

The Fatty Acid Synthase of the Basidiomycete *Omphalotus olearius* Is a Single Polypeptide

Luis Antelo^{a,*}, Angela Schlipp^a, Carolin Hof^a, Katrin Eisfeld^a, Holger Berg^b, Till Hornbogen^b, Rainer Zocher^{b,†}, and Heidrun Anke^a

^a IBWF, Institut für Biotechnologie und Wirkstoff-Forschung e. V., Erwin-Schrödinger-Str. 56, D-67663 Kaiserslautern, Germany. E-mail: antelo@ibwf.de

^b Technische Universität Berlin, Fakultät II – Institut für Chemie, AG Biochemie und Molekulare Biologie, Franklinstr. 29, D-10587 Berlin, Germany

* Author for correspondence and reprint requests

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Fatty acids are essential components of almost all biological membranes. Additionally, they are important in energy storage, as second messengers during signal transduction, and in post-translational protein modification. *De novo* synthesis of fatty acids is essential for almost all organisms, and entails the iterative elongation of the growing fatty acid chain through a set of reactions conserved in all kingdoms. During our work on the biosynthesis of secondary metabolites, a 450-kDa protein was detected by SDS-PAGE of enriched fractions from mycelial lysates from the basidiomycete *Omphalotus olearius*. Protein sequencing of this protein band revealed the presence of peptides with homology to both α and β subunits of the ascomycete fatty acid synthase (FAS) family. The FAS encoding gene of *O. olearius* was sequenced. The positions of its predicted 21 introns were verified. The gene encodes a 3931 amino acids single protein, with an equivalent of the ascomycetous β subunit at the N-terminus and the α subunit at the C-terminus. This is the first report on an FAS protein from a homobasidiomycete and also the first fungal FAS which is comprised of a single polypeptide.

Key words: *Omphalotus olearius*, Fatty Acid Synthase, Basidiomycete

Introduction

Fatty acids are essential components of life. They have very diverse biological functions in contrast to their structural simplicity. Fatty acids are constituents of neutral or polar lipids [except for archaea (Schweizer and Hofmann, 2004)] playing a role in the integrity and dynamics of biological membranes. They are also present as side chains in some coenzymes, secondary metabolites, as well as in post-translational modifications of proteins. Furthermore, they represent an important form of energy storage and, as parts of second-messenger molecules, are involved in the modulation of gene expression (Schweizer and Hofmann, 2004; Maier *et al.*, 2006).

Synthesis of fatty acids is present in almost all organisms. *Malassezia globosa* is one exception and its apparent lack of fatty acid synthase genes is compensated by the abundance of genes encoding hydrolases that might be involved in the re-

lease of fatty acids from lipids in its environment (Xu *et al.*, 2007).

De novo synthesis of fatty acids involves a set of conserved reactions for the cyclic elongation of precursors and requires a multienzyme complex. First, there is the acyl transfer from acetyl and malonyl coenzyme A to the prosthetic phosphopantetheine group of the acyl carrier protein (ACP) by an acetyl transferase (AT) and malonyl transferase (MT). The enzyme-bound substrates are then condensed by the ketoacyl synthase (KS) by decarboxylation of the malonyl to acetoacetyl-ACP, which is further reduced by a ketoacyl reductase (KR), dehydrated by a dehydratase (DH) and reduced by an enoyl reductase (ER) to form butyryl-ACP. These reactions are repeated six times. The mammalian fatty acid synthases (FASs) release the newly formed fatty acid by a thioesterase (TE) whereas in fungi it is transferred back from the ACP to coenzyme A by an MT (Jenni *et al.*, 2006). The fatty acids are further elongated by elongases (Rawlings, 1998).

† Rainer Zocher deceased on March 26, 2008.

Although the synthesis of fatty acids is similar in all organisms, three different molecular architectures of FASs have developed during evolution. The type II FASs, present in most bacteria and plants, consist of independent proteins for the different enzymatic activities encoded by discrete genes. In contrast, type I FASs are highly integrated multienzymes, which contain the various catalytic domains in one or two polypeptide chains. The type I FASs can be subdivided in two groups according to the domain organization and subunit stoichiometry (Schweizer and Hofmann, 2004). Animal FASs (type Ib) are α_2 dimers with the domain sequence KS-AT-DH-ER-KR-ACP-TE, and have an asymmetric X-shape. In ascomycetes, FASs are barrelshaped $\alpha_6\beta_6$ oligomers with the domain sequence ACP-KR-KS-PPT for the α subunit (PPT for phosphopantetheinyl transferase) and AT-ER-DH-MT for the β subunit (Fig. 1) (Lynen, 1980; Jenni *et al.*, 2006).

There are other examples of multienzyme complexes grouped in single polypeptides in fungi. Type I polyketide synthases (PKSs) are very large multifunctional proteins with individual functional domains, while the type II PKSs, present only in bacteria, are built up by individual proteins (Cox, 2007). Fungal nonribosomal peptide synthetases (NRPSs) usually consist of a single polypeptide containing all modules necessary for the synthesis of the corresponding secondary metabolite, whereas their bacterial counterparts, in most cases, are distributed on more than one peptide chain (Doekel and Marahiel, 2001).

During our work on proteins involved in the biosynthesis of secondary metabolites a 450-kDa protein was detected. To our surprise, this large protein was found to be a fatty acid synthase and

therefore, it is involved in primary metabolism. Since such large proteins are not common among fatty acid synthases from fungi, an attempt was made to further characterize this enzyme. Here we describe the partial purification, microsequencing and identification of the FAS protein of the homobasidiomycete *Omphalotus olearius*, as well as the identification of the FAS gene and the analysis of its cDNA.

Material and Methods

Organisms, media, and cultivation conditions

The mycelial culture of *O. olearius* TA90170 was obtained from a spore print of a fruiting body attached onto the lid of a Petri dish. Basidiospores were collected on YMG solid medium (4 g/l yeast extract; 10 g/l malt extract; 4 g/l glucose; pH 5.5) containing penicillin (100 mg/l), streptomycin (200 mg/l) and 1.5% of agar. The culture resulting from the germinated spores is deposited at the IBWF, Kaiserslautern, Germany. The strain was cultivated and maintained in YMG medium. Fermentations up to 2 l were carried out in 5-l Erlenmeyer flasks with agitation (120 rpm) at 28 °C. Larger fermentations were carried out in a Biostat A-20 fermenter (Braun, Melsungen, Germany) containing 20 l of YMG medium at 28 °C with aeration (3 l/min) and agitation (120 rpm).

The *Escherichia coli* strain DH5 α (Gibco BRL, Rockville, MD, USA) was used for cloning and plasmid propagation.

Enzyme purification

All operations were carried out at 4 °C. Lyophilized mycelium of *O. olearius* (10 g) was homog-

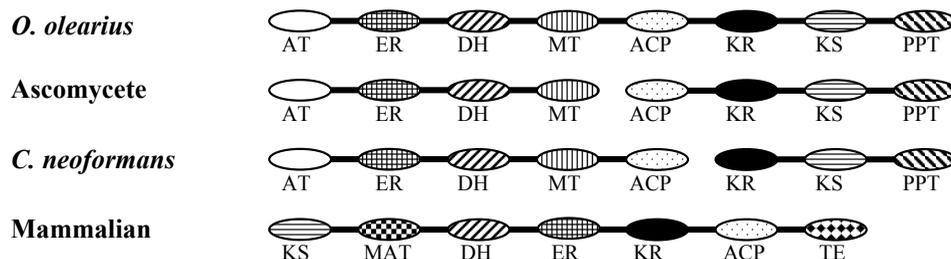


Fig. 1. Diagrammatic comparison of domain organization of *O. olearius*, ascomycete, *C. neoformans*, and mammalian type I FAS. KS, β -ketoacyl synthase; AT, acetyl-CoA-ACP transacylase; MT, malonyl-CoA-ACP-transacylase; DH, dehydratase; ER, β -enoyl reductase; KR, β -ketoacyl reductase; ACP, acyl carrier protein; PPT, phosphopantetheinyl transferase; MAT, malonyl-CoA-acetyl-CoA-ACP transacylase; TE, thioesterase.

enized in a mortar with liquid nitrogen and extracted with buffer A [200 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 8.0, 625 mM NaCl, 10% glycerol, 1 mM EDTA, 20 mM dithiothreitol, 5 mM benzamidine, 5 mM phenylmethylsulfonylfluoride] by gentle stirring for 1 h. The extract was centrifuged for 20 min at 15,000 rpm using an SS-34 rotor in a Beckmann JM-21 centrifuge. A solution of polyethylene imine (8.7%, pH 7) was added to the supernatant to give a final content of 0.3%; the mixture was kept on ice for 10 min. The extract was centrifuged for 10 min at 15,000 rpm. The supernatant was brought to a final ammonium sulfate saturation of 70% by adding saturated ammonium sulfate solution (pH 8.0), and was centrifuged for 10 min at 15,000 rpm. The resulting pellet was resuspended in buffer B (15% w/v glycerol, 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 4 mM DTE, 1 mM benzamidine, 1 mM PMSF) and applied to a Ultrogel AcA34 (Pall, East Hills, NY, USA) size exclusion column (50 cm × 3.0 cm). The proteins were eluted with buffer B and 5-ml fractions were collected. 2 ml of the fractions were precipitated with sodium deoxycholate-trichloroacetic acid according to Bensadoun and Weinstein (1976) and separated by a minifuge (Eppendorf, Hamburg, Germany). The resulting pellets were resuspended in sample buffer according to Piccioni *et al.* (1982) and applied to SDS-PAGE.

Protein determination

Protein concentrations were determined by using a modified Bradford procedure with bovine serum albumin as a standard (Bradford, 1976).

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Gels contained 4% acrylamide. The relative molecular mass of the protein was determined from its mobility related to those of the standard proteins cyclosporin synthase (1,700 kDa) and enniatin synthase (350 kDa) (Sigma, high molecular weight markers). Cyclosporin synthase was isolated from *Tolipocladium inflatum* mycelium and enniatin synthase from *Fusarium scirpi* mycelium as previously described (Lawen and Zocher, 1990; Zocher *et al.*, 1982). Gels were stained with Coomassie Brilliant Blue.

In-gel tryptic digestion

A stained protein band corresponding to a molecular mass of approximately 450 kDa was excised from the gel, cut into small pieces and destained with methanol/acetic acid/water (40:10:50). The gel pieces were incubated in acetonitrile for 10 min, the solvent was removed, and the gel pieces were dried in a vacuum centrifuge for 3 min. Reduction was performed by addition of 10 mM DTT in 100 mM NH_4HCO_3 for 30 min at room temperature. The solution was removed and 100 mM iodoacetamide in 100 mM NH_4HCO_3 was added during 30 min at room temperature for alkylation of the thiol groups. The gel pieces were dehydrated with acetonitrile and rehydrated with 100 mM NH_4HCO_3 twice. After a last dehydration step with acetonitrile, 30 μl of 20 ng/ μl trypsin (Sigma) in 50 mM NH_4HCO_3 were added to the gel pieces, and incubation was carried out overnight at 37 °C. After enzymatic digestion, 30 μl of 50 mM NH_4HCO_3 were added, the supernatant was collected, and the gel pieces were extracted three times with acetonitrile/formic acid/water (50:5:45). The collected samples were pooled and dried in a vacuum centrifuge. The peptides were dissolved in 0.1% formic acid and desalting was done using μC18 -Ziptips (Millipore, Eschborn, Germany) following the manufacturer's instructions.

Protein sequencing

The desalted sample was loaded into a nano-electrospray capillary (Waters Micromass, Manchester, UK). Measurements were performed in an electrospray (ESI) Q-TOF tandem mass spectrometer (Q-ToF 2, Waters Micromass). Peptides were fragmented in the collision chamber, using energies of 20–60 eV and argon as the collision gas. Peptide fragments were detected using a time of flight (ToF) analyzer. Data acquisition and processing were conducted using the MassLynx software package (Waters Micromass).

DNA and RNA isolation

For genomic DNA isolation, lyophilized mycelium of *O. olearius* was ground to a fine powder. Extraction buffer (1 M Tris-HCl, pH 8.0, 0.1 mM EDTA, 1% SDS, 200 $\mu\text{g}/\text{ml}$ proteinase K, 100 $\mu\text{g}/\text{ml}$ DNase-free RNase) was added and the mixture incubated for 50 min at 56 °C. The mixture

was extracted once with phenol and three times with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) followed by a final extraction with chloroform/isoamyl alcohol (24:1 v/v). To remove proteins, 0.1 vol of 5 M potassium acetate was added to the supernatant, the mixture was incubated for 1 h on ice and centrifuged. DNA was precipitated with 2 vol of ethanol and washed with 70% ethanol.

RNA was isolated by grinding lyophilized mycelium under liquid nitrogen. The fine powder was extracted with the Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's instructions. DNase treatment was performed by dissolving the RNA pellets in 200 μ l buffer (0.1 M sodium acetate, 5 mM MgSO₄, pH 5.0) containing 2 units of DNase and incubating for 20 min at room temperature, followed by a final phenol/chloroform extraction. RNA was precipitated with 2 vol ethanol and dissolved in 500 μ l of RNase-free water. mRNA was isolated from total RNA using the PolyAtract mRNA Isolation System (Promega).

Sequence analysis of FAS1

A genomic fosmid bank of *O. olearius* was constructed using the EpiFOS™ Fosmid Library Production Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions. A total of 1800 fosmid clones was examined by sequencing the 5' termini of the inserts. One clone exhibited homology to fungal fatty acid synthase genes. This gene fragment was used as a DIG-labelled probe (Roche, Basel, Switzerland) for screening of the fosmid bank. Three fosmids containing the FAS fragment were identified. The complete FAS gene was analyzed by sequencing

the subclones of the fosmid inserts which hybridized with the probe.

To identify the introns contained in the FAS1, cDNA was generated from *O. olearius* mRNA using the OneStep RT-PCR Kit (Quiagen, Hilden, Germany). Gene-specific primers which covered the genomic sequence of the FAS1 were used for amplification of the cDNA fragments which were subsequently compared with the genomic sequence using Seqman (DNASTAR, Madison, WI, USA).

Results and Discussion

Protein purification and sequencing

During our search for proteins involved in the secondary metabolism of *O. olearius*, we enriched a large protein that later was identified as a fatty acid synthase (FAS). The FAS of *O. olearius* was partially purified from the mycelium after ammonium sulfate precipitation and size exclusion chromatography. A protein band, with an apparent molecular weight of 450 kDa, was visualized after SDS-PAGE (Fig. 2). This protein band was excised from the gel and protein microsequencing was performed. A total of 23 peptides could be successfully sequenced (Table I), most of them with homology to either the β or the α subunits of known FAS from ascomycetes. Since electrophoresis was carried out under denaturing conditions, all peptides should belong to the same polypeptide chain. This finding correlates well with the putative FAS of the basidiomycetes *Ustilago maydis* and *Coprinus cinereus*, in which the α and β subunits are predicted to be condensed in a single polypeptide. These putative proteins had been derived from genome sequencing projects,

Table I. List of peptides sequenced from the FAS of *O. olearius*. Proper leucine or isoleucine have been corrected by comparison with the sequence of the gene.

Sequenced peptides from <i>O. olearius</i>	Equivalent position in ascomycetous FAS
VVVG YEGDVPSSVTIYGAAR; YIPNLIATPFEVSR; EYASIIYDQTSSPR; VPDG VGVSLNSLYINPR; SIFPESIDGDLK; LLTDLEGEELWK; EFDDTVFNLPK; ALIQNLLR; SYAGDASSVPVVDYLAGAR; PNALPLPVGISR; SIFPESIDGDLK; SEANIVSVVNSPAGK; HGNPVLAYLQR	β subunit
KLPGGFNNSAVK; ALYEGVVR; LPDLADITTR; LIQTVDVLPK; FNFPTLESSQSLSDLSTLR; LIEPELFR; YGLPEDILAQTDR; TALELGAPIR; TPGNAVPIIAQK; YLF GALDPAYYEAYK	α subunit

and no protein evidence has been presented so far. Thus, this is the first report, at the protein level, on a single polypeptide for the FAS in basidiomycetes.

Molecular characterization of the FAS gene of *O. olearius*

The sequence of the FAS gene of *O. olearius* (FAS1) (accession EU707901) was determined by overlapping and assembling the sequences of fosmide subclones, and it was found to have a length of 12962 bp. The entire cDNA sequence was analyzed by overlapping reverse transcribed PCR using RNA in order to determine the position and length of introns, as well as confirming the presence of a single mRNA. The comparison of cDNA and genomic sequences revealed the existence of 21 introns ranging in size from 50 to 67 bp, which are distributed over the whole length of the gene (Fig. 3). The 5' and 3' boundaries in most cases match the consensus sequences for fungal introns (Balance, 1986; Kupfer *et al.*, 2004). A high number of introns had previously been found in other genes of *O. olearius* (Welzel *et al.*, 2005) and in basidiomycetous genes in general (Larrondo *et al.*, 2004; Martinez *et al.*, 2004). Comparison of the relative position of the introns of FAS1 and the putative FAS gene of *C. cinereus* shows a high degree of conservation. The *O. olearius* gene contains three additional introns. Conserved intron locations among members of gene families are not unusual and, for example, have also been shown

for laccase genes and P450 multigene families (Hoegger *et al.*, 2004; Doddapaneni *et al.*, 2005).

The predicted protein encoded by the FAS1 gene is a very large protein comprising 3931 amino acids with a mass of approximately 431 kDa, which is in agreement with the detected 450-kDa protein band. It contains all peptides sequenced from the purified protein band (Table I), confirming that the FAS comprises a single polypeptide. The peptides sequenced represent 7.5% of the total protein sequence. The FAS1 possesses the highest similarity to the hypothetical FAS from *C. cinereus* (accession XP_001836417) with 75% amino acid identity, indicating that FASs are highly conserved proteins. This has also been demonstrated for the FASs of ascomycetes. Among the FAS peptide sequences from *Penicillium patulum*, *Candida albicans*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Aspergillus nidulans* FasA proteins share 60% identity and FasB proteins exhibit 70% identity (Brown *et al.*, 1996).

During fatty acid synthesis, substrates are tethered to the prosthetic phosphopantetheine group of the ACP. Elongation of the growing fatty acid chain operates by directional shuttling of the intermediates to active centres of the KS domain (Leibundgut *et al.*, 2007). The conserved motifs of this interaction could be identified in the *O. olearius* FAS as aa S2251, carrying the phosphopantetheine group, and aa C3347, H3584, H3620 and K3625, mediating the function of the KS domain (Leibundgut *et al.*, 2007; Rawlings, 1998).

The domain organization of the FAS1 protein (Fig. 1) is identical to those of the putative FAS protein of the basidiomycetes *C. cinereus* and *U. maydis*, indicating that FASs from basidiomycetes generally differ from those of ascomycetes. Their domain organization (AT-ER-DH-MT-ACP-KR-

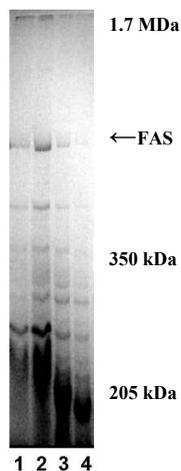


Fig. 2. SDS-PAGE analysis of four fractions from size exclusion chromatography (lanes 1–4). Molecular weight markers are indicated at the right-hand side. The putative FAS of 450 kDa size is indicated with an arrow. The 450-kDa protein band was excised from the gel and subjected to protein sequencing.

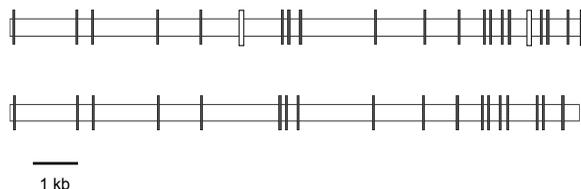


Fig. 3. Diagrammatic representation of intron distribution in the fatty acid synthases of *O. olearius* (top) and *C. cinereus* (bottom). Intron positions of both FAS genes are conserved, with the *O. olearius* gene containing three additional introns (empty bars).

KS-PPT), however, is similar to those of the latter (Schweizer and Hofmann, 2004) provided that the β subunit (AT-ER-DH-MT) is placed at the N-terminus and the α subunit (ACP-KR-KS-PPT) is placed at the C-terminus of the FAS of the ascomycete type. Actually, it has been shown through the elucidation of the crystal structure of the FAS of *Thermomyces lanuginosus* (Jenni *et al.*, 2007) that the N-terminus of the α chain and the C-terminus of the β chain are very close in space (8 Å apart).

Interestingly, the FAS of the basidiomycete *Cryptococcus neoformans* is composed of two subunits (Chayakulkeeree *et al.*, 2007). But in this case the domain distribution differs from that of

the ascomycetes: the β subunit contains at its C-terminus the ACP domain, which is the first domain at the N-terminus of the α subunit in the ascomycetes (Fig. 1).

Due to the similarity in domain organization of the *O. olearius* FAS to the ascomycetous FASs it is likely that the *O. olearius* FAS forms a homohexamer equivalent to the $\alpha_6\beta_6$ dodecamer described for ascomycetes (Jenni *et al.*, 2007).

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