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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.

2.5. Preparation of intact mitochondria

Intact mitochondria from *Y. lipolytica* were prepared essentially by the enzymatic digestion method described in [41]. Briefly, *Y. lipolytica* cells were harvested at the early logarithmic stage (OD ~ 3-4). The cell wall was weakened by 5 mM dithiothreitol and digested with Zymolyase 20T (from *Arthrobacter luteus*, ICN Biomedicals). The resulting spheroplasts were disrupted by 20 gentle strokes in a loosely fitting Dounce homogenizer and centrifuged at 2000*g* for 10 min. Mitochondrial fractions were collected by centrifugation of the supernatant at 7000*g* for 20 min. Crude mitochondria were further purified on a sucrose step gradient as described in Ref. [42].

2.6. Mitochondrial fractionation

Intact mitochondria corresponding to 500 μg of protein determined according to a standard protocol [43] were suspended in 170 μl 1 mM EDTA, 20 mM Na^+/Mops pH 7.2, subjected to 3 cycles of freezing and thawing using liquid nitrogen and centrifuged for 1 h at 100,000*g*. The supernatant (containing matrix and intermembrane space components) was transferred to another tube and 80 μl of gel loading buffer I (0.03% Serva Blue G, freshly added 6% 2-mercaptoethanol, 30% glycerol, 12% SDS, 150 mM Tris-Cl pH 7.0) was added. The pellet (containing inner and outer membrane components) was resuspended in 250 μl gel loading buffer I/3 (buffer I diluted 3 times in water). The final protein concentration of the supernatant and pellet samples was 2 mg/ml. Also a sample of total mitochondria with the same protein concentration was prepared.

2.7. Gel electrophoresis and western blotting

To investigate expression and localization of tagged ACPMs, soluble and membrane fractions of mitochondria were resolved by Tricine-SDS-PAGE according to [44]. 2D (BN/SDS) PAGE was used to visualize respiratory chain complexes. Mitochondrial membranes corresponding to 500 μg of total protein were solubilised with 3 g/g digitonin and 500 mM amino caproic acid. Native protein complexes were separated in the first dimension using 4–13% gradient gels [45]. Gel stripes were excised, incubated in 1% SDS solution for 30 min and put on 16% SDS gels. Tricine dSDS-PAGE was used to separate the subunits of complex I from *Y. lipolytica* [46]. Proteins were separated in the first dimension using 6 M urea and 10% polyacrylamide SDS-PAGE and in the second dimension using 16% polyacrylamide SDS-PAGE. Silver staining was performed according to Rabilloud [47].

Proteins were transferred to polyvinylidendifluoride membranes (Immobilon TMP, Millipore) using a semidry immunoblotting procedure. Membranes were incubated with the following antisera, diluted in PBS buffer (270 mM NaCl, 5 mM KCl, 15 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.5) with 0.1% (w/v) Tween20: ACPM1-strepII with mouse monoclonal anti-strepII-tag antibody (Qiagen); ACPM2-flag with anti-flag (Sigma); aconitase with rabbit polyclonal antiserum (kind gift of R. Lill, Marburg); hexokinase with rabbit polyclonal antiserum, directed against the peptide SRKGSMADVPRDLLC (Sigma), complex I subunits NESM, 39-kDa, 49-kDa and 51-kDa with mouse monoclonal antibody (kind gift of V. Zickermann). All secondary antibodies were peroxidase conjugated rabbit anti-mouse or anti-rabbit IgG. Signals were detected using enhanced chemiluminescence (Sigma).

2.8. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS)

Stained protein spots were excised from dSDS gels and treated following the protocol of [48]. The proteins were cleaved with trypsin (12.5 ng/μl) in digestion buffer containing 25 mM ammonium hydrogen carbonate, 5 mM $CaCl_2$ at 37 °C over night.

Spectra were recorded in the positive ion mode with a Ultraflex MALDI TOF-TOF MS Instrument (Bruker Daltonics). The samples were deposited on preparative plates by the fast evaporation method. DHB (2,5-dihydroxybenzoic acid) or HCCA (4-hydroxy-α-cyano-cinnamic acid) from Sigma were used as a matrix. Spectra were calibrated internally using bovine trypsin autolysis products (*m*/*z* 805.4167 and 2163.0567) or, if necessary, externally using a reference peptide mixture of bradykinin, angiotensin II, insulin (oxidized B chain), adrenocorticotropic hormone (ProteoMass Peptide MALDI-MS Calibration Kit, Sigma) covering the *m*/*z* 757.3997-3494.6513 range. MALDI spectra were analyzed by the Mascot software package (Matrix Science Ltd., London), Prowl software package (ProteoMetrics, LLC, New York, USA).

2.9. Sequence analysis

DNA and protein sequences were analyzed using the Vector NTI (Informax) software. Homology searches of mammalian and fungal databases were done using the BLAST server at http://www.ncbi.nlm.nih.gov/BLAST/. Alignments of fungal and mammalian proteins were generated using the program CLUSTALW at http://www.ebi.ac.uk/clustalw/index.html. Searches of the NCBI conserved domains database [49] were performed at the NCBI server (www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi).

2.10. HPLC analysis of mitochondrial phospholipids

Mitochondrial lipids were analyzed by high performance liquid chromatography (HPLC) as described by Richers and Hunte (manuscript in preparation). In short, lipids were extracted from mitochondrial membranes according to Folch [50] with two modifications. After mixing a 50-μl sample with 950 μl chloroform/methanol (2/1, v/v), phase separation was induced by the addition of 200 μl $HCl_{(aq)}$ (0.1 M) instead of the original recommended salt solutions. Furthermore two additional cycles of washing of the aqueous phase with the organic phase increased the yield. The combined organic phases were dried and redissolved in chloroform/methanol (4/1, v/v). The strains acpm2Δ and *acpm2*Δ, pACPM2-S88A were compared to the parental and the *nubm*Δ strain with a known complex I assembly defect. The comparison was performed with four different starting materials: *Y. lipolytica* cells, mitochondrial membranes, intact mitochondria and sucrose gradient purified intact mitochondria [51]. The phospholipids were separated by normal phase chromategraphy with a solvent system of chloroform/ methanol/water/ammonia based on the method described by [52]. An evaporative light scattering detector (Sedex 75, Sedere) supplied with 3.3 bar nitrogen and heated to 80 °C detected the phospholipids. Phospholipids were quantified by external calibration with commercially available standards: 1,1′,2,2′-tetraoleoyl cardiolipin; 1,2-dipalmitoyl-sn-glycero-phosphoethanolamine; L-α-phosphatidylinositol (soybean); 1,2-dipalmitoyl-sn-glycero-phosphate; 1,2-dipalmitoyl-sn-glycero-phosphocholine; LPE lysophosphatidylethanolamine; LPI lysophosphatidylinositol; LPA lysophosphatydic acid; LPC lysophosphatidylcholine (all from Sigma).

3. Results

3.1. Deletion of the genes for ACPM1 and ACPM2 has severe effects

In order to investigate the function of the ACPMs we deleted the two homologues of the *S. cerevisiae* gene for the mitochondrial acyl carrier protein (YKL192c) that we had identified in the *Y. lipolytica* genome as YALI0D14850 g (*ACPM1*) and YALI0D24629g (*ACPM2*). To minimize the risk of selection for second site repressors during homologous recombination, we transformed the linearized deletion constructs into the diploid *Y. lipolytica* strain GB1. In the case of *ACPM2*, the desired diploid strain was confirmed to be heterozygous

for the deletion allele and could be sporulated to yield the desired haploid *acpm2::URA3* deletion strain (data not shown).

While we also successfully generated a diploid *ACPM1*/*acpm1::URA3* deletion strain, subsequent extensive sporulation attempts failed to generate haploid strains carrying the *ACPM1* deletion allele. However, when a plasmid-borne, functional copy of the *ACPM1* gene was introduced into the *ACPM1*/ *acpm1::URA3* strain prior to the sporulation, haploid *acpm1Δ, pACPM1* strains could be isolated. This demonstrated successful complementation of the deletion allele and excluded that second-site genetic defects in the diploid heterozygous deletion strain had prevented formation of viable haploid deletion spores. We thus concluded that *ACPM1* is an essential gene in *Y. lipolytica*. To discriminate whether the ACPM1 protein as a whole or just its functional group was required for survival we exchanged serine-66 of ACPM1 with alanine. This serine is conserved in all acyl-carrier proteins and known to bind the phosphopantethein moiety. A plasmid-borne copy of *ACPM1-S66A* was transformed into the heterozygous *ACPM1*/*acpm1::URA3* strain, but in contrast to the wild-type copy it failed to complement the deletion phenotype and no *acpm1Δ*,pACPM1-S66A spores were obtained. Thus the single amino acid exchange seemed to have the same effect as complete removal of the *ACPM1* gene.

In contrast, *ACPM2* was found to be not essential in a strain carrying NDH2i to complement for complex I deficiency and a haploid *ACPM2* deletion strain (*acpm2Δ*) was obtained. To test whether complex I was affected by deletion of the *ACPM2* gene, we first measured its activities in mitochondrial membranes prepared from the deletion strain and compared them to the parental strain (Table 1). Specific inhibitor sensitive dNADH:DBQ oxidoreductase activity was lost completely. A strong decrease in dNADH:HAR oxidoreductase activity down almost to a level typically observed with other complex I deficient strains suggested that the loss of complex I activity was due to a very low content of complex I. Remarkably, essentially the same loss in activity was observed when a mutated copy of the *ACPM2* gene was introduced on a plasmid in which the phosphopantethein binding serine-88 was replaced by alanine. The loss of complex I was confirmed when mitochondrial membranes from the *acpm2Δ* strain and the parental strain were analyzed by two-dimensional BN/SDS-PAGE (Fig. 1A). In contrast to the parental strain no complex I could be detected by silver staining in the *ACPM2* deletion strain (Fig. 1B). The same result was obtained with the mutant version lacking serine-88 (Fig. 1C). The pattern of the other respiratory chain complexes V_D, V_M, III, IV, II and the presence of small amounts of subcomplex F_1, corresponding to the matrix part of complex V [53] were not affected by the deletion, except that complex IV was below the detection limit in the *acpm2Δ* strain (Fig. 1).

For higher sensitivity and to detect low amounts of possible subcomplexes of complex I we further analyzed mitochondrial membranes from the *acpm2Δ*strain by Western blotting of native gels with different antibodies directed against subunits of complex I (Fig. 2). In membranes from the parental strain fully assembled complex I was found to be the dominant form. In addition, several supercomplexes containing complex I together with complexes III and/or complex IV were detected as described recently [54]. Some disintegration of complex I into subcomplexes was also observed with monoclonal antibodies directed against the 51-kDa and 49-kDa subunits of the peripheral arm and the accessory NESM subunit of the membrane arm. With mitochondria from the *ACPM2* deletion and the S88A mutant strain a very small amount of fully assembled complex I was detected with all three antibodies. However, only with the antibody directed against the NESM subunit pronounced signals of two putative subcomplexes were observed. These subcomplexes migrated in a similar position as the disintegration products observed with the parental strain at about 700 (marked ❶) and 300 kDa (marked ❷), but the fact that they were not detectable by the anti-51-kDa and anti-49-kDa antibodies in the *ACPM2*-mutant strains suggested a different composition. Rather, the antibodies directed against the 51-kDa and 49-kDa subunits gave strong signals over a broad range of molecular masses that were even more pronounced in the *ACPM2*-mutants. These signals probably reflected the individual subunits and/or small subcomplexes containing the respective subunits. Unfortunately, due to the very small amounts available, it was not possible to further analyze the subunit composition of the subcomplexes.

3.2. Deletion of ACPM2 has no effect on the phospholipid composition of mitochondria

To see whether deletion and mutagenesis of ACPM2 had affected the lipid composition, we analyzed the phospholipid content of extracts from mitochondrial membranes of parental, *acpm2Δ*, *acpm2Δ*, pACPM2-S88A and the *nubmΔ* strains by HPLC. The *nubmΔ* strain carrying a deletion of the central 51-kDa subunit of complex I was included as additional control because of its severe complex I assembly defect [55]. Fig. 3 shows that all lipids were present at very similar ratios in intact, sucrose gradient-purified mitochondria from all strains. Prominent peaks were identified by MALDI-MS as cardiolipin, phosphatidyl-ethanolamine, phosphatidyl-inositol, lyso-phosphatidyl-ethanolamine, phosphatidyl-choline. It should be noted that the peak for LPE was found at a mass of 454.33 Da indicating substitution with a palmitoyl-chain (16:0; theoretical mass 454.29 Da) rather than an unsaturated acyl-chain. Moreover, a strong unidentified anion peak was observed at a mass of 924.81. Also the protein to phospholipid ratio was nearly identical in mitochondria from all strains (not shown). In addition, the analysis was performed with whole cells, mitochondrial membranes and crude intact mitochondria yielding very similar results (data not shown).

3.3. ACPM1 and ACPM2 are bona fide subunits of Y. lipolytica complex I

We next addressed the question whether one or both of the ACPM-proteins in *Y. lipolytica* were bound to complex I as accessory subunits. In a previous proteomic approach aimed at the identification of the accessory subunits of *Y. lipolytica* complex I we had already identified ACPM1 as a component of the purified enzyme by MALDI-TOF mass spectrometry [56]. However, the almost complete loss of fully

Table 1
Catalytic activities of mitochondrial membranes and complex I.

	Parental	*acpm2Δ*	*acpm2Δ*::pS88A	*ACPM1*-strepII	*ACPM2*-flag
	μmol $min^{-1}mg^{-1}$				
Mitochondrial membranes					
NADH:HAR oxidoreductase	0.75	0.18	0.17	0.81	0.77
dNADH:DBQ oxidoreductase	0.27	<0.1	<0.1	0.31	0.30
Purified complex I					
NADH:HAR oxidoreductase	45	n.d[a]	n.d	50	48
dNADH:DBQ oxidoreductase	3.4	n.d	n.d	3.1	3.0

[a] not determined.

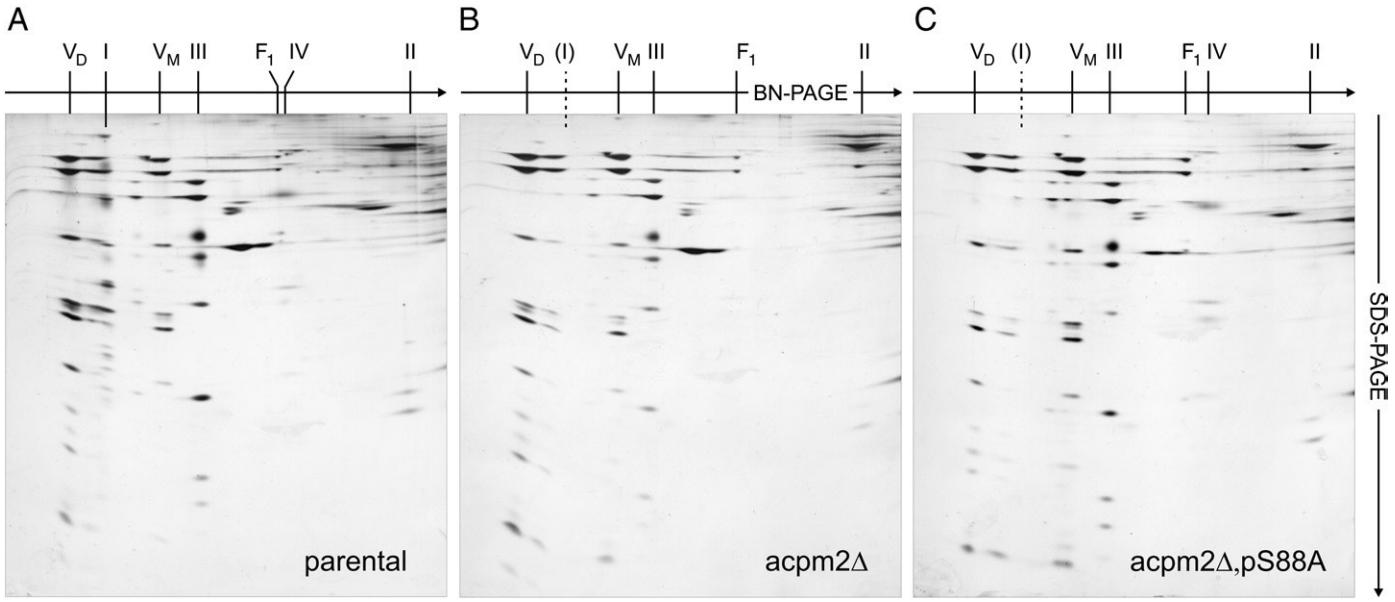

Fig. 1. 2D-BN/SDS PAGE analysis of *Y. lipolytica* mitochondrial membranes. Protein complexes were resolved by BN-PAGE in the first and by 16% SDS-PAGE in the second dimension. (A) A typical pattern of the parental strain respiratory chain complexes visible from left to right with decreasing molecular weight: ATP synthase dimer (V_D), complex I, complex V monomer (V_M), complex III, F_1 part of complex V (F_1), complex IV and complex II. (B) *acpm2Δ* strain and (C) *acpm2Δ*:: pACPM2-S88A strain exhibit lack of complex I assembly. Other respiratory complexes appear to assemble normally except that complex IV was below the detection limit in the *acpm2Δ* strain.

assembled complex I caused by the deletion of the *ACPM2*-gene suggested a tight link between complex I and ACPM2 as well. Indeed, thorough analysis of the double-spot in dSDS-PAGE that had previously been shown to contain ACPM1 (Fig 4A) allowed the unambiguous identification of both ACPM1 and ACPM2 by MALDI-MS/MS sequencing of peptides from both proteins (Supplemental Fig. S2).

To confirm this assignment we introduced tagged versions of the two proteins into the ACPM deletion strains. Both constructs appeared to be functional, since ACPM1-strepII permitted survival of strain *acpm1Δ* (Fig. 4B) and ACPM2-flag restored normal assembly of complex I (Fig. 4C). Also NADH:HAR oxidoreductase and dNADH:DBQ oxidoreductase activities of mitochondrial membranes from

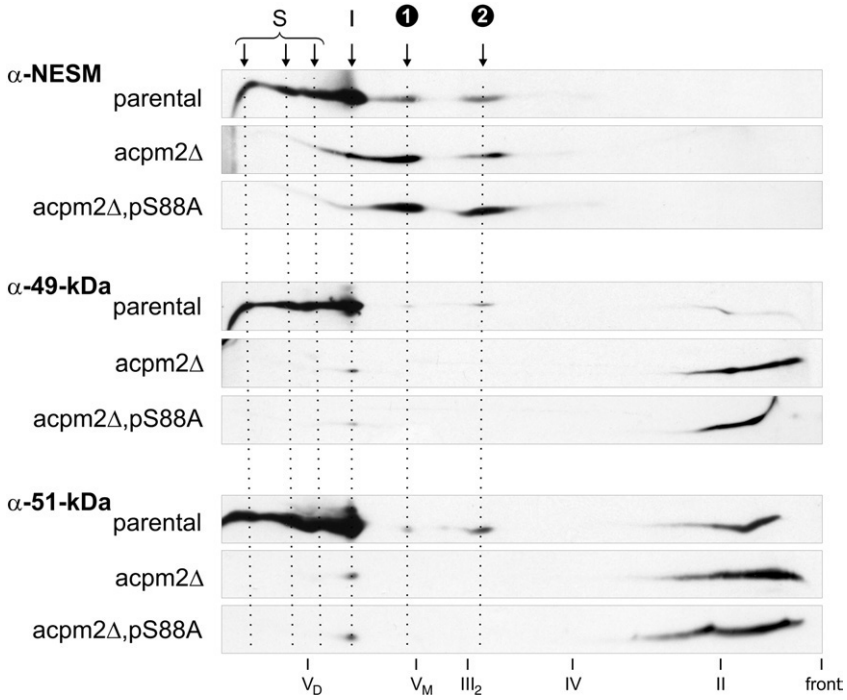

Fig. 2. Complex I subcomplexes in *acpm2Δ* and the *acpm2Δ,pACPM2-S88A* strains. Western blot analysis of mitochondrial membranes from the parental strain and strains *acpm2Δ* and *acpm2Δ*, pACPM2-S88A is shown. In the parental strain predominantly fully assembled complex I and several supercomplexes of complex I associated with complex III and complex IV were detected (S, [54]) but not analyzed in detail here. Subcomplexes were detected at positions marked ❶ and ❷ by antibodies against the 51-kDa, the 49-kDa and the NESM subunit in the parental strain. In strains *acpm2Δ* and pACPM2-S88A only the antibody against the membrane arm subunit NESM indicated subcomplexes in these positions suggesting that different subcomplexes of similar size but different composition were detected. In strains *acpm2Δ* and pACPM2-S88A the majority of the 49-kDa and 51-kDa subunits of the peripheral arm were detected close to the front of the gel and only minute amounts of fully assembled complex I were identified. Respiratory chain complexes are indicated by arrows: V_D, complex V dimer: I, complex I; V_M, complex V monomer; III_2, complex III dimer; IV, complex IV; II, complex II.

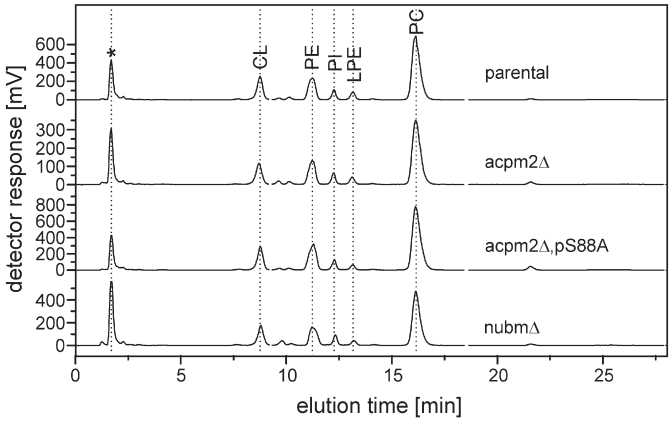

Fig. 3. HPLC profiles of phospholipids extracted from *Y. lipolytica* mitochondria. Extracts from sucrose gradient purified intact mitochondria (0.5 mg/ml) of the parental, *acpm2Δ acpm2Δ,pACPM2-S88A* and *nubmΔ* strains were analyzed. No significant difference in phospholipid composition among the analyzed strains was found. CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; *, pass through.

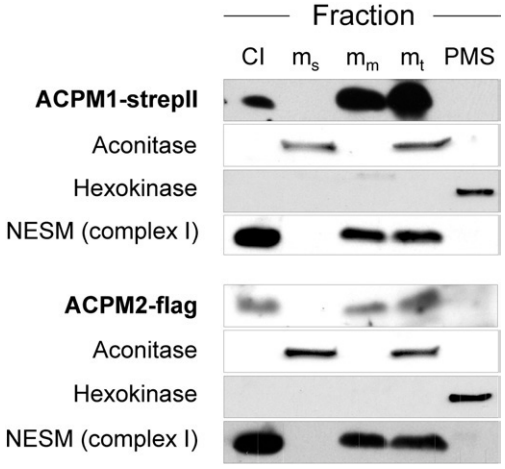

Fig. 5. Localization of tagged versions of ACPM in mitochondrial fractions. Intact mitochondria were fractionated into soluble (m_s) and membrane (m_m) part and analyzed for ACPM presence with antibodies specific to strepII- and flag-tags. Neither of them could be detected in the soluble fraction of mitochondria nor in the cytosolic (PMS) fraction. Both ACPMs were detectable in the membrane fraction. Purified complex I (CI) and whole mitochondria (m_t) were used as controls. Antiserum specific to representative proteins of each fraction were used: aconitase (soluble fraction), hexokinase (cytosol) and NESM (complex I, membrane fraction).

both complemented strains were restored to parental strain levels (Table 1). Moreover, also the purified complexes from both tagged strains exhibited wild-type catalytic activities indicating normal stability of the multiprotein complex. This allowed dSDS-PAGE analysis of the tagged complexes. Molecular mass shifts of ACPM1-strepII (Fig. 4B) and ACPM2-flag (Fig. 4C) subunits as compared to the untagged parental strain (Fig. 4A) confirmed the assignment of the two proteins that thus have both to be considered *bona fide* subunits of complex I in *Y. lipolytica*.

3.4. ACPM1 and ACPM2 are only found in the membrane fraction of mitochondria

We then asked whether the two ACPMs were exclusively present as complex I subunits in *Y. lipolytica* mitochondria. We isolated intact mitochondria from the *Y. lipolytica* strains containing the tagged versions of ACPM1 and ACPM2 and analyzed soluble and membrane fractions by Western blotting (Fig. 5). As controls for the identity and purity of the individual fractions we used aconitase for the mitochondrial matrix, hexokinase for the cytosol and the complex I subunit NESM for the mitochondrial membranes. Both ACPMs were exclusively detectable in the membrane fraction but absent in the soluble fraction of mitochondria and the cytosolic fraction (PMS). This strongly suggested that all ACPM1 and ACPM2 in mitochondria from these strains were completely bound to complex I in the inner mitochondrial membrane of *Y. lipolytica*.

4. Discussion

Individual deletion of the genes for the two mitochondrial acyl-carrier protein homologues ACPM1 and ACPM2 revealed that both proteins serve important functions in *Y. lipolytica*. While in the absence of ACPM2 only trace amounts of fully assembled complex I could be detected in mitochondrial membranes, ACPM1 even seems to be indispensable for survival. This clear difference is quite remarkable considering that the mature forms of the two proteins are of very similar size [56] and exhibit a sequence similarity of 81% with 63% identical amino acids (Supplemental Fig. S3). Despite of this similarity, no or only partial cross-complementation between the two genes was observed. The fact that ACPM1 turned out to be essential for survival of *Y. lipolytica* indicated an important role for viability that could not be compensated by ACPM2. In return the role of ACPM2 in assembly and stability of mitochondrial complex I could not be taken

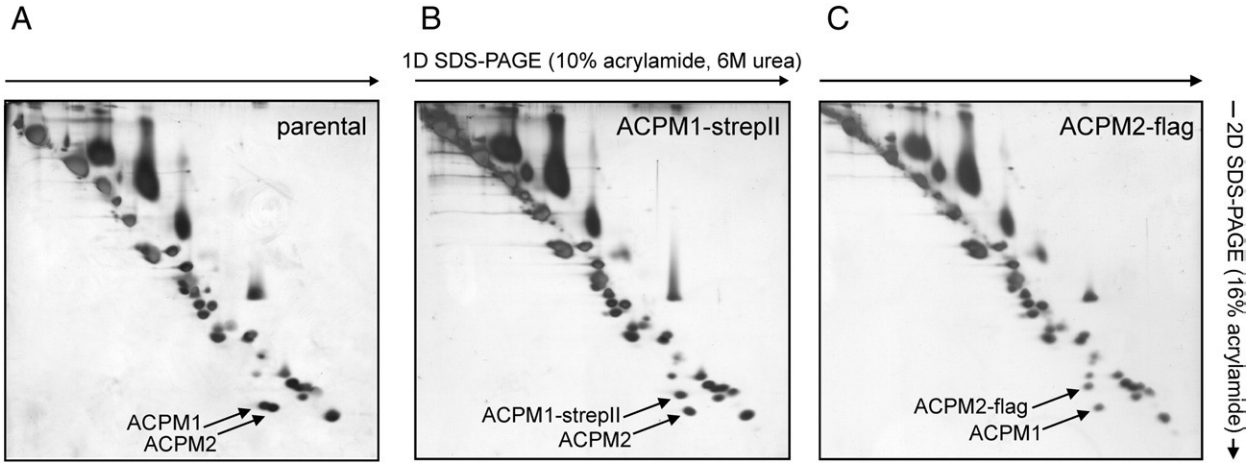

Fig. 4. Analysis of tagged versions of ACPMs in purified complex I by dSDS-PAGE. Both *ACPM* genes were deleted and replaced with plasmid-borne copies encoding tagged versions of the two proteins. Molecular mass shifts as compared to the parental strain (A) are visible in dSDS-PAGE of ACPM1-strepII (B) and ACPM2-flag (C) complex I (both marked by arrows).

over by ACPM1. Two scenarios could accommodate these findings. On one hand it could be envisioned that the two mitochondrial acyl-carrier proteins have entirely different and independent functions. Considering their very high similarity and the fact that neither of the proteins seems to contain functional domains not present in the other, this would be quite remarkable. Alternatively, the two proteins could operate together e.g. by forming a heterodimer responsible for both functions. In this case, one would have to postulate that in the deletion strains a homodimer of ACPM1 but not of ACPM2 could be formed to take over the function essential for survival and that on the other hand, both proteins would be necessary for complex I assembly and stability. Favouring a similar function for ACPM1 and ACPM2, we also found that removal of the conserved phosphopantethein carrying serines in either of the two proteins had the same effect as deletion of the complete reading frame. It should be noted here that we have shown in a previous study that both proteins carry an additional mass of about 560 Da consistent with the binding of phosphopantetheine-hydroxy-tetradecanoate [56].

The observation that the residue binding the phosphopantethein moiety is of critical importance was in line with the postulated role of the ACPMs in the lipid metabolism of mitochondria. Indeed, it has been reported that disruption of the ACPM subunit of complex I from *N. crassa* results in a four-fold increase of lysophospholipids in mitochondrial membranes [57]. However, in the *ACPM2* deletion strain of *Y. lipolytica* we could not observe any changes in the phospholipid composition of the mitochondria.

In *Y. lipolytica* strains containing complex I both ACPMs were found to be tightly and exclusively associated with complex I. Suggesting a localization near the surface of complex I, association of both proteins was not affected by attaching a C-terminal affinity-tag (Fig. 4) and no protein was detected in any mitochondrial compartment other than the mitochondrial membranes (Fig. 5). This is in striking contrast to the situation in other organisms analyzed so far. In mammalian mitochondria, where the ACPM protein was first identified as the SDAP subunit of complex I [10], the major portion of this protein is detectable in the mitochondrial matrix [28]. In *Arabidopsis* none of the three ACPM isoforms seems to bind tightly to complex I [27].

This suggests that the functionality of the ACPMs is independent of their location, i.e. whether they are bound as subunits to complex I or reside in the mitochondrial matrix. In fact, one has to conclude that the ACPM1 protein was still present and functional even in the absence of most of complex I in the *acpm2Δ* strain because *ACPM1* was found to be required for survival of *Y. lipolytica*. Notably, ACPM is also found in *S. cerevisiae* which does not contain mitochondrial complex I and is devoid of all other complex I genes [58].

While *ACPM1* seems to be an essential gene in *Y. lipolytica*, *N. crassa* carrying a disrupted *acp-1* gene–the only gene coding for an ACPM protein in this organism [59]–is viable. The functional basis of this observation remains unclear. With respect to assembly of complex I the effects are similar in both organisms. Disruption of the acp-1 gene in *N. crassa* results in lack of assembly of the peripheral arm and improper assembly of the membrane part [57] and deletion of the *ACPM2* gene in *Y. lipolytica* results in a severe assembly defect of complex I and appearance of subcomplexes (Fig. 2). In line with a role in the stabilization of complex I, at least the ACPM homologous subunit of bovine heart complex I seems to be associated with the membrane arm of complex I: treating the purified complex from this source with the chaotropic detergent lauryldimethylamine-oxide splits it into subcomplexes Iα and Iβ that both comprise part of the membrane arm and both contain SDAP [32].

Further studies will be required to understand why the deletion of the *ACPM1* gene is essential for survival and why deletion of the highly similar *ACPM2* gene is not. Differences between species seem to suggest that the exact role of ACPMs in mitochondrial fatty acid metabolism and complex I assembly may vary among species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.09.007.

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