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Characterization of two different acyl carrier proteins in complex I from *Yarrowia lipolytica*

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ABSTRACT

Acyl carrier proteins of mitochondria (ACPMs) are small (~10 kDa) acidic proteins that are homologous to the corresponding central components of prokaryotic fatty acid synthase complexes. Genomic deletions of the two genes *ACPM1* and *ACPM2* in the strictly aerobic yeast *Yarrowia lipolytica* resulted in strains that were not viable or retained only trace amounts of assembled mitochondrial complex I, respectively. This suggested different functions for the two proteins that despite high similarity could not be complemented by the respective other homolog still expressed in the deletion strains. Remarkably, the same phenotypes were observed if just the conserved serine carrying the phosphopantethein moiety was exchanged with alanine. Although this suggested a functional link to the lipid metabolism of mitochondria, no changes in the lipid composition of the organelles were found. Proteomic analysis revealed that both ACPMs were tightly bound to purified mitochondrial complex I. Western blot analysis revealed that the affinity tagged ACPM1 and ACPM2 proteins were exclusively detectable in mitochondrial membranes but not in the mitochondrial matrix as reported for other organisms. Hence we conclude that the ACPMs can serve all their possible functions in mitochondrial lipid metabolism and complex I assembly and stabilization as subunits bound to complex I.

1. Introduction

Acyl carrier proteins (ACPs) are small (~10 kDa) acidic proteins with highly conserved amino acid sequences. They are central components of fatty acid synthase (FAS) complexes from bacteria and organelles of bacterial origin (plant chloroplasts and mitochondria), classified as FAS type II. In such enzymes, all steps in fatty acid synthesis are carried out by separate polypeptides [1]. In this type of FAS, acyl carrier protein functions to shuttle intermediates among enzymes. The FAS type I complex is present in the cytosol of eukaryotes [2,3] and is composed of one or two giant polypeptides [4] containing all necessary enzymes for fatty acid synthesis with ACP being its central domain. In both systems ACP or ACP-like domains contain a centrally located, serine-bound phosphopantethein prosthetic group. This group is post-translationally attached and serves as a platform for the acyl intermediates during fatty acid synthesis. Additional postulated functions for bacterial ACPs involve synthesis of polyketides [5], peptide antibiotics [6] and acylation of toxins [7].

One or several ACPs of the bacterial FAS type II have been found in mitochondria of several species, including *Neurospora crassa* [8,9], bovine heart [10] and *Arabidopsis thaliana* [11]. These proteins are denoted ACPM for "*A*cyl *C*arrier *P*rotein, *M*itochondrial". Mitochondria of *N. crassa* were found to contain a protein which was labelled with [^{14}C] pantothenic acid and carried an acyl group [8]. Evidence for a covalently attached pantetheine-4′-phosphate in bovine ACPM [10] was obtained by electrospray mass spectrometry, before and after incubation of the protein at alkaline pH conditions. A number of bacterial ACP structures are known: *Escherichia coli, Bacillus subtilis* [12] and *Mycobacterium tuberculosis* [13]. So far, only one structure of a mitochondrial ACP is available from *Toxoplasma gondii* [14].

Following discovery and identification of ACPMs, further studies suggested that mitochondria contain all necessary enzymes for fatty acid biosynthesis and that they are able to synthesize short-chain fatty acids [15,16]. ACPM was postulated to be involved in the synthesis of octanoic acid as a precursor for lipoic acid in pea [17], *A. thaliana* [18],

Abbreviations: 2D BN/SDS PAGE, two dimensional blue native/sodium dodecyl sulfate polyacrylamide gel electrophoresis; ACP, acyl carrier protein; ACPM, mitochondrial acyl carrier protein; DBQ, n-decylubiquinone; dNADH, deamino nicotinamide adenine dinucleotide, reduced form; FAS, fatty acid synthase; FMN, flavin mononucleotide; HAR, hexamine-ruthenium(III)-chloride; LHON, Leber's hereditary optic neuropathy; MALDI-MS, matrix assisted laser desorption/ionisation mass spectrometry; ORF, open reading frame; V_D, complex V dimer

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N. crassa [19,20], *Saccharomyces cerevisiae* [21] and *Trypanosoma brucei* [22]. Lipoic acid is an important prosthetic group of some mitochondrial enzymes such as the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes [23,24]. There are also indications for the synthesis of longer fatty acids [17,18]. It was speculated that this activity plays a vital role, most likely for repair of mitochondrial lipids [15].

Typically, the number of mitochondrial acyl carrier proteins in eukaryotes ranges from one to three. However it seems that in most eukaryotes two isoforms are found [25]. In mammalian [10] and *N. crassa* [26] mitochondria one ACP homologue is found to be tightly associated with mitochondrial complex I, whereas in *Arabidopsis* none of three ACPM isoforms seems to be tightly associated with complex I [27]. Also in mammalian mitochondria a fraction of the mitochondrial ACP has been found in the mitochondrial matrix separate from complex I [28].

Complex I (NADH:ubiquinone oxidoreductase) is the first electron transferring protein complex of the mitochondrial respiratory chain and is one of the largest and most intricate membrane bound protein complexes known [29]. The L-shaped molecule is composed of an intrinsic membrane arm extending into the lipid bilayer and a peripheral arm protruding into the matrix space. The atomic structure of the hydrophilic part of the homologous enzyme from *Thermus thermophilus* has been solved [30]. Nonetheless, the catalytic mechanism of complex I is still not understood. Mitochondrial complex I catalyses transfer of two electrons from reduced nicotinamide adenine dinucleotide (NADH) via flavin mononucleotide (FMN) and eight iron-sulfur clusters to ubiquinone. This transfer is coupled to the translocation of four protons across the inner mitochondrial membrane. The minimal set of 14 central subunits that makes up the prokaryotic enzyme is extended significantly by up to 31 accessory subunits in eukaryotes adding up to a mass of about 1 MDa [31,32]. In mammals and fungi, seven hydrophobic central subunits are encoded in the mitochondrial genome while the rest of the subunits are nuclear coded. A number of mutations in complex I subunit genes are known to be associated with neurodegenerative diseases, including Parkinson's disease, LHON and Leigh syndrome [33,34].

We have established *Yarrowia lipolytica* [35] as a useful yeast genetic model to study mitochondrial complex I. To obtain further insight into the role of ACPMs and their relationship to mitochondrial complex I we analyzed the function of the two *ACPM* homologues found in the genome of the strictly aerobic yeast *Y. lipolytica*.

2. Material and methods

2.1. Deletion of ACPM genes in Y. lipolytica

Using the sequences from the Genolevures website (http://cbi.labri.fr/Genolevures/ index.php), we identified the *ACPM* homologues YALI0D14850g (*ACPM1*) and YALI0D24629g (*ACPM2*). 2.70 kb of genomic DNA, comprising the complete *ACPM1* open reading frame (two exons) and 0.97 kb of 5′ and 0.96 kb of 3′ flanking DNA was amplified with primers TACTCGGCTGAACTGCAAG and ATCTCACGGCTTCACAACG. 3.08 kb of genomic DNA, comprising the complete *ACPM2* open reading frame (three exons) and 0.67 kb of 5′ and 0.66 kb of 3′ flanking DNA, was amplified using primers GTACAGAAGTGTCG GCGCAG and CTCGCTCTAACGCCGTACTC. Both PCR products were made using Taq DNA polymerase (Sigma) and genomic DNA from *Y. lipolytica* strain E129 and cloned into plasmid pCR2.1 (Invitrogen). Deletion strains for the ACPM subunits of complex I from *Y. lipolytica* were generated by homologous recombination using the one-step transformation method as described [36]. Deletion alleles in which the *ACPM* ORFs are replaced with the *URA3* marker gene were created as follows: First, genomic DNA fragments were transferred into the single *EcoRI* site of vector pUB44 (Supplemental Fig. S1). Then, the *ACPM1* construct was gapped by PCR, using primers AAGTCGACACGGACAGGATTCGAAC and ATGGATCCAGCGTTAAGGCCACAG, thereby removing the ORF together with 143 bp of 5′ flanking sequence and 22 bp of 3′ flanking sequence. Similarly, the *ACPM2* construct was gapped by PCR, using primers AAGTCGACAGTGTGGAGGATGGTGTGG and AAGGATCCTCAAACAGACTAAAGCCCTG, thereby removing the ORF together with 16 bp of 5′ flanking sequence and 4 bp of 3′ flanking sequence. PCR products were digested with *Bam*HI and *Sal*I (sites underlined) and ligated with the *Y. lipolytica URA3* marker gene as a 1.7 kb *Bam*HI/*Sal*I fragment, such that the orientation of the marker was opposite to the original ORFs. The resulting constructs were linearized with *Not*I and transformed into the diploid *Y. lipolytica* strain GB14 (*NUCM*-Htg2, *NDH2i*, *ura3*-302, *leu2*-270, *lys11*/+, *his1*/+). Heterozygous deletion strains in which one of the chromosomal copies of either *ACPM1* or *ACPM2* had been replaced with the *URA3* marker by double homologous recombination (Supplemental Fig. S1) were selected for their ability to grow in the absence of uracil and their inability to grow in the presence of hygromycin B. Sporulation, followed by random spore selection for the same markers was performed both in the presence and absence of 10 μg/ml lipoic acid. Deletion strains carrying a functional copy of the respective *ACPM* gene on replicative plasmid pUB4 [37] were used as controls and will be referred to as parental strains here.

2.2. Exchange of the phosphopantethein binding serines

ACPM ORFs with flanking regions were amplified as described above and cloned into plasmid pUB4. Mutations leading to the exchange of the phosphopantethein binding serines for alanines were carried out by PCR using primers **GCC**TTAGACACCGTCGAGGTTG and GTCGAGGTTGAGGTCCTTGG (ACPM1-S66A); **GCT**TTGGATGTCGTCGAGGTG and GTCCAGACCAAGGTCGGAAG (ACPM2-S88A). Subsequently, the mutated genes were transformed into *ACPM1*/*acpm1*Δ and *acpm2*Δ strains, respectively.

2.3. Tagged versions of ACPMs

For production of *ACPM1-strepII* primers AGGATGAGACCATTTAGCATCGGGCTGG and CAATTTGAAAAATAAGCAAGCTGTATATAATAG, and for production of *ACPM2-flag* primers GTCTTTGTAATCAACGGCTGGGTTAGTAGG and GATGATGATAAATAATCAACC TCAAACAGAC were used. As a result *ACPM* ORFs were extended by 8 amino acids at the C-termini: ACPM1 by WSHPQFEK and ACPM2 by DYKDDDDK. Plasmids carrying tagged versions of *ACPM* genes were transformed into diploid *acpm*Δ strains carrying one wild-type and one *acpm*::*URA3* allele. Complemented haploid deletion strains were obtained by sporulation and random spore selection.

2.4. Preparation and analysis of complex I

For large scale purification of complex I, *Y. lipolytica* was grown at 27 °C in a 10-litre Biostat E fermenter (Braun, Melsungen, Germany). Freshly harvested cells, or cells shock frozen at −80 °C, were used for mitochondrial membrane preparations as described [38] with the modification that cells were broken up in a cooled glass bead mill (Bernd Euler Biotechnology, Frankfurt, Germany). Complex I was purified from mitochondrial membranes that were solubilised with n-dodecyl-β-D-maltoside as described [39] with slight modifications. Purification was achieved by Ni^{2+}-affinity chromatography with a modest reduction of the imidazole concentration from 60 mM to 55 mM in the equilibration and washing buffer and subsequent gel filtration using a TSK4000 column. Measurements of NADH:HAR and dNADH:DBQ oxidoreductase activities were carried out as detailed in [40]. The mean values of three measurements are given.