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Peroxisomal Fatty Acid Uptake Mechanism in *Saccharomyces cerevisiae*[*]

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Background: Pxa1p-Pxa2p transports cytosolic acyl-CoA to the peroxisomal lumen, but the actual mechanism is unknown.
Results: Acyl-CoAs are hydrolyzed and re-esterified prior to their oxidation, and Pxa1p-Pxa2p functionally interacts with acyl-CoA synthetases Faa2p and Fat1p.
Conclusion: Cytosolic acyl-CoAs are hydrolyzed prior to their transport and are subsequently re-esterified by acyl-CoA synthetases.
Significance: We propose a new peroxisomal acyl-CoA transport mechanism.

Peroxisomes play a major role in human cellular lipid metabolism, including fatty acid β-oxidation. The most frequent peroxisomal disorder is X-linked adrenoleukodystrophy, which is caused by mutations in *ABCD1*. The biochemical hallmark of X-linked adrenoleukodystrophy is the accumulation of very long chain fatty acids (VLCFAs) due to impaired peroxisomal β-oxidation. Although this suggests a role of ABCD1 in VLCFA import into peroxisomes, no direct experimental evidence is available to substantiate this. To unravel the mechanism of peroxisomal VLCFA transport, we use *Saccharomyces cerevisiae* as a model organism. Here we provide evidence that in this organism very long chain acyl-CoA esters are hydrolyzed by the Pxa1p-Pxa2p complex prior to the actual transport of their fatty acid moiety into the peroxisomes with the CoA presumably being released into the cytoplasm. The Pxa1p-Pxa2p complex functionally interacts with the acyl-CoA synthetases Faa2p and/or Fat1p on the inner surface of the peroxisomal membrane for subsequent re-esterification of the VLCFAs. Importantly, the Pxa1p-Pxa2p complex shares this molecular mechanism with *Hs*ABCD1 and *Hs*ABCD2.

Peroxisomes perform a range of different metabolic functions, including β-oxidation of fatty acids and synthesis and degradation of bioactive, lipid-derived molecules (1). Substrates for peroxisomal catabolism, *e.g.* free fatty acids (FFA), can enter the organelles through passive diffusion (2) or by means of ATP-binding cassette (ABC)[2] transporters, belonging to subclass D (Refs. 3 and 4; see also Fig. 1) or via the recently identified porin Pxmp2 (5). Functional ABC transporters are composed of a minimum of four functional domains: two transmembrane domains involved in substrate binding and translocation and two nucleotide binding domains that bind and hydrolyze ATP, thus providing energy for the transport step (6, 7). These domains may be present in a single polypeptide, *i.e.* a full-size transporter, or as two polypeptides, each composed of a transmembrane domain fused to a nucleotide binding domain, *i.e.* half-size transporters, that hetero- or homodimerize to form a functional transporter.

The yeast *Saccharomyces cerevisiae* contains two genes that encode peroxisomal half-size ABC proteins: Pxa1p (peroxisomal ABC transporter 1) and Pxa2p (8–10). The single *pxa1*Δ or *pxa2*Δ deletion mutants are unable to grow on oleate (C18:1) as the sole carbon source and exhibit reduced β-oxidation of this long chain fatty acid. It has been proposed that Pxa1p and Pxa2p operate as a heterodimer to form a functional transporter for long chain CoA esters (*e.g.* C18:1) (8, 9, 11, 12). Short chain fatty acids as well as medium chain fatty acids (*e.g.* C12:0) presumably enter yeast peroxisomes by passive diffusion as free acids independently of Pxa1p-Pxa2p and are subsequently activated by the peroxisomal acyl-CoA synthetase Faa2p, which is localized on the peroxisomal periphery, prior to β-oxidation (8). In addition, there is a second acyl-CoA synthetase, Fat1p, that is not only present on the peroxisomal membrane but also on the plasma membrane, endoplasmic reticulum, and lipid bodies.

In a previous report (13), we have shown that the phenotype of the *pxa1*/*pxa2*Δ yeast mutant, *i.e.* impaired growth on oleate-containing medium and deficient in oxidation of oleic acid, can be partially rescued by human ABCD1 (*Hs*ABCD1) and human ABCD2 (*Hs*ABCD2). Full rescue of β-oxidation activity in cells expressing human ABCD2 was observed with C22:0 and various unsaturated very long chain fatty acids. In

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[2] The abbreviations used are: ABC, ATP-binding cassette; ABCD, ATP-binding cassette transport family D; CPT2, carnitine palmitoyltransferase 2; CTS, Comatose (*At*PXA1); Faa2, peroxisomal acyl-CoA synthetase 2; Fat1, fatty acid transporter 1; FOX2, peroxisomal bifunctional protein; FOX3, peroxisomal 3-ketoacyl-CoA thiolase; Pxa1, peroxisomal ABC transporter 1; Pxa2, peroxisomal ABC transporter 2; VLCFA, very long chain fatty acid, X-ALD, X-linked adrenoleukodystrophy; *Hs*, *Homo sapiens*; *At*, *A. thaliana*.

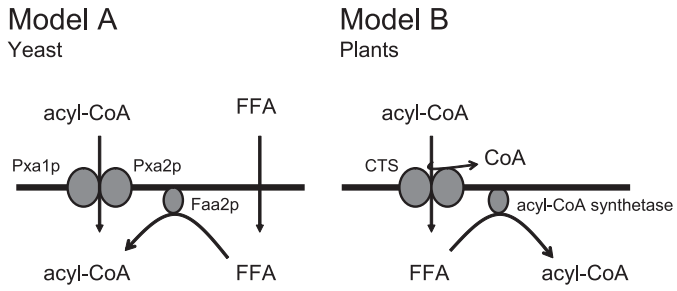

FIGURE 1. **Two current models for fatty acid transport across peroxisomal membrane.** Model A, uptake of fatty acids into yeast peroxisomes occurring as FFAs requiring intraperoxisomal activation by Faa2p or as fatty acyl-CoA esters facilitated by the ABCD transporters Pxa1p and Pxa2p in *S. cerevisiae*. Model B, in *A. thaliana*, the ABC transporter CTS (*At*PXA1) delivers non-esterified fatty acids to the peroxisomal matrix. These are subsequently activated by peroxisomal acyl-CoA synthetases.

contrast, full rescue was observed for C24:0 and C26:0 β-oxidation in cells expressing *Hs*ABCD1, the protein involved in X-linked adrenoleukodystrophy (X-ALD), indicating that both proteins are functional homologues of Pxa1p-Pxa2p and both can function as homodimers.

Two different models for peroxisomal fatty acid transport are described in literature (see Fig. 1 and Refs. 14 and 15) for yeast and plant cells, respectively. In model A, the ABC transporters deliver esterified fatty acids directly to the peroxisomal matrix while in parallel free fatty acids may enter the peroxisomal matrix via passive diffusion after which they are subjected to esterification. According to model B, the ABC transporters hydrolyze the CoA esters prior to their entry into the peroxisomes after which the latter are re-esterified by a peroxisomal synthethase (16–20).

In *Trypanosoma brucei*, the glyoxysomal full ABC transporter GAT1 serves to transport oleoyl-CoA and presumably other long chain acyl-CoAs from the cytosol to the glyoxysomal lumen (21), which is in line with the findings described above for yeast. Also in the plant *Arabidopsis thaliana*, a functional equivalent to Pxa1p-Pxa2p has been detected, named Comatose (CTS; *At*ABCD1; *At*PXA1) (16–20). Unlike the yeast and the mammalian peroxisomal ABC proteins, *CTS* is a full-size ABC transporter with dissimilar N- and C-terminal halves. CTS was able to rescue the oleate growth phenotype of the *pxa1*/*pxa2*Δ mutant and restored β-oxidation of fatty acids, showing that this transport system is a functional homologue of Pxa1p-Pxa2p too. Interestingly, when expressed in yeast peroxisomal membranes, the basal ATPase activity of CTS could be stimulated by fatty acyl-CoAs but not by fatty acids (22). However, genetic evidence with mutants in which peroxisomal acyl-CoA synthetases were deleted (14, 15, 23) suggested that CTS delivers fatty acid moieties to the peroxisomal lumen (Fig. 1*B*).

The above results prompted us to investigate the molecular mechanism of transport as facilitated by Pxa1p-Pxa2p and its orthologues from higher eukaryotes. The conclusion from our experiments is that the ABC transporters accept acyl-CoA esters as their transport substrate but that only the fatty acid moiety is actually transported after a hydrolysis step. Upon entry into the peroxisomes, the translocated fatty acids are re-esterified with CoA by the CoA-ester synthetase(s) Faa2p and/or Fat1p.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—S. cerevisiae BJ1991 (*Mat-α*, *leu2*, *trp1*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*) was used as the wild type strain (WT). The following derivatives of this strain, all impaired in β-oxidation, were used: *fox2*Δ and *fox3*Δ (carrying a deletion of the peroxisomal bifunctional protein or 3-ketoacyl-CoA thiolase, respectively), *pxa1*Δ and *pxa2*Δ (deletion mutants of the peroxisomal ABC transporters 1 and 2, respectively), and *faa2*Δ (carrying a deletion of the peroxisomal acyl-CoA synthetase). These mutants were constructed from BJ1991 as described previously (8, 11). The double mutant *pxa1*/*pxa2*Δ and ABCD1 and ABCD2 plasmids used in this study were as described before (13). The *pxa1*/*pxa2*/*faa2*Δ (*LEU*:*KAN*:*BLE*) triple mutant was made by replacing the whole *FAA2* ORF from the *pxa1*/*pxa2*Δ (*LEU*:*KAN*) mutant by the *BLE* gene. The *FAA2* deletion construct was made by PCR with pUG66 plasmid (*BLE* gene) as a template, the forward primer 5′-TGATGGGTTGGAACTATATAAAGCATCGGAAACGATGGCTAAGGGAAGTCAGCTGAAGCTTAGACGC, and the reverse primer 5′-TGCATAGGGATATCCTACATCAAAGTTTTTTCTAGTTTGAAGTGTTCCAGCATAGGCCACTAGTGGATCTG. BLE+ transformants were selected for integration in the *FAA2* gene by PCR analysis.

Yeast strains were grown in minimal medium containing 6.7g/liter yeast nitrogen base without amino acids (YNB-WO) supplemented with 3g/liter glucose plus amino acids (20 mg/liter) when required. For the induction of peroxisome proliferation, cells were shifted to YPO+ medium containing 5 g/liter potassium phosphate buffer, pH 6.0, 3 g/liter yeast extract, 5g/liter peptone supplemented with 1.2 g/liter oleate, 10 g/liter glycerol, and 2g/liter Tween-80. Prior to shifting to these media, the cells were grown in minimal medium with 3 g/liter glucose for at least 24 h. One *A* unit is the amount of cells from 1 ml cell cultures with an $A_{600\ nm}$ of 1 and containing 2×10^7 cells.

Carnitine Palmitoyltransferase 2 (CPT2) Expression Plasmids— The ORF encoding mature CPT2 (*i.e.* with its mitochondrial targeting sequence deleted) was amplified using forward primer TATAGGATCC ATG AGC GCC GGC TCC GGG CCC GGC CAG TAC CTG CAG and reverse primer TATAGCATGC TTA AAG CTT ACT TTT GAT GGA TTT GCC TTC containing a KL* extension (restriction sites are underlined). An additional HindIII site, which facilitates 3′ modifications, was included in the reverse primer. As a template for the PCR, we used the pYES2-CPT2 plasmid containing the ORF of *HsCPT2* (generous gift from Dr. F. Taroni, Milano, Italy). The resulting PCR product was cloned in pGEM-T to generate pGEM-T-CPT2-SKL.

The plasmid pCPT2per1 was made by subcloning the BamHI-SphI fragment of pGEM-T-CPT2-SKL into the yeast expression vector Pca31, which contains the oleate-inducible catalase promoter (24) plus an oligonucleotide adaptor encoding the N terminus of the mature hemagglutinin protein as described in detail in Elgersma *et al.* (25). For expression of CPT2 in the cytosol (pCPTcyt), the peroxisomal targeting signal (SKL) was replaced by the C-terminal end of the native CPT2. To this end, the NcoI-SphI fragment from pYES2-CPT2

was cloned into pCPT2per1, which was digested by NcoI and SphI. The HindIII site (upstream of the stop codon) in pCPT2per1 was used to enhance the effectiveness of the peroxisomal targeting signal (SKL) by insertion of a linker (sense, AG CTT GGT TCC GGA GGT GAA GCT GCA GTT AAG CTT TCT CAA GCT AAA TCT AAA CTT TAATCTAGAT; antisense, AG CTA TCT AGA TTA AAG TTT AGA TTT AGC TTG AGA AAG CTT AAC TGC AGC TTC ACC TCC GGA ACC A) that encodes the 12 C-terminal amino acids of FOX2 (peroxisomal targeting signal 1) preceded by a flexible loop, resulting in pCPT2per2.

In pCPT2per3, the flexible loop was replaced by an α-helical linker (26) to modify the accessibility of the PTS1 signal. To this end, pCPT2per2 was cleaved by HindIII, and the following linker was inserted: sense, AG CTA GCA GCT AAA GAA GCC GCA GCT AAA GAA GCC GCA GCT AAA GAA GCC GCA GCT AAA GAA GCT GCA GTT A; antisense, AG CTT AAC TGC AGC TTC TTT AGC TGC GGC TTC TTT AGC TGC GGC TTC TTT AGC TGC GGC TTC TTT AGC TGC T. All inserts were verified by DNA sequencing. In summary, the C terminus of these four CPT2 derivatives is as follows (extensions in bold face): pCPT2cyt, EGKSIKS; pCPT2per1, EGKSIKS**KL**; pCPT2per2, EGKSIKS**KLGSGGEAAVKLSQAKSKL**; pCPT2per3, EGKSIKS**KLAAKEAAAKEAAAKEAAAKEAAVKLSQAKSKL**.

Mass Spectrometric Metabolite Analyses—To compare the acyl-CoA levels in different cellular compartments, we first transformed the yeast cells with human carnitine palmitoyltransferase 2 (*Hs*CPT2) with or without a peroxisomal targeting signal. After growing for at least 24 h on 0.3 g/liter glucose plus amino acids (20 mg/liter), yeast cells were grown overnight on YPO+ medium. Isolated yeast cells were washed and incubated ($A = 2.5$) for 60 or 180 min with 400 μM caprylic (C8:0) acid and 0.33 g/liter Tween-80 or 2.4 mM oleic acid (C18:1) and 2 g/liter Tween-80, respectively, in 3 g/liter yeast extract, 5 g/liter Bacto Peptone, 25 mM potassium phosphate, pH 6.0, and 2 mM carnitine. Of this mixture, 2 ml were spun down, and cell pellets were stored at −80 °C. For measurements of O^{18} incorporation into 3-OH-acylcarnitine intermediates, cells were incubated as described above with 50% $[^{18}O]H_2O$ (in $[^{16}O]H_2O$) as the only adjustment. For acylcarnitine measurements, pellets were taken up in 500 μl of 70% (v/v) acetonitrile. Acylcarnitine levels were determined by liquid chromatography-tandem mass spectrometry as described previously (27, 28) using $[^2H_3]$palmitoylcarnitine as an internal standard.

Subcellular Fractionation and Nycodenz Gradient Centrifugation—To prepare a peroxisome-enriched fraction, subcellular fractionation by differential centrifugation of *S. cerevisiae* homogenates was performed as described before (10). Organellar pellets were layered on top of a 150–350 g/liter Nycodenz gradient (12 ml) with a cushion of 1.0 ml of 500 g/liter Nycodenz solution. All Nycodenz solutions contained 5 mM MES, pH 6.0, 1 mM EDTA, 1 mM KCl, and 85 g/liter sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8x35) at 19,000 rpm at 4 °C. After centrifugation, gradients were aliquoted into different fractions, which were assayed for activity of various marker enzymes as described below.

Enzyme Assays—β-Oxidation assays in intact cells were performed as described previously by van Roermund *et al.* (29) with slight modifications. Cells were grown overnight in media containing oleate to induce β-oxidation. The β-oxidation capacity was measured in 50 mM MES, pH 6.0 supplemented with 10 μM 1-^{14}C-fatty acids. Subsequently, $[^{14}C]CO_2$ was trapped with 2 M NaOH and used to quantify the rate of fatty acid oxidation. Results are presented as percentages relative to the rate of oxidation of wild type cells. In WT cells the rates of lauric acid (C12:0; 1-h incubation) and behenic acid (C22:0; 2-h incubation) oxidation were 7.24 ± 0.80 and 0.62 ± 0.08 nmol/min·10^7 cells, respectively, whereas lignoceric acid (C24:0; 2.5-h incubation) was oxidized at a rate of 4.46 ± 0.15 pmol/h·10^7 cells.

Acyl-CoA synthetase activity was measured as described by Knoll *et al.* (30) with the following modifications. The reaction was started by adding cell extract to a mixture of 10 μM [1-^{14}C]C22:0, 150 mM Tris, pH 8.5, 10 mM ATP, 10 mM $MgCl_2$, 200 μM CoA, and 1 g/liter Triton X-100 and incubated for 10 min at 28 °C. The activity of the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase was measured on a Cobas-Fara centrifugal analyzer by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm (31).

Fumarase activity was measured on a Cobas-Fara centrifugal analyzer by monitoring the increase in absorbance at 365 nm for 5 min at 37 °C. In the fumarase assay, 3-acetylpyridine adenine dinucleotide rather than NAD was used as the cofactor. Its reduced form absorbs at 365 nm (29). The reaction was started by adding 10 mM fumarate into an incubation mixture containing the following components: 100 mM Tris, pH 9.0, 1 g/liter Triton X-100, 4 units/ml malate dehydrogenase, and 1 mM 3-acetylpyridine adenine dinucleotide. Alkaline phosphatase and esterase activities were measured on a Cobas-Fara centrifugal analyzer according to published procedures (32). Protein concentrations were determined by the bicinchoninic acid method described by Smith *et al.* (33).

RESULTS

Evidence That Faa2p Is Involved in HsABCD-dependent Acyl-CoA Transport across Peroxisomal Membrane—To determine the chemical nature of the product delivered by the *Hs*ABCD complex into the peroxisomes, we measured the β-oxidation of C22:0 and C24:0 in *pxa1*/*pxa2*Δ and *pxa1*/*pxa2*/*faa2*Δ cells, both expressing either the human *Hs*ABCD1 or *Hs*ABCD2 (see Fig. 2, *B* and *A*, respectively). Full rescue was observed in *pxa1*/*pxa2*Δ cells expressing *Hs*ABCD1 or *Hs*ABCD2 for C24:0 and C22:0 β-oxidation activity, respectively, whereas no rescue was detected in yeast strains in which (in addition to *pxa1*/*pxa2*) *faa2* also was deleted. These results imply that the β-oxidation activity in *pxa1*/*pxa2*Δ cells that express *Hs*ABCD1 or *Hs*ABCD2 is dependent on the intraperoxisomal acyl-CoA synthetase Faa2p.

As a control for proper induction of the β-oxidation enzymes, we measured the activity of 3-hydroxyacyl-CoA dehydrogenase (Fig. 2*C*). The results indicate that *Hs*ABCD1p and *Hs*ABCD2p do deliver free fatty acids into the peroxisomal matrix that after their import into the peroxisomal lumen need to be activated into CoA esters to become substrates for β-oxidation.

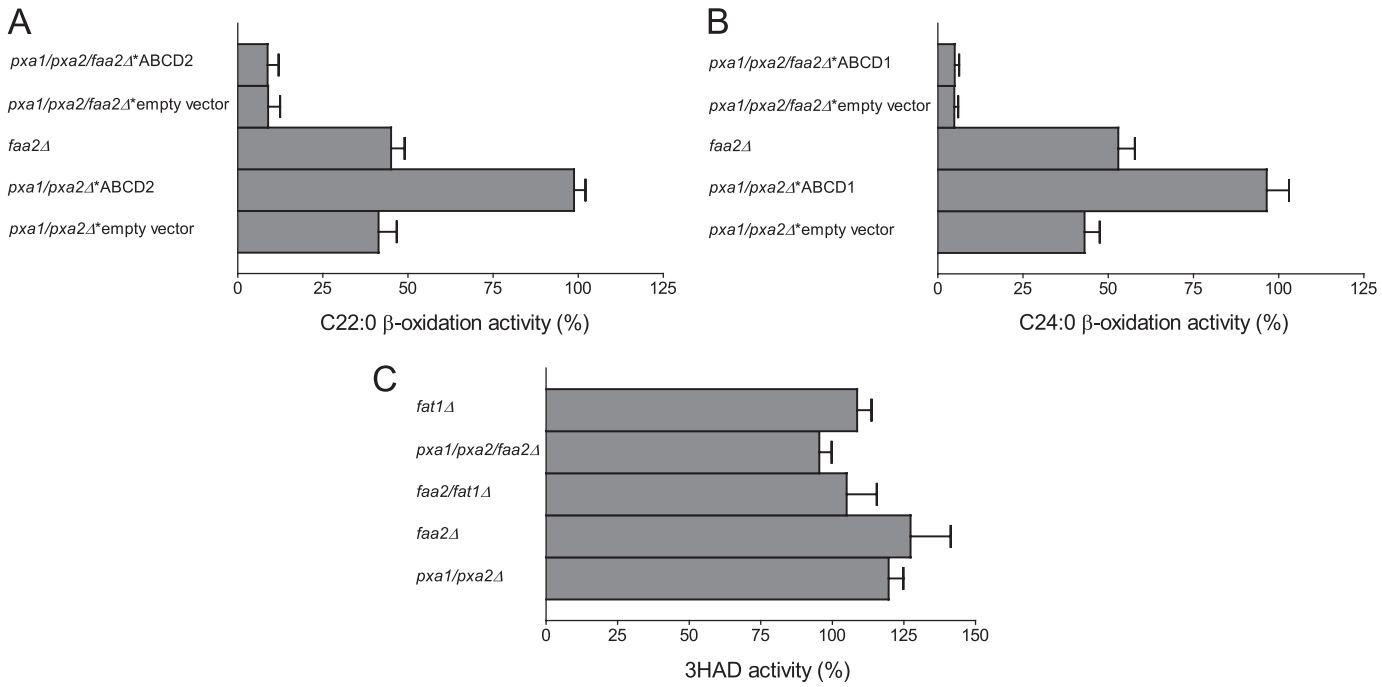

FIGURE 2. **Evidence that Faa2p is involved in *Hs*ABCD-dependent acyl-CoA transport across peroxisomal membrane.** Cells grown on oleate/glycerol were incubated with [1-^{14}C]behenic acid (C22:0) or [1-^{14}C]lignoceric acid (C24:0), and β-oxidation rates were measured (*A* and *B*; see "Experimental Procedures"). *C*, as a control for induction of β-oxidation, 3-hydroxyacyl-CoA dehydrogenase (*3HAD*) activity was measured. The activity in wild type cells was taken as reference (100%). This experiment was performed at least three times; *error bars* indicate mean ± S.D. See "Experimental Procedures" for strains used and experimental details.

Measurement of Peroxisomal and Cytosolic Acyl-CoA Pool—To gain more insight into the mechanism of acyl-CoA transport across the peroxisomal membrane, it would be important to be able to determine and compare the acyl-CoA levels in different cellular compartments, *e.g.* in the cytosol and in the peroxisomal matrix. Measuring acyl-CoA levels in isolated peroxisomes *versus* cytosol, however, will not provide such information because peroxisomes become leaky (and therefore permeable for acyl-CoA esters) upon isolation from the cells. We therefore developed an assay to determine these levels *in vivo*.

To this end, we used *Hs*CPT2, which can convert acyl-CoAs into the corresponding acylcarnitine esters, which then can be readily measured by LC-tandem mass spectrometry (34). Targeting of *Hs*CPT2 to either the cytosol or to the peroxisomes will provide an assay for determining the amount of acyl-CoA formed in either compartment.

To develop this experimental system, we designed four different CPT2 constructs, all lacking the authentic mitochondrial targeting signal: one construct was targeted to the cytoplasm (CPT2cyt) and three constructs, each containing a peroxisomal targeting signal 1 in a different context, were targeted to peroxisomes (CPT2per1–3; for further details see "Experimental Procedures"). To study the subcellular localization of CPT2cyt and CPT2per1–3 in oleate-grown cells, we transformed these CPT2 constructs into our wild type yeast strain. After fractionation of oleate-grown cells into organellar fractions, we found that CPT2cyt was present exclusively in the supernatant, and CPT2per1 was in the supernatant as well as in the organellar pellet, whereas CPT2per2 and CPT2per3 were mainly present in the organellar pellet. To separate peroxisomes from mitochondria, the organellar pellets were further fractionated by Nycodenz density gradient centrifugation. Immunoblot analysis of the gradient fractions using CPT2 antibodies revealed that CPT2cyt co-localized with the cytosolic marker phosphoglucose isomerase, and both CPT2per2 and CPT2per3 co-localized with the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase, thus indicating a cytosolic and peroxisomal localization, respectively (data not shown). Localization of CPT2per1 was 50% peroxisomal and 50% cytosolic, indicating that a flexible loop (CPT2per2) or a helical linker (CPT2per3) together with a peroxisomal targeting sequence is necessary for efficient peroxisomal localization. Fig. 3*A* shows that CPT2cyt and CPT2per2 constructs *in situ* are actively expressed as deduced from the measured oleoylcarnitine levels (for further details see "Experimental Procedures") in wild type cells growing on oleate/glycerol and expressing CPT2cyt and CPT2per2, respectively. This activity was not observed in wild type cells transformed with an empty vector (not shown).

To investigate *in vivo* the acyl-CoA transport across the peroxisomal membrane, we measured the peroxisomal β-oxidation intermediate 3-OH-oleoyl-CoA by comparing the levels of 3-OH-oleoylcarnitine in oleate/glycerol-grown yeast cells expressing CPT2per2 and CPT2cyt. Expression of CPT2per2 resulted in markedly higher levels of 3-hydroxyoleoylcarnitine in oleate/glycerol-grown *fox3*Δ cells (carrying a deletion of the peroxisomal 3-ketoacyl-CoA thiolase) when compared with wild type cells or *fox2*Δ cells (carrying a deletion of the peroxisomal bifunctional protein), indicating that peroxisomally expressed CPT2 can indeed be used to determine acyl-CoA levels in the peroxisomes (Fig. 3*B*).

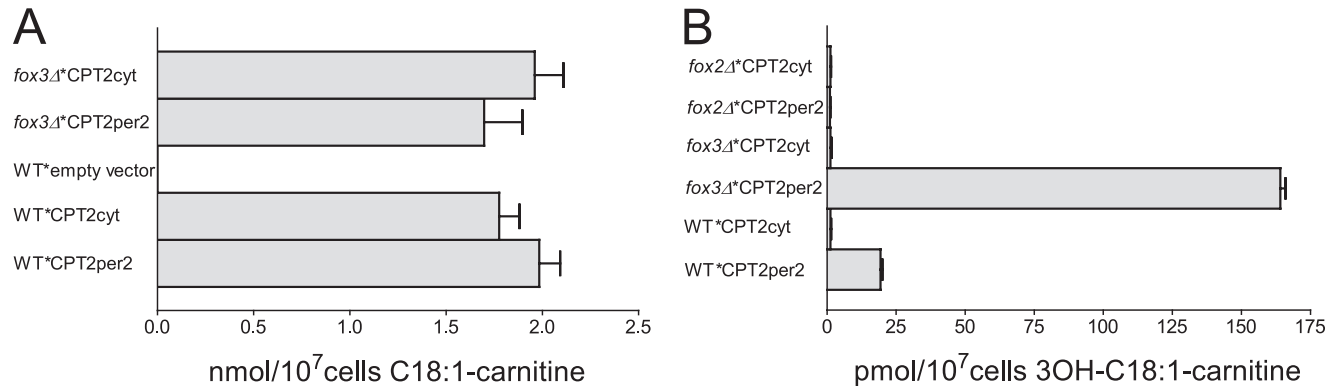

FIGURE 3. **Acyl-CoA hydrolysis is obligatory step prior to β-oxidation.** Wild type and *fox3*Δ cells transformed with CPT2per2 and CPT2cyt were grown on oleate/glycerol. *A*, oleoylcarnitine levels were measured using tandem mass spectrometry (see "Experimental Procedures"). *B*, the levels of 3-OH-oleoylcarnitine were measured in wild type and *fox3*Δ cells expressing CPT2cyt or CPT2per2, both grown on oleate/glycerol. This experiment was performed at least three times; *error bars* indicate mean ± S.D. See "Experimental Procedures" for strains used.

CoA Ester Hydrolysis Occurs before Fatty Acid Oxidation—The above described approach to assay acyl-CoA levels provides a tool to elucidate the mechanism of the acyl-CoA synthetase-dependent acyl-CoA transport process across the peroxisomal membrane. When acyl-CoA esters are hydrolyzed during or after the uptake process into the peroxisomes, a water molecule is added to the substrate. In the presence of 50% $[^{18}O]H_2O$, a theoretical ratio of no ^{18}O incorporation to single ^{18}O incorporation to double ^{18}O incorporation into the 3-hydroxyacyl-CoA ester of 0.75:1.0:0.25 is expected in the *fox3*Δ mutant (Fig. 4*A*).

Fig. 4*B* shows the 3-OH-oleoylcarnitine levels with different degrees of ^{18}O incorporation in wild type and *fox3*Δ cells transformed with CPT2per2 grown in oleate/glycerol/$[^{18}O]H_2O$ medium. The observed ratios of 0.90:1.0:0.24 and 0.85:1.0:0.19 in wild type and *fox3*Δ cells, respectively, are close to the theoretically predicted ratio when the uptake process involves hydrolysis and subsequent re-formation of the acyl-CoA ester. Moreover, the observation of a double ^{18}O incorporation in wild type cells suggests that the hydrolysis step (and subsequent esterification) is an obligatory step prior to β-oxidation. As a control, we incubated WT and *fox3*Δ cells, both transformed with CPT2per with octanoic acid (C8:0) (Fig. 4*C*), and observed no incorporation and single ^{18}O incorporation into the β-oxidation intermediate 3-hydroxyoctanoylcarnitine but no double ^{18}O incorporation, indicating that C8:0 presumably enters yeast peroxisomes by passive diffusion as a free acid independently of Pxa1p-Pxa2p.

Pxa1p-Pxa2p Heterodimer Forms a Functional Unit with Additional Acyl-CoA Synthetase—The C24:0 and C22:0 β-oxidation activity in *faa2*Δ cells is ~50% of the activity in the corresponding wild type strain (Fig. 2, *A* and *B*), whereas no activity can be measured in *faa2*/*pxa1*/*pxa2*Δ cells. However, when the yeast transporters Pxa1p and Pxa2p are replaced by the human orthologues *Hs*ABCD1 and *Hs*ABCD2 in *faa2*/*pxa1*/*pxa2*Δ cells, the residual β-oxidation activity is still less than 5% (Fig. 2, *A* and *B*). This implies that the C24:0 or C22:0 β-oxidation flux redirected by *Hs*ABCD1 and *Hs*ABCD2, respectively, is strictly dependent on the presence of the peroxisomal Faa2p. This is somewhat surprising because we expected, based on the homology between yeast and *Hs*ABCD transporters, that the essence of the mechanism of acyl-CoA transport across the peroxisomal membrane would be the same, although their chain length specificity for CoA esters may differ. One explanation for this is that the deletion of *PXA1*/*PXA2* also affects a peroxisomal acyl-CoA synthetase activity other than Faa2p. Because there are no recognizable sequence features in the ABCD proteins that suggest that these proteins also display acyl-CoA synthetase activity, we hypothesize that this acyl-CoA synthetase forms a functional protein complex with Pxa1p-Pxa2p but cannot form such a complex with *Hs*ABCD1 or *Hs*ABCD2.

To determine the existence of an additional peroxisomal acyl-CoA synthetase for very long chain fatty acids, we first optimized the C22:0-CoA synthetase assay in isolated peroxisomes from wild type cells (see "Experimental Procedures") and then measured the subcellular localization of C22:0-CoA synthetase activity in wild type, *faa2*Δ, *pxa1*/*pxa2*Δ, and *pxa1*/*pxa2*/*faa2*Δ cells grown on oleate/glycerol. In all deletion strains we measured C22:0-CoA synthetase activity in the organellar fraction as well as in the supernatant fraction (Fig. 5*A*). After separation of peroxisomes from other organelles by equilibrium-density gradient centrifugation, we observed that part of the C22:0-CoA synthetase activity of wild type yeast cells co-fractionated with the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase, whereas the remaining activity co-fractionated with the mitochondrial marker fumarase, the endoplasmic reticulum marker esterase, and the plasma membrane marker alkaline phosphatase (Fig. 5*B*). Interestingly, no peroxisomal C22:0-CoA synthetase activity was present in *pxa1*/*pxa2*/*faa2*Δ yeast cells, which is in marked contrast to the presence of this activity in *faa2*Δ and *pxa1*/*pxa2*Δ cells. This suggests not only that Faa2p contributes to the activation of C22:0 in peroxisomes but also points to the existence of an additional acyl-CoA synthetase activity associated directly or indirectly with the ABC transporters Pxa1p-Pxa2p.

Fat1p Is Involved in ABC Transporter-dependent Acyl-CoA Transport—One of the candidates for this presumed additional peroxisomal acyl-CoA synthetase is the previously reported fatty acid transporter protein Fat1p. Fat1p contains the peroxisomal targeting signal IKL and is localized not only in peroxisomes but also in the plasma membrane, in lipid bodies, and in the endoplasmic reticulum (35–38). Fat1p functions in vectorial acylation, which couples the uptake of exogenous fatty acids

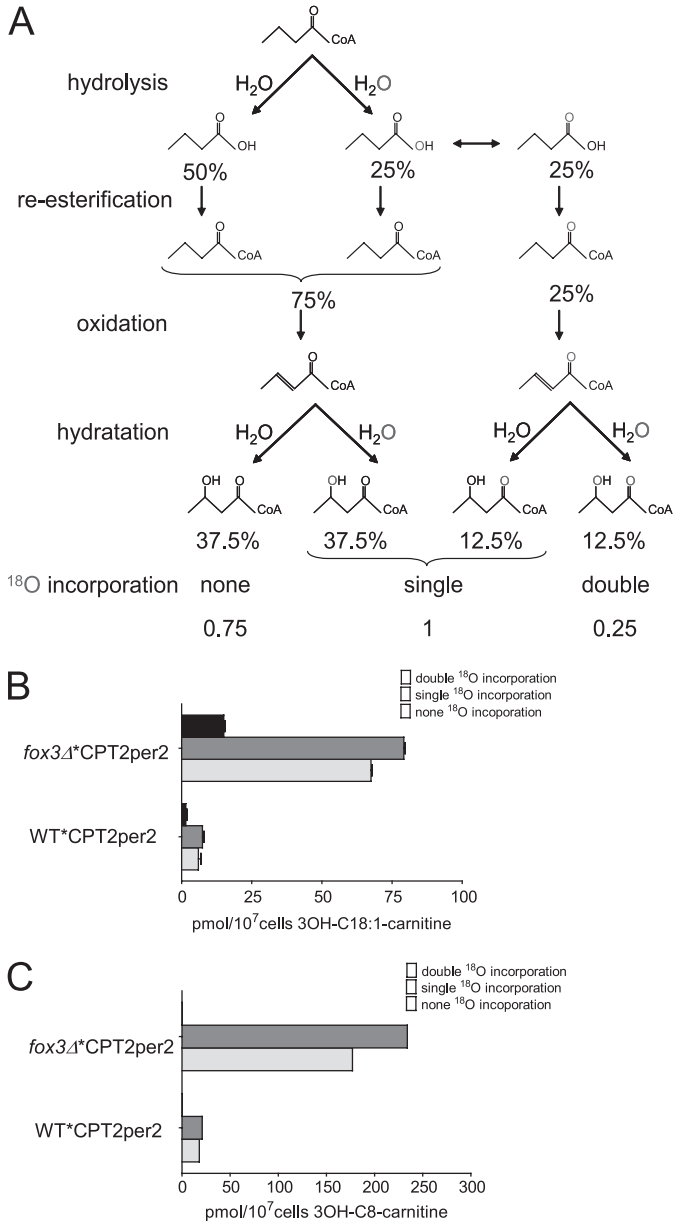

FIGURE 4. **Incorporation of ^{18}O into carboxyl group of acyl-CoA indicates that acyl-CoAs are hydrolyzed and reactivated prior to β-oxidation.** *A*, upon hydrolysis of an acyl-CoA ester, a water molecule is added to the acyl chain to produce a free fatty acid. Thus, when hydrolysis occurs in the presence of $[^{18}O]H_2O$, the carboxyl group of the fatty acid will be labeled with ^{18}O, and this can be used directly to determine whether or not hydrolysis occurs during or prior to uptake of acyl-CoA esters into the peroxisomes. The carboxyl group has two oxygen atoms, which are in a tautomeric equilibrium upon hydrolysis of the acyl-CoA ester into the free fatty acid. After re-esterification of the free fatty acid to the corresponding CoA ester, one of these oxygen atoms will be lost, resulting in a 50% change in incorporation of the ^{18}O atom. In the hydration step of β-oxidation, another water molecule is added to the 2-enoyl-CoA derivative to form 3-hydroxy acyl-CoA. This second $[^{18}O]H_2O$ addition, therefore, has to be taken into account to calculate the enrichment of ^{18}O in the final product. To study these events *in vivo*, we cultured yeast in 50% $[^{18}O]H_2O$ (in $[^{16}O]H_2O$). This means that 50% of the 3-OH groups will be labeled. Finally, a theoretical ratio of no ^{18}O incorporation to single ^{18}O incorporation to double ^{18}O incorporation into the 3-hydroxy acyl-CoA ester of 0.75:1.0:0.25 is expected. *B*, wild type and *fox3*Δ cells transformed with CPT2per2 were incubated in oleate medium with 50% $[^{18}O]H_2O$, and 3-OH-oleoylcarnitine levels were measured. The observation of double ^{18}O incorporation in the carnitine ester in wild type and *fox3*Δ cells indicates that the acyl-CoA hydrolysis step is an obligatory step prior to β-oxidation. *C*, wild type and *fox3*Δ cells transformed with CPT2per2 were incubated in octanoic acid medium with 50% $[^{18}O]H_2O$, and 3-OH-octanoylcarnitine levels were measured. The observation of single but no double ^{18}O incorporation in the carnitine ester in wild type and *fox3*Δ cells indicates that ^{18}O is not incorporated into the carboxyl group of C8:0 and that C8:0 presumably enters yeast peroxisomes by passive diffusion as free acid independently of Pxa1p-Pxa2p. Each of these experiments except for the experiment with octanoic acid was performed at least three times (see "Experimental Procedures" for further details); *error bars* indicate mean ± S.D.

to their activation into the corresponding CoA esters. Furthermore, it carries a very long chain acyl-CoA synthetase motif with its active site on the inner face of the plasma membrane (37).

To confirm that Fat1p indeed is also present in the peroxisomal membrane, double mutants were generated in which both *FAA2* and *FAT1* were deleted, and C22:0-CoA synthetase activity was measured in isolated peroxisomes of *fat1*Δ and *faa2*/*fat1*Δ cells. Fig. 6*A* shows that the isolated peroxisomal fraction of *fat1*/*faa2*Δ cells is deficient for C22:0-CoA synthetase activity, indicating that Fat1p is responsible for the peroxisomal C22:0-CoA synthetase activity in the wild type and *faa2*Δ strain. To study the involvement of Fat1p in acyl-CoA transport across the peroxisomal membrane, *fat1*Δ, *faa2*Δ, and *faa2*/*fat1*Δ cells were subsequently analyzed for C22:0 β-oxidation activity. Importantly, *fat1*Δ cells showed normal C22:0 β-oxidation activity, suggesting that Fat1p is not the rate-limiting step of C22:0 β-oxidation in wild type yeast (Fig. 6*B*). Furthermore, by measuring the C22:0 carnitine levels in *fat1*Δ cells expressing CPT2cyt and incubated with C22:0, we could show that the cytosolic concentration of C22:0-CoA was not affected by this deletion (data not shown), indicating that the transport of C22:0 across the plasma membrane in *fat1*Δ cells is normal. As a control for constitutive induction of β-oxidation enzymes under these conditions, we measured the 3-hydroxyacyl-CoA dehydrogenase activity (Fig. 2*C*), the third step of the β-oxidation pathway. We found that the C22:0 β-oxidation was severely affected in *faa2*/*fat1*Δ cells (Fig. 6*B*), which indicates that both acyl-CoA synthetases, Faa2p and Fat1p, contribute to the peroxisomal β-oxidation of C22:0. Furthermore, whereas C12:0 β-oxidation activity is not impaired, consistent with previous observations (7), we observed that C12:0 oxidation is fully dependent on Faa2p (Fig. 6*B*).

DISCUSSION

In this study, we show that uptake of (very) long chain acyl-CoAs into yeast peroxisomes via the Pxa1p-Pxa2p complex is accompanied by hydrolysis of their CoA ester followed by re-esterification with coenzyme A in the peroxisomal lumen. As observations have been made with plant- and mammal-derived homologues of this system that can be interpreted in a similar way (10, 32), this mechanism of uptake into the peroxisomes presumably is functional in all eukaryotes. Whereas the plant homologue, Comatose, is a complex, four-domain-containing protein, the human homologues are able to function in this uptake process as a homodimer.

At first sight, such a hydrolysis/re-esterification process may seem energetically unfavorable. However, *in vitro* biochemical studies will be needed to reveal the details of the free energy requirements of the various steps in this process. CoA is only used catalytically in the β-oxidation cycle. Hydrolysis of the

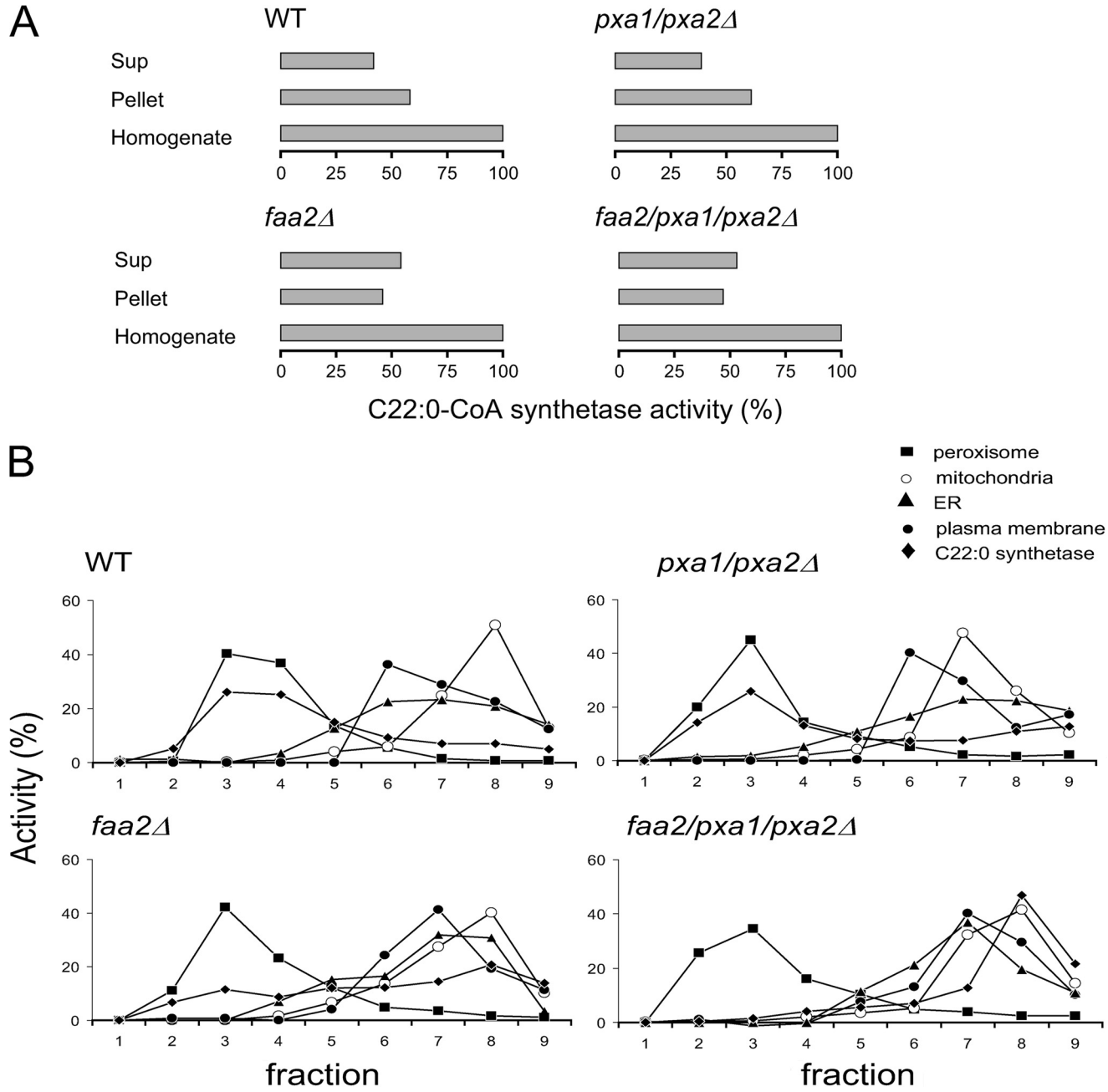

FIGURE 5. **Peroxisomal C22:0 acyl-CoA synthetase activity is dependent on presence of Pxa1p-Pxa2p.** *A*, subcellular fractionation of wild type, *pxa1*/*pxa2*Δ, *faa2*Δ, and *faa2*/*pxa1*/*pxa2*Δ cells in oleate/glycerol medium. C22:0-CoA synthetase activity was measured in homogenates, organellar pellets, and the supernatant fraction (*Sup*) (see also "Experimental Procedures"). *B*, an organellar pellet fraction prepared by differential centrifugation (see Fig. 4*A*) was subjected to Nycodenz gradient centrifugation. Each fraction was analyzed for C22:0-CoA synthetase activity (♦) and for the marker enzymes fumarase (mitochondria; ○), 3-hydroxyacyl-CoA dehydrogenase (peroxisomes; ■), esterase (endoplasmic reticulum (*ER*); ▲), and alkaline phosphatase (plasma membrane; ●). The experiment was conducted three times. The data from a representative experiment are shown.

acyl-CoA prior to transport prevents accumulation of CoA in the peroxisome and makes export of CoA superfluous. Because of this, functional reconstitution of the Pxa1p-Pxa2p complex with and without a coupled acyl-CoA synthetase in a liposomal system is required to resolve this issue.

The observed biochemical mechanism of the uptake process differs significantly from most transport processes, which are mostly energized by either a chemiosmotic mechanism (symport, antiport, etc.) or ATP hydrolysis (see *e.g.* Ref. 39). The current process shows some similarities with group translocation mechanisms albeit it is almost the inverse of the best known example from this class, the phosphoenolpyruvate phosphotransferase system from enterobacteria (40). However, rather than receiving an additional moiety, here the substrate is reduced in size during the transport step.

A key consequence of the mechanism of uptake of very long chain fatty acids into peroxisomes that we report here is that the availability of coenzyme A in the peroxisomal lumen has to be regulated separately by the eukaryotic cell. While this paper was under review, Agrimi *et al.* (42) provided evidence in favor of a peroxisomal CoA transporter, which could be involved in this regulation.

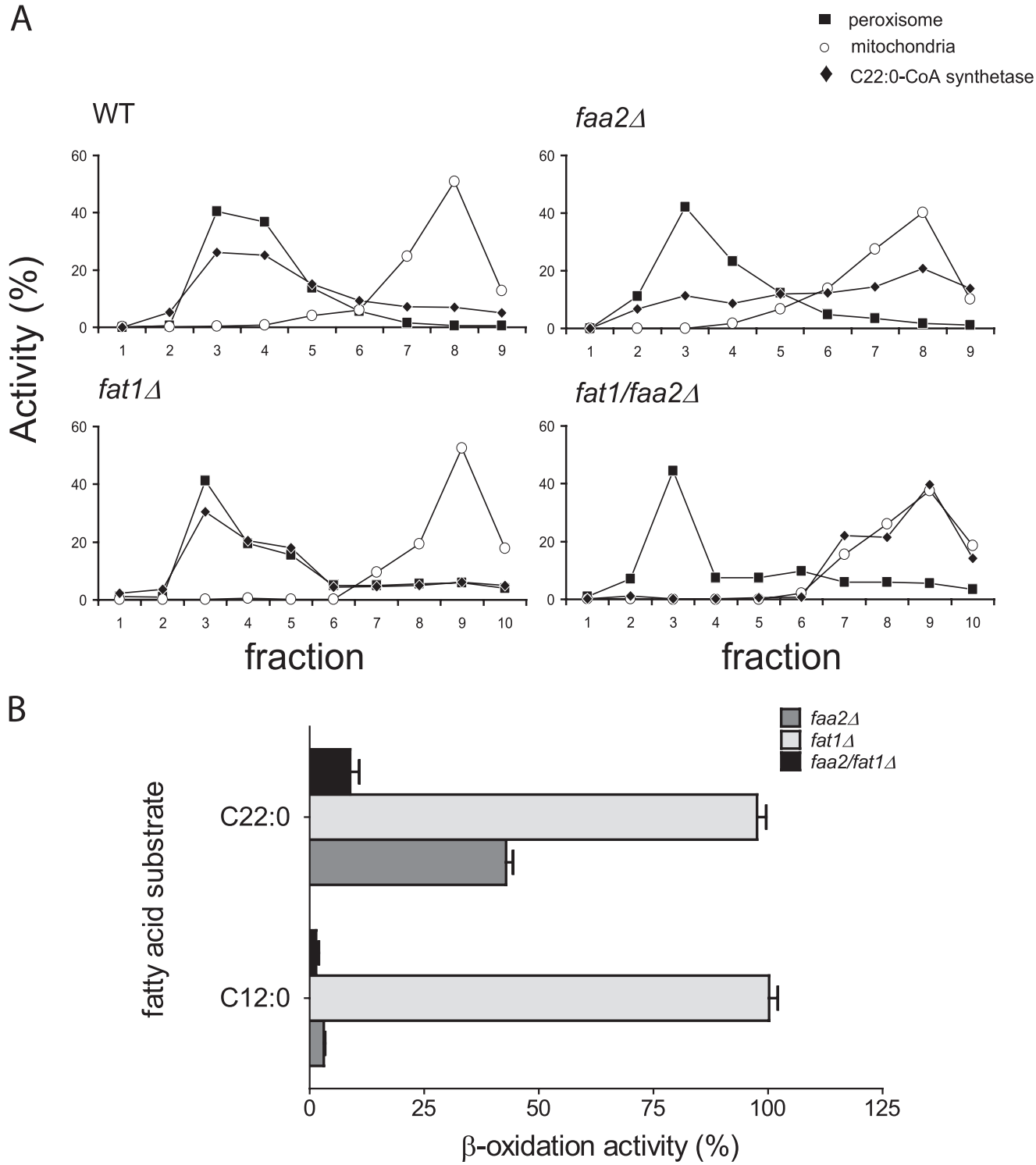

FIGURE 6. **Fat1p is peroxisomal acyl-CoA synthetase involved in C22:0 β-oxidation.** *A*, distribution of C22:0-CoA synthetase activity in *fat1*Δ and *fat1*/*faa2*Δ cells. The activities of mitochondrial (fumarase; ○) and peroxisomal (3-hydroxyacyl-CoA dehydrogenase; ■) marker enzymes and C22:0-CoA synthetase (◆) were measured in the fractions of a Nycodenz gradient. The experiment was carried out three times. The results from a representative experiment are shown. *B*, cells grown on oleate/glycerol were incubated with [1-^{14}C]behenic acid (C22:0) or [1-^{14}C]laureate acid (C12:0), and β-oxidation rates were measured (see "Experimental Procedures"). β-Oxidation rates in wild type cells were taken as a reference (100%). This experiment was performed at least three times; *error bars* indicate mean ± S.D. See "Experimental Procedures" for the strains used.

We also showed (see Figs. 6 and 7) that Fat1p has a role in the ABC transporter-dependent C22:0 fatty acid uptake into peroxisomes. Detection of functional activity of this protein is dependent on the presence of Pxa1p-Pxa2p. This may be because the Fat1 protein is associated with the ABC transporters Pxa1p-Pxa2p or because there is an optimal chain length match only with these two transporters. The model proposed in Fig. 7 shows fatty acid interaction with proteins at the peroxisomal membrane either as an FFA or as an acyl-CoA ester. We do not have solid evidence for free fatty acid transport across the peroxisomal membrane via Fat1p

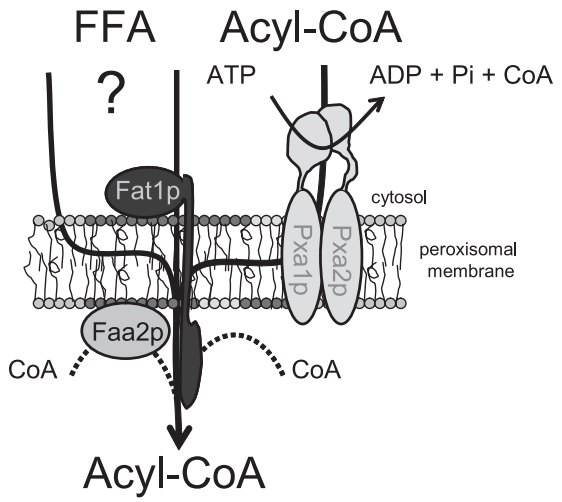

FIGURE 7. **New model for fatty acid transport across peroxisomal membrane in *S. cerevisiae*.** The uptake of fatty acids into peroxisomes may occur via two routes: either as free fatty acid thus requiring intraperoxisomal activation by the peroxisomal acyl-CoA synthetase(s) Faa2p and/or Fat1p into acyl-CoA or as acyl-CoA ester. The latter route involves the two yeast half-transporters Pxa1p-Pxa2p, which deliver the acyl group from acyl-CoA esters to the peroxisomal matrix. The corresponding free fatty acids are reactivated to their CoA ester by Faa2p and/or Fat1p in the peroxisomal lumen prior to β-oxidation.

(peroxisomal), but there is evidence that Fat1p is functional as a bifunctional protein and plays a central role in fatty acid trafficking at the level of long chain fatty acid transport and very long chain fatty acid activation (34–37). This Fat1p-mediated transport is much more energetically favorable than the ABC transporter-mediated pathway.

The biochemical hallmark of patients suffering from X-ALD is the significant accumulation of saturated, straight chain, very long chain fatty acids (VLCFAs; C24:0 and C26:0) and monounsaturated VLCFA (C26:1) in plasma and various tissues. Initially, the elevated VLCFA levels that occur in X-ALD were predicted to be caused by a reduced activity of VLCFA acyl-CoA synthetase, and thus it was thought that VLCFA acyl-CoA synthetase was the candidate gene to be defective in X-ALD. However, positional cloning identified the *Hs*ABCD1 gene as defective in X-ALD. Although the involvement of VLCFA acyl-CoA synthetase has been disputed (41), our current findings explain why co-expression of adrenoleukodystrophy protein and VLCFA acyl-CoA synthetase has a synergistic effect on β-oxidation and VLCFA acyl-CoA synthetase activity and leads to a functional rather than a physical interaction of VLCFA acyl-CoA synthetase and ABC transporters.

Confusingly, there is no clear-cut correlation between genotype and phenotype in X-ALD patients. For example, no correlations could be identified between the nature of the ABCD1 mutations, level of VLCFA accumulation, and severity of the pathology of X-ALD. In our transport model (Fig. 7), we can divide the process of acyl-CoA transport across the peroxisomal membrane into three steps: 1) hydrolysis of the acyl-CoA ester, 2) transport of the fatty acid across the peroxisomal membrane, and 3) re-esterification of the fatty acid to an acyl-CoA ester. Not only could a defect of one of these three steps lead to an accumulation of VLCFAs and/or VLCFA-CoA esters, but also the level of the peroxisomal acyl-CoA synthetase activity or the cytosolic CoA concentration could influence the accumulation of VLCFAs and/or VLCFA-CoA esters. Each of these could have a different influence on the pathology of individual X-ALD patients, thereby giving rise to multiple phenotypes. The mechanism of hydrolysis of VLCFA-CoA esters and the transport and re-esterification of the VLCFAs will be investigated in future studies by using humanized yeast as an *in vivo* model system or *in vitro* by functional reconstitution of the Pxa1p-Pxa2p complex in a liposomal system.

In summary, we provide evidence that in *S. cerevisiae* acyl-CoA esters are hydrolyzed prior to the actual transport into the peroxisomes by the Pxa1p-Pxa2p complex with the CoA presumably being released into the cytoplasm. The Pxa1p-Pxa2p complex functionally interacts on the inner surface of the peroxisomal membrane with the acyl-CoA synthetase(s) Faa2p and/or Fat1p for subsequent re-esterification of the VLCFAs.

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