation window of 2 *m/z* and an automatic gain control value set to 10^5 ions with a maximal ion injection time of 150 ms. Full Scan MS-Data were acquired in profile mode.

MS Data from gel slices were analyzed by Peaks7 Studio software for proteomics (Bioinformatics Solutions, Canada). MS Data from chromatographically purified samples and native gels for complexome profiling