# Brody, S., Oh, C., Hoja, U. & Schweizer, E. Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in Saccharomyces cerevisiae. FEBS Lett. 408, 217–220

**ARTICLE** *in* FEBS LETTERS · JUNE 1997

Impact Factor: 3.17 · DOI: 10.1016/S0014-5793(97)00428-6 · Source: PubMed

# Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in *Saccharomyces cerevisiae*

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Received 17 March 1997; revised version received 2 April 1997

**Abstract The yeast gene, *ACP1*, encoding the mitochondrial acyl carrier protein, was deleted by gene replacement. The resulting acp1-deficient mutants had only 5–10% of the wild-type lipoic acid content remaining, and exhibited a respiratory-deficient phenotype. Upon meiosis, the lipoate deficiency co-segregated with the acp1 deletion. The role of ACP1 in long-chain fatty acid synthesis was studied in fas1 and fas2 null mutants completely lacking cytoplasmic fatty acid synthase. When grown on odd-chain (13:0 and 15:0) fatty acids, these cells showed less than 1% of C-16 and C-18 acids in their total lipids. Mitochondrial ACP is therefore suggested to be involved with the biosynthesis of octanoate, a precurser to lipoic acid.**



*Key words:* Lipoic acid; Acyl carrier protein; ACP mutation; Mitochondrial FAS; Yeast

## 1. Introduction

Acyl carrier protein (ACP) is a characteristic, low molecular mass component of non-aggregated type II fatty acid synthase (FAS) and polyketide synthase (PKS) systems [1,2]. It acts as central carrier of substrates, intermediates and products in the FAS and PKS biosynthetic sequences. The respective acyl groups are covalently linked, as thioesters, to the 4-phosphopantetheine prosthetic group of ACP. In addition, ACP participates in bacterial phospholipid biosynthesis [3] as well as in the formation of membrane-bound oligosaccharides in *Escherichia coli* [4]. A specific ACP variant is required, in *Rhizobium leguminosarum*, for the production of a host specificity-conferring lipooligosaccharide [5]. In plant chloroplasts, multiple ACP isoforms are possibly responsible for differential and tissue-specific FAS activities [6]. Depending on the activity of distinct accessory enzymes, specific side products of bacterial fatty acid synthesis such as 3-hydroxymyristic or lipoic acid are presumed to be synthesized as ACP-bound derivatives [1]. The functional diversity of ACP in bacterial cells is underscored further by the finding that, in *E. coli*, it even appears to be required for correct chromosome partitioning during cell division [7].

Due to the integrated structure of eucaryotic type I FAS multienzymes, there is no distinct, low molecular mass ACP in the eucaryotic cytoplasm. However, a bacterial-type ACP is found in eucaryotic organelles such as chloroplasts and mitochondria [8–13]. The organellar localization of these acyl carrier proteins is manifested by the occurrence of typical organellar import sequences in front of the known ACP core sequence. In the chloroplasts of green plants, ACP is part of an organellar type II FAS system providing the rest of the cell with the required long-chain fatty acids [14]. In contrast, no significant amount of de novo long-chain fatty acid synthesis has been demonstrated in mitochondria and, therefore, the biochemical function of mitochondrial ACP (mtACP) is unknown, so far. In *Neurospora crassa*, mtACP is associated with complex I of the respiratory chain and appears to be essential for both its structural and functional integrity [15]. In yeast, mutational inactivation of mtACP also leads to a respiratory-deficient phenotype although there exists no mitochondrial NADH–ubiquinone oxidoreductase, in the organism [15]. Another type II FAS component enzyme, β-ketoacyl synthase, has recently been localized to yeast mitochondria, too [16]. Since mutants of both the mitochondrial ACP and β-ketoacyl synthase elicit the same, respiratory-deficient phenotype it is suggested that both proteins primarily represent components of a mitochondrial FAS system and only indirectly affect mitochondrial respiration. The availability of yeast mutants defective in cytoplasmic de novo fatty acid synthesis [17] together with the identification of the mtACP-encoding DNA sequence [11] provided the opportunity to study this function in more detail.

## 2. Materials and methods

### 2.1. Strains and media

The haploid *Saccharomyces cerevisiae* strains SC 1259 (MATα pra1-1 prb1-1 pre1-1 cps1-3 ura3 leu2 his112 Δfas1::LEU2), SC 1260 (MATα pra1-1 prb1-1 pre1-1 cps1-3 ura3 leu2 his112 Δfas2::LEU2), SC 1294 (MATa leu2 his3 trp1 ura3 Δacp1::URA3), SC 1332 (Matα Δfas2::LEU2 Δacp1::URA3) were from our own collection. Strains MYY 110 (MATa leu2 his3 rho$^-$; M. Yaffe, La Jolla, CA), D8-22A/2 (MATα ade4 rho°; G. Michaelis, Düsseldorf, Germany) and JS 89.16 (diploid, ura3/ura3 leu2/leu2 his3/HIS3 trp1/TRP1; H.-J. Schüller, Erlangen, Germany) were kindly provided from other sources. For lipoic acid bioassays the *E. coli* mutant JRG33/CGSC No. 4286 was used [18]. Yeast cells were routinely grown in either fatty acid-free YEP media containing 0.5% yeast extract (Difco), 0.7% peptone (Difco) and 2% sucrose. Fatty acid-supplemented YEP-FA media contained, in addition, 0.5% Brij 58 (Fluka) and 0.015% of each 13:0 and 15:0 fatty acids (Sigma). Radioactive labelling of in vivo synthesized fatty acids was performed in YEP-FA medium containing 100 μCi [1-$^{14}$C] acetic acid (NEN; 2.0 GBq/mmol in ethanol). For lipoic acid bioassays, the basal media indicated by Herbert and Guest [18] was used with the additions listed by Sulo and Martin [19]. Respiratory competence of yeast strains was examined on YPG-FA solid media containing 1% yeast extract, 2% peptone, 3% glycerol, 1% Tween-40 and 2% agar.

### 2.2. Plasmids and DNA manipulations

The *S. cerevisiae ACP1* gene was isolated, as 2.7 kb *Xba*I/*Bam*HI fragment, from the plasmid pLMY 141 which was kindly provided by

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*Abbreviations:* FAS, fatty acid synthase; mtACP, mitochondrial acyl carrier protein; PKS, polyketide synthase; PCR, polymerase chain reaction


*PII* S0014-5793(97)00428-6

L. Mattheakis, Palo Alto, CA [11]. After subcloning into pUCBM (Boehringer, Mannheim) the ACP1 coding sequence was eliminated, together with its surrounding DNA, as a 0.87 kb *Cla*I/*Spe*I fragment and replaced by the yeast *URA3* gene. This was derived from YDp-U [20] with intermediate subcloning into pBluescript (Stratagene). Subsequently, the *Xba*I/*Bam*HI DNA fragment containing the acp1::URA3 replacement was incorporated into the yeast genome by integrative transformation of the diploid strain, JS 89.16 [21]. After sporulation, the acp1 deletion mutant, SC 1294, was identified by its URA3 prototrophy and subsequently verified by PCR analysis. PCR analysis of ACP1 wild-type and mutant alleles was performed using the oligonucleotides 5′-GATGAAGTCAGGATTTGTATC-3′ (ACP-B) and 5′-ACGATCTTTCAACGCTGCCAC-3′ (ACP-A) as primers. The respective products eventually exhibiting a length difference of 0.5 kb were inspected by agarose gel electrophoresis.

*2.3. Lipoic acid analysis*

Yeast cultures were grown for 24–36 h at 30°C in 25 ml of YEP media under occasional shaking. The harvested cells were thoroughly washed and subsequently hydrolyzed with 1.0 ml of 9 N $H_2SO_4$ for 2 h at 120°C. The hydrolysate was neutralized with 10 N NaOH and subsequently centrifuged for 30 min at 15000×*g*. Remaining insoluble material was removed by filtration through glass wool. Aliquots of the filtrate were used for lipoic acid bioassays employing the lipoic acid requiring *E. coli* strain, JRG33/CGSC 4286 [18]. Assays were conducted with duplicates of two different aliquots from each sample. The aliquots were chosen to fit into the initial, linear range of the lipoic acid standard plot. Extracts containing very low levels of lipoic acid were re-assayed in the presence of known amounts of lipoic acid thereby excluding the interference of inhibitors. The hydrolysis and storage procedures used were tested with known amounts of lipoic

Table 1
Meiotic co-segregation of the acp1 deletion with lipoic acid deficiency

| | Tetrad No. | Spore No. | ACP1 | URA3 | Lipoic acid (ng/g wet weight) |
|---|---|---|---|---|---|
| Complete tetrad | 4 | A | Δ | + | 48 |
| | | B | Δ | + | 15 |
| | | C | + | − | 270 |
| | | D | + | − | 140 |
| ACP-negative spores from incomplete tetrads | 2 | A | Δ | + | 16 |
| | 8 | B | Δ | + | 15 |
| | 9 | C | Δ | + | 22 |
| | 10 | D | Δ | + | 17 |
| | 17 | D | Δ | + | 20 |
| | 18 | C | Δ | + | 17 |
| | 20 | B | Δ | + | 14 |
| | 22 | A | Δ | + | 11 |
| | 23 | B | Δ | + | 7 |
| | | | | average | 18 |
| ACP-positive spores from incomplete tetrads | 2 | D | + | − | 340 |
| | 6 | A | + | − | 320 |
| | 8 | A | + | − | 310 |
| | 8 | C | + | − | 190 |
| | 9 | A | + | − | 210 |
| | 9 | D | + | − | 220 |
| | 10 | A | + | − | 170 |
| | 10 | C | + | − | 180 |
| | 11 | A | + | − | 490 |
| | 11 | D | + | − | 130 |
| | 13 | A | + | − | 180 |
| | 13 | B | + | − | 220 |
| | 14 | B | + | − | 530 |
| | 14 | D | + | − | 330 |
| | 15 | C | + | − | 210 |
| | 17 | A | + | − | 160 |
| | 17 | C | + | − | 190 |
| | 18 | A | + | − | 480 |
| | 19 | A | + | − | 150 |
| | 20 | C | + | − | 440 |
| | 21 | A | + | − | 160 |
| | 21 | C | + | − | 160 |
| | 21 | D | + | − | 460 |
| | 23 | C | + | − | 450 |
| | 26 | A | + | − | 290 |
| | 26 | B | + | − | 220 |
| | 27 | C | + | − | 310 |
| | 28 | C | + | − | 440 |
| | | | | average | 280 |
| Controls | | | | | |
| SC 1216 | (ACP1, rho$^+$) | | | | 275 |
| MYY 110 | (ACP1, rho$^-$) | | | | 250 |
| D8-22A/2 | (ACP1, rho$^0$) | | | | 250 |
| SC 1294 | (Δacp1, rho$^-$) | | | | 34 |

Diploids of the cross SC 1294×SC1296 were sporulated and subjected to tetrad analysis. Upon outgrowth on YEP-FA agar, viable spores were screened for respiratory competence and growth on uracil-free SC-media. Wild-type and deleted ACP1 alleles were identified by PCR analysis. Lipoic acid bioassays were performed as described in Section 2.

acid and proved to show excellent recoveries. Lipoic acid concentrations were correlated to cell densities ($OD_{600}$) of the individual yeast cultures. It was found that $OD_{600}$=1.0 corresponded to 1.8 mg of wet weight yeast cells.

*2.4. Fatty acid analyses*

The fatty acid composition of total yeast lipids was determined using two different procedures depending on whether the cells had been radioactively labelled (B) or not (A).

*2.4.1. Procedure A.* Lipids were extracted with chloroform/methanol according to Folch et al. [22]. Subsequently, fatty acids were converted into methyl esters by treatment with methanol/$BCl_3$ and analyzed on a 5890A Hewlett Packard gas chromatograph equipped with a HP5 capillary column (nonpolar, 0.2 mm×25 m).

*2.4.2. Procedure B.* Freeze-dried cells were hydrolyzed with 2 N HCl in methanol at 90°C for 5 h. The methyl esters were extracted with hexane, the hexane layer was dried over anhydrous $MgSO_4$ and then evaporated to dryness. The methyl esters were redissolved in 50 μl $CS_2$ and analyzed by radio-gas chromatography as stated previously [23].

## 3. Results and discussion

*3.1. Inactivation of the Saccharomyces cerevisiae ACP1 gene*

The putative mtACP coding sequence, ACP1, present on *S. cerevisiae* chromosome XI was replaced by the yeast *URA3* gene using the one-step gene disruption method of Rothstein [21]. Chromosomal integration of the replacement construct into the diploid strain, JS 89-16, was verified by Southern blotting with both URA3 and ACP1 hybridization probes. Sporulation of the heterozygous diploid resulted in viable spores containing the acp1 null mutation. One of the acp1 null mutants, SC 1294, was backcrossed to the ACP1-positive strain, SC 1216, and after renewed sporulation, meiotic segregation of the defective acp1 allele was followed by tetrad analysis. Even though the number of complete tetrads comprising four viable spores was low, the acp1 mutation clearly segregated 2:2 as was verified by both the URA3 phenotype and the ACP-1-specific PCR fragment patterns of the spores. Independent of the complexity and composition of the growth media used, the acp1 null mutants were respiratory deficient exhibiting the well-known pleiotropy of rho-negative petites [24]. Neither individual fatty acids of chain lengths between 8 and 18 carbon atoms nor a mixture of the fatty acids contained in wild-type yeast lipids, were able to suppress this phenotype (data not shown). The acp1-induced rho-negative character exhibited a moderate suppressivity since not only the acp1-defective segregants but also about 50% of the ACP1-positive spores in the above cross were rho-negative petites. In accordance with their pleiotropic respiratory deficiency, the low-temperature cytochrome spectra of acp1 mutants indicated, apart from a marginal amount of cytochrome *c*, the loss of essentially all mitochondrial cytochromes (data not shown).

*3.2. Fatty acid synthesis in acp1 null mutants*

In yeast, the cytoplasmic type I fatty acid synthase is considered to be responsible for the bulk of cellular fatty acid synthesis [25]. Nevertheless, the existence of a mitochondrial FAS system contributing to this process to a certain extent could not be excluded, a priori. In order to identify any possible products of this mitochondrial system, the fatty acid profile of an acp1-negative mutant was compared, in the background of a cytoplasmic FAS deficiency (Δ fas2), to that of an ACP1-positive strain. Supplementation of the fatty acid-requiring mutants with the odd-chain-length acids, tridecanoic (13:0) and pentadecanoic (15:0) acid was expected to uncover any even-chain-length fatty acids synthesized, de novo, by the mitochondrial FAS system. As is indicated in Table 1, the relative content of even-chain-length fatty acids in the fas2 mutants was extremely low (1–2%), under these conditions, and proved to be independent of a functional ACP1 gene. This could be due to traces of even-chain-length fatty acids being present in the growth media or to the introduction of these acids during the workup procedure. Experiments employing the fas1-mutant cells yielded a content of below 0.3% even-chain fatty acids. No significant labelling of these traces of even-chain acids by [$^{14}$C]acetate could be detected (Table 1). On the other hand, enough label was incorporated into the odd-chain fatty acids (C-15 and C-17) to allow detection of even-chain fatty acid labelling at the 0.5–1% level (data not shown).

These results strongly argue against the significance of mitochondrial ACP and of the putative mitochondrial FAS system for overall yeast fatty acid synthesis. It should be stressed, however, that uncommon fatty acids such as hydroxy-acids or shorter-chain-length homologues with less than 12 carbon atoms were not included in this analysis. Apart from fatty acid synthesis, phospholipid synthesis was also found to be qualitatively unaffected by the yeast acp1 mutation (data not shown). Similar results have recently been reported by Harington et al. [16] for the yeast mitochondrial β-ketoacyl synthase mutant, cem1, and by Schneider et al. [15] for the mitochondrial ACP mutants of *Neurospora crassa* and yeast. In contrast to the above in vivo studies on yeast mitochondrial FAS, [$^{14}$C]malonic acid was incorporated, in vitro, into long-chain fatty acyl ACP esters when incubated with purified mi-

Table 2
Effect of ACP1 on de novo fatty acid synthesis in FAS-deficient yeast strains

| Strain | Fatty acids (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 13:1 | 13:0 | 15:1 | 15:0 | 16:1 | 16:0 | 17:1 | 17:0 | 18:1 | 18:0 | odd | even |
| SC 1260 (Δfas2 ACP1) | – | 24 | 7 | 45 | 0.3 | – | 7 | 15 | – | 15 | 98 | 1.8 |
| SC 1332 (Δfas2 Δacp1) | – | 41 | 17 | 35 | 0.5 | – | 4 | 2 | – | 0.5 | 99 | 1.0 |
| SC 1259 (Δfas1 ACP1) | 3 | 27 | 28 | 28 | 0.2 | – | 10 | 3 | – | 0.1 | 99 | 0.3 |

Strains SC 1260 and SC 1332 were grown in YEP media supplemented with 0.5% Brij 58 and 0.03% of each tridecanoic (13:0) and pentadecamoic (15:0) acid. Fatty acid composition was analyzed according to procedure A.
Strain SC 1259 was grown in YEP-FA media containing per litre, 100 μCi sodium [1-$^{14}$C] acetate (2.0 GBq/mmol). Radioactive fatty acids were analyzed according to procedure B.
All cultures were grown on a shaker at 30°C and harvested at an $OD_{600}$ between 4 and 5.

tochondria from *Neurospora* [23] or plants [28]. It remains to be shown whether these differences are due to the use of different organisms or to the different experimental conditions employed.

### 3.3. *Lipoic acid content of wild type and acp1-deficient yeast cells*

The failure to detect any long-chain fatty acyl derivatives, in vivo, as potential products of the yeast mitochondrial FAS system prompted us to concentrate on possible shorter-chain-length products. Among these, octanoic acid and its derivative, lipoic acid, were considered as the most prominent candidates since in earlier studies of Mikolajczyk and Brody [23,26], octanoic acid was identified as one of the major products associated with *N. crassa* mtACP. Furthermore, data recently reported by Morris et al [27] suggested that in *E. coli*, lipoic acid biosynthesis implies ACP-bound intermediates. Thus, the lipoic acid content of yeast and its correlation to the functioning of mitochondrial ACP was investigated. For this, a large collection of ACP-positive and acp-1-defective segregants from a ACP1/acp1 heterozygous cross were analyzed. The data obtained from one complete tetrad and from a large number of vital spores provided by a series of incomplete tetrads are listed in Table 2. Among a total of 11 acp1-negative and 30 ACP1-positive spores investigated, a strict correlation between the functional status of ACP1 and the cellular lipoic acid concentration was observed. The average lipoic acid content of acp1-defective cells was about 10–20-fold lower than that of ACP1-positive cells. The residual lipoic acid found in the acp1 deletion mutants is likely to originate from the growth medium which contained about 60 ng/ml of the cofactor. As expected, the acp1-defect being determined by PCR analysis co-segregated in all cases with the URA3 wild-type allele which had been used for insertional inactivation of ACP1. As is evident from the normal lipoic acid level of the ACP1-positive petite mutants included in this study as a control, the rho-negative character, per se, is obviously not responsible for the observed lipoic acid deficiency. Therefore, it is concluded that mitochondrial ACP, together with other components of the putative mitochondrial type II FAS system, is involved in lipoic acid biosynthesis. Very recently, Wada et al. [28] came to the same conclusion when studying the incorporation of [$^{14}$C]malonic acid into the H-protein of mitochondria from *Neurospora* and plants.

In yeast, the addition of lipoic acid to the growth medium cannot compensate for the block in endogenous lipoic acid synthesis. Other than in *E. coli*, free lipoic acid is either not activated in yeast, or it is not taken up into the mitochondrial compartment. Recently, Sulo and Martin [19] working with a lipoic acid-deficient yeast mutant affected in the lipoate synthase gene, *LIP5*, came to the same conclusion. Both acp1 and lip5 defective yeast mutants exhibit the same respiratory-deficient and non-supplementable phenotype. The role of lipoic acid as an essential cofactor of the two α-ketoacid dehydrogenase reactions in the tricarboxylic acid cycle sufficiently explains the observed respiratory defect. The loss of mitochondrial DNA as the consequence of a nuclear pet-mutation is a well-known phenomenon and may be correlated to the low-energy status of the mutant mitochondria. Considering the lack of NADH–ubiquinone oxidoreductase in yeast mitochondria, mtACP is excluded from functioning as an essential structural component of this complex, as it is reported for *N. crassa* mtACP [15]. A possible involvement of yeast mtACP in 3-hydroxymyristic acid biosynthesis as was postulated earlier for *N. crassa* mtACP [23,26] has not been examined, in this study. Thus, it remains to be investigated whether mitochondrial ACP still serves additional functions other than the biosynthesis of lipoic acid and its putative precursor, octanoic acid.

*Acknowledgements:* This work was supported by the Deutsche Forschungsgemeinschaft.

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