

Function of human mitochondrial 2,4-dienoyl-CoA reductase and rat monofunctional Δ^3 - Δ^2 -enoyl-CoA isomerase in β -oxidation of unsaturated fatty acids

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Human 2,4-dienoyl-CoA reductase (2,4-reductase; DECR) and rat monofunctional Δ^3 - Δ^2 -enoyl-CoA isomerase (rat 3,2-isomerase; ECI) are thought to be mitochondrial auxiliary enzymes involved in the β -oxidation of unsaturated fatty acids. However, their function during this process has not been demonstrated. Although they lack obvious peroxisomal targeting signals (PTSs), both proteins have been suggested previously to also occur in the mammalian peroxisomal compartment. The putative function and peroxisomal location of the two mammalian proteins can be examined in yeast, since β -oxidation of unsaturated fatty acids is a compartmentalized process in *Saccharomyces cerevisiae* requiring peroxisomal 2,4-dienoyl-CoA reductase (Sps19p) and peroxisomal 3,2-isomerase (Eci1p). A yeast *sps19Δ* mutant expressing human 2,4-reductase ending with the native C-terminus could not grow on petroselinic acid [*cis*-C_{18:1(6)}] medium but could grow when the protein was extended with a PTS tripeptide, SKL (Ser-Lys-Leu). We therefore

reason that the human protein is a physiological 2,4-reductase but that it is probably not peroxisomal. Rat 3,2-isomerase expressed in a yeast *eci1Δ* strain was able to re-establish growth on oleic acid [*cis*-C_{18:1(9)}] medium irrespective of an SKL extension. Since we had shown that $\Delta^{2,4}$ double bonds could not be metabolized extra-peroxisomally to restore growth of the *sps19Δ* strain, we postulate that rat 3,2-isomerase acted on the Δ^3 unsaturated metabolite of oleic acid by replacing the mutant's missing activity from within the peroxisomes. Immunoblotting of fractionated yeast cells expressing rat 3,2-isomerase in combination with electron microscopy supported our proposal that the protein functioned in peroxisomes. The results presented here shed new light on the function and location of human mitochondrial 2,4-reductase and rat monofunctional 3,2-isomerase.

Key words: Eci1p, human DECR, rat ECI, Sps19p.

INTRODUCTION

In higher eukaryotes, β -oxidation of fatty acids occurs in both mitochondria and peroxisomes. Although the two processes are not identical (e.g. mitochondrial β -oxidation acts solely on L-3-hydroxyacyl-CoA esters, whereas mammalian peroxisomes additionally metabolize D-3 intermediates [1]), the β -oxidation spiral in both systems can only metabolize double bonds in the Δ^3 position and in the *trans*-configuration [2]. To reposition the double bonds of unsaturated fatty acids (either at odd or even-numbered positions and mostly in the *cis*-configuration) prior to β -oxidation, both compartments engage a comparable set of reaction steps executed by β -oxidation auxiliary enzymes [3,4]. The position of the mammalian auxiliary enzymes Δ^3 - Δ^2 -enoyl-CoA isomerase [3,2-isomerase (ECI); EC 5.3.3.8], 2,4-dienoyl-CoA reductase [2,4-reductase (DECR); EC 1.3.1.34] and $\Delta^{3,5}$ - $\Delta^{2,4}$ -dienoyl-CoA isomerase (di-isomerase) in both mitochondrial and peroxisomal β -oxidation is shown in Scheme 1.

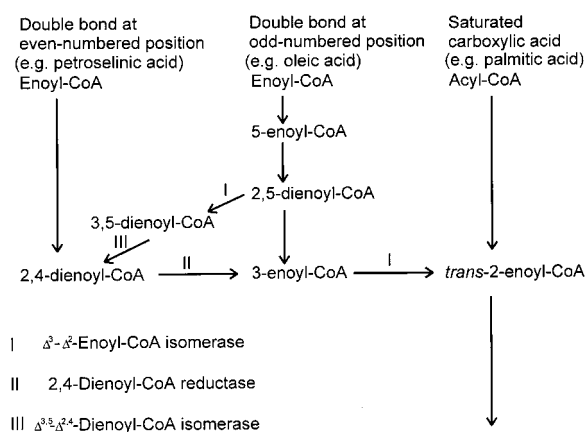
The present work is concerned with mammalian 3,2-isomerase (and 2,4-reductase, see below). Two rat 3,2-isomerases have been characterized at the molecular level; one being an integral part of the peroxisomal multifunctional enzyme type I (multifunctional

enzyme, [5]) whereas the other is a monofunctional enzyme [6]. Because monofunctional 3,2-isomerase contains a mitochondrial leader sequence and has been shown to be overwhelmingly mitochondrial [6], multifunctional enzyme is thought to represent the only 3,2-isomerase in rat peroxisomes. However, this postulate is disputed in light of other ultrastructural evidence indicating that rat monofunctional 3,2-isomerase could represent a dually compartmentalized protein [7–9]. This raises the question of whether mammalian peroxisomes contain additional physiological 3,2-isomerases to that integrated in multifunctional enzyme.

The additional concern of this work with human mitochondrial 2,4-reductase has an important medical aspect since a deficiency in 2,4-reductase activity (Online Mendelian Inheritance in Man, OMIM™, Johns Hopkins University, Baltimore, MD OMIM number 222745:22/12/1997; <http://www.ncbi.nlm.nih.gov/omim/>) is fatal in humans [10,11]. The case of a patient with a disorder in which degradation of linoleic acid [*cis*, *cis*-C_{18:2(9,12)}] was impaired has recently been reported [10]. The current theory that the disease is caused by a defective mitochondrial 2,4-reductase is based on the pattern of tissue distribution of a residual 2,4-reductase activity thought to

Abbreviations used: rat 3,2-isomerase (ECI), rat monofunctional Δ^3 - Δ^2 -enoyl-CoA isomerase; 2,4-reductase (DECR), 2,4-dienoyl-CoA reductase; rat di-isomerase, rat $\Delta^{3,5}$ - $\Delta^{2,4}$ -dienoyl-CoA isomerase; rat multifunctional enzyme, rat peroxisomal multifunctional enzyme type I; PTS, peroxisomal targeting signal; GFP, green fluorescent protein.

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Scheme 1 The positions of 3,2-isomerase (I), 2,4-reductase (II) and diisomerase (III) in fatty acid β -oxidation

The enzymes are required in eukaryotes for degrading unsaturated fatty acids but are dispensable for degrading saturated fatty acids. The vertical lines represent reactions catalysed by enzymes of the β -oxidation spiral.

originate from an intact peroxisomal isoform. The gene and cDNA encoding human mitochondrial 2,4-reductase as well as the cDNA of the corresponding rat analogue have previously been identified [12–14]; however, a physiological function has not been demonstrated for these proteins.

Although analysis of the nucleotide sequences of the human and rat cDNAs had shown these to encode proteins with putative mitochondrial leader sequences, nevertheless the subcellular distribution of mitochondrial 2,4-reductase remains unclear. Rat 2,4-reductase has been localized to mitochondria using affinity purified anti-(rat 2,4-reductase) antibody [15]; however, other work [16] has shown that a polyclonal antibody enriched for the fraction binding mitochondrial 2,4-reductase cross-reacts with a peroxisomal antigen. A mammalian gene coding for a physiologically functional peroxisomal 2,4-reductase has not yet been identified. Therefore, although both rat and human mitochondrial 2,4-reductases lack an obvious peroxisomal targeting signal (PTS), it cannot be ruled out that they are additionally partitioned to peroxisomes. Hence it is important to elucidate both the function and subcellular location of mammalian mitochondrial 2,4-reductase.

The potential function of rat monofunctional 3,2-isomerase and human 2,4-reductase in the β -oxidation of unsaturated fatty acids could be determined in yeast since *Saccharomyces cerevisiae* is able to grow on unsaturated fatty acids [17] that require 3,2-isomerase and 2,4-reductase for their breakdown. Yeast strains devoid of either peroxisomal 2-enoyl-CoA hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase (Fox2p) or peroxisomal 3,2-isomerase (Eci1p) had been used previously to demonstrate the function of rat multifunctional enzyme during the degradation of oleic acid [1,18]. In addition, since in fungi β -oxidation is solely a peroxisomal process [19] the purported extra-mitochondrial location of the two mammalian proteins could be investigated. Despite the pivotal role of unsaturated fatty acids in human nutrition, only very few of the enzymes postulated to be entrained in the process of their breakdown have been demonstrated to function *in vivo*.

To determine the physiological function of the two mammalian proteins and gain insight into their subcellular location, they were expressed in yeast. We report on the functional complementation of a yeast *eci1* Δ strain lacking peroxisomal 3,2-

isomerase activity [18,20] using rat monofunctional 3,2-isomerase (GenBank accession number M61112). In addition, growth of an *sps19* Δ mutant devoid of peroxisomal 2,4-reductase activity [21] was restored using human 2,4-reductase (GenBank accession number L26050) extended by a PTS.

MATERIALS AND METHODS

Growth media

For oleic acid induction, late log-phase cultures obtained from overnight pre-cultures grown in 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 5% (w/v) D-glucose (at 30 °C with vigorous shaking) were added to 100-ml conical flasks containing 50 ml of medium to give an $A_{600\text{ nm}} = 0.2$. This medium consisted of 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and a final concentration of 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80 adjusted to pH 7.0 with NaOH, 75 $\mu\text{g/ml}$ ampicillin and 0.05% (w/v) D-glucose. The cultures were then incubated further, with shaking, and samples were removed for analysis after 21 h. For electron microscopy, yeast cells were induced in liquid oleic acid medium as described in [22]. To assess utilization of fatty acids via the formation of clear zones, solid media were used that contained oleic acid, petroselinic acid, or palmitic acid ($C_{16:0}$). These media consisted of 0.67% (w/v) yeast nitrogen base with amino acids, 0.1% (w/v) yeast extract, 0.5% (w/v) potassium phosphate buffer, pH 6.0, 2% (w/v) agar, with 0.125% (w/v) oleic acid, 0.125% (w/v) petroselinic acid or 0.125% (w/v) palmitic acid, and 0.5% (w/v) Tween 80 to solubilize the fatty acid. The media were poured in a thin layer at a temperature of 45 °C. Growth conditions used in the qualitative assay for two-hybrid interaction were as described previously [23].

Strains and expression plasmids

The *S. cerevisiae* strains used are described in Table 1 and the plasmids and oligonucleotides used are listed in Table 2. Standard recombinant DNA techniques were used throughout this work [24]. Construction of plasmids expressing C-terminal green fluorescent protein (GFP) fusions of human 2,4-reductase was performed as follows: PCR was applied to template cDNA from plasmid pUC18::DECRC[12] using *KpnI*-terminated primers RED F and R. The reaction produced a *KpnI*-terminated fragment containing DECRC from codon 25 (without the mitochondrial leader signal) to the penultimate codon that was inserted in-frame into a *KpnI* site in plasmid pJR233 [23]. This plasmid contains the gene encoding a modified *Aequorea victoria* GFP extended by a C-terminal PTS, SKL (Ser-Lys-Leu); the GFP-SKL gene fusion is under the control of the yeast malate synthase (*MLS1*) promoter in the yeast-*Escherichia coli* shuttle vector YEp352 [23]. The resulting recombinant plasmid, pAG510, was used to express a peroxisomally-targeted human 2,4-reductase-GFP-SKL fusion protein. Application of a further PCR to template DNA from plasmid pAG510 using oligonucleotides CTA-RED F and CTA-RED-GFP R produced an *XbaI*- and *XhoI*-terminated fragment representing DECRC with the nucleotides ATG at codon 24. This fragment contained DECRC fused in frame with GFP. The fragment was inserted in an *EcoRV*-linearized pBluescript[®] SK(+) vector (pSK) to yield plasmid pAG625 and, following digestion with *XbaI* and *XhoI*, it was ligated in the appropriate sites of plasmid pYE352-CTA1 [1], resulting in plasmid pAG631. This construct was used to express a non-targeted human 2,4-reductase-GFP under the control of the oleic acid-inducible yeast catalase A (*CTA1*) promoter. Plasmids pAG510 and pAG631 were used to transform a homozygous *sps19* Δ strain yAG161 [21], resulting in strains

Table 1 *S. cerevisiae* strains used

TPS, the present study.

Strain	Description	Source or Reference
(1) BJ1991	<i>MATα leu2 ura3-52 trp1 pep4-3 prb1-1122</i>	[51]
(2) NKY857	<i>MATα leu2 ura3-52 his4X lys HO::LYS</i>	N. Kleckner†
(3) yAG141 ^{1*}	<i>sps19Δ::LEU2</i>	[21]
(4) yAG146 ²	<i>sps19Δ::LEU2</i>	[21]
(5) yAG161 ^{3\times4}	<i>MATα/α homozygous <i>sps19Δ</i> disruptant</i>	[21]
(6) PCY3	<i>MATα gal4Δ gal80Δ URA3::GAL1-lacZ LYS2::GAL1-HIS3 his3Δ200 trp1Δ63 leu2 ade2-101ochre; derivative of PCY2</i>	[42,52]
(7) BJ1991 <i>eci1Δ</i> ¹	<i>eci1Δ::kanMX4</i>	[18]
(8) NKY857 <i>eci1Δ</i> ²	<i>eci1Δ::kanMX4</i>	[18]
(9) yAG760 ^{8\times9}	<i>MATα/α homozygous <i>eci1Δ</i> disruptant</i>	[18]
(10) BJ1991 <i>pex6Δ</i> ¹	<i>pex6Δ::LEU2</i>	[53]
yAG824 ⁵	<i>sps19Δ</i> with pAG620 expressing human 2,4-reductase-SKL	TPS
yAG825 ⁵	<i>sps19Δ</i> with pAG777 expressing human 2,4-reductase	TPS
yAG853 ⁵	<i>sps19Δ</i> expressing catalase A from pYE352-CTA1	TPS
yAG854 ⁹	<i>eci1Δ</i> expressing rat 3,2-isomerase from pAG847	TPS
yAG855 ⁹	<i>eci1Δ</i> expressing rat 3,2-isomerase-SKL from pAG849	TPS
yAG826 ⁹	<i>eci1Δ</i> expressing yeast 3,2-isomerase Eci1p from pAG766	[18]
yAG827 ⁹	<i>eci1Δ</i> harbouring YEplac181 (plasmid vector)	[18]
yAG874 ⁹	<i>eci1Δ</i> expressing catalase A from pYE352-CTA1	TPS
yAG903 ⁷	<i>eci1Δ</i> expressing rat 3,2-isomerase from pAG847	TPS
yAG904 ⁷	<i>eci1Δ</i> expressing rat 3,2-isomerase-SKL from pAG849	TPS
yAG905 ⁶	PCY3 containing pAH950 and pAG897	TPS
yAG906 ⁶	PCY3 containing pAH950 and pAG900	TPS
yAG907 ⁶	PCY3 containing pAH950 and pGBT9	TPS
yAG908 ⁶	PCY3 containing pAH951 and pAG897	TPS
yAG909 ⁶	PCY3 containing pAH951 and pAG900	TPS
yAG910 ⁶	PCY3 containing pAH951 and pGBT9	TPS
yAG911 ⁶	PCY3 containing pFAN26 and pAG897	TPS
yAG912 ⁶	PCY3 containing pFAN26 and pAG900	TPS
yAG913 ⁶	PCY3 containing pFAN26 and pGBT9	TPS
yAG1012 ⁵	with pAG510 expressing human 2,4-reductase-GFP-SKL	TPS
yAG1013 ⁵	with pAG631 expressing human 2,4-reductase-GFP	TPS
yAG1014 ⁵	with pJC18 expressing peroxisomal Sps19p	TPS
yAG1025 ¹	with pAG510 expressing human 2,4-reductase-GFP-SKL	TPS
yAG1026 ¹⁰	with pAG510 expressing human 2,4-reductase-GFP-SKL	TPS
yAG1027 ¹	with pAG631 expressing human 2,4-reductase-GFP	TPS
yAG1028 ¹⁰	with pAG631 expressing human 2,4-reductase-GFP	TPS

* The numbers in superscript following the strains' designation refer to their parental genotypes, e.g. yAG141¹ was derived from (1) BJ1991.

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yAG1012 and yAG1013 respectively. As a control, strain yAG161 was transformed with plasmid pJC18 expressing yeast peroxisomal 2,4-reductase (Sps19p) to create yAG1014. For fluorescence microscopy, plasmids pAG510 and pAG631 were used to transform strains BJ1991 and BJ1991 *pex6 Δ* , resulting in strains yAG1025 to yAG1028.

Construction of plasmids expressing a modified human 2,4-reductase lacking the putative mitochondrial leader sequence or one that was additionally extended by SKL was performed as follows. Oligonucleotides CTA-RED F and CTA-RED R or CTA-RED R2 were used with *Pfu* high-fidelity DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) in a PCR that was applied to template cDNA from plasmid pUC18::*DECR* [12] to generate *Xba*I- and *Xho*I-terminated products representing the genes for human 2,4-reductase-SKL and human 2,4-reductase respectively. In both products, the translational start codon was followed by the phenylalanine codon that is present in the native human 2,4-reductase gene at the site corresponding to position 25 immediately after the mitochondrial-targeting-signal cleavage site [12]. The PCR products were ligated to an *Eco*RV-digested pSK vector resulting in plasmids pAG616 and pAG755. Following *Xho*I- and *Xba*I-double digestion of the plasmids, the

released inserts were purified and inserted in plasmid pYE352-CTA1, yielding plasmids pAG620 and pAG777. These were used to transform a homozygous *sps19 Δ* strain yAG161 [21], resulting in strains yAG824 and yAG825 respectively. As a control, strain yAG161 was transformed with plasmid pYE352-CTA1 overexpressing yeast catalase A to create strain yAG853.

Plasmids expressing rat 3,2-isomerase or rat 3,2-isomerase-SKL were similarly generated by inserting *Xba*I- and *Xho*I-terminated DNA fragments that were amplified with oligonucleotides CTA1-ISO F and CTA1-ISO R or CTA1-ISO R-SKL using *Pfu* high-fidelity DNA polymerase and template cDNA from plasmid pUEX1::*ECI* [25]. The translational start codon was followed by the phenylalanine codon present in the mature 3,2-isomerase at position 1 thereby removing the mitochondrial-targeting-signal cleavage site [25]. The blunt-ended fragments were ligated to an *Eco*RV-digested pSK vector, resulting in plasmids pAG841 and pAG844, and inserts were verified by nucleotide sequencing. Following *Xho*I- and *Xba*I-double digestion, the released fragments were inserted in a similarly digested plasmid pYE352-CTA1, resulting in plasmids pAG847 and pAG849 respectively. These expression plasmids were introduced into a homozygous *eci1 Δ* strain yAG760 [19]

Table 2 Plasmids and oligonucleotides used

TPS, the present study.

	Description	Source or Reference
Plasmid		
pAG510	2,4-reductase-GFP-SKL regulated by the <i>MLS1</i> promoter in YE352	TPS
pAG625	2,4-reductase-GFP in pSK/ <i>EcoRV</i> for insertion in pYE352-CTA1	TPS
pAG631	2,4-reductase-GFP regulated by the <i>CTA1</i> promoter in pYE352-CTA1	TPS
pAG616	2,4-reductase-SKL in pSK/ <i>EcoRV</i> for insertion in pYE352-CTA1	TPS
pAG755	2,4-reductase in pSK/ <i>EcoRV</i> for insertion in pYE352-CTA1	TPS
pAG620	2,4-reductase-SKL in pYE352-CTA1	TPS
pAG777	2,4-reductase in pYE352-CTA1	TPS
pAG841	3,2-isomerase in pSK/ <i>EcoRV</i> for insertion in pYE352-CTA1	TPS
pAG844	3,2-isomerase-SKL in pSK/ <i>EcoRV</i> for insertion in pYE352-CTA1	TPS
pAG847	3,2-isomerase in pYE352-CTA1	TPS
pAG849	3,2-isomerase-SKL in pYE352-CTA1	TPS
pAG887	3,2-isomerase in pSK/ <i>EcoRV</i> for insertion in pGBT9	TPS
pAG890	3,2-isomerase-SKL in pSK/ <i>EcoRV</i> for insertion in pGBT9	TPS
pAG897	3,2-isomerase in pGBT9	TPS
pAG900	3,2-isomerase-SKL in pGBT9	TPS
pGBT9	2-hybrid vector with the Gal4p DNA-binding domain	[54]
pAH950	pGAD424 containing Pex5p	[42]
pAH951	pGAD424 with the Pex5p tetratricopeptide repeat domain	[42]
pFAN26	pGAD424 containing Pex7p	A. Firzinger*
pGAD424	2-hybrid vector with the Gal4p activation domain	[54]
pUC18::DECR	human mitochondrial 2,4-reductase cDNA in pUC18	[12]
pUEX1::ECI	rat monofunctional 3,2-isomerase cDNA in pUEX1	[25]
pYE352-CTA1	multi-copy plasmid expressing peroxisomal catalase A	[1]
pAG766	YEplac181-based plasmid for expressing yeast peroxisomal Eci1p	[18]
pJR233	GFP-SKL regulated by the <i>MLS1</i> promoter in YE352	[23]
YEplac181	<i>LEU2</i> -containing multi-copy plasmid vector	[55]
pJC18	multi-copy plasmid expressing Sps19p	[56]
pSK	pBluescript®SK(+) cloning vector	Stratagene
Oligonucleotide		
RED F	5'-AATTGGTACCGGAACCTTTTG-3'	TPS
RED R	5'-AATTGGTACCTTCAGTTATG-3'	TPS
CTA-RED F	5'-CGAGCTCTAGAAGATGTTTCAGTTATGGGAC-3'	TPS
CTA-RED-GFP2 R	5'-CCTCGAGTTATTTGTATAGTTTCATCC-3'	TPS
CTA-RED R	5'-CCTCGAGATCAGAGTTTTGAGGAACCTTTTGTC-3'	TPS
CTA-RED R2	5'-CCTCGAGATCAGGAACCTTTTGTC-3'	TPS
CTA-ISO F	5'-CGAGCTCTAGAAGATGTTCTTAACAAGCGGGTG-3'	TPS
CTA-ISO R	5'-CCTCGAGATCAGCCCTTCTTTGCTTGAG-3'	TPS
CTA-ISO R-SKL	5'-CCTCGAGATCAGAGTTTTGAGCCCTTCTTTGCTTGAG-3'	TPS
BAMHI-ISO F	5'-GGGATCCTGTTCTTAAACAGCGGGTG-3'	TPS

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resulting in strains yAG854 and yAG855. To create the control strains yAG826, yAG827, and yAG874, strain yAG760 was transformed with plasmid pAG766 expressing yeast Eci1p [19], the corresponding YEplac181 plasmid vector, and plasmid pYE352-CTA1 respectively. Expression plasmids pAG847 and pAG849 were additionally introduced into a BJ1991*eci1Δ* strain [19], resulting in the respective strains yAG903 and yAG904.

Plasmids for the two-hybrid assay were constructed by generating *Bam*HI- and *Xho*I-terminated amplification products with oligonucleotides BAMHI-ISO F and CTA-ISO R using pAG841 DNA as template, or with BAMHI-ISO F and CTA-ISO R-SKL using template DNA from plasmid pAG844. The respective products were inserted into an *EcoRV*-digested pSK vector, resulting in plasmids pAG887 and pAG890. Following *Bam*HI- and *Xho*I-double digestion of the plasmids, the released inserts were purified and ligated to a *Bam*HI- and *Sal*I-digested pGBT9 vector containing the transcription factor Gal4p DNA-binding domain, yielding plasmids pAG897 and pAG900. The recombinant plasmids and the pGBT9 vector were introduced into PCY3-based two-hybrid strains carrying, in addition to the

GAL10-lacZ reporter construct, the Gal4p activation domain fused with either the yeast type 1 PTS receptor (Pex5p) [26] (amino acids 78 to 612; pAH950), the tetratricopeptide repeat domain of Pex5p (amino acids 252 to 612; pAH951), or the complete yeast type 2 PTS receptor (Pex7p) [27] (pFAN26). Plasmid pFAN26 contains a 1.8 kb *Sma*I- and *Pst*I-terminated DNA fragment encoding the complete Pex7p (from pRS414-Gal4BD-PEX7) fused in-frame to the Gal4p activation domain in pGAD424.

SDS/PAGE and Western blot analysis

Soluble protein extracts derived from oleic acid-induced yeast cells were prepared as described previously [21,28]. Fractionation of yeast spheroplasts was conducted according to a published protocol [29]. Protein extracts were resolved using an SDS/12% (w/v) polyacrylamide gel [30] and transferred to a nitrocellulose membrane. Detection of human 2,4-reductase was performed following incubation for 1 h in a 1:1000 dilution of anti-(rat 2,4-reductase) primary antibody [31], and for 1 h with a 1:10000 dilu-

tion of a secondary antibody consisting of goat anti-(rabbit IgG) conjugated to horseradish peroxidase (BioRad). Rat 3,2-isomerase was detected with a rabbit anti-(rat 3,2-isomerase) primary antibody followed by a secondary goat anti-rabbit antibody. Analysis using enhanced chemiluminescence was performed according to the manufacturer's instructions (SuperSignal, Pierce, Rockford, IL, U.S.A.). Catalase A was detected by adding a 1:1000 dilution of anti-(catalase A) antibody raised in rabbit.

Enzyme assays

2,4-Reductase activity was measured spectrophotometrically as reductase-dependent NADPH consumption at 23 °C in an assay mixture that consisted of 50 mM potassium phosphate buffer, pH 7.2, 125 μ M NADPH and 60 μ M 2,4-hexadienoyl-CoA as substrate [32]. The assay for 3,2-isomerase was conducted according to published methods [33] using 60 μ M *trans*-3-hexenoyl-CoA as substrate.

Electron microscopy

Yeast cells were processed essentially as described in [22]. Briefly, cells were harvested and fixed with 4% (w/v) formaldehyde and 0.1% (w/v) glutaraldehyde in PBS (0.15 M NaCl, 0.04 M K_2HPO_4 , 0.01 M KH_2PO_4 , pH 7.4). Oxidation of cell-wall carbohydrates was performed by applying 1% (w/v) sodium meta-periodate followed by 50 mM ammonium chloride, and cells were serially dehydrated at low temperature, embedded in Lowicryl HM20, and the resin polymerized by UV light. Ultrathin sections were incubated with anti-(rat 2,4-reductase), anti-(rat 3,2-isomerase) or anti-(yeast thiolase; Fox3p) antibodies (raised in rabbits and diluted 1:1000 in PBS) and then with a Protein A-gold (14 nm) complex. Following counterstaining with uranyl acetate and lead acetate grids were inspected using a Zeiss EM900 microscope.

For subcellular localization of rat di-isomerase by electron microscopy using rat tissue from control and treated Sprague-Dawley rats fed a 0.5% (w/w) clofibrate-supplemented chow for 2 weeks, livers were harvested after vascular perfusion fixation through the left ventricle and ascending aorta as described previously [34]. For thin sections, tissues were embedded in Micro-Bed resin (Electron Microscopy Sciences; Fort Washington, PA, U.S.A.) according to the manufacturer's instructions except that the tissues were infiltrated from 70% (v/v) ethanol instead of completion of the dehydration protocol. The resin was polymerized at 4 °C under UV light (365 nm) for 72 h.

To determine the subcellular location of rat 3,2-isomerase, liver tissue from clofibrate-treated and control rats was similarly prepared, except that, prior to perfusion with the fixative, livers were perfused for 2 min with PBS containing 2.5% (w/v) sucrose at pH 7.4. This was followed by perfusion for 5 min with a solution consisting of 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde. Small blocks (1 mm³) of liver were immersed for 15 min in a solution containing 4% (w/v) paraformaldehyde and 0.07% (v/v) glutaraldehyde and 30 min in 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde. Tissue blocks were washed three times, each for 30 min, at room temperature in PBS containing 7% (w/v) sucrose and serially dehydrated at 4 °C in 50, 70 and 90% (v/v) ethanol (15 min in each dilution).

For immunolabelling, thin sections were blocked for 15 min in PBS containing 1% (w/v) BSA, 0.075% (w/v) fish skin gelatin and 0.05% (w/v) Tween 20, pH 7.4. Immunogold labelling using anti-(rat di-isomerase) antibody as primary antibody was performed as described previously [34]. For labelling using anti-(rat

3,2-isomerase) antibody, grids were incubated overnight at 4 °C with the primary antibody diluted 1:70 in the blocking buffer. After washing in PBS containing 1% (w/v) BSA and in PBS, grids were incubated for 1 h at room temperature with a Protein A-gold (20 nm) complex diluted 1:75 in the above buffer. The grids were washed three times (each for 10 min) in PBS and three times (each for 2 min) in filtered distilled water and counterstained with uranyl acetate and lead citrate.

RESULTS

Expression of human mitochondrial 2,4-reductase in yeast

A human gene encoding mitochondrial 2,4-reductase with the potential for representing the defective protein leading to human 2,4-reductase deficiency has been identified previously [10]. However, obstacles remain in the way of attributing the cause of the disease to this candidate protein, including (i) demonstration of a physiological role for this enzyme in β -oxidation, and (ii) its exclusion as a peroxisomal protein. Because human 2,4-reductase is similar (28% identical) to the product of the yeast *SPS19* gene representing peroxisomal NADPH-dependent 2,4-dienoyl-CoA reductase [21], the yeast *sps19* Δ mutant was chosen as a test organism in which to express the human enzyme. Expression of many yeast peroxisomal proteins, including the β -oxidation auxiliary enzyme Sps19p (as well as Eci1p and catalase A), in media containing oleic acid is regulated by a gene promoter sequence termed the oleate response element [35,36]. Therefore, abundant expression of human 2,4-reductase (and rat 3,2-isomerase; see below) in cells grown on fatty acids was ensured by fusing their corresponding cDNAs to the catalase A promoter in a multi-copy plasmid.

To determine whether human mitochondrial 2,4-reductase represents a protein that can enter peroxisomes, its subcellular location was examined in yeast. In fungi β -oxidation is confined to peroxisomes [19] and there is currently no evidence to indicate that Sps19p and Eci1p [21,18,20], or other yeast peroxisomal enzymes with β -oxidation functions [37], additionally act else-

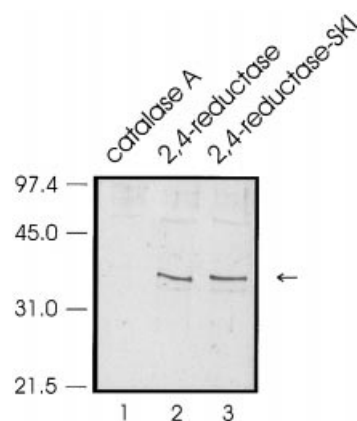


Figure 1 Expression of human 2,4-reductase in yeast

Immunoblotting of soluble protein extracts derived from homogenized *sps19* Δ cells expressing yeast catalase A, human 2,4-reductase, or human 2,4-reductase extended with SKL, using antibody raised against rat 2,4-reductase. Lane 1, oleic acid-induced cells expressing yeast peroxisomal catalase A (yAG853; 10 μ g); lanes 2 and 3, similarly induced cells expressing human 2,4-reductase (yAG825; 10 μ g) or human 2,4-reductase-SKL (yAG824; 10 μ g) respectively. Arrowhead indicates human 2,4-reductase. Numbers in the left margin refer to the migration of marker proteins (kDa): 21.5, trypsin inhibitor; 31.0, carbonic anhydrase; 45.0, ovalbumin; 97.4, phosphorylase.

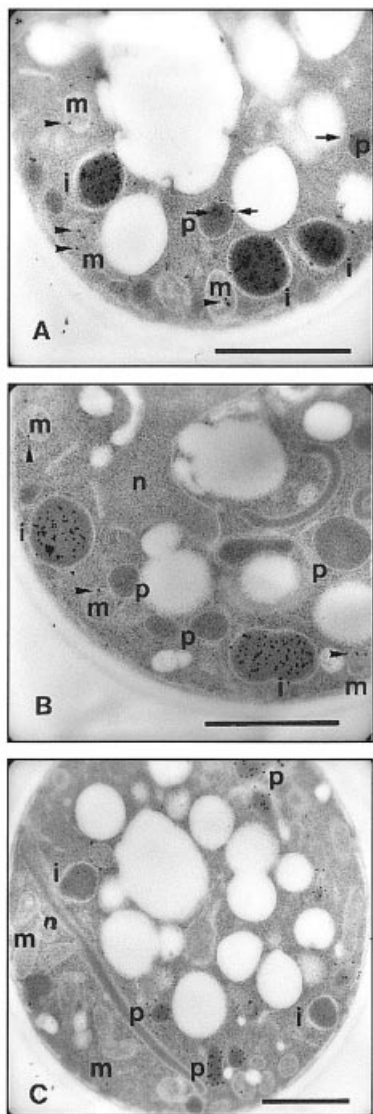


Figure 2 Localization of human 2,4-reductase in yeast cells

Immunoelectron micrographs demonstrating aggregation of human 2,4-reductase in inclusion bodies. Oleic acid-induced *sps19Δ* cells were labelled with anti-(rat 2,4-reductase) antibody. (A) A yAG824 cell producing excess SKL-extended human 2,4-reductase. (B) A yAG825 cell expressing human 2,4-reductase. (C) A yAG824 cell labelled with anti-(yeast thiolase) antibody. Arrows indicate labelled peroxisomes and arrowheads indicate labelled mitochondria. i, membrane-bounded inclusion body; m, mitochondrion; n, nucleus; p, peroxisome. The bar is 1 μ m.

where. Hence, to prevent transport of human 2,4-reductase to mitochondria (which in yeast are not engaged in β -oxidation), an N-terminally truncated variant missing the first 25 amino acids representing the putative mitochondrial leader sequence was constructed. This protein ended with the native C-terminus. A second human 2,4-reductase acting as a control construct was generated so that it was similarly devoid of the N-terminal leader sequence but was additionally extended by a C-terminal PTS1 tripeptide, SKL [38–40]. Immunoblotting of soluble protein extracts from homogenized oleic acid-induced yeast *sps19Δ* cells expressing human 2,4-reductase using antibodies raised against the rat analogue which cross-react with human 2,4-reductase [10,13] demonstrated that both forms of the human 2,4-

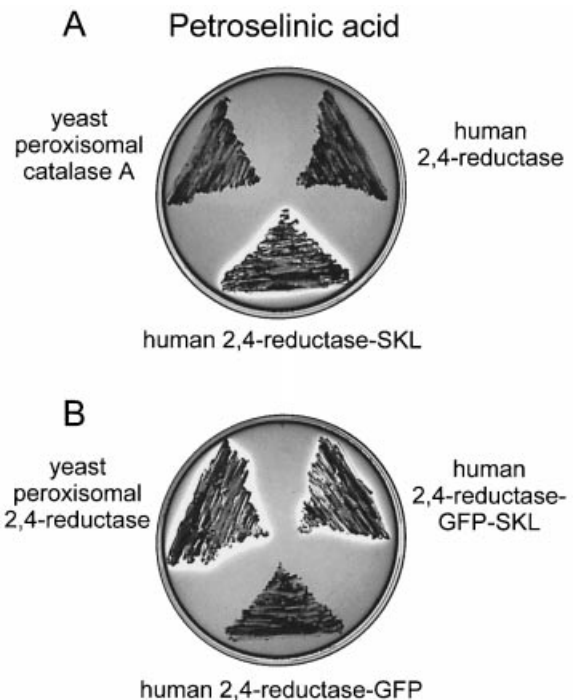


Figure 3 Growth of *sps19Δ* cells expressing human 2,4-reductase–SKL on petroselinic acid medium

Plate assay for the ability of human 2,4-reductase to restore growth of *sps19Δ* cells on petroselinic acid medium. The strains used were (A) yAG824 (expressing human 2,4-reductase–SKL), yAG825 (expressing human 2,4-reductase), and yAG853 (expressing yeast peroxisomal catalase A), and (B) yAG1012 (expressing human 2,4-reductase–GFP–SKL), yAG1013 (expressing human 2,4-reductase–GFP), and yAG1014 (expressing *Sps19p*).

reductase were expressed in the transformants (Figure 1, lanes 2 and 3). Similarly propagated isogenic control cells over-expressing yeast catalase A did not give rise to a specific signal (Figure 1, lane 1).

The subcellular location of the heterologously expressed proteins was determined by electron microscopy. Examination of oleic acid-induced cells expressing either SKL-extended human 2,4-reductase (Figure 2A) or the non-extended variant (Figure 2B) demonstrated large, membrane-bounded structures that were intensively labelled with anti-(rat 2,4-reductase) antibody. Application of anti-(yeast thiolase) antibody revealed that these membrane-bounded structures were not labelled, indicating that they represented inclusion bodies and not peroxisomes (Figure 2C). Despite truncation of the postulated mitochondrial leader sequence, both variants of human 2,4-reductase were clearly detectable in the mitochondria (arrowheads; Figures 2A and B). Cells over-expressing yeast catalase A revealed no structural anomalies and application of anti-(rat 2,4-reductase) antibody did not result in specific labelling (results not shown). Only very occasionally was SKL-extended human 2,4-reductase detectable in the peroxisomal matrix (arrows; Figure 2A). However, despite an extensive investigation of fields consisting of cells producing an excess of non-extended human 2,4-reductase, we could not detect peroxisomes that were labelled with anti-(rat 2,4-reductase) antibody.

As an alternative to using the rat antibody in localizing human 2,4-reductase in yeast cells, fluorescence microscopy was performed. Previous work had shown that tethering the gene for a GFP extended with PTS1 (GFP–SKL) to the yeast malate-

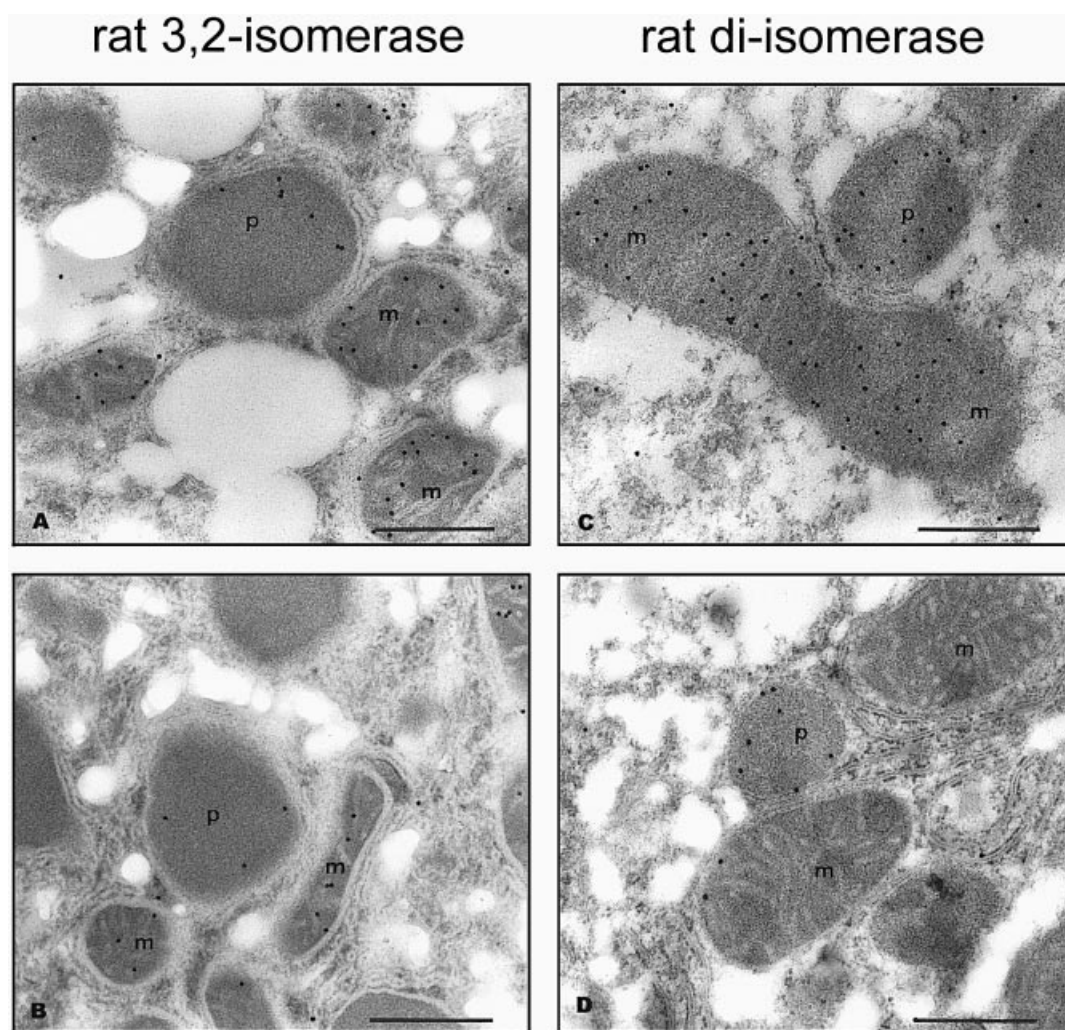


Figure 4 Localization of rat 3,2-isomerase and rat di-isomerase in rat liver cells

Labelling of liver tissue from (A and C) clofibrate-treated or (B and D) control rats using anti-(rat 3,2-isomerase) or anti-(rat di-isomerase) antibodies, as noted. m, mitochondrion; p, peroxisome. The bar is 0.5 μ m.

synthase promoter results in ample expression in yeast cells, yielding punctate fluorescence in wild-type cells and diffuse fluorescence in cells devoid of peroxisomes [23]. To determine the subcellular location of the human protein using fluorescent microscopy, N-terminal GFP fusion proteins were generated. Wild-type yeast cells were transformed with plasmids expressing human 2,4-reductase-GFP-SKL or human 2,4-reductase-GFP (yAG1025 and yAG1027 respectively). However, this did not result in fluorescence that was sufficiently abundant to record photographically. Moreover, the pattern of faint punctate fluorescence observed in wild-type cells was also seen in mutant cells devoid of peroxisomal structures (yAG1026 and yAG1028). This low level of green fluorescence indicated that much of the over-expressed protein had misfolded or aggregated, and underscored the previous results using immunoelectron microscopy.

SKL-extended human 2,4-reductase functionally complements a yeast *sps19* Δ strain

Entry of human 2,4-reductase into yeast peroxisomes could take place at a level that is below the detection limits of electron-

fluorescence microscopy. If the putative unknown human PTS also works in yeast and an amount of 2,4-reductase activity that is sufficient for function gets into peroxisomes, then this could be determined by testing for complementation of the *sps19* Δ mutant on petroselinic acid medium [21]. It has been shown previously for mitochondrial mutants that very low rates of import of cytoplasmically expressed proteins may be sufficient for complementing the mutant phenotype [41]. Were the human protein to replace the missing 2,4-reductase in the *sps19* Δ mutant by entering the peroxisomes in an enzymically active form, this could restore growth of the mutant cells.

To determine whether the heterologously expressed human protein was enzymically active in yeast cells, assays for 2,4-reductase activity were performed on soluble protein extracts from homogenized oleic acid-induced *sps19* Δ cells. This demonstrated that despite aggregating in inclusion bodies a portion of both forms of human 2,4-reductase was active (human 2,4-reductase-SKL, 28 nmol/min per mg of protein; human 2,4-reductase, 23 nmol/min per mg of protein). Soluble protein extracts from similarly propagated control *sps19* Δ cells over-

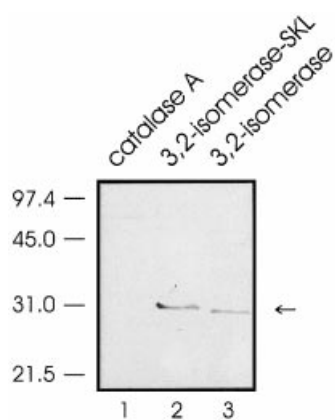


Figure 5 Expression of rat 3,2-isomerase in yeast

Immunoblotting of soluble protein extracts derived from homogenized *eci1Δ* cells expressing yeast catalase A, rat 3,2-isomerase, or SKL-extended rat 3,2-isomerase, using anti-(rat 3,2-isomerase) antibody. Lane 1, oleic acid-induced cells expressing yeast catalase A (yAG874; 10 μg); lanes 2 and 3, similarly induced cells expressing rat 3,2-isomerase–SKL (yAG855; 10 μg) or rat 3,2-isomerase (yAG854; 10 μg) respectively. Arrowhead indicates rat 3,2-isomerase.

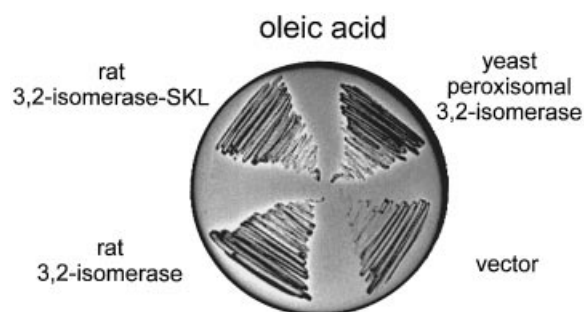


Figure 6 Growth of *eci1Δ* cells expressing rat 3,2-isomerase on oleic acid medium

Plate assay for the ability of rat 3,2-isomerase to restore the competence of *eci1Δ* cells to degrade oleic acid as the sole carbon source. The strains used were yAG854 (expressing rat 3,2-isomerase), yAG855 (expressing rat 3,2-isomerase–SKL), yAG826 (expressing *Eci1p*), and yAG827 (harbouring the vector).

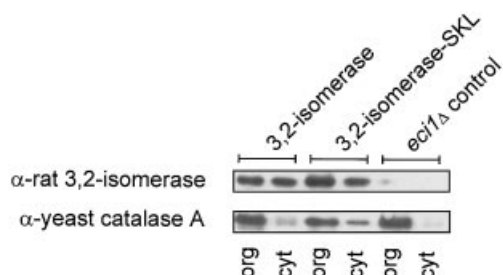


Figure 7 Presence of rat 3,2-isomerase in yeast cell fractions

Immunoblotting of organellar (org) and cytoplasmic (cyt) fractions of spheroplasted yeast cells expressing rat 3,2-isomerase using anti-(rat 3,2-isomerase) antibody. Oleic acid induction was assured by monitoring yeast peroxisomal catalase A in the organellar pellets using anti-(catalase A) antibody applied to a control Western blot containing the corresponding amount of protein. The strains used were yAG904 (expressing SKL-extended rat 3,2-isomerase), yAG903 (expressing non-extended rat 3,2-isomerase) and the parental strain BJ1991 *eci1Δ*.

expressing yeast catalase A did not contain a detectable level of 2,4-reductase activity. Using this approach, 2,4-reductase activity cannot be detected in soluble protein extracts derived from homogenized wild-type cells grown in oleic acid [21].

To examine whether the human protein could compensate for the loss of 2,4-reductase activity missing in the peroxisomes of the *sps19Δ* mutant, cells were grown on petroselinic acid as the sole carbon source (Figure 3A). In this and subsequent growth assays, solid fatty acid media were used that additionally contained Tween 80 which acted to form an emulsion but also as a poor carbon source. Hence, mutant cells could grow to some extent on these plates but opaque zones in the medium indicate utilization of the fatty acid substrate. Expressing human 2,4-reductase–SKL in the *sps19Δ* mutant substituted for the missing peroxisomal 2,4-reductase activity since the strain was able to form clear zones, whereas non-extended human 2,4-reductase or yeast peroxisomal catalase A failed to do so. On solid medium containing saturated fatty acids such as palmitic acid for which 2,4-reductase activity is not required, no differences in growth or formation of clear zones were detected among the strains tested (results not shown). The GFP fusion proteins of human 2,4-reductase were also examined for their ability to replace yeast peroxisomal 2,4-reductase (Figure 3B). This demonstrated that the SKL-extended GFP fusion was able to restore growth of the mutant on petroselinic acid since the strain could form a clear zone in the medium, whereas the SKL-less protein failed to complement the mutant phenotype.

Hence, two instances are provided whereby, with a PTS, human 2,4-reductase could functionally replace *Sps19p*. Therefore, the 2,4-reductase activity contained in the human protein *in vitro* was physiologically functional in yeast β -oxidation, and probably also in that in humans. Without the SKL extension, the human protein failed to restore the missing compartmentalized activity, and this indicated that it could not enter yeast peroxisomes. If both SKL-extended human 2,4-reductase variants had acted extra-peroxisomally to restore growth of the mutant, then this would be difficult to reconcile with the fact that the non-extended protein failed to act in the same way. Therefore, in yeast, expression of a missing auxiliary enzyme activity outside of the peroxisomes does not restore the carbon flux through β -oxidation. The significance of this observation will become apparent in the following sections.

Localization of 3,2-isomerase in rat liver

Rat 3,2-isomerase is devoid of an obvious PTS and previous immunocytochemistry using liver tissue from clofibrate-treated rats had shown it to be predominantly mitochondrial [6]. However, a close scrutiny of tissues obtained from untreated control rats revealed at the time that the density of gold particles representing rat 3,2-isomerase antigens in peroxisomes was 7-fold that of non-compartmentalized antigens considered as background [6]. This minor extra-mitochondrial labelling might indicate a peroxisomal location for rat 3,2-isomerase or, alternatively, the existence of other immunologically cross-reactive protein that is induced by feeding the animals with clofibrate.

The subcellular location of the rat 3,2-isomerase antigen was re-examined in tissue from control and clofibrate-fed rats. Application of anti-(rat 3,2-isomerase) antibody (Figures 4A and 4B) demonstrated a dual partitioning of the cognate protein, with a pattern of distribution that was similar to that of rat di-isomerase (Figures 4C and 4D), a protein present in both peroxisomes and mitochondria [34]. To investigate whether the peroxisomal location of rat monofunctional 3,2-isomerase could

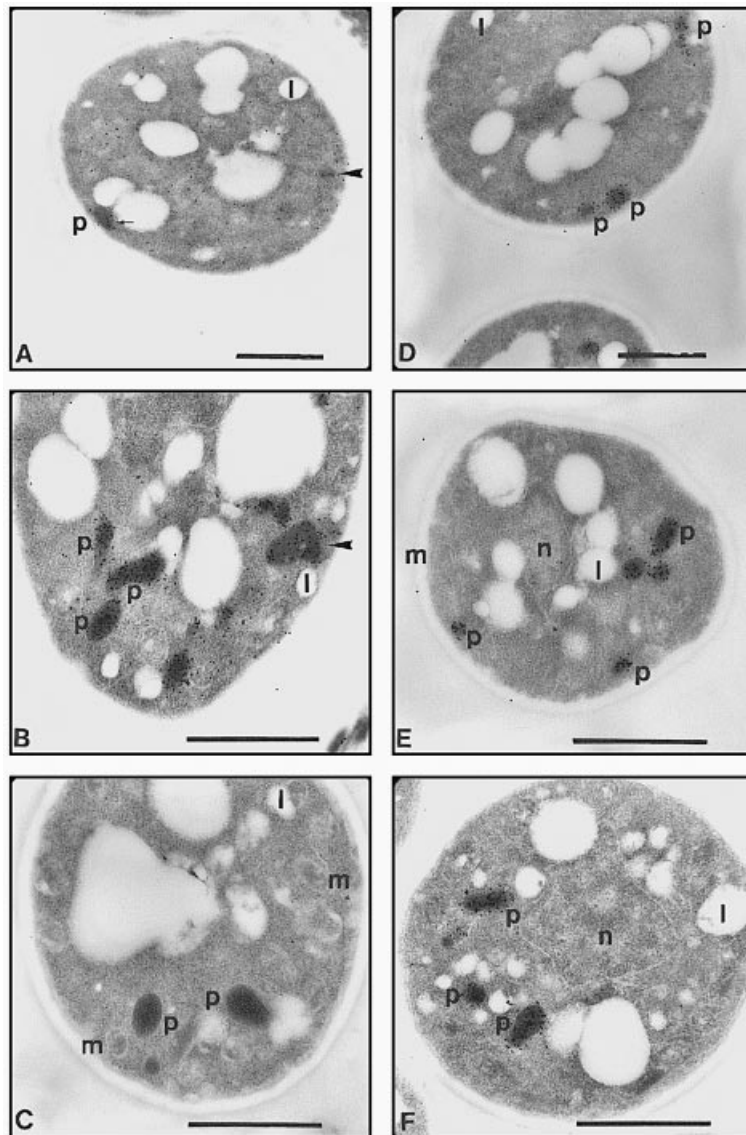


Figure 8 Localization of rat 3,2-isomerase in yeast cells

Immunoelectron micrographs of oleic acid-induced *eci1Δ* cells labelled with anti-(rat 3,2-isomerase) antibody. (A) A yAG854 cell expressing rat 3,2-isomerase. An arrow points to label within the peroxisomal matrix, and an arrowhead indicates an unidentified labelled structure. (B) A yAG855 cell producing excess SKL-extended rat 3,2-isomerase. (C) A yAG874 cell over-expressing catalase A. (D–F) Micrographs of the respective previous cells labelled with anti-(yeast thiolase) antibody. m, mitochondrion; p, peroxisome; l, lipoidal inclusion; n, nucleus. The bar is 1 μ m.

have a physiological relevance it was expressed in *S. cerevisiae*, since yeast has previously been shown to be a convenient model system in which to demonstrate that a heterologous 3,2-isomerase activity can substitute for the corresponding yeast peroxisomal protein [18].

Rat 3,2-isomerase complements the phenotype of a yeast *eci1Δ* mutant

Two forms of rat 3,2-isomerase were expressed in a yeast *eci1Δ* mutant devoid of peroxisomal 3,2-isomerase activity. One protein terminating with the native C-terminus was devoid of a complete mitochondrial leader sequence, whereas the other, in addition to being N-terminally truncated, was extended by SKL. An immunoblot assay using soluble protein extracts derived from

homogenized oleic acid-induced cells and an antibody raised against purified rat 3,2-isomerase [6] revealed that the mutant strains expressed the rat proteins (Figure 5). Enzyme assays for 3,2-isomerase activity demonstrated that both variants of the rat protein contained a high specific activity (3,2-isomerase, 65 nmol/min per mg of protein; 3,2-isomerase-SKL, 96 nmol/min per mg of protein). Protein extracts derived from similarly propagated control *eci1Δ* cells over-expressing yeast catalase A gave rise to values that were below the detection limit of the assay used (< 1 nmol/min per ml of sample). Using this assay, 3,2-isomerase activity cannot be detected in soluble protein extracts made from oleic acid-grown wild-type cells [18].

To examine whether rat 3,2-isomerase could replace its yeast peroxisomal counterpart *in vivo*, growth on oleic acid of *eci1Δ* mutants producing either rat 3,2-isomerase or rat 3,2-isomerase-

SKL was compared with those either over-expressing yeast 3,2-isomerase [18] or harbouring the plasmid vector (Figure 6). The mutant expressing rat 3,2-isomerase utilized the fatty acid as efficiently as the strain producing rat 3,2-isomerase-SKL, with both strains forming a zone of clearing in the solid medium that was comparable with that formed by the mutant producing excess yeast peroxisomal 3,2-isomerase. Mutant cells harbouring the plasmid vector did not generate opaque zones in the medium. This demonstrated that like rat peroxisomal multifunctional enzyme [18], rat 3,2-isomerase could replace yeast Eci1p *in vivo*. Control growth assays on non-selective solid medium containing palmitic acid showed no differences in growth or formation of clear zones between the strains tested (results not shown). Hence, despite lacking an obvious PTS, rat 3,2-isomerase restored the missing peroxisomal function. Since in the first section of this work, non-extended human 2,4-reductase failed to complement the phenotype of the *sps19Δ* mutant because it did not enter the peroxisomes, we reasoned that the similarly non-extended rat 3,2-isomerase lead to growth of the *eci1Δ* strain by entering the β -oxidation compartment to restore the carbon flux. This was probably due to a cryptic targeting signal capable of directing the rat protein to the peroxisomes in yeast.

Rat 3,2-isomerase enters yeast peroxisomes

To provide further evidence for the presence of rat 3,2-isomerase in yeast peroxisomes, fractionation of yeast cells was performed. Oleic acid-induced *eci1Δ* cells expressing the rat protein were spheroplasted and homogenized, and cytoplasmic and organellar fractions (containing both mitochondria and peroxisomes) were resolved using SDS/PAGE and subjected to Western analysis (Figure 7). Application of anti-(yeast catalase A) antibody to a control membrane demonstrated that the corresponding protein was most abundant in the organellar pellet (approx. 5- to 10-fold compared with the cytosolic fraction). Addition of anti-(rat 3,2-isomerase) antibody showed no signal in lanes containing protein from the control BJ1991*eci1Δ* strain. However, specific signals were seen in lanes corresponding to the organellar pellets from isogenic cells over-expressing rat 3,2-isomerase or rat 3,2-isomerase-SKL, with the latter being stronger.

The organellar signal obtained using the non-extended 3,2-isomerase could have been due to the presence of rat 3,2-isomerase in mitochondria or inclusion bodies. To exclude this possibility, the yeast strains were examined by immunoelectron microscopy. Staining with anti-(rat 3,2-isomerase) antibody of cells producing the non-extended 3,2-isomerase demonstrated mostly cytoplasmic labelling, although in rare instances some label was also seen in single-membrane bounded structures (arrow; Figure 8A). Cells containing excess rat 3,2-isomerase-SKL showed abundant labelling that was compartmentalized (Figure 8B). In both cases, only rarely could unidentified labelled structures be seen (arrowheads). Since the N-terminal leader sequences were removed from the two proteins, mitochondria were not labelled, and it was concluded that unlike human 2,4-reductase, rat 3,2-isomerase did not contain an additional cryptic mitochondrial targeting signal that could function in yeast. Specific labelling was not seen in control cells over-expressing catalase A (Figure 8C). Staining with anti-(yeast thiolase) antibody demonstrated that all of the structures bounded by a single-membrane were labelled (Figures 8D-8F), indicating that these were peroxisomes and not inclusion bodies. Hence, since rat 3,2-isomerase did not enter mitochondria or aggregate in inclusion bodies, we reason that the presence of the non-extended enzyme in the organellar pellet (Figure 7) was due to a peroxisomal location.

Rat 3,2-isomerase could have entered yeast peroxisomes with the help of a cryptic PTS by engaging the known components of the peroxisomal import machinery. To determine whether import of the rat protein was linked to this machinery, it was assayed for two-hybrid interaction with the PTS1 and PTS2 receptors (yeast Pex5p and Pex7p respectively). A qualitative two-hybrid assay using strains yAG905 to yAG913 (results not shown) demonstrated that cells containing SKL-extended rat 3,2-isomerase produced an interaction signal with Pex5p as well as its tetrapeptide repeat domain which is sufficient for PTS1 interaction [42]. This confirmed the immunoblotting and electron microscopical findings that the SKL extension was functional. On the other hand, control cells or those containing non-extended rat 3,2-isomerase did not result in interaction following extended incubation. Neither rat variant protein interacted with Pex7p. This indicated that if rat 3,2-isomerase was imported into peroxisomes by interacting with Pex5p or Pex7p, then this interaction was either at a level below the detection limit of the two-hybrid assay or that it did not work in this artificial system. Alternatively, rat 3,2-isomerase might have used unknown peroxisomal import factors either replacing Pex5p and/or Pex7p, piggy backed into the compartment on another peroxisomal enzyme, or engaged an adaptor-like intermediate protein that had masked the Pex5p or Pex7p two-hybrid interaction.

DISCUSSION

Using *S. cerevisiae* as a model system, we demonstrate for the first time that human mitochondrial 2,4-reductase functions in the β -oxidation of unsaturated fatty acids. In rodents, mitochondrial 2,4-reductase has been suggested to additionally represent the posited peroxisomal isoform [16]. However, since we showed here that human mitochondrial 2,4-reductase did not enter peroxisomes in yeast without an SKL extension, and in light of very recent discoveries of several novel proteins potentially representing physiological 2,4-reductases in mammalian peroxisomes (see below), we reason that mitochondrial 2,4-reductase probably does not enter peroxisomes in man.

In reference to the fatal human disorder associated with a dysfunctional metabolism of unsaturated fatty acids with double bonds at even-numbered positions, some reductase activity (probably peroxisomal) could be measured in the patient's liver (40%) and muscle (17%) [10]. The finding that human mitochondrial 2,4-reductase does not additionally represent a peroxisomal isoform strengthens the hypothesis that the disease is due to a defect in this particular protein, since it is unlikely that a defect in an exclusively mitochondrial 2,4-reductase would lead to a complete loss of activity.

From the outset of the present work it was at least conceivable that the SKL-less human 2,4-reductase could restore growth of the *sps19Δ* strain from outside the compartment. Were the accumulated C_{16} intermediate of petroselinic acid to exit peroxisomes, its $\Delta^{2,4}$ conjugated double bonds could have been metabolized extra-peroxisomally by the SKL-less human 2,4-reductase. The corresponding Δ^3 -enoyl-CoA intermediate could have then re-entered the peroxisomes to restore the carbon flux through β -oxidation, since such activated long-chain fatty acids have been shown previously to enter yeast peroxisomes from the cytoplasm [43]. However, based on our current observations, C_{16} fatty dienoyl-CoAs do not permeate out and subsequently back into this compartment in sufficient quantities to support growth of a mutant expressing the missing peroxisomal activity in a mislocalized form. Commensurate with this assertion, previous work demonstrated that expression of a normally peroxisomal yeast malate dehydrogenase without its PTS does not restore

growth of a mutant lacking the corresponding activity on oleic acid medium [44].

We also demonstrated for the first time that rat monofunctional 3,2-isomerase functions during the breakdown of oleic acid. In addition, our findings confirm that rat monofunctional 3,2-isomerase is a dually compartmentalized protein [7–9]. We find the possibility that rat 3,2-isomerase could have acted cytoplasmically to restore growth of the yeast mutant difficult to reconcile with the collective data presented here as well as with current understanding of a compartmentalized β -oxidation process. Even if activated C_{12} fatty acids such as the Δ^3 unsaturated intermediate expected to accumulate due to incomplete metabolism of oleic acid were to permeate out of peroxisomes and be metabolized by a mislocalized 3,2-isomerase to the corresponding Δ^2 intermediate, unlike C_{16} metabolites, C_{12} fatty acyl-CoAs would (re-) enter peroxisomes *in vivo* only poorly [43].

While this manuscript was being prepared, a number of novel mammalian proteins posited to represent peroxisomal 2,4-reductase have been identified. A screen for proteins selectively retained on immobilized *E. coli* HSP70 (DnaK) identified rat mitochondrial 2,4-reductase (whose human analogue was expressed here in yeast) and a potentially peroxisomal protein suspected to be an isoform of it (GenBank accession number AF021854), i.e. a novel rat peroxisomal 2,4-reductase [45]. However, the subcellular location and enzymic properties of the putative isoform remain to be determined. By binding multimeric proteins such as the homotetrameric rat mitochondrial 2,4-reductase, HSP70 is thought to shield hydrophobic surfaces prone to aggregation [45]. It is tempting to speculate that the reason for the extensive aggregation of the human mitochondrial 2,4-reductase in inclusion bodies within yeast cells, as well as the vanishingly small amount of fluorescence produced by the GFP fusion proteins, was due to inappropriate levels of chaperone needed for correct folding and oligomerisation.

A second potential rat peroxisomal 2,4-reductase (GenBank accession number AF044574) was identified using phage display [46]. Subcellular localization using a GFP fusion protein as well as expression in bacterial cells revealed that the novel protein was targeted to peroxisomes and contained 2,4-reductase activity [46]. The corresponding protein in mouse, PDCR (Genbank accession number AF155575), was also shown to be a potentially physiological peroxisomal 2,4-reductase [47]. Since current knowledge stipulates that mammals possess only a single peroxisomal 2,4-reductase, it is important to determine which of the novel proteins is physiologically entrained in peroxisomal β -oxidation of unsaturated fatty acids thereby representing peroxisomal 2,4-reductase. The *sps19* Δ strain is ideally placed to serve as a test organism for this purpose since it could be used to examine whether these proteins could substitute for yeast peroxisomal 2,4-reductase.

Another exciting finding to arise at the time of preparation of this manuscript was the identification of a novel mammalian gene *PECI* [48] (GenBank accession number AA188052). Immunofluorescence microscopy and expression in bacterial cells revealed that *PECI* encodes a peroxisomal protein containing 3,2-isomerase activity. Although *PECI* did not contain detectable levels of 2-enoyl-CoA hydratase or di-isomerase activities [48], other enzyme activities associated with the low-similarity hydratase/isomerase protein family were not tested. A thorough examination for additional activities in such proteins is important because rat ECH1, which contains hydratase 1 activity, actually represents a di-isomerase [34].

In addition to resembling yeast Eci1p, *PECI* is also similar to yeast Yor180cp [48,49]. All three proteins lack the motif for an

isomerase [4] but nonetheless contain at least some 3,2-isomerase activity. However, similarly to the situation with rat ECH1, Yor180cp is actually a yeast peroxisomal di-isomerase (Dci1p), and not a physiological 3,2-isomerase [50]. *PECI* may finally be demonstrated to represent a monofunctional peroxisomal 3,2-isomerase *in vivo*, but at the moment a different peroxisomal hydratase/isomerase-like physiological function for *PECI* cannot be entirely ruled out. If *PECI* could alleviate the requirement for Eci1p during growth of yeast cells on oleic acid, as was shown previously with rat multifunctional enzyme [19] and here with rat monofunctional 3,2-isomerase, this would prompt an important investigation into the relative contributions of multifunctional enzyme, ECI and *PECI* to total 3,2-isomerase activity in mammalian peroxisomes as well as their substrate specificities. Demonstration that mammalian proteins with β -oxidation enzyme activities have a physiological function in the degradation of fatty acids will increase our understanding of this metabolic process. At least in a number of cases this could be done conveniently using yeast mutants as a relevant model system.

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