Studies on the effect of an heterologous fatty acid-binding protein on acyl-CoA oxidase induction in *Saccharomyces cerevisiae*

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The participation of fatty acid-binding protein (FABP) in the induction of peroxisomal β -oxidation of fatty acids was investigated *in vivo* in an heterologous system. Bovine heart FABP was expressed in *Saccharomyces cerevisiae* under the control of two different promoters: a constitutive one and an oleic acid-inducible one. Constructs were introduced into yeast cells on

multicopy and integrating plasmids. The heterologous FABP was present in yeast cells in two isoforms having pI values of about 5 and was able to bind oleic acid. The heterologous FABP had no significant effect on acyl-CoA oxidase activity at various concentrations of the inducing agent.

INTRODUCTION

Proliferation of peroxisomes coupled to the induction of peroxisomal β -oxidation of fatty acids has been intensely studied in recent years, mostly in mammalian and yeast cells (for reviews see [1–3]). The enzymic reactions and the structures of this organelle are similar in both systems. However, they respond to different inducing agents. In mammalian cells, peroxisome proliferation is typically induced by hypolipidaemic drugs, toxic compounds (e.g. herbicides) and temperature shifts [4–6], whereas in yeast it may be caused by the presence of *n*-alkanes (*Candida tropicalis*, *Yarrowia lipolytica*) [7] or long-chain fatty acids (*Saccharomyces cerevisiae*) [8,9]. Transferring the glucose-grown *S. cerevisiae* to an oleic acid-containing medium results in the synthesis of all peroxisomal β -oxidation enzymes and peroxisome-localized catalase A.

Although numerous details concerning peroxisome structure and its enzymic components are known [3,10–12], there is almost no data concerning the uptake of oleic acid from the environment and its transport to the nucleus where it could act as a signal for the induction of β -oxidation enzymes and proliferation of peroxisomes. The mechanism for delivering of oleic acid to the peroxisome, which is the site of its metabolism, is also not fully understood.

Several models for intracellular transport of fatty acids have been proposed: (i) by diffusion; (ii) by lateral movement in a continuum of membranes [13]; and (iii) by specific carrier proteins [14,15]. Most of the experimental data support the participation of fatty acid-binding proteins (FABPs) in this process. There are also suggestions that these cytosolic low-molecular-mass proteins play a role in lipid metabolism, as they are abundant in many tissues involved in intensive fatty acid metabolism [14,15]. However, despite many experimental approaches there is no unequivocal explanation of the function of these proteins.

In this study we investigated the possible participation of FABPs in the induction of β -oxidation enzymes in *S. cerevisiae*. The presence of an FABP-type protein in *S. cerevisiae* has not, as yet, been reported, although such a protein was detected in *n*-alkane-utilizing *Y. lipolytica* [16] and there are data suggesting

the existence of such a protein in *C. tropicalis* [17]. Taking into account that uptake and flux of fatty acids within the yeast cell is a prerequisite for their action as the induction signal, we assumed that FABP-like protein exists in *S. cerevisiae* and we checked whether the presence of an heterologous FABP (bovine heart FABP, H-FABP) in *S. cerevisiae* cells would have any effect on induction of peroxisomal enzymes by oleic acid. This heterologous system gives a unique opportunity to study the FABP– β -oxidation relationship *in vivo*.

EXPERIMENTAL

Strains

Escherichia coli strain DH5 α was used for bacterial transformations. *S. cerevisiae* strains used were WS17-5D (α *leu2 arg1 ura3 trp1*) [18] and DCT30-5A (α *leu1 his3 ura3 ctt1-1*) (our laboratory collection).

Growth conditions

Strains were grown in complete [YP; 1% (w/v) yeast extract, 1% (w/v) peptone] or minimal [ω_c ; 0.67% (w/v) yeast nitrogen base supplemented with required amino acids and nucleotides] media supplemented with various carbon sources: 2% (v/v) ethanol (medium thereafter referred to as a ‘control medium’), 0.25% (w/v) oleic acid in 0.25% (w/v) Tween 80 (medium thereafter referred to as an ‘inducing medium’), 5% (w/v) glucose, or 1% (w/v) glucose plus 1% (v/v) ethanol.

DNA manipulations and plasmids

Standard techniques were used for DNA manipulations [19]. Plasmids described in this study were constructed as derivatives of: YEp352 [20], pFL34 [21], pEVPI1 [containing the constitutive promoter of the *Schizosaccharomyces pombe* alcohol dehydrogenase gene (*ADH1*)] [22], pGEM-F720 [containing H-FABP cDNA insert; this cDNA being originally obtained in Professor F. Spener’s laboratory (University of Muenster, Muenster, Germany) and kindly provided by Professor F. Paltauf (University of Gratz, Gratz, Austria)] [23], and 20L [containing the

Abbreviations used: FABP, fatty acid-binding protein; H-FABP, bovine heart FABP; IEF, isoelectrofocusing; *ADH1*, alcohol dehydrogenase gene; *CAT1*, oleic acid-inducible catalase A gene.

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Figure 1 Multicopy (pSI3, pSI5) and integrating (pSI10-1, pSI9-1) plasmids

pSI3 (a) and pSI10-1 (b) have an FABP-coding cDNA insert (H-FABP) under the control of the constitutive alcohol dehydrogenase promoter (*ADH1p*). pSI5 (c) and pSI9-1 (d) have the same insert under the control of the inducible promoter of catalase A (*CTA1p*).

promoter of the *S. cerevisiae* oleic acid-inducible catalase A gene (*CTA1*) [24].

Construction of multicopy and integrating plasmids

Two multicopy and two integrating plasmids containing the H-FABP insert under the control of the *S. pombe* gene *ADH1* promoter were constructed as follows: first, the plasmid pEVP11 containing *ADH1* was digested with *SalI* and *EcoRI* and the 800 bp fragment containing the promoter was isolated and introduced into vector pFL34 digested with the same endonucleases. The *EcoRI*–*BamHI* fragment from pGEM-F720 (594 bp) was then introduced into the above construct, giving the low-expression integrating plasmid pSI10-1 (Figure 1b). Finally, the *SalI*–*BamHI* fragment (1.4 kb) from pSI10-1 containing *ADH1* promoter in front of H-FABP cDNA was ligated into YEp352, giving the multicopy plasmid pSI3 (Figure 1a).

An 800 bp fragment of the plasmid 20L, containing the promoter of the *S. cerevisiae* catalase A gene, was cut out with *EcoRI* and *SacI* and ligated with the multicopy shuttle vector YEp352, digested with the same nucleases. The 594 bp fragment coding for H-FABP cDNA was excised from the pGEM-F720

plasmid with *EcoRI* and *BamHI* and ligated with the previously constructed plasmid containing the *CTA1* promoter, giving the multicopy pSI5 vector (Figure 1c).

To obtain the integrating plasmid with the FABP cDNA insert under the control of the *CTA1* promoter, the pSI5 vector described above was cut with *EcoRI* and *BamHI* and the resulting 1.4 kb fragment containing the *CTA1* promoter in front of FABP cDNA was isolated and introduced into plasmid pFL34, giving the integrating vector pSI9-1 (Figure 1d).

Construction of yeast strains with integrated and multicopy plasmids

To construct strains carrying pSI10-1 or pSI9-1 plasmids integrated into the genome, *S. cerevisiae* strain DCT30-5A was transformed to uracil prototrophy by using the above-described and control (without the insert) plasmid DNA cleaved at the *EcoRV* site within the *URA3* gene. Transformed cells were isolated on selective medium and transformants were checked for the presence of immuno-cross-reacting FABP by Western blotting with rabbit anti-(H-FABP) serum [19].

Strains carrying multicopy plasmids were obtained by trans-

forming *S. cerevisiae* strain WS17-5D with plasmids pSI3, pSI5 and the control plasmid YEp352. Transformed cells were isolated and checked as above.

Transformations

E. coli DH5 α cells were transformed using the CaCl₂ method according to Maniatis et al. [19]. Yeast cells were transformed using the LiCl method according to Ito et al. [25].

Enzymic assays

For enzymic assays cells were grown as follows: strains with the integrated H-FABP cDNA insert were pre-grown overnight in non-selective (YP) medium with 1% (w/v) glucose plus 1% (v/v) ethanol as carbon sources; strains with the insert on a multicopy plasmid were grown in selective ω_c -uracil medium with the same carbon sources. Overnight cultures of cell densities of approx. 1×10^7 cells/ml were spun down and transferred into inducing (oleic acid-containing) or control (ethanol-containing) media and routinely incubated for 8 h, unless described otherwise.

Acyl-CoA oxidase and catalase activities were measured in homogenates obtained by disrupting cells in 50 mM potassium phosphate buffer, pH 7.0, in a Braun homogenizer as described in [26]. Phenylmethanesulphonyl fluoride was added to a final concentration of 1 mM. Acyl-CoA oxidase activity was assayed spectrophotometrically [27] and catalase activity as described in [28]. Protein contents were determined by the method of Lowry [29].

Antibody-affinity chromatography

Recombinant FABP expressed in *S. cerevisiae* was purified on an antibody-affinity column kindly provided by Søren Nielsen (Institute of Biochemistry, University of Münster, Münster, Germany). This column contained rabbit anti-(rat H-FABP) antibodies conjugated to Protein-A-Sepharose (Pharmacia-LKB). Yeast cytosol was applied to this column in 0.2 M Tris/HCl/0.15 M NaCl/1% (w/v) Triton X-100, pH 7.5. The column was washed with 20 vol. of PBS (pH 7.4) to remove unbound proteins. Specifically bound proteins were eluted with 10 mM citric acid/20 mM NaH₂PO₄, pH 2.8.

Isotopic labelling of FABP *in vitro* and isoelectrofocusing (IEF)

Before labelling, yeast homogenate was centrifuged at 105 000 *g* for 1 h to give the cytosolic fraction. Immunoaffinity-purified recombinant FABP was labelled before IEF analysis with [1-¹⁴C]oleic acid (58 Ci/mol) as described in [30].

Flat-bed IEF was carried out in 5% (w/v) polyacrylamide gels over the pH range 3–10 in Pharmacia-LKB ampholytes [31]. After focusing, gels were dried and exposed directly. After the exposure gels were silver-stained for proteins.

RESULTS

Using the constructs with constitutive or inducible promoters made it possible to check the influence of FABP expressed constitutively in yeast cells (*ADHI* constitutive promoter) or expressed exclusively with the induction of the β -oxidation pathway (*CTAI* oleic acid-inducible promoter) [24].

Expression of H-FABP in *S. cerevisiae* cells

Multicopy (pSI3, pSI5) and integrating (pSI10-1, pSI9-1) plasmids constructed for studying the influence of H-FABP on β -oxidation of fatty acids are shown in Figure 1. Strains transformed with a multicopy plasmid were grown in selective medium (ω_c -uracil) with three different carbon sources: 5% (w/v) glucose, 2% (v/v) ethanol or 0.25% oleic acid. Equal numbers of cells were lysed and analysed by Western blotting with anti-FABP serum (Figure 2). H-FABP expression was strong for both types of promoters. The synthesis of FABP under the control of *CTAI* reaches the maximum after 8 h incubation in the presence of oleic acid (data not shown).

Strains transformed with integrating plasmid were grown in complete medium containing yeast extract, peptone and 2% (v/v) ethanol or 2% (v/v) ethanol supplemented with 0.25% oleic acid, but the expression of H-FABP, as assayed by Western blotting, was very low. We also checked strains transformed with the original plasmid obtained from Professor Paltauf (H-FABP under *GALI0* promoter), which was grown in medium containing 5% (w/v) galactose; however, in this case also the H-FABP expression was very low.

The original H-FABP has two isoforms of pI 4.9 and 5.1 [31]. In order to check the number of isoforms of the yeast-expressed H-FABP we analysed it by IEF. H-FABP was purified from the strain transformed with the multicopy pSI3 plasmid in a one-step procedure on an antibody-affinity column. A protein specifically retained on the column gave one band of 15 kDa on SDS/PAGE (results not shown). When analysed by IEF the protein shows two isoforms, both of pI about 5 (Figure 3a). This result was rather unexpected as only one cDNA was introduced into yeast cells. However, it has already been reported that the same cDNA expressed in *E. coli* causes the appearance of six isoforms [32] resulting from post-translational modifications [33].

Figure 2 Western-blot analysis of yeast strains transformed with multicopy and integrating plasmids

Whole-cell lysates were applied to SDS/polyacrylamide gels, electroblotted on Hybond C-extra (Amersham) and probed with rabbit anti-FABP serum. The specific binding was visualized by reaction with goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase. Each sample was derived from the same total number of cells (1×10^8). Lane 1, cells transformed with the control plasmid without the H-FABP insert and grown in minimal medium supplemented with 5% (w/v) glucose as a carbon source; lane 2, same as lane 1, but with 0.25% oleic acid instead of glucose; lane 3, cells transformed with the multicopy plasmid pSI5 and grown in minimal medium supplemented with 5% (w/v) glucose; lane 4, same as lane 3, but with 2% (v/v) ethanol; lane 5, same as lane 3, but with 0.25% oleic acid; lane 6, cells transformed with the multicopy plasmid pSI3 and grown in minimal medium supplemented with 5% (w/v) glucose; lane 7, same as lane 6, but with 2% (v/v) ethanol; lane 8, same as lane 6, but with 0.25% oleic acid; lane 9, cells transformed with multicopy plasmid YEp-F600 containing the H-FABP insert under the control of *GALI0* promoter [21] and grown in minimal medium supplemented with 5% (w/v) glucose; lane 10, same as lane 9, but with 5% (w/v) galactose; lane 11, H-FABP purified from transformed *E. coli* cells (a gift from Professor F. Spener). The arrow indicates the H-FABP band.

Figure 3 IEF and oleic acid-binding analysis of FABP expressed in yeast cells transformed with the multicopy plasmid pSI3

H-FABP was purified by immunoaffinity chromatography and labelled *in vitro* with $[1-^{14}\text{C}]$ oleic acid. After IEF the gel was dried and autoradiographed (c). After exposure the gel was fixed in 20% trichloroacetic acid and silver stained (Sigma kit) (a). (b) Silver-stained pI standards.

***In vitro* fatty acid-binding activity of H-FABP synthesized in yeast cells**

The antibody-affinity-purified H-FABP from transformed yeast cells was labelled *in vitro* with $[1-^{14}\text{C}]$ oleic acid and the co-migration of the protein and the ligand was checked on an IEF gel. Results presented in Figure 3 show that both isoforms bind

oleic acid. This result proves that H-FABP synthesized in transformed yeast cells retains its fatty acid-binding activity.

Enzymic activities

As already mentioned, the heterologous system described in this paper made it possible to study the influence of FABP on the β -oxidation pathway *in vivo*. We measured activities of two marker enzymes: acyl-CoA oxidase, which is an enzyme of the β -oxidation pathway, and catalase A, which is localized in peroxisomes and co-induced with the β -oxidation pathway. Routine measurements of β -oxidation induction were performed after incubation for 8–9 h in a medium containing 0.25% oleic acid. Normally, this length of incubation is sufficient to detect 5- to 10-fold increases in the activities of marker enzymes. However, such measurements of enzyme activities in extracts derived from cells transformed with pIS3, pIS4, pIS9-1, pIS10-1 and control plasmids failed to show any influence of FABP on the induction of marker enzymes (results not shown). Also, we did not find any influence of H-FABP expression on growth rate or growth efficiency.

Therefore we decided to look for a possible regulatory function of FABP in H-FABP-expressing versus non-expressing yeast cells incubated in media with low concentrations of oleic acid. We expected to notice stronger or more rapid induction of marker enzymes in H-FABP-expressing cells, due to increased supply of the substrate for the β -oxidation pathway. Three concentrations of oleic acid were tested: 0.001%, 0.01% and 0.05% and the results are shown in Figure 4. As previously reported [26], the level of induction of acyl-CoA oxidase activity depended on the concentration of oleic acid and increased with incubation time. In cells incubated in medium containing 0.001% oleic acid the maximal induction was reached after 4 h and remained constant for the next 3 h (Figure 4a), whereas in cells incubated in medium containing 0.05% oleic acid the induction was still increasing after 8 h (Figure 4c). We did not find significant differences between H-FABP-expressing and control strains.

In cells incubated in 0.01% oleic acid-containing medium

Figure 4 Acyl-CoA oxidase activity in yeast cells

(X), Cells transformed with the control plasmid (without H-FABP insert). (O), Cells transformed with the multicopy plasmid pSI3 carrying the H-FABP insert under the control of the constitutive promoter of the *ADH1* gene. Cells were incubated in medium containing: 0.05% oleic acid (a); 0.01% oleic acid (b); and 0.001% oleic acid (c). Samples were taken every 2 h. Data for two independent experiments are shown.

(Figure 4b) the maximal acyl-CoA oxidase activity in the control strain was observed after 6 h and remained constant for the next 2 h, whereas in the strain expressing H-FABP the activity of the marker enzyme was still on the increase at 8 h of incubation. To verify these observations we also measured the activity of another marker enzyme, catalase, (results not shown) and its activity paralleled that of acyl-CoA oxidase.

DISCUSSION

The scarcity of literature data shows that the nature of the relationship between induction of peroxisome proliferation, β -oxidation and FABP is unclear [34–36]. The concept of FABP participation in β -oxidation arose mainly from the observation of a parallel increase in the level of this protein and induction of β -oxidation enzymes by hypolipidaemic drugs and peroxisome inducers. It was found that distinct types of FABP are synthesized specifically in response to individual inducing agents [37]. Based on experiments *in vitro*, it was suggested that FABPs might be involved in the transport of fatty acids into peroxisomes [36]. These studies also indicated that FABP might constitute a factor regulating the activities of β -oxidation enzymes under differing physiological conditions. However, studies with isolated mammalian peroxisomes indicated that even though FABP can act as a fatty acid donor, it has no influence on the activity of a β -oxidation marker enzyme i.e. acyl-CoA oxidase [38].

In this study the effect of FABP on induction of the synthesis of peroxisomal enzymes involved in β -oxidation of fatty acids was investigated *in vivo* in a heterologous system (*S. cerevisiae* cells). An earlier study reported that in yeast cells expressing H-FABP the uptake and incorporation into glycerolipids of exogenous [14 C]palmitic acid was identical with that in control cells. However, the efficiency of unsaturation of the exogenous palmitate was about 2-fold lower [23].

In this study we confirmed the expression of H-FABP in *S. cerevisiae* and demonstrated that it is present in two isoforms having pI values of about 5 (see Fig. 3). This protein is able to bind fatty acid as can the authentic one. Since only one cDNA sequence was introduced into *S. cerevisiae* cells the appearance of two isoforms of H-FABP must have resulted either from different post-translational modifications, as reported for the same protein expressed in *E. coli* [33], or from mRNA editing as discussed in [39]. We did not find a distinct influence of H-FABP on the induction of the synthesis of β -oxidation enzymes. In cells expressing heterologous FABP we expected to detect higher activity of marker enzymes compared with control cells, especially in very low (0.001 %) concentrations of fatty acid, as a consequence of the increased supply of fatty acid. For the same reason we also hoped to observe more rapid induction of these enzymes in cells expressing H-FABP. However, no significant effect of heterologous FABP was observed. The lack of the expected influence of H-FABP on induction of the synthesis of β -oxidation enzymes by oleic acid is probably caused by a different metabolic role for this protein than that postulated [36]. It seems that FABP only functions as a fatty acid-storage molecule, capable of binding and releasing fatty acid and moderating its intracellular concentration, but does not influence acyl-CoA oxidase. Another reason for the apparent lack of influence of the heterologous FABP on fatty acid β -oxidation could be the presence of a very high level of endogenous FABP in yeast cells; the increase in the total amount of FABP due to the expression of heterologous protein would then be negligible. This explanation seems highly unlikely, since we could not detect the presence of endogenous FABP in *S. cerevisiae* cytosol using the Lipidex 1000 assay [40]

for total cytosolic proteins and for cytosolic proteins sub-fractionated by gel-filtration and ion-exchange chromatography. This assay was successfully used previously for detecting FABP-like proteins in the cytosol of *Y. lipolytica* [16]. In that case, however, a high level of fatty acid binding was detected after the induction of FABP-like protein by palmitate. In *S. cerevisiae* we did not notice a difference in the binding of radioactive fatty acid by the cytosol of yeast grown in inducing and non-inducing media. The possibility that H-FABP cannot fulfil its physiological function in an heterologous system cannot be excluded.

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