The peroxisomal lumen in *Saccharomyces cerevisiae* is alkaline

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Summary

Peroxisomes have a central function in lipid metabolism, including the β-oxidation of various fatty acids. The products and substrates involved in the β-oxidation have to cross the peroxisomal membrane, which previously has been demonstrated to constitute a closed barrier, implying the existence of specific transport mechanisms. Fatty acid transport across the yeast peroxisomal membrane may follow two routes: one for activated fatty acids, dependent on the peroxisomal ABC half transporter proteins Pxa1p and Pxa2p, and one for free fatty acids, which depends on the peroxisomal acyl-CoA synthetase Faa2p and the ATP transporter Ant1p. A proton gradient across the peroxisomal membrane as part of a proton motive force has been proposed to be required for proper peroxisomal function, but the nature of the peroxisomal pH has remained inconclusive and little is known about its generation. To determine the pH of *Sacharomyces cerevisiae* peroxisomes in vivo, we have used two different pH-sensitive yellow fluorescent proteins targeted to the peroxisome by virtue of a C-terminal SKL and found the peroxisomal matrix in wild-type cells to be alkaline (pH_{per} 8.2), while the cytosolic pH was neutral (pH_{cyt} 7.0). No ΔpH was present in *ant1Δ* cells, indicating that the peroxisomal pH is regulated in an ATP-dependent way and suggesting that Ant1p activity is directly involved in maintenance of the peroxisomal pH. Moreover, we found a high peroxisomal pH of >8.6 in *faa2Δ* cells, while the peroxisomal pH remained 8.1±0.2 in *pxa2Δ* cells. Our combined results suggest that the proton gradient across the peroxisomal membrane is dependent on Ant1p activity and required for the β-oxidation of medium chain fatty acids.

Key words: Peroxisomes, β-oxidation, pH, Fatty acid transport, Carrier, Yeast

Introduction

Peroxisomes are essential subcellular organelles involved in a variety of metabolic processes. Their importance is underlined by the identification of a number of inherited human diseases in which one or more peroxisomal functions are impaired (Moser et al., 1991; van den Bosch et al., 1992; Wanders et al., 1995). One of the main functions of peroxisomes is the degradation of fatty acids. In mammals, including humans, this takes place not only in peroxisomes but also in mitochondria. Long-chain fatty acids (LCFA) and medium-chain fatty acids (MCFA) are oxidized in mitochondria whereas very long-chain fatty acids and certain branched-chain fatty acids are first chain-shortened in peroxisomes and subsequently oxidized to completion in mitochondria. This and other metabolic functions of peroxisomes (van den Bosch et al., 1992; Reddy and Mannaerts, 1994; Wanders and Tager, 1998) imply the existence of transport proteins in the peroxisomal membrane to shuttle metabolites from the interior of peroxisomes to the cytosol and vice versa. Indeed, several candidates for such transport proteins have been identified, including the ABC half transporters Aldp, Aldrp, Pmp70p and Pmp69p (Mosser et al., 1993; Liu et al., 1999). Moreover, we recently demonstrated that, similar to its yeast orthologue Ant1p, the human peroxisomal membrane protein Pmp34p functions as an adenine nucleotide transporter (Visser et al., 2002).

We and others have been using the yeast *Saccharomyces cerevisiae* as a model organism to study the function of peroxisomes for a number of reasons. Firstly, in contrast to mammalian cells, peroxisomes in yeast are the only organelles in which β-oxidation of fatty acids takes place (Kunau et al., 1995). Secondly, *S. cerevisiae* is an organism easy to manipulate genetically and its entire genome sequence is available to enable specific studies. Thirdly, *S. cerevisiae* can use fatty acids as sole carbon source and, therefore, mutants disturbed in fatty acid β-oxidation can be readily identified by their growth characteristics in media supplied with different fatty acids.

In the last few years much insight into the specific roles of peroxisomal (membrane) proteins involved in peroxisome biogenesis has been obtained (Subramani, 1998; Tabak et al., 1999; Gould and Valle, 2000). By contrast, there is still only little detailed information on peroxisomal membrane proteins involved in metabolite transport despite in vivo evidence – at least in yeast – for their existence (van Roermund et al., 1995;

van Roermund et al., 1998; Henke et al., 1998; Hettema and Tabak, 2000). Earlier we reported the existence of two independent pathways for fatty acid transport across the peroxisomal membrane (Hettema et al., 1996): one for activated fatty acids, which depends on the peroxisomal ABC transporter proteins Pxa1p and Pxa2p (Shani et al., 1995; Shani and Valle, 1996; Swartzman et al., 1996) that probably act as acyl-CoA ester transporters (Verleur et al., 1997), the other for free fatty acids, which depends on the peroxisomal acyl-CoA synthetase Faa2p and Pex11p (van Roermund et al., 2000). Furthermore, we and others demonstrated the existence of a peroxisomal ATP transporter (Nakagawa et al., 2000; van Roermund et al., 2001; Palmieri et al., 2001).

A controversial issue with respect to peroxisomal metabolite transport remains. Does a proton gradient across the peroxisomal membrane indeed exist? Such a gradient might provide a proton motive force that promotes the transport of certain metabolites such as fatty acids. In 1987, Douma and co-workers (Douma et al., 1987) reported the existence of a putative proton-translocating ATPase in the peroxisomal membrane of the methylotrophic yeast *Hansenula polymorpha*. The role of this ATPase might be to serve as an ATP-driven pump to acidify the peroxisomal lumen, which in this yeast was shown by ^{31}P-NMR to have an acidic pH of 5.8-6.0 (Nicolay et al., 1987) as opposed to a neutral cytosolic pH (pH_{cyt}). In contrast to these results obtained in yeast, Dansen et al. recently reported that peroxisomes in mammalian cells are alkaline (Dansen et al., 2000), while Jankowski et al. claimed that the peroxisomal pH (pH_{per}) in such cells was similar to the pH_{cyt} (i.e. neutral) (Jankowski et al., 2001). Finally, Van der Lende et al. reported a slightly alkaline pH in peroxisomes of the fungus *Penicillium chrysogenum* (Van der Lende et al., 2002).

Since most in vivo evidence for peroxisomal metabolite transporters has been obtained in *S. cerevisiae*, we decided to determine the peroxisomal pH in vivo in oleate-grown *S. cerevisiae* cells. We used two different, pH-sensitive yellow fluorescent proteins (YFPs), enhanced YFP (EYFP) and EYFP(H148G), that have p*K*a values of 7.0 and 8.0, respectively (Elsliger et al., 1999), and that are either targeted to peroxisomes by a peroxisomal targeting signal or are used as cytosolic proteins when lacking these targeting signals. Similar proteins were previously used to determine the pHs of the cytosol, golgi and mitochondria (Robey et al., 1998; Llopis et al., 1998; Kneen et al., 1998; Elsliger et al., 1999; Matsuyama et al., 2000; Awaji et al., 2001).

In line with the results of Dansen and collegues in mammalian cells (Dansen et al., 2000), we found a ΔpH across the peroxisomal membrane with a peroxisomal pH of 8.2, as opposed to a pH_{cyt} of 7.0. This implies that in vivo the peroxisomal membrane provides a barrier for protons. We also measured the peroxisomal pH in different mutants and under different growth conditions and found some remarkable differences compared with wild-type cells. Our combined results suggest that the proton gradient across the peroxisomal membrane depends on the activity of Ant1p and acts as a driving force for MCFA β-oxidation.

Materials and Methods

Yeast strains and culture conditions

The wild-type strain used in this study was *S. cerevisiae* BJ1991 (Mat α, *leu*2, *trp*1, *ura*3-251, prb1-1122, *pep*4-3, *gal*2). The β-oxidation mutants *fox1Δ, pxa2Δ, ant1Δ* and the mutants with a defect in peroxisomal import of either PTS1 (*pex5Δ*) or PTS2 (*pex7Δ*) proteins, were constructed from BJ1991 as described previously (van Roermund et al., 1998; Hettema et al., 1996). Yeast transformants were selected and grown in minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco), supplemented with 0.3% glucose and amino acids (20 μg/ml) as needed. Liquid-rich media to grow cells for β-oxidation assays, confocal microscopy and enzyme assays were composed of 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone supplemented with 3% glycerol or 0.12% oleate with 0.2% Tween-80, or a combination of both carbon sources. Before shifting to these media, the cells were grown in minimal 0.3% glucose medium for at least 24 hours.

Construction of EYFP-expression plasmids

To enable the expression of EYFP in *S. cerevisiae*, the encoding open reading frame was amplified by standard PCR methods. Forward primer: 5′-TTTTggATCCATgAgCAAgggCgAggA, reverse primer: 5′-TTTTTCTAgATTACTTgTACAgCTCgTCC. To create EYFP-SKL, the peroxisomal targeting signal SKL was introduced to the C-terminus of the protein with a different reverse primer, 5′-TTTTC-TAgTTAAAgCTTTgACTTgTACAgCTCgTCCATgC. EYFP(H148G) and EYFP(H148G)-SKL were obtained using a two step PCR in which the first PCR product was used as a megaprimer in the second PCR. The first PCR was done with the forward primer 5′-TTTTggATCCATgAgCAAgggCgAggA and the reverse primer 5′-CATgATATAgACgTTgCCgCTgTTgTAgTTgTACTC. This latter primer introduces the H148G mutation. Full-length EYFP(H148G) and EYFP(H148G)-SKL were made by a second PCR amplification using the first PCR product as the megaprimer and either the reverse primer 5′-TTTTTCTAgATTACTTgTACAgCTCgTCC [to obtain EYFP(H148G)] or by using a different reverse primer, 5′-TTTTCTAgTTAAAgCTTTgACTTgTACAgCTCgTCCATgC to obtain EYFP(H148G)-SKL.

The PCR products were cloned into the *S. cerevisiae* expression plasmid pEL30 (Pca31) (van Roermund et al., 1999) to be under the transcriptional control of the oleate-inducible catalase promoter. All PCR fragments were sequenced to ensure that the sequences contained no PCR-introduced errors.

Intracellular pH measurements

S. cerevisiae cells expressing EYFP, EYFP-SKL, EYFP(H148G) or EYFP (H148G)-SKL were cultured overnight in oleate-medium, harvested and resuspended to an OD_{600}=1 in 0.65 M sorbitol supplemented with 100 μg/ml digitonin. After a 10-minunte incubation, cells were pelleted, washed and resuspendend to an OD_{600}=1 into a buffer solution of 50 mM MES, 50 mM MOPS, 50 mM Bicine and 0.65 M sorbitol. Calibration pH curves were generated by determining the fluorescence of digitonin-permeabilized transformants incubated in buffers with pH values of 4 to 9. The emission was determined by confocal microscopy and the median of the fluorescence of at least 100 different cells was calculated. To determine pH_{cyt} and pH_{per}, the fluorescence was measured in at least 100 non-permeabilized cells expressing EYFP-SKL and EYFP(H148G)-SKL or EYFP and EYFP(H148G).

Imaging was performed with a Leica SP2 confocal system. Excitation of EYFP was done with the 514 nm line of the Ar-Ion laser and the emission was detected between 525 nm and 580 nm. The detection pinhole setting equalled 1 Airy disc for all experiments. A 63×/1.32NA HCX PL APO Oil UV objective was used. Images (8 bit) were acquired in a 512×512 format with a pixel size of 0.46 μm^2. For a series of experiments the sample expected to give the brightest fluorescence was imaged first and the image was adjusted to the full dynamic range (8 bit) of the system. For all following samples of this

series these instrument settings were strictly adhered to. Consequently, the intensities of all images within a series can be compared directly. The Leica QWin image analysis software was used to determine the intensity of the fluorescence in the images.

Enzyme assays

β-oxidation assays in intact cells were performed as previously described by Van Roermund et al. (Van Roermund et al., 1999). Cells were grown overnight in media containing oleate and glycerol to induce fatty-acid β-oxidation.

Results

Subcellular localisation of EYFP and EYFP(H148G) and their SKL-tagged variants

To enable the in vivo measurement of pH_{cyt} and pH_{per} in oleate-grown *S. cerevisiae* cells, we expressed EYFP and an H148G mutant version of EYFP plus SKL-tagged versions of these proteins (see Materials and Methods). To verify that under the culture conditions chosen, EYFP and EYFP(H148G) are confined to the cytoplasm and EYFP-SKL and EYFP (H148G)-SKL to the peroxisomes, we determined the subcellular localisation of the various EYFPs using confocal laser scanning microscopy. In all yeast cells expressing EYFP-SKL and EYFP(H148G)-SKL, a punctate fluorescence pattern consistent with a peroxisomal localisation was observed (Fig. 1a and b). Cells expressing the untagged forms of these two EYFPs showed a diffuse fluorescence pattern consistent with a cytosolic localization (Fig. 1c and 1d).

As an additional control for the peroxisomal localization, we also expressed the two SKL-tagged EYFPs in *pex5Δ* cells, which are defective in the peroxisomal import of proteins with a peroxisomal targeting signal 1 (PTS1) including SKL (Fig. 1e,f). As expected, we observed a cytosolic labelling in line with the fact that the SKL-tagged EYFPs are imported via the PTS1-mediated import pathway. In further agreement with this notion, a normal punctate peroxisomal fluorescence pattern of the SKL-tagged EYFPs was observed in *pex7Δ* cells. These cells are defective in the peroxisomal import of a different subset of proteins that harbour a PTS2 signal, but still import proteins with a PTS1 signal (Fig. 1g,h). These results show that the SKL-tagged versions of EYFP behave like authentic peroxisomal proteins, whereas the untagged versions remain cytosolic.

pH_{cyt} and pH_{per} in wild-type *S. cerevisiae*

Using cells that express EYFP and EYFP(H148G), we determined the pH_{cyt} in oleate-grown cells based on the median of the fluorescence as measured in >100 individual intact cells imaged by confocal laser scanning microscopy. To be able to relate the amount of fluorescence to a certain pH value, we first generated pH calibration curves for the two proteins using digitonin-permeabilized cells that expressing EYFP or EYFP(H148G) and buffers ranging from pH 4 to pH 9. Digitonin is known to bind cholesterol and ergosterol leading to the subsequent permeabilization of the membranes (Verleur et al., 1997; Artuch et al., 2000). Consistent with its previously determined p*Ka* value of 7.0 (Valenciano et al., 1998), we observed that EYFP can only be used reliably for pH measurements between 6.4 and 7.6 (Fig. 2A), whereas EYFP (H148G) with a p*Ka* value of 8.0 (Matsuyama et al., 2000) can only be used reliably for pH measurements between 7.4 and 8.6. (Fig. 2B). Based on the fluorescence in intact cells expressing EYFP we determined a pH_{cyt} of 7.0±0.1. In accordance with this, we did not observe fluorescence in intact cells expressing EYFP (H148G) (i.e. below pH=7.4).

The same set of experiments was repeated for the cells expressing the SKL-tagged versions of the EYFPs to determine the pH_{per}. In intact cells expressing EYFP-SKL we observed a maximum fluorescence, implying a pH>7.5 (Fig. 2C). Consistent with this, we were able to determine a pH_{per} of 8.2±0.2 in intact cells expressing the EYFP(H148G)-SKL variant. These experiments thus indicate that peroxisomes

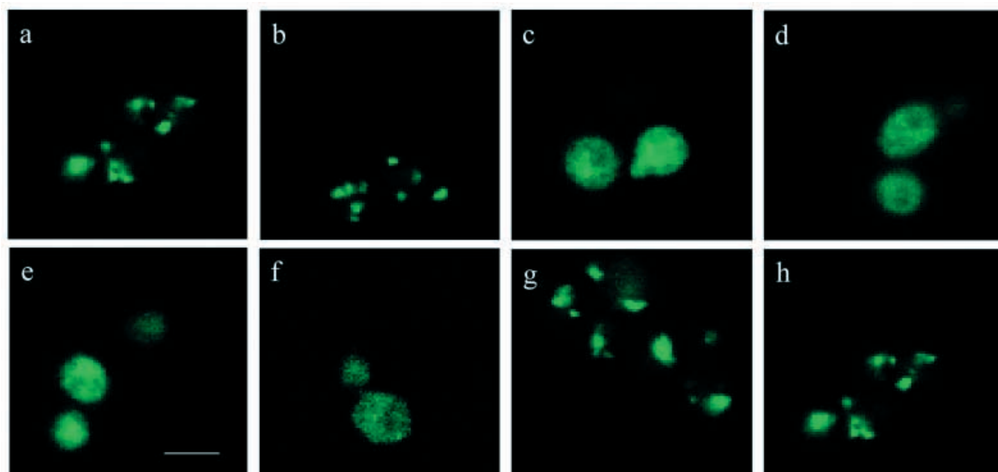

Fig. 1. Subcellular localisation of EYFP, EYFP(H148G), EYFP-SKL and EYFP(H148G)-SKL expressed in *S. cerevisiae* and assessed by confocal laser scanning microscopy. Expression of EYFP-SKL (a) and EYFP (H148G)-SKL (b) in wild-type cells results in a peroxisomal localisation of EYFPs, whereas expression of EYFP (c) and EYFP(H148G) (d) results in a cytosolic localisation. Expression of EYFP-SKL (e) and EYFP (H148G)-SKL (f) in *pex5Δ* cells results in a cytosolic localisation, whereas in *pex7Δ* cells both EYFP-SKL (g) and EYFP(H148G)-SKL (h) are localised to peroxisomes. Bar: 5 μM.

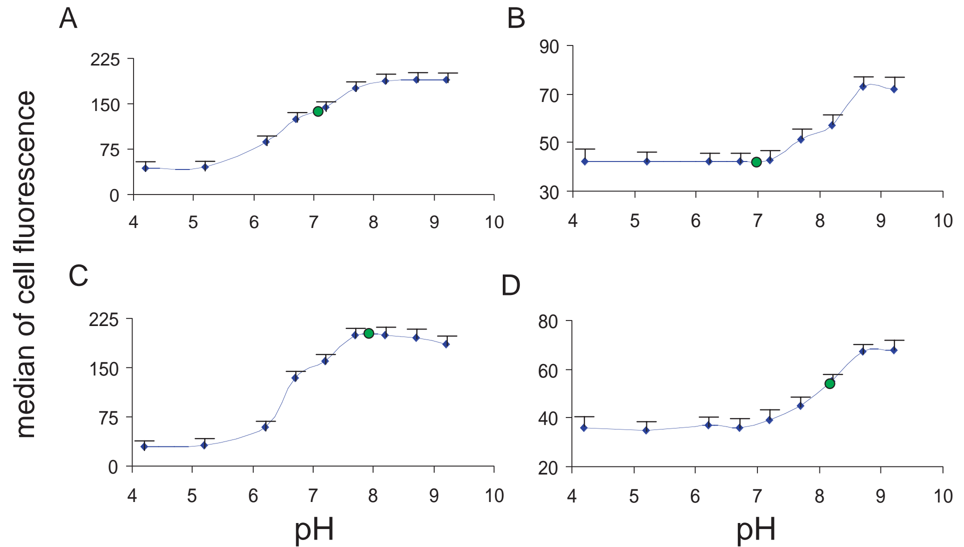

Fig. 2. Determination of cytosolic (pH_{cyt}) and peroxisomal (pH_{per}) pH. Wild-type cells were transformed with plasmids containing the coding sequence of EYFP (A), EYFP(H148G) (B), EYFP-SKL (C) and EYFP(H148G)-SKL (D), and were grown in oleate-containing medium. Calibration pH curves were generated by determining the fluorescence of digitonin-permeabilized transformants incubated in buffers with pH values ranging from 4.2 to 9.2 The emission was determined by confocal microscopy and the median of the fluorescence of at least 100 different cells was calculated (see Materials and Methods). To determine the pH_{cyt}, the fluorescence was measured in at least 100 non-permeabilized cells expressing EYFP and EYFP(H148G) [green circles in (A) and (B)]. To determine the pH_{per}, the fluorescence was measured in at least 100 non-permeabilized cells expressing EYFP-SKL and EYFP(H148G)-SKL [green circles in (C) and (D)]. Each experiment was performed at least three times and the standard deviations from the mean values are indicated by error bars.

have an alkaline lumen compared with the cytosol with a neutral pH.

Reduced pH_{per} in pex7Δ cells and in the β-oxidation mutant fox1Δ

Using a different approach, Dansen et al. reported that the pH_{per} in cultured human cells is also approximately 8.2 as opposed to a pH_{cyt} of approximately 7.2 (Dansen et al., 2000). Moreover, the group reported that the pH_{per} in cells from patients with Rhizomelic Chondrodysplasia Punctata (RCDP) is lower than in control cells and in fact similar to the pH_{cyt}. Such RCDP patients have a functional deficiency of the PTS2 receptor peroxin 7 because of mutations in the *PEX7* gene (Motley et al., 1997; Motley et al., 2002; Braverman et al., 1997; Purdue et al., 1997). To study whether the same is true in *S. cerevisiae*, we measured the pH_{per} in *pex7Δ* cells transformed with either EYFP-SKL or EYFP(H148G)-SKL. In contrast to wild-type cells, we no longer measured fluorescence in the cells expressing EYFP (H148G)-SKL, indicating a pH <7.4. Indeed, the measurements of the *pex7Δ* cells expressing EYFP-SKL revealed a pH_{per} of approximately 7.0, which is similar to the pH of the cytosol (Table 1).

Since disruption of *PEX7* leads to the inability of yeast cells to grow on fatty acids, one explanation for the reduced pH_{per} in *pex7Δ* cells could be that the block in fatty acid β-oxidation has a direct effect on the pH_{per}. We therefore performed similar experiments in *fox1Δ* cells in which acyl-CoA oxidase, the first enzyme in peroxisomal β-oxidation, is deficient. Also in these mutant cells, the pH_{per} was approximately 7.0 (Table 1).

Table 1. Measurements of the peroxisomal and cytosolic pH in different β-oxidation mutants cultured on oleate

Yeast cells	Deleted gene	pH_{per}	pH_{cyt}
BJ1991	None (wild type)	8.2±0.2	7.0±0.1
fox1Δ	Acyl-CoA oxidase	6.9±0.1	7.1±0.1
pex7Δ	PTS2 receptor	7.0±0.1	6.9±0.1
ant1Δ	ATP/AMP carrier	7.0±0.1	7.1±0.1

Mutant and wild-type cells were transformed with EYFP-SKL or EYFP(H148G)-SKL for peroxisomal pH and EYFP or EYFP(H148G) for cytosolic pH measurements. Fluorescence was quantified using confocal laser scanning microscopy (see Materials and Methods).

ATP is necessary to maintain the pH_{per}

The absence of a high pH_{per} of 8.2 in the *pex7Δ* and *fox1Δ* cells might be owing to a lowered energy status of the cells caused by the inability to oxidise fatty acids. We therefore repeated the experiments but also added glycerol to the cells, since glycerol is readily oxidised by mitochondria, thereby leading to an increased energy status. Oleate was included to maintain maximal induction of peroxisomes. It appears that under these conditions the pH_{per} shifts to >8.6 in both mutants (Table 2), as concluded from the maximum fluorescence observed both in the cells expressing EYFP-SKL and EYFP(H148G)-SKL. In wild-type cells grown under these conditions the pH_{per} remains 8.2.

To study whether the pH in the peroxisomes depends on the cytosolic or the peroxisomal ATP pools, we also measured the pH_{per} in cells whose *ANT1* gene had been disrupted. Previous studies have shown convincingly that Ant1p functions as an

Table 2. Measurements of the peroxisomal and cytosolic pH in different β-oxidation mutants cultured on oleate supplemented with glycerol

Yeast cells	Deleted gene	pH_{per}	pH_{cyt}
BJ1991	None (wild type)	8.2±0.2	7.0±0.1
fox1Δ	Acyl-CoA oxidase	>8.6	7.1±0.1
pex7Δ	PTS2 receptor	>8.6	7.1±0.1
ant1Δ	ATP/AMP carrier	7.0±0.1	7.1±0.1
faa2Δ	Acyl-CoA synthetase	>8.6	6.9±0.1
pxa2Δ	ABC transporter	8.1±0.2	7.0±0.1

Mutant and wild-type cells were transformed with EYFP-SKL or EYFP(H148G)-SKL for peroxisomal pH and EYFP or EYFP(H148G) for cytosolic pH measurements. Fluorescence was quantified using confocal laser scanning microscopy (see Materials and Methods).

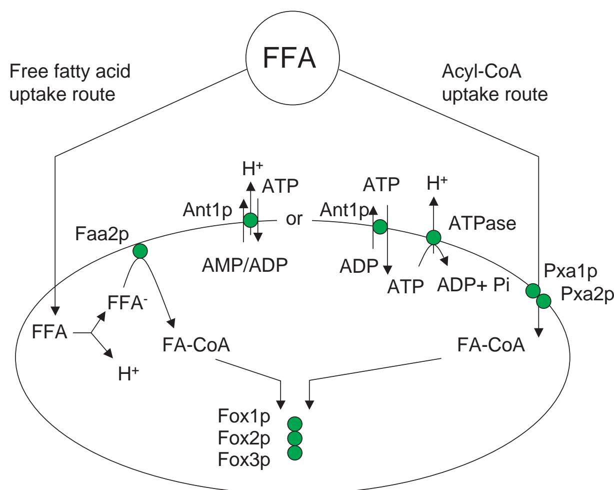

Fig. 3. Putative scheme of peroxisomal fatty acid β-oxidation with the free fatty acid and the acyl-CoA uptake route plus the role of Ant1p in ΔpH generation. FFA, free fatty acid; Fox1p, Fatty acyl-Coenzyme A oxidase; Fox2p, 3-hydroxyacyl-CoA dehydrogenase; Fox3p, 3-oxoacyl-CoA thiolase; Ant1p, Adenine nucleotide transporter; Pxa1p, peroxisomal ATP-binding cassette (ABC) transporter 1; Pxa2p, peroxisomal ATP-binding cassette (ABC) transporter 2, putative ATPase (postulated).

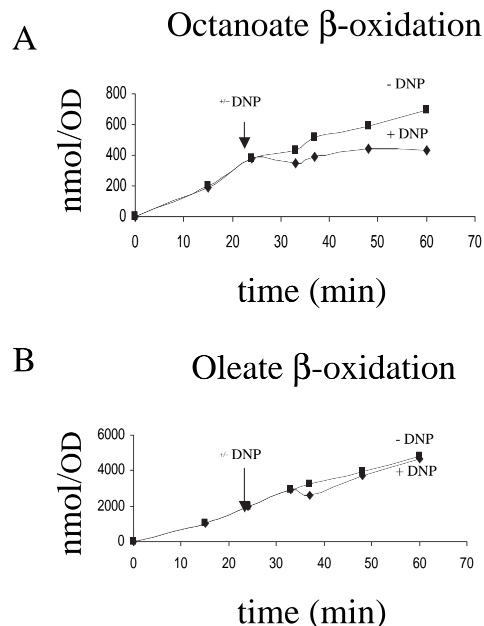

Fig. 4. Octanoate β-oxidation (A) and oleate β-oxidation (B) in wild-type yeast cells. The medium-chain fatty acid β-oxidation was completely deficient after 5 mM dinitrophenol had been added to dissipate the ΔpH, whereas oleate β-oxidation remained the same. Arrows indicate the time point were DNP was either added (+) or not (–).

ATP carrier in the peroxisomal membrane (Palmieri et al., 2001; van Roermund et al., 2001) and thus links the cytosolic ATP pool to the peroxisomal pool. In *ant1Δ* cells, we observed a pH_{per} of 7.0 when cells were grown in the medium containing only oleate but also when cells were grown in the medium containing both oleate and glycerol (Tables 1 and 2). This indicates that the ATP carrier activity is required for establishing the pH gradient across the peroxisomal membrane.

pH_{per} gradient: a driving force for MCFA β-oxidation

Previous work has indicated that fatty acids can enter the peroxisomes via two different pathways (Hettema et al., 1996) (Fig. 3). One pathway accepts the CoA-esters of fatty acids, resulting from the activation of fatty acids by the cytosolic synthetases Faa1p and/or Faa4p (Knoll et al., 1994), and is dependent on the peroxisomal ABC-half-transporter proteins Pxa1p and Pxa2p (Shani et al., 1995; Hettema et al., 1996; Shani and Valle, 1996; Swartzman et al., 1996). The other pathway accepts free fatty acids followed by the intraperoxisomal activation to their CoA-esters by the peroxisomal acyl-CoA synthetase Faa2p. LCFAs, including oleate, can be transported via both import pathways although under physiological conditions the predominant pathway is via Pxa1/Pxa2p as LCFA-CoA. Medium-chain fatty acids, such as octanoate, are primarily transported as free fatty acid across the peroxisomal membrane, an import process that not only requires the involvement of Faa2p, but also that of Ant1p and Pex11p (Nakagawa et al., 2000; van Roermund et al., 2000; van Roermund et al., 2001; Palmieri et al., 2001).

To determine whether there is a relationship between fatty acid transport and the pH_{per}, we determined the pH_{per} in mutants disturbed in either of these pathways (Table 2). To this end, the various mutants were grown in oleate-glycerol medium to avoid problems in the energy status as discussed above.

When the transport pathway for FA-CoA esters is blocked by disruption of *PXA2*, the pH_{per} remained 8.2, suggesting that this pathway is not needed for maintenance of the pH_{per}. By contrast, when the free-fatty-acid-import pathway is disrupted through deletion of the *FAA2* gene, the pH_{per} increased to >8.6, suggesting that the establishment of a pH_{per} of 8.2 is dependent on the activity of this pathway.

To provide additional evidence for this, we measured the octanoate and oleate β-oxidation in wild-type cells after adding 5 mM of the uncoupler dinitrophenol (DNP) to dissipate the ΔpH across the peroxisomal membrane. Fig. 4 shows that MCFA β-oxidation was completely blocked whereas oleate β-oxidation was normal. This data indicate that a drop in the pH_{per} value has no effect on LCFA β-oxidation, and that a high pH_{per} is required for the oxidation of free fatty acids.

Discussion

In this study we have shown that in vivo, the pH_{cyt} in wild-type *S. cerevisiae* yeast cells growing on oleate is approximately 7.0±0.1 and the pH_{per} is approximately 8.2±0.2. This indicates that under these conditions a proton gradient exists across the peroxisomal membrane in yeast, which provides additional support for the fact that in vivo the peroxisomal membrane constitutes an impermeable barrier to metabolites. Furthermore, we have shown that peroxisomes in *pex7Δ* and *fox1Δ* cells cultured in oleate medium have a neutral pH. This suggests that a disruption of genes involved in the β-oxidation of fatty acids influences the pH_{per} most probably because a block in β-oxidation leads to reduced cellular ATP levels.

Indeed, when the *pex7Δ* and *fox1Δ* cells were grown on oleate-glycerol mixture, the pH_{per} became more than 8.6, whereas the pH_{per} of wild-type cells under these growth conditions remained 8.2. Moreover, when we measured the pH_{per} in *ant1Δ* cells, which are defective in the peroxisomal ATP carrier Ant1p (Palmieri et al., 2001; van Roermund et al., 2001; Nakagawa et al., 2000), we observed no effect of the cell's energy status on the establishment of a pH_{per}; on both oleate and oleate-glycerol the pH_{per} remained neutral and similar as the pH_{cyt}. Combined, these data indicate that the capability of the yeast cell to generate a pH difference between the cytosol and the peroxisomal lumen during growth on oleate is coupled to the activity of the ATP carrier Ant1p. In principle, there are two possible scenarios for this: either the uptake of ATP via Ant1p is directly coupled to transmembrane proton-transport or intraperoxisomal ATP is required to drive a proton-translocating ATPase (see Fig. 3). Future studies using reconstituted Ant1p should resolve this issue.

When we determined the pH_{per} in mutants disturbed in either of the two import pathways for fatty acids, we noted an interesting difference. In *pxa2Δ* cells, whose peroxisomal import pathway of FA-CoA esters is disturbed, no effect on the pH_{per} is observed: like in wild-type cells, the pH_{per} is approximately 8.2. In the *faa2Δ* mutant, however, in which the free-fatty-acid oxidation pathway is disturbed since the free fatty acids can no longer be activated by the intraperoxisomal acyl-CoA synthetase, we observed a high pH_{per} (>8.6). One explanation for this might be that the high pH_{per} is facilitating the import of free fatty acids, or perhaps is even required for this. As discussed by Kamp et al., transport of FFA involves the adsorption of ionised FFAs to a membrane followed by protonation, flip-flop within the membrane and finally desorption at the other side of the membrane where the FFAs will be deprotonated (Kamp et al., 2003). Hence, the peroxisomal import of FFAs may very well be coupled to the transport of protons and might be further facilitated by coupling transport of FFAs to the esterification by the peroxisomal acyl-CoA synthetase (Schaffer, 2002). Thus, when the transport is hampered because the peroxisomal acyl-CoA synthetase is absent in the *faa2Δ* mutant, the basic pH_{per} will no longer be compensated for by the co-import of protons as a consequence of transmembrane free-fatty-acid transport and, consequently, will increase. This suggests that a sufficiently high free-fatty-acid flux across the peroxisomal membrane will lead to a partial collapse of the pH_{per}.

In case of the *pxa2Δ* mutant, the free-fatty-acid import pathway is still functional and, as a consequence, no effect on the pH_{per} is observed. This was confirmed when we added the uncoupler DNP to wild-type yeast cells and found that the MCFA (octanoate) oxidation was affected while the LCFA (oleate) β-oxidation remained normal, indicating that oleate β-oxidation does not dependent on a ΔpH but is necessary for MCFA oxidation.

Based on studies with a pH-sensitive fluorescent probe tagged with a peroxisomal targeting signal, Dansen et al. concluded that peroxisomes in human cells also have a pH of approximately 8.2 as opposed to a neutral pH of 7.2 in the cytosol (Dansen et al., 2000). More recently, however, Jankowski et al. disputed these findings and claimed that peroxisomes in human cells are not basic but have a neutral pH between 6.9 and 7.1 (Jankowski et al., 2001). They based this conclusion on the results with a pH-sensitive green fluorescent protein (GFP) mutant called pHluorin, originally constructed by Miesenbock and collegues (Miesenbock et al., 1998). Unfortunately, it appears that the pKa of this pHluorin is 6.5, which is too low to enable a reliable determination in the case of a high pH, similar as we observed with the EYFP-SKL protein, which even had a higher pKa of 7.0. Finally, based on studies with only the EYFP protein, van der Lende et al. also obtained indications for a slightly alkaline pH in peroxisomes of the fungus *Penicillium chrysogenum (*van der Lende et al., 2002).

Remarkably, it had been reported previously that peroxisomes in the methylotrophic yeast *Hansenula polymorpha* grown on methanol are acidic in nature, with a pH of 5.8 to 6.0 (Douma et al., 1987; Nicolay et al., 1987). This was concluded from ^{31}P-NMR studies, which revealed a strong correlation of a separate phosphate pool at a pH of approximately 6.0 with the presence of peroxisomes (Nicolay et al., 1987). Support for a lower pH_{per} in this and another methylotrophic yeast came from studies with a weak base DAMP probe, which was reported to accumulate in peroxisomes (Waterham et al., 1990). Additional studies will be required to determine whether the pH_{per} is different under different growth conditions. In this respect it should be noted that the pH optimum of most peroxisomal enzymes is between pH 8 and 9.

In conclusion, making use of two different yellow fluorescent proteins with different p*Ka* values that are specifically targeted to peroxisomes, we have demonstrated that peroxisomes in oleate-grown *S. cerevisiae* have an alkaline pH as opposed to a neutral pH_{cyt} and that Ant1p plays a crucial role in maintenance of this ΔpH.

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