MOLECULAR CHARACTERIZATION
OF PEROXISOMAL
MULTIFUNCTIONAL 2-ENOYL-COA
HYDRATASE 2/(3R)-
HYDROXYACYL-COA
DEHYDROGENASE (MFE TYPE 2)
FROM MAMMALS AND YEAST

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1999
MOLECULAR CHARACTERIZATION OF PEROXISOMAL MULTIFUNCTIONAL 2-ENOYL-COA HYDRATASE 2/(3R)-HYdroxyacyl-COA DEHYDROGENASE (MFE TYPE 2) FROM MAMMALS AND YEAST

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Raahensali (Auditorium L 10), Linnanmaa, on August 13th, 1999, at 12 noon.
To Qiang and Mandi
Qin, Yong-Mei, Molecular characterization of peroxisomal multifunctional 2-enoyl-CoA hydratase 2/(3R)-hydroxyacyl-CoA dehydrogenase (MFE type 2) from mammals and yeast
Biocenter Oulu and Departments of Biochemistry and Medical Biochemistry, University of Oulu, FIN-90570 Oulu, Finland
Acta Univ. Oul. D 537, 1999
Oulu, Finland
(Manuscript received 23 June 1999)

Abstract

Fatty acid degradation in living organisms occurs mainly via the β-oxidation pathway. When this work was started, it was known that the hydration and dehydrogenation reactions in mammalian peroxisomal β-oxidation were catalyzed by only multifunctional enzyme type 1 (MFE-1; Δ^2-Δ^2-enoyl-CoA isomerase/2-enoyl-CoA hydratase 1/(3S)-hydroxyacyl-CoA dehydrogenase) via the S-specific pathway, whereas in the yeast peroxisomes via the R-specific pathway by multifunctional enzyme type 2 (MFE-2; 2-enoyl-CoA hydratase 2/(3R)-hydroxyacyl-CoA dehydrogenase).

The work started with the molecular cloning of the rat 2-enoy-CoA hydratase 2 (hydratase 2). The isolated cDNA (2205 bp) encodes a polypeptide with a predicted molecular mass of 79.3 kDa, which contains a potential peroxisomal targeting signal (AKL) in the carboxy terminus. The hydratase 2 is an integral part of the cloned polypeptide, which is assigned to be a novel mammalian peroxisomal MFE-2.

The physiological role of the mammalian hydratase 2 was investigated with the recombinant hydratase 2 domain derived from rat MFE-2. The protein hydrates a physiological intermediate (24E)-3α, 7α, 12α-trihydroxy-5β-cholest-24-enoyl-CoA to (24R, 25R)-3α, 7α, 12α, 24-tetrahydroxy-5β-cholestanoyl-CoA in bile acid synthesis.

The sequence alignment of human MFE-2 with MFE-2(s) of different species reveals 12 conserved protic amino acid residues, which are potential candidates for catalysis of the hydratase 2. Each of these residues was replaced by alanine. Complementation of Saccharomyces cerevisiae fox-2 (devoid of endogenous MFE-2) with human MFE-2 provided a model system for examining the in vivo function of the variants. Two protic residues, Glu366 and Asp510, of the hydratase 2 domain of human MFE-2 have been identified and are proposed to act as a base and an acid in catalysis.

Mammalian MFE-2 has a (3R)-hydroxyacyl-CoA dehydrogenase domain, whereas the yeast MFE-2 has two dehydrogenase domains, A and B. The present work, applying site-directed mutagenesis to dissect the two domains, shows that the growth rates of fox-2 cells expressing a single functional domain are lower than those of cells expressing S. cerevisiae MFE-2. Kinetic experiments with the purified proteins demonstrate that domain A is more active than domain B in catalysis of medium- and long-chain (3R)-hydroxyacyl-CoA, whereas domain B is solely responsible for metabolism of short-chain substrates. Both domains are required when yeast cells utilize fatty acids as the carbon source.

Keywords: β-oxidation, fatty acid, bile acid synthesis, enzymology
Acknowledgements

The present study was carried out at Biocenter Oulu, the Department of Biochemistry and Department of Medical Biochemistry, University of Oulu during the years 1993-1999. The accomplishment of this work would be impossible without the help of so many people.

I wish to express my deepest gratitude to my supervisor, Professor Kalervo Hiltunen, for his hospitality to give me the opportunity to do my thesis work as a member of his research group. His everlasting enthusiasm, open mind and never-failing spirit in science have indefinitely encouraged me to fulfill this work. I also wish to thank Professors Taina Pihlajaniemi, Ilmo Hassinen, Kari Kivirikko, Raili Myllylä, Seppo Vainio, Rik Wierenga and Johan Kemmink for providing excellent working facilities and creating a stimulating atmosphere in our weekly seminars at the both Departments.

I wish to acknowledge Professors Juhani Syväoja and Pekka Mänepää for their critical appraisal of the manuscript, and Dr. Sidney Higley for her careful revision of the language. Dmitry Novikov, Ph.D., deserves my warmest thanks for his critical view on the subject and a pleasant collaboration. I am indebted to my co-authors, Ulf Hellman, Ph.D., Dean Cuebas, Ph.D., Werner Schmitz, Ph.D., Matti Poutanen, Ph.D., Tuomo Glumoff, Ph.D., Kirsí Siivari, M.Sc., Antti Haapalainen, M.Sc., and Mari Marttila, B.Sc. for their tremendous contribution to this work. Former and present members of "KH team", especially, Sirpa Filppula, Heli Helander, Kari Koivuranta, Tiila-Riikka Kiema, Tanja Kokko, Ahmed Yagi, and Tiina Kotti are appreciated for their friendship and fruitful discussion during and after the work.

I wish to extend my sincere thanks to whole staff of the Departments. I am much obliged to Aila Holappa, Irma Vuoti, Anja Mattila, Raija Pietilä, Tanja Kokko, Ville Ratas and Marika Kamps for their skillful technical assistance, Anna-Leena Hietajärvi for making our precious substrates, Eeva-Liisa Stefanius for synthesizing oligonucleotides, Maire Jarva for doing the automatic sequencing, Jaakko Keskitalo and Kyösti Keränen for developing the photos and fixing Schimadzu spectrophotometer. Special thanks also go to Seppo Kilpeläinen, M.Sc., for setting up an excellent computer network, Auli Kinnunen, Marja-Leena Kivelä, Anneli Kaattari and Tuula Koret for their kind help with secretarial and many practical tasks.

I would like to thank my father, mother and brothers for encouraging me to study abroad and their support in each step of my life. I am also grateful to my parents and sister in-law for their help whenever I need. Finally, I owe my heartfelt thanks to my husband
Qiang for his support in all matters of life and our daughter Mandi who always keeps me in touch with the true life.

This work was financially supported by grants from the Sigrid Jusélius foundation, Academy of Finland and University of Oulu foundation.

Oulu, June 1999

Yong-Mei Qin
Abbreviations

ABC ATP-binding cassette
X-ALD X-linked adrenoleukodystrophy
ATP adenosine triphosphate
bp base pair(s)
cDNA complementary DNA
CoA coenzyme A
FAD flavine adenine dinucleotide
FADH2 flavine adenine dinucleotide (reduced)
LCAD long-chain acyl-CoA dehydrogenase
kDa kilodalton(s)
MFE multifunctional enzyme
MFE-1 peroxisominal multifunctional Δ⁴-Δ⁵-enoyl-CoA isomerase/2-enoyl-CoA hydratase 1/(3S)-hydroxyacyl-CoA dehydrogenase
MFE-2 peroxisominal multifunctional 2-enoyl-CoA hydratase 2/(3R)-hydroxyacyl-CoA dehydrogenase
mRNA messenger RNA
NAD+ nicotinamide adenine dinucleotide (oxidized)
NADH nicotinamide adenine dinucleotide (reduced)
NADPH nicotinamide adenine dinucleotide phosphate (reduced)
PCR polymerase chain reaction
peroxin protein involved in peroxisome biogenesis
pI isoelectric point
PPAR peroxisomal proliferator activated receptor
PPRE peroxisomal proliferator responsive element
PTS peroxisomal targeting signal
RACE rapid amplification of cDNA ends
RXR retinoid X receptor
SDR short-chain alcohol dehydrogenase/reductase family
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
THCA 3α, 7α, 12α-trihydroxy-5β-cholestanolic acid
Xaa any amino acid
List of original articles

This thesis is based on the following articles, which are referred to the text by their Roman numerals:


III Qin Y-M, Marttila MS, Haapalainen AM, Glumoff T, Novikov DK & Hiltunen JK Human peroxisomal multifunctional enzyme type 2: site-directed mutagenesis studies show the importance of two protic residues for 2-enoyl-CoA hydratase 2 activity. Submitted.

IV Qin Y-M, Marttila, MS, Haapalainen AM, Siivari KM, Glumoff T & Hiltunen JK Yeast peroxisomal multifunctional enzyme: (3R)-hydroxyacyl-CoA dehydrogenase domains A and B are required for optimal growth on oleic acid. Submitted.
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1. Introduction

The name peroxisome was proposed in 1965 by De Duve who discovered a subcellular compartment containing oxidases and catalase that participate in the production and consumption of hydrogen peroxide. This far, over 50 enzymes have been found to be involved in a variety of peroxisomal metabolic processes, including the β-oxidation of fatty acids and fatty acid derivatives; β-oxidation of the side chain of cholesterol that results in the formation of bile acids; fatty acid elongation; synthesis of plasmalogen, cholesterol and dolichols; catabolism of amino acids, purines and polyamines; metabolism of glyoxylate; degradation of glucose via the hexose-monophosphate pathway; inactivation of reactive oxygen species and the process of α-oxidation (for a review, see Mannaerts & Van Veldhoven 1996).

The peroxisomal β-oxidation pathway in mammals was discovered by Lazarow and De Duve in 1976. The co-existence of both mitochondrial and peroxisomal β-oxidation and the consequences of β-oxidation deficiencies have revealed the significance of fatty acid oxidation in mammals (Kunau et al. 1988). Peroxisomal β-oxidation plays a key role in the metabolism of polyunsaturated fatty acids (for a review, see Hiltunen et al. 1996). The (2E)-enoyl-CoA ester is the only unsaturated intermediate in the β-oxidation spiral and therefore, double bonds of unsaturated fatty acids mainly in the cis configurations require additional auxiliary enzymes in order to be removed.

The present work is concerned with the molecular characterization of the 2-enoyl-CoA hydratase 2 (hydratase 2) from rat liver that was previously found to participate in the epimerization of 3-hydroxyacyl-CoA ester between the R- and S-form (Hiltunen et al. 1989, Smeland et al. 1989). According to the original hypothesis, this epimerization was supposed to be involved in the β-oxidation of cis-2-enoyl-CoA esters (Stoffel et al. 1964) and at that time hydratase 2 was listed as an auxiliary enzyme of β-oxidation. The physiological function of hydratase 2 in yeast peroxisomes was elucidated to be in the catabolism of (2E)-enoyl-CoA to a 3-keto complex via a (3R)-hydroxyacyl-CoA intermediate catalyzed by a multifunctional enzyme (MFE) possessing (3R)-hydroxyacyl-CoA dehydrogenase and hydratase 2 activities (Hiltunen et al. 1992). The processing of (2E)-enoyl-CoA to the 3-keto complex occurs in mammalian peroxisomes via a (3S)-hydroxyacyl-CoA intermediate. It is carried out by a peroxisomal multifunctional enzyme with three activities: (3S)-hydroxyacyl-CoA dehydrogenase, 2-enoyl-CoA hydratase 1
(Osumi & Hashimoto 1979), and $\Delta^1$-$\Delta^2$-enoyl-CoA isomerase (Palosaari & Hiltunen 1990). However, the function of hydratase 2 in mammals remained unknown. The aim of this work was the molecular cloning of mammalian hydratase 2, identification of potential catalytic amino acid residues contributing to the hydration reaction, and functional complementation of mammalian hydratase 2 in \textit{Saccharomyces cerevisiae}. In order to further elucidate the function of peroxisomal MFE, yeast (3\textit{R})-hydroxyacyl-CoA dehydrogenase was also investigated in the present work.
2. Review of the literature

2.1. Fatty acids

Fatty acids are simple lipids that have a hydrophilic carboxyl group at one end of a hydrocarbon chain. They are mainly classified as saturated or unsaturated fatty acids with many important species of the latter form being in the cis-configuration. α-Linolenic (C\textsubscript{18:3, n-3}) and linoleic (C\textsubscript{18:2, n-6}) acids are known as essential fatty acids (EFAs), since they are important for the well-being of individuals and must be present in the diet. EFAs serve several vital functions as nutrients, an energy source, structural components of cells and precursors of prostanoids and leukotrienes. In polyunsaturated fatty acids, the double bonds usually alternate between odd- and even-numbered carbon atoms and are separated by a methylene group.

2.1.1. Uptake and transport of fatty acids into the mammalian cell

The mechanism of the transport of long-chain fatty acids across the cellular membrane has been proposed as being either a passive or carrier-mediated transmembrane translocation. The passive mechanism is a non-saturable diffusion governed by the transmembrane gradient of fatty acid concentration (De Grela & Light 1980). The carrier-mediated process of migration of fatty acid through the plasma membrane is either mediated by a Na⁺/fatty acid co-transport system (Stremmel 1987) or by membrane-associated fatty acid-binding proteins (mFABPs). The intracellular transport of fatty acids from the plasma membrane either to the sites of metabolic conversion or to the subcellular target is facilitated by cytoplasmic fatty acid binding proteins (cFABPs) (for a review, see Glatz et al. 1997). cFABPs are abundant low-molecular-mass proteins (14-16 kDa), whose levels are responsible for nutritional, endocrine and a variety of pathological states.

To date, several mFABPs have been identified and these are implicated in transmembrane transport of long-chain fatty acids. In general, three classes of mFABPs have been described, and these include fatty acid translocase (FAT; Abumrad et al. 1993), membrane-bound fatty acid-binding proteins (FABPpm; Stremmel et al. 1985) and fatty
acid transport protein (FATP; Schaffer & Lodish, 1994). The expression of the genes encoding FAT and FATP is regulated by peroxisome proliferator activated receptors (PPARs), indicating that in addition to other PPAR function discussed below, peroxisome proliferators influence the cellular uptake of fatty acids (Motojima et al. 1998, Frohnert et al. 1999). In *S. cerevisiae*, the gene encoding a putative membrane-bound long-chain fatty acid transport protein (FAT1) was identified (Færgeman et al. 1997) and possessed very long-chain acyl-CoA synthetase activity (see Section 2.2.4.; Watkins et al. 1998). FABPs were proposed to solubilize and compartmentalize fatty acid, to facilitate the cellular uptake and intracellular trafficking of fatty acids, as well as to modulate cell growth and differentiation (for a review, see Glatz et al. 1997).

2.1.2. Pathways for fatty acid oxidation

There are three different pathways for oxidizing fatty acids and they are described as α-, β-, and ω-oxidation. Quantitatively, the major pathway is β-oxidation which is located in both mitochondria and peroxisomes of mammalian cell. In contrast, in lower eukaryotes such as yeast and filamentous fungi, as well as in most plants, β-oxidation occurs solely in peroxisomes (Kunau et al. 1988). Fatty acid α-oxidation is a minor pathway that is involved in the catabolism of branched isoprenoid-derived 3-methyl substituted fatty acids, i.e. phytic acid (3,7,11,15-tetramethylhexadecanoic acid). It includes four reactions, namely activation, hydroxylation, release of formyl-CoA resulting in formation of pristanal followed by a dehydrogenation reaction yielding pristanic acid (Croes et al. 1996). 2-Methyl branched fatty acid is then further β-oxidized predominantly in peroxisomes (Schepers et al. 1990, Vanhove et al. 1991). The importance of α-oxidation in humans has been recognized by Refsum’s disease, which is characterized by an accumulation of large amounts of phytic acid. The intracellular site of α-oxidation is not yet clear. Evidence suggests that it occurs either in peroxisomes (Mihalik et al. 1995, Croes et al. 1996), in mitochondria (Skjeldal & Stokke 1987, Watkins & Mihalik 1990), in the endoplasmic reticulum (ER) (Huang et al. 1992), or in both mitochondria and peroxisomes (Fingerhut et al. 1993). Ordinary straight-chain fatty acids may also undergo ω-oxidation, resulting in the formation of medium- and long-chain dicarboxylates (Van Hoof et al. 1988). Monocarboxylic acids are first ω-hydroxylated to form ω-hydroxymonocarboxylic acids by a lauric acid ω-hydroxylase (E.C. 1.14.14.1.). These intermediates can be either ω-oxidized in mitochondria and peroxisomes or further oxidized to ω-oxomonomocarboxylic acids and dicarboxylic acids by cytosolic alcohol dehydrogenase (E.C. 1.1.1.71) and aldehyde dehydrogenase (E.C. 1.2.1.3), respectively. On the other hand, dicarboxylic acids are activated to their CoA esters in microsomes and subsequently β-oxidized to succinate (for a review, see Osmundsen et al. 1991). For xenobiotic fatty acids that can not be β-oxidized, i.e. 3-thia fatty acids, ω-oxidation is the main catabolic pathway (for a review, see Skrede et al. 1997).
2.2. Peroxisomes

A peroxisome is spherical or ovoid with a diameter of 0.1 to 1.5 \( \mu \text{m} \) and consists of a single membrane delimiting a granular matrix. Peroxisomes are versatile in most eukaryotic cells, and depending on the organism, participate in a variety of metabolic processes, including lipid metabolism; \( \text{H}_2\text{O}_2 \)-based respiration; oxidation of unsaturated very long-chain fatty acids, branched fatty acids and dicarboxylic acids; synthesis of plasmalogens, cholesterol and bile acids; and catabolism of purines, polyamines, and amino acids (for a review, see Mannaerts & Van Veldhoven 1996).

Peroxisome biogenesis consists of three aspects: membrane synthesis, import of matrix proteins, and proliferation. Over 20 peroxins (proteins involved in peroxisomal import, biogenesis, proliferation and inheritance) have been identified, and the corresponding PEX genes have been cloned and characterized (for reviews, see Kunau & Erdmann 1998, Titorenko & Rachubinski 1998). The view of peroxisome biogenesis has reached a crossroad. The original model proposed that peroxisomes arose through budding from the ER (Novikoff & Shinn, 1964). By the end of the 1980s, the ‘growth and division’ model became accepted which postulated that new peroxisomes were formed by the fission of pre-existing ones, and that this process is accomplished by the post-translational import of membrane and matrix proteins from the cytosol (Lazarow & Fujiki 1985). Recent observations that the ER plays a direct role both in transport of membrane proteins to peroxisomes and in supplying peroxisomes with phospholipids via ER-derived vesicles have challenged the current theory of peroxisome biogenesis (for reviews, see Kunau & Erdmann 1998, Titorenko & Rachubinski 1998). Peroxisome biogenesis in the absence of pre-existing organelles was also addressed by determining the function of Pex16p, when overexpressed, restored the de novo formation of peroxisomes in cells of a Zellweger syndrome patient (South & Gould 1999). Other important progress has been made by identifying specific signal sequences and the receptors responsible for targeting proteins to the peroxisomes, but this will not be discussed here (for a review, see Subramani 1998).

2.2.1. Peroxisomal proliferation in mammalian cells

An outstanding feature of peroxisomal \( \beta \)-oxidation is its powerful induction by a number of peroxisome proliferators. More than ten-fold induction in the number and size of peroxisomes is observed in rodents in response to peroxisomal proliferators (Lazarow & De Duve 1976, Lazarow 1977). Peroxisomal proliferators include a broad group of chemicals such as fibrate hypolipidemic drugs, i.e. clofibrate (ethyl-\( p \)-chlorophenoxyisobutyrate), nafenopin, Wy 14,643, industrial plasticisers, i.e. di-(2-ethylhexyl) phthalate, herbicides and leukotrene antagonists. In addition, thia fatty acids, which block \( \beta \)-oxidation, are also powerful inducers of peroxisomal \( \beta \)-oxidation (for a review, see Skrede et al. 1997). Induction of peroxisomes is found primarily in liver and kidney and is species specific, i.e. rodents are more susceptible than other organisms. In addition to the increase in the size and number of peroxisomes, most of the peroxisomal enzyme activities are also dramatically induced. Thus, peroxisomal proliferation provides
a tool for investigation of the biological aspects of peroxisomal function (for reviews, see Osmundsen et al. 1991, Schoonjans et al. 1997).

Peroxisome proliferation is a pleiotropic cellular response following the specific binding moiety is demonstrated to peroxisome proliferators. This receptor-mediated mechanism for proliferation was first proposed in 1983 by Lalwani and co-workers. Peroxisomal proliferator-activated receptors (PPARs) have been identified as products of separate genes from rodents (Issemann & Green 1990, Göttlicher et al. 1992), *Xenopus laevis* (Dreyer et al. 1992), and humans (Sher et al. 1993, Greene et al. 1995). PPARs in different vertebrates form a distinct subfamily of nuclear receptors and have been classified as PPARα, β (δ) and γ (for a review, see Schoonjans et al. 1997). PPARα is predominantly expressed in liver, heart, kidney, and the intestinal mucosa, all of which display high catabolic rates for peroxisomal metabolism of fatty acids (Braissant et al. 1996). Knockout mice deficient in PPARα do not respond to peroxisome proliferators and transcriptional activation of the target genes is not observed in these animals (Lee et al. 1995). PPARβ is ubiquitously and highly expressed in the central nervous system, however, its role is not known (Braissant et al. 1996). PPARγ is predominantly expressed in adipose tissue and is responsible, at least in part, for adipocyte differentiation (for a review, see Spiegelman 1998).

Activation of PPARs is initiated by lipophilic ligands that bind to a ligand-binding site in their carboxyl-terminal region. PPARα is activated either by peroxisomal proliferators (Issemann & Green 1990, Dreyer et al. 1993) or by a variety of long-chain fatty acid and, in particular, polyunsaturated fatty acids (Keller et al. 1993, Bocos et al. 1995). The assays based on *in vitro* binding, conformational change, or receptor-coactivator transactivation indicate that the synthetic peroxisome proliferators and fatty acids, i.e. 18:2 (*n*-6), 18:3 (*n*-3 and *n*-6) and 20:4 (*n*-6), as well as leukotriene B4 and prostaglandin I₂ analogs act as direct ligands for PPARs (Forman et al. 1997, Kliewer et al. 1997, Krey et al. 1997). The binding of fatty acids to the PPAR subtypes reveals that PPARγ interacts more efficiently with unsaturated fatty acids than with saturated fatty acids (Xu et al. 1999). The crystal structure of the PPARβ ligand-binding domain demonstrates that the hydrophobic tail of the fatty acid adopts different conformations within the large hydrophobic cavity, which can explain the propensity for PPARs to interact with a variety of fatty acids (Xu et al. 1999).

A cognate DNA sequence that is recognized by and binds to PPARs has been identified to be a *cis*-acting peroxisome proliferator-responsive element (PPRE). It consists of a degenerate direct repeat of the canonical AGG(A/T)CA sequence separated by one base pair. PPREs were identified in the 5' flanking region of genes coding for many proteins implicated in intra- and extracellular lipid metabolism, and most of those involved in peroxisomal β-oxidation, such as, palmitoyl-CoA oxidase (Tugwood et al. 1992), multifunctional enzyme (Zhang et al. 1993) and thiolase (Kliewer et al. 1992). Upon activation of the ligand, PPARs dimerize with the 9-*cis* retinoic acid receptor (RXRα) to form a PPAR-RXRα complex that binds to the PPRE motif to regulate the transcription of the target genes (for a review, see Schoonjans et al. 1997). The nucleotides in the flanking region upstream of the PPRE also play an important role in regulation of the affinity of PPAR-RXRα complex to its target site (Juge-Aubry et al. 1997).
2.2.2. Induction of genes encoding peroxisomal proteins in yeast

In *S. cerevisiae*, fatty acids are the only exogenous substances known to influence the levels of peroxisomal enzymes and the number and size of peroxisomes (Veenhuis et al. 1987), whereas in other yeast species such as the methylotrophic yeast *Pichia pastoris*, methanol is a potent inducer of peroxisomes.

Promoters of genes encoding peroxisomal matrix proteins contain a positive *cis*-acting element termed the oleate response element (ORE) that mediates the induction of these genes in the presence of a fatty acid such as oleic acid (cis-C18:1(9)) (Einerhand et al. 1993, Filipits et al. 1993). The ORE is an imperfect repeat CCGG(Xaa)15/18CCG containing two conserved CGG triplets that are separated by 15-18 nucleotides (Rottensteiner et al. 1996). In addition, there are conserved A and T nucleotides in the consensus half site 5'-CGGN;T(Xaa)A-3', which are important for ORE function (Einerhand et al. 1993, Filipits et al. 1993). The ORE is the target for transcription factors consisting of Oaf1p (oaf: oleate-activated transcription factor) and Pip2p (pip: peroxisome induction pathway), both of which possess a Zn2Cys6 DNA-binding motif and bind to the consensus ORE (Karpichev et al. 1997, Luo et al. 1996, Rottensteiner et al. 1997). Heterodimerization of Pip2p and Oaf1p regulates induction of peroxisomal β-oxidation when yeast cells are grown in oleic acid medium. When yeast cells are grown on glucose, Oaf1p is constitutively expressed and binds to ORE as a homodimer, whereas Pip2p is specifically induced in the presence of oleic acid (Rottensteiner et al. 1997). In addition to Pip2p and Oaf1p, other transcription factors are involved in the oleate-dependent induction of peroxisomal proteins, most notably Adr1p (a regulator of alcohol dehydrogenase II synthesis; Simon et al. 1991).

2.2.3. Activation and transportation of fatty acids into peroxisomes

Mammalian peroxisomes are involved in the β-oxidation of long-chain and very long-chain fatty acids (LCFAs and VLCFAs). Initiation of the β-oxidation pathway starts with activation of fatty acids to their fatty acyl-CoA thioesters by fatty acyl-CoA synthetases. The peroxisomal membrane contains at least three acyl-CoA synthetases depending on chain length specificity and species. The first one is a long-chain acyl-CoA synthetase (LACS, E.C. 6.2.1.3) which activates straight long-chain fatty acids (Shindo & Hashimoto 1978, Krisan et al. 1980, Mannaerts et al. 1982, Bronfman et al. 1984). The rat enzyme additionally activates pristanic acid, a 2-methyl branched fatty acid originating from the α-oxidation of phytanic acid (Vanhove et al. 1991, Wanders et al. 1992, Watkins et al. 1996). The activity of the enzyme was also found in the membrane of ER (Kornberg & Pricer 1953) as well as in the outer membrane of the mitochondria (Norum et al. 1966). The mammalian gene for this acyl-CoA synthetase has been cloned (Suzuki et al. 1990, Abe et al. 1992) and characterized (Suzuki et al. 1995).

The second enzyme is a very long-chain acyl-CoA synthetase (VLACS), which activates very-long-chain (C20-C26) fatty acids (Laposata et al. 1985, Singh & Poulos 1988). The enzyme is located in the membrane of the peroxisomes and the ER, but not in mitochondria (Singh & Poulos, 1988, Lazo et al., 1990a, Singh et al., 1993). The gene for
the enzyme was cloned from rat liver (Uchiyama et al. 1996) and the predicted amino acid sequence had a high sequence similarity to a fatty acid transport protein from *S. cerevisiae* (Fat1p, Schaffer & Lodish, 1994). Disruption of the *S. cerevisiae* *FAT1* gene decreases VLACS activity and elevates intracellular concentrations of very long-chain fatty acids (Watkins et al. 1998). *FAT1* defective cells were found to grow poorly in medium supplemented with oleic acid when the known fatty acid synthases (FAAs) were inactivated, whereas fatty acid import into the cytosol was not significantly affected (Choi & Martin 1999). This result suggests that *FAT1* codes for peroxisomal VLACS and that the primary cause of the growth-defective phenotype is probably due to a failure in fatty acid β-oxidation rather than a defect in fatty acid transport. In humans, a phytanoyl-CoA ligase of peroxisomal α-oxidation isolated from fibroblasts is a third acyl-CoA synthetase distinct from the long-chain- and the very-long-chain- acyl-CoA synthetases. (Pahan et al. 1993).

The site of VLCFA activation is still a matter of debate (Lazo et al. 1990b, Lageweg et al., 1991). LCFAs are activated outside peroxisomes, whereas medium-chain fatty acids are activated inside peroxisomes (Krisans et al. 1980, Mannaerts et al. 1982). A new mechanism of activation and transportation of LCFAs in *S. cerevisiae* has been proposed following the discovery of two peroxisomal ATP-binding cassette (ABC) proteins, Pat1p (Bossier et al. 1994) and Pat2p (Shani et al. 1995), that form a heterodimer facilitating transportation of cytosolic LACS across peroxisomal membranes (Hettema et al. 1996). One of the peroxisomal disorders, X-linked adrenoleukodystrophy (ALD), characterized by accumulation of VLCFAs, was previously regarded to be caused by VLACS deficiency. When the gene for adrenoleukodystrophy protein (ALDp) was cloned, it showed no homology to the VLACs, but instead to the mammalian gene *PMP70* whose product is a peroxisomal membrane protein belonging to the family of ABC transporter proteins (Mosser et al. 1993). ALDp was reported to assist VLACS to localize in peroxisomes (Yamada et al. 1999).

### 2.2.4. The peroxisomal β-oxidation spiral

#### 2.2.4.1. Acyl-CoA oxidation

The first reaction of peroxisomal β-oxidation of fatty acids is the rate-limiting step catalyzed by an acyl-CoA oxidase, a FAD-containing enzyme that transfers electrons from acyl-CoA to oxygen, producing H$_2$O$_2$ as well as desaturation of acyl-CoA to (2E)-enoyl-CoAs (Fig. 1). Rat liver contains at least three peroxisomal acyl-CoA oxidases that
Fig. 1. The peroxisomal β-oxidation cycle consists of two partially parallel pathways following reciprocal stereochemistry. The mammalian mitochondrial and bacterial β-oxidation pathways occur via (3S)-hydroxyacyl-CoA.
facilitate the metabolism of a great variety of substrates in peroxisomes. The first enzyme is clofibrate-inducible palmitoyl-CoA oxidase which oxidize the CoA esters of medium-, long- and very long- straight chain fatty acids, medium- and long-chain dicarboxylic fatty acids and eicosanoids (Osumi & Hashimoto 1978, Schepers et al. 1990, Van Veldhoven et al. 1992). The low activity of the enzyme toward short-chain substrates may explain why \( \beta \)-oxidation of fatty acids in mammalian peroxisomes is not complete (Osumi et al. 1980).

The second enzyme is pristanoyl-CoA oxidase which oxidizes the CoA esters of 2-methyl-branched fatty acids and the CoA esters of long chain mono- and dicarboxylic fatty acids, especially very-long-chain monocarboxylic acids (Van Veldhoven et al. 1991 & 1994a). The third acyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase is found in hepatic tissue (Schepers et al. 1990) and oxidizes the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids (Schepers et al. 1989a & 1990, Van Veldhoven et al. 1994b). However, only two acyl-CoA oxidases have been detected in human liver. One is a palmitoyl-CoA oxidase with the same function as its rat counterpart. The other is a branched acyl-CoA oxidase, which has both functions of the two other rat oxidases (Casteels et al. 1990, Wanders et al. 1990, Vanhove et al. 1993).

The cDNAs of palmitoyl-CoA oxidases have been cloned from mammals (Miyazawa et al. 1987, Aoyama et al. 1994, Fournier et al. 1994, Varanasi et al. 1994). Either the rat or human cDNA encodes a protein of 661 amino acid residues, containing a SKL carboxy terminal tripeptide. The corresponding genes have also been characterized and both have been shown to be alternatively spliced (Osumi et al. 1987, Fournier et al. 1994, Varanasi et al. 1994). The 5’ untranslated region of both of the genes contains a GC-rich region and a peroxisomal proliferator response element (Osumi et al. 1987, Varanasi et al. 1994 & 1996). It has been reported that mice deficient in palmitoyl-CoA oxidase suffer from a liver disorder and spontaneous peroxisome proliferation. This implicates the oxidase as a key regulator of PPAR\( \alpha \) function and acyl-CoAs as potent biological ligands which are responsible for the transcriptional activation of PPAR\( \alpha \) in vivo (Fan et al. 1996 & 1998).

In several yeast species such as Candida tropicalis and Candida maltosa, there are multiple isoforms of acyl-CoA oxidases (Okazaki et al. 1986 & 1987, Murray & Rachubinski, 1987, Hill et al. 1988). In S. cerevisiae, however, there is only one acyl-CoA oxidase (Pox1p) and the expression of the \( \text{POX1} \) gene is induced when the yeast cells are grown in the presence of oleic acid (Dmochowska et al. 1990). The regulation of the \( \text{POX1} \) gene is mediated by the heterodimerization of Oaf1p and Pip2p, which bind to the ORE as described in section 2.2.2. (Rottensteiner et al. 1996, Karpichev et al. 1997).

2.2.4.2. Hydration of trans-2-enoyl-CoA and subsequent dehydrogenation

The second reaction in the \( \beta \)-oxidation is the reversible hydration of (2E)-enoyl-CoA to (3S)-hydroxyacyl-CoA (Fig. 1). In mitochondria, the hydratase reaction catalyzed by a monofunctional 2-enoyl-CoA hydratase 1 (hydratase 1 or crotonase) follows \textit{syn} stereochemistry and is concerted. Two protic amino acid residues are required for catalysis. In rat hydratase 1, the protonated Glu-164 acts as a catalytic acid providing a
Fig. 2. The proposed reaction mechanism of rat mitochondrial 2-enoyl-CoA hydratase 1. The hydration and dehydration reactions are catalyzed by amino acid residues, Glu164 and Glu144, at the active site.

proton to the α-carbon of the (2E)-enoyl-CoA, whereas the deprotonated Glu-144 accepts a proton from water as a catalytic base and the formed hydroxide anion adds to the β-carbon of the substrate (Fig. 2.; Engel et al. 1996, Kiema et al. 1999). The crystal structure of the protein has been solved and reveals a core domain consisting of repeating ββα-units that form a characteristic right handed spiral structure (Engel et al., 1996).

In addition to 2-enoyl-CoA hydratase 1, 2-enoyl-CoA hydratase 2 in rat liver catalyzes reversible hydration/dehydration between (2E)-enoyl-CoA and (3R)-hydroxyacyl-CoA, which is supposed to arise from a minor pathway associated with the metabolism of polyunsaturated fatty acids with cis double bonds, i.e. trans-2, cis-4-decadienoyl-CoA (Stoffel et al. 1964). The “epimerase-pathway” is only present in peroxisomes and would be essential for preventing the accumulation of (3R)-hydroxoyctanoyl-CoA (Chu & Schulz 1985, Yang et al. 1986). The 3-hydroxyacyl-CoA epimerase reaction in mammalian cells is not catalyzed by a single enzyme, but by cooperation of two stereospecific hydratases: 2-enoyl-CoA hydratase 1 and a 2-enoyl-CoA hydratase 2 (Hiltunen et al. 1989). The epimerization mechanism in a bacterial system also confirms that 3-hydroxyacyl-CoA esters are epimerized by the β-oxidation complex of Escherichia coli via a dehydration-hydration mechanism (Smeland et al. 1991). It is still not clear whether monofunctional epimerase exists. However, the cloning and expression of a plant glyoxysomal multifunctional protein (MFE) demonstrated that the (3R)-hydroxyacyl-CoA converting activity was actually an epimerase rather than a part of a combindend water eliminating and water attaching system (Preisig-Müller et al. 1994). Inhibition studies showed that the hydratase 2 activity was inhibited by a sulphydryl inhibitor (N-methylmaleimide) and acetocetacetyl-CoA. The studies of the chain length specificity of the enzyme showed that the ratio of hydration rates \(C_{10}/C_4\) was 14.4, indicating the low activity toward short-chain substrates. At least two hydratase 2 species have been purified from rat liver. The isoforms
of 44 kDa and 31.5 kDa were found to be located in peroxisomes (Li et al. 1990) and microsomes (Malila et al. 1993), respectively.

The third reaction is the dehydrogenation of (3S)-hydroxyacyl-CoA to ketoacyl-CoA by (3S)-hydroxyacyl-CoA dehydrogenase (E.C. 1.1.3.5). In mammalian peroxisomes, the second and third reactions of β-oxidation are catalyzed by a single multifunctional protein (MFE-1) which has 2-enoyl-CoA hydratase 1, (3S)-hydroxyacyl-CoA dehydrogenase and Δ2-Δ3-enoyl-CoA isomerase activities (Osumi & Hashimoto 1979, Palosaari & Hiltunen 1990). In the yeast peroxisomes, the second and third reactions of β-oxidation proceed via a (3R)-hydroxyacyl-CoA intermediate and these two reactions are catalyzed by yeast peroxisomal MFE (MFE-2) possessing (3R)-hydroxyacyl-CoA dehydrogenase and hydratase 2 activities (Hiltunen et al. 1992).

2.2.4.3. Cleavage of 3-ketoacyl-CoA

The fourth reaction of β-oxidation is thiolytic cleavage of a 3-ketoacyl-CoA thioester, which produces an acyl-CoA shortened by two carbon atoms and acetyl-CoA (Fig.1). At least three peroxisomal 3-ketoacyl-CoA thiolase genes exist. They are thiolase A (encoded by gene A) containing a pre-sequence of 36 amino acids (Hijikata et al. 1987 & 1990), thiolase B (encoded by gene B) containing a pre-sequence of 26 amino acid residues (Miyazawa et al. 1980) and a sterol carrier protein 2/3-ketoacyl-CoA thiolase (Seedorf et al. 1994, Antonenkov et al. 1997). The pre-sequences at the amino terminus of both rat liver thiolases harbor the peroxisomal targeting signal PTS-2 (Osumi et al. 1991, Swinkels et al. 1991, Miura et al. 1994, Tsukamoto et al. 1994). Gene B was markedly activated by treatment with peroxisome proliferators whereas gene A was only slightly or not activated (Hijikata et al. 1990). Thiolase A and thiolase B isolated from rat liver are virtually identical in their substrate specificity for the thiolytic cleavage of straight-chain 3-ketoacyl-CoAs (Antonenkov et al. 1997 & 1999). In humans, only one peroxisomal 3-ketoacyl-CoA thiolase has been identified and comparison with two rat thiolases reveals 78% identity at the amino acid level (Bout et al. 1988, Fairbarin & Tanner 1989, Bondar et al. 1990).

The mRNA encoding sterol carrier protein 2/3-ketoacyl-CoA thiolase, has two initiation sites giving polypeptides with molecular masses of 58 kDa (SCPx) and 15 kDa (pro-SCP2). The open reading frame encodes a fusion protein containing a C-terminal 143-amino acid lipid transfer domain (SCP2 domain) and an N-terminal domain of 404 amino acids, displaying thiolase activity toward 2-methyl-branched 3-ketoacyl-CoA and the bile acid intermediate (Antonenkov et al. 1997). In line with this observation, catabolism of methyl-branched fatty acyl CoAs is found to be impaired in SCP2 (-/-) mice, exemplified by the accumulation of the tetramethyl-branched fatty acid phytanic acid due to defective thiolytic cleavage of 3-ketopristanoyl-CoA (Seedorf et al. 1998).

In S. cerevisiae, 3-ketoacyl-CoA thiolase has been isolated (Erdman & Kunau, 1994) and its structure has been determined to 1.8Å resolution (Mathieu et al. 1994). Peroxisomal aceoacetat-CoA thiolase (thiolase I) and 3-ketoacyl-CoA thiolase (thiolase II) were found and purified from C. tropicalis (Kurihara et al. 1989, Tanaka et al. 1995). The kinetic studies showed that the long-chain substrates were degraded exclusively by
thiolase II, while acetoacetyl-CoA was degraded preferentially by thiolase I. The *POT1* gene encoding 3-ketoacyl-CoA-thiolase from *Yarrowia lipolytica* has been isolated and characterized (Berninger *et al.* 1993). As there are no other catabolic thiolases in *Y. lipolytica*, the inability of this yeast to grow on oleic acid when the *POT1* gene is disrupted implies that Pot1p plays an important role in β-oxidation.

2.2.5. Oxidation of the cholesterol side chain

Primary bile acids are synthesized from cholesterol in the liver by the actions of at least 14 different enzymes (for reviews, see Russel & Setchell 1992, Perdersen 1993). The sequence of reactions involved in the formation of bile acids may be seen as a series of detoxification steps transforming the highly hydrophobic cholesterol molecules into water-soluble products. The reactions are divided into two main stages: i) oxidation and modification of the ring system; and ii) oxidation and cleavage of the side chain followed by conjugation with glycine or taurine.

The terminal steps of side-chain oxidation take place mainly in the peroxisome and are thought to follow a pathway similar to that of the β-oxidation of fatty acids in this organelle (Fig. 2.). The 3α, 7α, 12α-trihydroxy-5β-cholestanolic acid (THCA) is initially activated to 3α, 7α, 12α-trihydroxy-5β-cholestanoyl-CoA (THCA-CoA) by a trihydroxycoprostanoyl-CoA synthetase (Schepers *et al.* 1989b), which is subsequently acted upon by an acyl-CoA oxidase to yield (24E)-Δ24-THCA-CoA. Followed by hydration and dehydrogenation catalyzed by a peroxisomal MFE, 24-keto-THCA-CoA is formed and thiolytically cleaved, yielding a propionyl-CoA and the formation of a bile acid-CoA product that is shortened by three carbons.

For the first reaction of peroxisomal β-oxidation, three different acyl-CoA oxidases have been described in rat and humans (see section 2.2.5.1; Van Veldhoven *et al.* 1991 & 1992, Schepers *et al.* 1990). Whereas, only rat trihydroxycoprostanoyl-CoA oxidase or human branched acyl-CoA oxidase is responsible for oxidation of THCA-CoA to (24E)-Δ24-THCA-CoA (Casteels *et al.* 1990, Schepers *et al.* 1990). The substrate specificity of the oxidase indicates that the 25S isomer of THCA-CoA is a preferential substrate, implying that 2-methylacyl-CoA rasemase exists in peroxisomes (Ikegawa *et al.* 1995). Previously, it was believed that the mammalian MFE-1 catalyzed the hydration and dehydrogenation of (24E)-Δ25-THCA-CoA to a 24-keto-THCA-CoA. However, Xu & Cuebas (1996) found that the product of hydration of (24E)-Δ25-THCA-CoA by MFE-1 was (24S, 25S)-24-OH-THCA-CoA, which was not a proper substrate for the dehydrogenase component of the enzyme. Thus, there is a paucity of data regarding which enzyme can act on these two reactions leading to biosynthesis of bile acid in the peroxisomes.
Fig. 3. The terminal steps in oxidation of a cholesterol side chain.
2.3. Auxiliary enzymes involved in β-oxidation

The only double bond of unsaturated acyl-CoA that is catabolized via normal β-oxidation is at the Δ2-position and in a trans configuration. Therefore, the removal of other double bonds requires the auxiliary enzymes, including 2, 4-dienoyl-CoA reductase, Δ3-Δ2-enoyl-CoA isomerase and Δ3,5-Δ2,4-dienoyl-CoA isomerase (Fig. 4.). Fatty acids with a double bond at even-numbered position yield 2, 4-dienoyl-CoAs, which can be converted to (2E)-enoyl-CoAs by 2, 4-dienoyl-CoA reductase and Δ3-Δ2-enoyl-CoA isomerase. In contrast, 2, 5-dienoyl-CoAs, which arise from fatty acids with double bonds at odd-numbered positions, enter β-oxidation by two distinct pathways (see Section 2.3.3.).

α-Methyl-acyl-CoA racemase is a recently discovered auxiliary enzyme required for β-oxidation of branched fatty acids and bile acid intermediates (Schmitz et al. 1994, Ikegawa et al. 1995).

2.3.1. 2, 4-Dienoyl-CoA reductase

An NADPH-dependent 2, 4-dienoyl-CoA reductase (E.C. 1.3.1.34) participates in the β-oxidation of fatty acids having double bonds at even-numbered positions (Fig. 4.; Kunau & Dommes 1978, Dommes & Kunau 1984a). It catalyzes the reduction of 2, 4-dienoyl-CoAs to trans-3-enoyl-CoAs in eukaryotes and trans-2-enoyl-CoAs in E. coli (Dommes & Kunau 1984b). Trans-3-enoyl-CoAs are converted to (2E)-enoyl-CoAs by Δ3-Δ2-enoyl-CoA isomerase (Cuebas & Schulz 1982, Osmundsen et al. 1982, Hiltunen et al. 1983) with the final product entering the β-oxidation spiral (Fig. 4.). Peroxisomes are impermeable to NADPH (Van Roermund et al. 1995), which is, at least in yeast, provided by an NADP⁺-dependent isocitrate dehydrogenase (Idp3) functioning in an NADP⁺ redox shuttle across the peroxisomal membrane (Van Roermund et al. 1998, Henke et al. 1998).

Mammals possess at least two isoforms of mitochondrial 2, 4-dienoyl-CoA reductases with different molecular masses (120 kDa and 60 kDa) and a third peroxisomal one (Dommes et al. 1981, Hakkola & Hiltunen 1993). The 120-kDa isoform has been cloned from rat and humans, (Hirose et al. 1990, Koivuranta et al. 1994), and the human gene has been characterized and assigned to the chromosome 8q21.3 (Helander et al. 1997). In S. cerevisiae, the product of the SPS19 gene (Coe et al. 1994) was identified to be a peroxisomal 2, 4-dienoyl-CoA reductase with a molecular mass of 69 kDa (Gurvitz et al. 1997).

2.3.2. Δ3-Δ2-Enoyl-CoA isomerase

Fatty acids with a double bond extending from an odd-numbered carbon atom were thought to proceed stepwise via β-oxidation until the Δ3-double bond was introduced. Δ3-Δ2-Enoyl-CoA isomerase (E.C. 5.3.3.8) converts both cis- and trans-3-enoyl-CoA esters to their trans-2-counterparts, which are metabolized further by regular fatty acid β-oxidation (Stoffel et al 1964). The known isomerases belong to the low-similarity
Fig. 4. Auxiliary enzymes are required for the β-oxidation of unsaturated fatty acids. The bacterial 2, 4-dienoyl-CoA reductase has trans-2-enoyl-CoA as an end product. Δ^3, Δ^2, Δ^3, Δ^4-Dienoyl-CoA isomerase activity has not yet been found in prokaryotes.
isomerase/hydratase protein superfamily with a conserved fingerprint –Val-Ser-Xaa-Ile-Asn-Gly-Xaa-Xaa-Ala-Gly-Gly-Xaa-Leu-Xaa-Xaa-Xaa-Cys-Asp-Tyr- (Müller-Newen & Stoffel 1993). Members include isomerases and hydratases acting on either the Δ² or the Δ³-double bond, and additionally include 4-chlorobenzoyl-CoA dehalogenase (Babbitt et al. 1992), naphthoate synthase (Sharma et al. 1992), β-hydroxyisobutyryl-CoA hydrolase (Hawes et al. 1996), Δ¹₂-Δ²₄-dienoyl-CoA isomerase (Filppula et al. 1998), carnitine racemase (Eichler et al. 1994) and other uncharacterized proteins. They are thought to have evolved from a common ancestor since they were found in a variety of species including bacteria and mammals. Interestingly, hydratase 1 has been reported to contain an intrinsic isomerase activity in addition to its known hydratase activity (Kiema et al. 1999). There are two separate mitochondrial short-chain and long-chain isomerases in rat liver (Palosaari et al. 1990, Kilponen et al. 1990). The mitochondrial short-chain isomerase has been characterized (Stoffel & Grol 1978, Palosaari et al 1990 & 1991, Tomioaka et al. 1991 & 1992, Müller-Newen et al. 1991, Stoffel et al. 1993, Janssen et al. 1994) and crystallized (Zeelen et al. 1992). The isomerase activity is markedly induced by the peroxisome proliferators. Theoretically, two protic amino acid residues are required to participate in catalysis, as in rat mitochondrial hydratase 1 (see Section 2.2.5.2). However, only one conserved protic residue in the isomerase, Glu165, was identified to be responsible for the isomerase activity (Müller-Newen et al. 1995). The second protic residue is unknown and could be a water molecule or side chain of an unidentified protic residue. In addition to the two mitochondrial isomerases, a third isoform of mitochondrial isomerase, that does not show any substrate length specificity, has been characterized in humans (Kilponen & Hiltunen 1993, Kilponen et al. 1994). The peroxisomal Δ³-Δ⁵-enoyl-CoA isomerase is a part of the peroxisomal MFE-1 in mammals (Palosaari & Hiltunen 1990). An oleate-inducible peroxisomal monofunctional isomerase has been identified in S. cerevisae and is also a member of the isomerase/hydratase superfamily (Gurvitz et al. 1998, Geisbrecht et al. 1998). This protein does not contain a conserved glutamate (Glu 165) at its active site and therefore lacks the conserved fingerprint. The significance of this divergence remains to be studied.

2.3.3. Δ⁵⁻Δ⁴-Dienoyl-CoA isomerase

The β-oxidation of unsaturated fatty acids with double bonds in odd-numbered positions proceeds to produce trans-2-enoyl-CoA via a Δ⁵-intermediate by Δ³-Δ⁵-enoyl-CoA isomerase (Fig. 4.; reductase-independent pathway; Stoffel et al. 1964). An additional NADPH-dependent reductive metabolism pathway of unsaturated fatty acids with double bonds at the Δ⁵ position has been described (Tserng & Jing 1991), and occurs through an intermediate of trans-2-Δ⁵-dienoyl-CoA (Fig. 4.; reductase-dependent pathway; Smeland et al. 1992). In the proposed sequence of reactions, Δ⁵-enoyl-CoA is dehydrogenated to trans-2, Δ⁵-dienoyl-CoA, which is isomerized to Δ¹₂-Δ⁵-dienoyl-CoA by Δ³-Δ⁵-enoyl-CoA isomerase or peroxisomal MFE-1. The Δ¹₂-Δ⁵-dienoyl-CoA is subsequently isomerized to trans-2, trans-4-dienoyl-CoA by Δ³-Δ⁴-dienoyl-CoA isomerase. (Luo et al. 1994, Chen et al. 1994, Luthria et al. 1995). The metabolic sequence from trans-2, trans-4-dienoyl-CoA operates by the NADPH-dependent pathway as proposed for unsaturated fatty acids
with even-numbered double bonds. The contribution of these two pathways in mitochondria is still a debate (Tserng et al. 1996, Shoukry & Schulz 1998). \( \Delta^{3,5}-\Delta^{2,4} \) Dienoyl-CoA isomerase has been purified from rat liver and is localized in both mitochondria (Smeland et al. 1992) and peroxisomes (He et al. 1995). Hiltunen’s group (Filppula et al. 1998) expressed a cDNA encoding a protein of 36 kDa, rECH1 (FitzPatrick et al. 1995), which belongs to the hydratase/isomerase superfamily. The expressed recombinant protein shows \( \Delta^{3,5}-\Delta^{2,4} \)-dienoyl-CoA isomerase activity. Subcellular fractionation and immunoelectronmicroscopy of rat liver localizes the polypeptide to both mitochondria and peroxisomes. Consistent with these observations, the open reading frame of rECH1 has a potential N-terminal mitochondrial targeting signal as well as a C-terminal peroxisomal PTS-1. The crystal structure of rat \( \Delta^{3,5}-\Delta^{2,4} \)-dienoyl-CoA isomerase has been solved at 1.5 Å resolution and it resembles that of 2-enoyl-CoA hydratase 1 and 4-chlorobenzoyl-CoA dehalogenase. The amino acid residue Asp204 has been revealed to be essential for catalysis (Modis et al. 1998). Recently, a peroxisomal \( \Delta^{3,5}-\Delta^{2,4} \)-dienoyl-CoA isomerase has been identified from \( S. \) cerevisiae (Gurvitz et al. 1999), but the physiological role of its participation in the reductase-dependent pathway in oxidation of unsaturated fatty acids with double bonds at odd-numbered position seems not important.

### 2.3.4. 2-Methylacyl-CoA racemase

Branched fatty acids arise from the metabolism of isoprenoids. For example, accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is observed in Refsum’s disease and in a number of peroxisomal diseases with defects in \( \alpha \)-oxidation. Phytanic acid is a 3-methyl-branched fatty acid and is formed from phytol, which is present in chlorophyll in an esterified form. Phytanic acid is converted to pristanic acid (2-methyl-branched fatty acid, 2,6,10,14-tetramethylpentadecanoic acid) via \( \alpha \)-oxidation, which shortens it by one carbon atom, and allows further degradation by \( \beta \)-oxidation. The resulting pristanic acid is a racemic mixture of 2S- and 2R-enantiomers. The racemase allows the \( \beta \)-oxidation of both enantiomers of 2-methyl-branched fatty acids by converting (2R)-enantiomer to (2S)-enantiomer, which is accepted as the substrate for mitochondrial acyl-CoA dehydrogenases or peroxisomal oxidases (Schmitz & Conzelmann 1997, Van Veldhoven et al. 1996). The bile acid intermediate, (25R)-trihydroxyprostanoyl-CoA is required to be racemized to the (25S)-enantiomer by the racemase in bile acid formation. The cDNA of the racemases from rat or mouse does not resemble any other known amino acid sequences of \( \beta \)-oxidation proteins (Schmitz et al. 1997). The racemases have been shown to be present in mitochondria, peroxisomes and the cytosol of human and rat livers (Schmitz et al. 1995, Van Veldhoven et al. 1997).

### 2.4. Multifunctional enzymes (MFEs) of \( \beta \)-oxidation

Multifunctional enzymes (MFEs) are associated with all known \( \beta \)-oxidation systems
characterized thus far and comprise the second and the third reactions of the β-oxidation spiral. In mammalian mitochondria, these two reactions can also be catalyzed by monofunctional enzymes. MFEs have been purified from bacteria, fungi, plants and mammals, and their activities depend on the subcellular location and vary from one species to another (Table 1; for a review, see Hiltunen et al. 1993).

2.4.1. Bacterial β-oxidation multienzyme complex

*E. coli* is used as a model system for studying fatty acid degradation. It contains a β-oxidation complex with a native molecular mass of 240 kDa, which is composed of two different subunits, α and β, that form a tetramer α₂β₂. α and β are encoded by the *fadBA* operon (Binstock et al. 1977, Binstock & Schulz 1981, Pawar & Schulz 1981). 2-Enoyl-CoA hydratase 1, (3S)-hydroxyacyl-CoA dehydrogenase and Δ¹-Δ²-enoyl-CoA isomerase activities are catalyzed by the 79 kDa-α subunit encoded by the *fadB* gene and 3-ketoacyl-CoA thiolase, encoded by *fadA* gene, is located in the 42 kDa β subunit (Yang & Schulz 1983, Yang et al. 1988, Yang et al. 1991). The epimerization reaction occurs via a hydration/dehydration mechanism (Smeland et al. 1991), which is the same as that found in mammals (see Section 2.2.5.2; Hiltunen et al. 1989). It has been proposed that *E. coli* hydratase 1 might function as a 3-hydroxyacyl-CoA epimerase possessing hydratase 2 and hydratase 1 activities (Yang & Elzinga 1993). Site-directed mutagenesis of the amino-terminal domain of the α-subunit and kinetic studies of the expressed recombinant proteins have revealed that both Glu119 and Glu139 are the catalytic residues for the hydration reaction following an acid-base mechanism (Yang et al. 1995, He & Yang 1997), which has been elucidated from rat mitochondrial 2-enoyl-CoA hydratase 1 (Kiema et al. 1999). His450 was assigned to be the catalytic residue of (3S)-hydroxyacyl-CoA dehydrogenase (He & Yang 1996) and this observation confirms that the *E. coli* dehydrogenase has a proposed NAD⁺-binding site in its aminoterminal domain and structurally resembles the porcine heart dehydrogenase (EC 1.1.1.35; Yang et al. 1991).

2.4.2. Fungal multifunctional enzymes

Alkane-assimilating yeast has been proposed to be an excellent model with which to investigate fatty acid degradation in mammalian cells. The capacity to degrade fatty acids is confined to peroxisomes rather than mitochondria. In yeast peroxisomes, unlike in those of mammals and bacteria, β-oxidation of fatty acids proceeds only via R-hydroxy intermediates catalyzed by a dimeric MFE-2 (Hiltunen et al. 1992). The genes encoding the MFE-2 in yeast *C. tropicalis* and *S. cerevisiae* have been characterized
Fig. 5. The proposed reaction mechanism of (3R)-hydroxyacyl-CoA dehydrogenase of yeast peroxisomal MFE-2.

(Nuttley et al. 1988, Hiltunen et al. 1992). Amino acid and nucleotide sequence analysis of the yeast MFE-2 suggested a division of the polypeptide into amino terminal, middle and carboxy terminal domains and a partial gene duplication in the first two domains during the evolution (Nuttley et al. 1988). A truncated version of the MFE-2 lacking 271 amino acids in carboxy terminal domain retains only the dehydrogenase activity, suggesting that the catalytic domains of the dehydrogenase are located in the amino terminus, whereas the hydratase domain is located in the carboxy terminus (Hiltunen et al. 1992). Limited proteolysis of MFE-2 isolated from both *C. tropicalis* and *Neurospora crassa* (Thieringer & Kunau 1991) yields two major fragmentation products, supporting the occurrence of two superdomain structures.

The duplicated dehydrogenase domains of MFE-2 belong to a short-chain alcohol dehydrogenase/reductase family (SDR), which includes phylogenetically related groups of enzymes acting on as diverse substrates as sugars, steroids, prostaglandins, aromatic hydrocarbons, antibiotics and compounds involved in nitrogen metabolism (for a review, see Jörnvall et al. 1995). A characteristic segment of the nucleotide [NAD(H) or NADP(H)] binding fold Gly-Xaa-Xaa-Gly-Xaa-Gly ("Rossmann fold") is largely conserved in the SDR family. Sequence analysis has revealed that the members of the SDR family contain a highly conserved pentapeptide motif of Tyr-Xaa-Xaa-Xaa-Lys. The tyrosine’s deprotonated phenolic group is proposed to catalyze hydride transfer with the aid of the positively charged lysine residue that decreases the pKa of the tyrosine’s phenolic group from 10 to about 7 (Fig. 5.). However, the significance of the existence of two dehydrogenase domains in yeast MFE-2 is unknown.
2.4.3. Plant multifunctional enzymes

Linoleic acid and other highly unsaturated fatty acids amount to up to 70% of the fatty acid moieties in plant lipids. In plants, the fatty acids are mainly degraded in glyoxysomes or peroxisomes. Mitochondrial β-oxidation is generally regarded to be absent or to have only a minor contribution. The peroxisomal chain shortening of acyl-CoA requires three enzymes: acyl-CoA oxidase, MFEs and thiolase. In the plant β-oxidation pathway, (3R)-hydroxyacyl-CoA is an intermediate in the conversion between cis-2-enoyl-CoA and trans-2-enoyl-CoA by (3R)-hydroxyacyl-CoA hydrolase (Engeland & Kindl 1991). Three isoenzymes of the MFEs have been found and they differ in the number of active sites, catalytic properties, size and other molecular characteristics (Gühnemann-Schäfer & Kindl 1995). One of these isoenzymes has been found to have a molecular mass of 79 kDa and contains the four activities of (3S)-hydroxyacyl-CoA hydrolyase, (3S)-hydroxyacyl-CoA dehydrogenase, (3R)-hydroxyacyl-CoA epimerase, and $\Delta^3 - \Delta^2$-enoxy-CoA isomerase (Preisig-Müller et al. 1994).

2.4.4. Mammalian multifunctional enzymes

The peroxisomal MFE-1 was induced by peroxisomal proliferators and purified from rat (Osumi & Hashimoto 1979) and humans (Reddy et al. 1987). Palosaari and Hiltunen (1990) showed that the enzyme possesses $\Delta^1 - \Delta^2$-enoyl-CoA isomerase activity in addition to 2-enoyl-CoA hydratase 1 and (3S)-hydroxyacyl-CoA dehydrogenase activities. The rat MFE-1 gene encodes 722 amino acid residues (Osumi et al. 1985), whereas the human gene encodes 723 amino acid residues (Hoefler et al. 1994) and both contain SKL peroxisomal targeting signals at the carboxy terminus. The amino acid identity between the rat and human enzyme sequences is 78%. Sequence comparison of MFE-1 with mitochondrial 3-hydroxyacyl-CoA dehydrogenase (Minami-Ishii et al. 1989) and E. coli fadB (Yang et al. 1991) reveals that the hydratase and isomerase reside in the aminoterminal domain and share the same CoA binding site (Palosaari et al. 1991), whereas the dehydrogenase is located in the carboxyterminus (Osumi & Hashimoto 1979). The MFE-1 gene has been characterized from rat (Ishii et al. 1987) and a PPRE has been identified in its promoter region (Zhang et al. 1992).

In addition to peroxisomal MFE-1, there is a mitochondrial trifunctional enzyme that has a structure of $\alpha_4\beta_4$ and catalyzes the second, third and fourth reaction of the β-oxidation spiral (Uchida et al. 1992). Amino acid sequence analysis of the enzyme indicates that the $\alpha$-subunit harbors a 2-enoyl-CoA hydratase in the aminoterminal domain and a 3-hydroxyacyl-CoA dehydrogenase in the carboxyterminal domain, while a 3-ketoacyl-CoA thiolase resides in the $\beta$-subunit (Kamijo et al. 1993).
<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular Mass</th>
<th>Activities</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>260,000</td>
<td>H₁, H₂, I, D₃, T</td>
<td>α₂β₂</td>
<td>Yang &amp; Schultz 1983</td>
</tr>
<tr>
<td>Pseudomonas fragi</td>
<td>240,000</td>
<td>H, E, I, D, T</td>
<td>α₃β₂</td>
<td>Imamura et al. 1990</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>365,000</td>
<td>H₂, D₆</td>
<td>α₄</td>
<td>Fossà et al. 1995</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>186,000</td>
<td>H₂, D₆</td>
<td>α₂</td>
<td>De La Garza et al. 1985, Nuttley et al. 1988</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>170,000</td>
<td>H₂, D₆</td>
<td>α₃</td>
<td>Hiltunen et al. 1992</td>
</tr>
<tr>
<td>Cucumis sativus</td>
<td>74,000*</td>
<td>H, D, E</td>
<td>α</td>
<td>Gühnemann-Schäfer et al. 1994</td>
</tr>
<tr>
<td></td>
<td>76,000**</td>
<td>H, D, I, E</td>
<td>α</td>
<td>Gühnemann-Schäfer et al. 1994</td>
</tr>
<tr>
<td></td>
<td>81,000***</td>
<td>H₁, D₃, I, E</td>
<td>α</td>
<td>Gühnemann-Schäfer &amp; Kindl 1995</td>
</tr>
<tr>
<td>Rat (peroxisomal)</td>
<td>79,047</td>
<td>H₁, D₃, I, E</td>
<td>α</td>
<td>Preisig-Müller et al. 1994</td>
</tr>
<tr>
<td></td>
<td>78,511</td>
<td>H₁, D₃, I</td>
<td>α</td>
<td>Osumi &amp; Hashimoto 1979, Palosaari &amp; Hiltunen 1990</td>
</tr>
<tr>
<td>Rat (mitochondrial)</td>
<td>460,000</td>
<td>H₁, D₃, T</td>
<td>α₄β₄</td>
<td>Uchida et al. 1992, Kamijo et al. 1993</td>
</tr>
<tr>
<td>Mouse (peroxisomal)</td>
<td>5,000</td>
<td>H₁, D₃, I</td>
<td>α</td>
<td>Stark &amp; Meijer 1994</td>
</tr>
<tr>
<td>Human (peroxisomal)</td>
<td>78,000</td>
<td>H₁, D₃, I</td>
<td>α</td>
<td>Reddy et al. 1987, Hoefler et al. 1994, Kilponen &amp; Hiltunen 1993</td>
</tr>
<tr>
<td>Human (mitochondrial)</td>
<td>230,000</td>
<td>H₁, D₃, T</td>
<td>α₄β₄</td>
<td>Carpenter et al. 1992, Kamijo et al. 1994</td>
</tr>
</tbody>
</table>

Abbreviations: H, 2-enoyl-CoA hydratase (stereochemistry of the reaction not specified); H₁, 2-enoyl-CoA hydratase 1; H₂, 2-enoyl-CoA hydratase 2, D, 3-hydroxyacyl-CoA dehydrogenase (stereochemistry of the reaction not specified); D₆, (3S)-hydroxyacyl-CoA dehydrogenase; D₆₀, (3R)-hydroxyacyl-CoA dehydrogenase; I, Δ₁⁻Δ₂-enoic-CoA isomerase; T, 3-ketoacyl-CoA thiolase; E, 3-hydroxyacyl-CoA epimerase. When present, 3-ketoacyl-CoA thiolase activity is located in the β-subunit. *MFE I, **MFE II, ***MFE III.
3. Outline of the present study

Although the main pathways of the β-oxidation of fatty acids has been investigated extensively, many questions still remain. When this work was started, it was assumed that the peroxisomal β-oxidation of fatty acids occurred only via MFE-1 in mammals and via MFE-2 in yeast. Rat 2-enoyl-CoA hydratase 2 that hydrates trans-2-enoyl-CoA to (3R)-hydroxyacyl-CoA had been purified from liver (Malila et al. 1993), however, the physiological function of the enzyme was not known. Furthermore, the reason for existence of the duplicate dehydrogenase domains in yeast MFE-2 was not clear. The specific aims of the present studies were:

1) to clone 2-enoyl-CoA hydratase 2 from rat liver,
2) to investigate the physiological role of 2-enoyl-CoA hydratase 2,
3) to study the catalytic properties of 2-enoyl-CoA hydratase 2,
4) to elucidate the significance of duplication of the gene encoding the (3R)-hydroxyacyl-CoA dehydrogenase domains of yeast peroxisomal MFE-2.
4. Materials and methods

Materials and methods are described in detail in the original articles (I-IV).

4.1. Biological sources

The rats used for isolation of 2-enoyl-CoA hydratase 2 were Wistar strains obtained from the Laboratory Animal Center of the University of Oulu. In order to study induction of peroxisomal proliferators (hypolipidaemic agents), the rats were fed on a standard pelleted diet containing 0.5 % clofibrate (w/w) for two weeks before removal of the livers.

*E. coli* strain DH5α was used for all plasmid transformations and isolations. The yeast *S. cerevisiae* wild-type strain UTL-7A is ura3-52, trp1, leu2,3-112 and the *fox-2* mutant is ura3-52, trp1, leu2,3-112, ade, fox2.

4.2. Culture media

Yeast *S. cerevisiae* strains were cultured in the following media, YPD (1% yeast extract, 2% Bacto peptone and 2% D-glucose), synthetic complete medium without uracil (SD/uracil, 0.67% Bacto-yeast nitrogen base without amino acids, 0.2% dropout powder without uracil, 2% D-glucose), oleic acid medium, YPOD (0.5% yeast extract, 0.5% Bacto peptone, 0.1% oleic acid, 0.1% glucose, 0.5% Tween 40, pH 7.0) and YNO (0.1% yeast extract, 0.67% yeast nitrogen base with amino acids, 0.1% oleic acid and 0.5% potassium phosphate, pH 6.0).
4.3. Synthesis of substrates

4.3.1. Synthesis of straight-chain fatty acyl-CoA esters

(3R)-Hydroxybutyric acid, (2E)-hexenoic acid, (2E)-decenoic acid and crotonyl-CoA are commercially available from Sigma. Acyl-CoA esters of trans-3-decenoic, (3R, 35)-hydroxydecanoic and (3R)-hydroxybutyric acids are prepared by the mixed anhydride method (Rasmussen et al. 1991) and purified on a µBondapak™C18 column (Waters, Milford, MA) by applying an acetonitrile gradient (15%-55%) for elution of the substrates. The structures of the substrates were verified by determining their masses with MALDI-TOF mass spectrometer (Ompact MALDI III, Kratos Analytical, Manchester, UK) and by testing with appropriate enzymes.

4.3.2. Synthesis of 2-pristenic acid

The detailed procedure of the synthesis of 2-pristenic acid and 2-methyltetradec-2-enoic acid is described in Paper I. CoA esters were prepared from the corresponding carboxylic acids by a modified mixed-anhydride method (Rasmussen et al. 1991).

4.3.3. Synthesis of 24-OH-3α,7α,12α-trihydroxy-5β-cholestanolic acid and (24E, Z)-Δ3-3α,7α,12α-trihydroxy-5β-cholestanolic acid

A mixture of the four (C24, 25) diastereomers of 3α,7α,12α,24ζ-tetrahydroxy-5β-cholestanolic acid (24-OH-THCA) was prepared using procedures previously described by condensing 3α,7α,12α-triformyloxy-5β-cholan-24-al with 2-methyl-bromopropionate (Batta. et al. 1982). (24E, Z)-3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid [(24E, Z)-Δ3-THCA] was prepared from 3α,7α,12α-triformyloxy-5β-cholan-24-al according to the published procedures (Iqbal et al. 1991). CoA derivatives were prepared by the mixed anhydride method and purified by HPLC (Schulz 1974).

4.4. Enzyme assays

4.4.1. Enzymes of fatty acid β-oxidation

The activities of 2-enoyl-CoA hydratase 2, 2-enoyl-CoA hydratase 1, (3R)- and (35)-hydroxyacyl-CoA dehydrogenases, 3-hydroxyacyl-CoA epimerase, Δ3-Δ5-enoyl-CoA isomerase and combined activities [metabolism of (2E)-enoyl-CoA esters to 3-ketoacyl-CoA esters] were measured as previously described (Hiltunen et al. 1989, Palosaari &
Hiltunen 1990, Filppula et al. 1995). The enzyme activities were measured using Shimadzu UV 3000 spectrophotometer.

4.4.2. Hydration and dehydration between (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA and 24-hydroxy, 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA

The incubation mixture for the reactions catalyzed by the recombinant 46 kDa hydratase 2 contained either 45 µM (24E)-Δ24-THCA or an 80 µM mixture of the (C24, 25)-diastereomers of 24-OH-THCA-CoA as substrate. The reactions were initiated by the addition of the enzyme. At various time intervals, a 150 µl- aliquot of the incubation mixture was withdrawn and the reaction stopped by the addition of 2M HCl, and the mixture was subsequently neutralized with 2M KOH. Aliquots of 150 µl were analyzed using HPLC and were performed on a Water dual-pump gradient with a YMC-Pack ODS-A reverse-phase column applying a linear gradient of methanol.

4.4.3. Enzyme kinetics

Kinetic constants (kcat and Km) of dehydrogenase activity were determined toward oxidation of (3R)-hydroxyacyl-CoA and formation of the Mg2+ complex of 3-ketoacyl-CoA was detected at 303 nm. The reaction buffer consisted of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM NAD+, 1 mM sodium pyruvate, and 2.5 mM MgCl2, and 21 units of L-lactic dehydrogenase (Sigma Chemicals) as an auxiliary enzyme. The measurement was performed at different substrate concentrations in a final volume of 0.5 ml at 22°C. The kinetic constants for hydratase 2 activity were measured with (2E)-decenoyl-CoA by a direct assay method (Binstock & Sculz 1981). The pH-dependence curve of hydratase 2 was determined by monitoring the hydration reaction at 263 nm over a broad range of pH values in 200 mM potassium phosphate buffer with (2E)-decenoyl-CoA as substrate in varying concentrations.

4.5. Isolation of peroxisomes

Peroxisomes were isolated from the light mitochondrial fraction of rat liver with a continuous Nycodenz density gradient (15-40%, w/w) as previously described (Ghosh & Hajra 1986).
4.6. Isolation and characterization of cDNA clones

mRNA from rat liver was isolated with Dynabead oligo (dT)$_{25}$ (Dynal, Oslo, Norway) according to the manufacturer’s instructions. The isolated mRNA was transcribed to the cDNA by reverse transcription. The PCR amplification was performed by using degenerate oligonucleotides deduced from the amino acids sequences of the isolated peptides as primers, and using the resultant cDNA as the template. The amplified PCR fragment was subcloned into pUC 18 plasmids with a SureClone ligation kit. The plasmid was used to transform E. coli DH5α. The subcloned fragments were sequenced by the dideoxynucleotide sequencing method.

4.7. Northern blot analysis

mRNA species were fractionated in 1% (w/v) agarose gel in the presence of formaldehyde, transferred to a nylon membrane by capillary blotting overnight and fixed by UV cross-linking. The membrane was hybridized with the labeled rat MFE-2 cDNA and exposed to X-ray film.

4.8. Expression of recombinant proteins

4.8.1. Expression of recombinant proteins in E. coli

The pET system is a powerful system developed for the expression of recombinant proteins in E. coli (Novagen). Target genes were cloned in pET plasmids under the control of the strong bacteriophage T7 promoter. The resulting constructs were transformed into an E. coli expression strain (BL21plysS or HMS174plysS) and plated on LB-ampicillin plate. Single colonies from the plates were used to inoculate M9ZB medium containing carbenicillin (50 µg/ml) and chloramphenicol (34 µg/ml). The cultures were grown at 37°C under aerobic conditions until an OD$_{600}$ of 0.4-0.6 was reached. The expression of the recombinant protein was induced by addition of IPTG to a final concentration of 0.4 mM. After 2 hours of induction at 33°-35°C, the cells were harvested and washed with phosphate-buffered saline (10 mM sodium phosphate, 2 mM potassium phosphate, 140 mM NaCl, 3 mM KCl, 5 mM β-mercaptoethanol, pH 7.4) and the pellet was stored at -70°C until used for protein purification.

4.8.2. Expression of recombinant proteins in P. pastoris

P. pastoris, representing one of four different genera of methylotrophic yeast, is capable of using methanol as a sole carbon source. Alcohol oxidase catalyzes the oxidation of
methanol to formaldehyde. Expression of this enzyme, encoded by the *AOX1* gene, is tightly regulated and induced by methanol to very high levels, typically ≥ 30% of the total soluble protein in cells grown with methanol as the carbon source. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of a gene of interest for heterologous protein expression. The coding sequence for a protein of interest was cloned into the intracellular expression vector pHILD2 behind *AOX1* promoter (Invitrogen). In addition, the vector, pHILD2, has a *HIS4* gene to facilitate the screening of recombinant strains. The resulting construct was linearized and integrated into the *AOX1* locus by transforming the *P. pastoris* HIS-deicient strain, GS115, with the expression vector by electroporation (Gene Pulser, Bio-Rad Laboratories) and allowing for homologous recombination to occur. Recombinant transformants were selected from solid histidine-deficient media and those with *AOX1*-disrupted were screened by poor growth in the presence of methanol. For expression, cells were grown on glycerol as a carbon source and were shifted to methanol for induction.

### 4.9. Site-directed mutagenesis

*In vitro* site-directed mutagenesis is a widely used technique for studying protein structure-function relationships. The QuickChange site-directed mutagenesis kit (Stratagene) was used to introduce separate point mutations into proteins. In this method, PCR reactions were performed using a supercoiled, double-stranded DNA plasmid as a template with two synthetic oligonucleotide primers containing the desired mutation encoding a mutated amino acid residue. *pfu* DNA polymerase was used in PCR reaction to replicate both DNA strands with high fidelity, according to the manufacturer's instructions.

### 4.10. Protein purification

Various chromatographic methods were applied to purify the enzymes. The columns were generally a dye ligand column, Matrex Red A, hydroxyapatite (HA), hydrophobic butyryl-sepharose, anion-exchange DEAE-Sephacel and Resource Q, cation-exchange Resource S and size exclusion Superdex™ 200 HR 10/30. The purification protocols are described in more detail in papers I-IV.

### 4.11. Analysis of proteins

In-Gel protein digestion was performed as previously described (Hellman *et al.* 1995). The stained protein band was cut from the SDS-PAGE gel and digested with trypsin. The resulting peptides were separated by reversed-phase liquid chromatography using a µRPC C2/C18 SC 2.1/10 column connected to a SMART micropurification system (Pharmacia Biotech). The peptides were eluted in a linear gradient of acetonitrile in 0.05% (v/v)
trifluoroacetic acid. The selected peptides were subjected to automatic Edman degradation in an Applied Biosystems model 470A sequencer.

The molecular weight of a native protein was determined on a size exclusion column Superdex™ 75 using bovine liver catalase (235 kDa), rabbit muscle lactase dehydrogenase (140 kDa), pig heart malate dehydrogenase (70 kDa) and cytochrome C (12.5 kDa) as standards. SDS-PAGE was run to determine the subunit molecular size in the presence of 6M urea, and the gels were stained with 0.25 % Coomassie Brilliant blue in methanol/acetic acid/water (5:1:5).

Dynamic light scattering measurements were performed with a DynaPro-MSTC dynamic light scattering device (Protein Solution, Charlottesville, VA). The measurement was started after incubation of the sample for 5 minutes at 6°C in the sample cell and hydrodynamic radius was determined.

Circular dichroism (CD) spectroscopy measurements were carried out at 22°C using a Jasco JHO spectropolarimeter. Adsorption at 280 nm was measured and used for fine adjustment of the protein concentration of the sample used for CD spectroscopy. The far-UV spectra of the proteins were measured from 200 to 250 nm in 80 mM potassium phosphate, pH 7.0, with the following instrument settings: response 1s, sensitivity 100 mdeg, speed 50 nm/min, average of 30 scans.

Protein concentrations were determined with the Bio-Rad protein assay reagent, using bovine serum albumin as the standard.

### 4.12. Complementation of *S. cerevisiae* fox-2 with MFE-2(s) and their variants

*S. cerevisiae* fox-2 cells (Hiltunen et al. 1992) that are devoid of endogenous MFE-2 served as a tool for examining the *in vivo* function of *S. cerevisiae* MFE-2 variants, heterologous human MFE-2 and its variants. The gene of interest was cloned into the *S. cerevisiae* expression vector pYE352 behind the catalase A1 promoter, which contains an ORE. *fox-2* cells were transformed with the resultant construct by a lithium acetate method (Gietz & Schiestl 1995) and selected for on SD/uracil plates. The transformants were streaked onto solid medium containing oleic acid. Utilization of fatty acids by the transformed *fox-2* cells was observed by zones of clearing on the oleic acid plates.

### 4.13. Immunoblotting

The proteins from SDS-PAGE were electroblotted onto nitrocellulose filters. Detection of the blotted/transfered proteins was performed using the rabbit IgG fraction of the antiserum as the primary antibody and goat antirabbit IgG coupled to horseradish peroxidase as the secondary antibody (Bio-Rad Laboratories). The recognized epitopes were detected by either 4-chloro-1-naphthol (Sigma Chemicals) or an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).
4.14. Software

Using the BLAST network service (Altschul et al. 1990) performed amino acid homology comparisons with the GenBank, EMBL, PIR and Swiss-Prot data base at the NCBI. Multiple sequence comparisons of polypeptides were carried out with multiple alignment program CLUSTAL_X using the default parameters. The kinetic constants ($K_m$ and $k_{cat}$), pH dependence curves of the $k_{cat}/K_m$ for hydratase 2 reaction, pKa values, and their errors were calculated with the GraFit computer software (Sigma Chemicals).
5. Results

5.1. A novel peroxisomal multifunctional enzyme type 2

2-Enoyl-CoA hydratase 2 with a molecular mass of 31.5 kDa was purified from rat liver (Malila et al. 1993) and was subjected to trypsin digestion. The resulting peptides were isolated and sequenced (Fig. 2 of paper I). The cloning of hydratase 2 reveals that the full length cDNA is 2535 bp that encodes a 735-residue polypeptide containing a potential peroxisomal targeting signal (AKL) in the C-terminus (Fig. 2 of paper I). The molecular mass of the polypeptide is 79.3 kDa, which is 2.5 fold higher than that predicted for the purified rat hydratase 2. To explain the apparent discrepancy between the purified hydratase 2 and the cloned polypeptide, immunoblotting of rat liver extract and pure peroxisomes was performed using the antibody to the rat hydratase 2 (Malila et al. 1993). The results revealed three bands, 79 kDa, 66 kDa, 46 kDa both in the liver cell extract and the peroxisomes, and a 31.5 kDa band that was present only in the liver cell extract (Fig. 5 of paper I), suggesting that hydratase 2 is actually a fragmentation product from the polypeptide and cannot target into peroxisomes without a peroxisomal targeting signal.

The enzyme activities catalyzed by the polypeptide were studied with a recombinant protein obtained via expression of the cDNA in P. pastoris cells. To confirm expression of the rat cDNA that was integrated into the P. pastoris genome, Northern blot analysis was performed and a 2.9 kb-signal was detected (Fig. 4 of paper I). Immunoblotting showed that a 79 kDa signal and two smaller signals (66 kDa, 31.5 kDa) were detected with antibody to the rat hydratase 2 (Fig. 4 of paper I). The partially purified recombinant protein was used for substrate specificity studies (Table 2 of paper I). The protein catalyzes hydration and dehydrogenation reactions with (2E)-decenoyl-CoA (C_{10}) and crotonyl-CoA (C_{4}) substrates, the C_{10}/C_{4} activity ratio being 6.6 and 1.4 for the hydration and the dehydrogenation respectively. The 2-methyl-branched esters were slowly metabolized by the protein. The protein did not have detectable activities of 2-enoyl-CoA hydratase 1, (3S)-specific dehydrogenase, Δ^{1}-Δ^{2}-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase. The first three activities are associated with the known mammalian peroxisomal MFE-1. Thus, the cloned polypeptide is assigned to be a novel mammalian peroxisomal multifunctional enzyme type 2 (MFE-2).
Amino acid sequence comparisons of rat MFE-2 revealed that it has 81.3% identity with porcine 17β-HSD type IV (Leenders et al. 1994). Rat MFE-2 shows a very low catalytic velocity for oxidation of estradiol to estrone (Table 2 in paper I). The N-terminal domain of rat MFE-2 (residues 1-292) shows a high degree of identity with the two dehydrogenase domains of MFE-2 from *C. tropicalis* (Fig. 3. of paper I; Nuttley et al. 1988) and *S. cerevisiae* (Fig. 3. of paper I; Hiltunen et al. 1992). The middle domain of MFE-2 (residues 315-600) is similar to the hydratase region of yeast MFE-2(s). The extreme C-terminus of rat MFE-2 (residues 612-731) is similar to rat sterol carrier protein 2 (SCP-2; Seedorf & Assman 1991).

5.2. Recombinant 2-enoyl-CoA hydratase 2 derived from rat MFE-2 (II)

The region of cDNA encoding amino acid residues 318–735 of rat MFE-2 was cloned into the *E. coli* expression vector pET-3a, yielding the plasmid pET-Hyd2. The 1279 bp insert in the plasmid was expressed in *E. coli* BL21 (DE3) plysS cells and the recombinant protein was purified (Table 1 of paper II). The molecular mass of the protein was determined to be 46 kDa by SDS-PAGE (Fig. 1 of paper II), which agrees with 46.58 kDa predicted from the amino acid sequence. Immunoblotting showed that an antibody to the rat hydratase 2 (Malila et al. 1993) recognizes the 46 kDa-hydratase 2 (Fig. 1 of paper II).

When the 46 kDa-hydratase 2 was incubated with 30 nmol crotonyl-CoA, the product of the hydration reaction serves as a substrate for (3R)-hydroxyacyl-CoA dehydrogenase but not for the (3S)-hydroxyacyl-CoA dehydrogenase. The kinetic constants of the 46 kDa-hydratase 2 with (2E)-enoyl-CoA substrates were determined (Table 2 of paper II). The \( K_m \) value with (2E)-decenoyl-CoA (C10) was one tenth of that with crotonyl-CoA (C4). The catalytic efficiency with C10/C4 substrates is 11.3, which was consistent with a previously reported value (14.1) for rat liver hydratase 2 (Hiltunen et al. 1989). The specificity constant \( (k_{cat}/K_m) \) of the hydratase 2 with crotonyl-CoA was about 10^4 times lower than that of mitochondrial short chain 2-enoyl-CoA hydratase 1 (for a review, see Schulz 1987). The enzyme preparation retained > 90% of the original activity when it was stored in the buffer containing 200 mM potassium phosphate at pH 7.4 at 4°C for a year.

Since Xu and Cuebas (1996) reported that the product of hydration of (24E)-Δ\(^{24}\)-THCA-CoA by peroxisomal MFE-1 is not a physiological isomer, hydratase 2 derived from rat MFE-2 was investigated. The protein hydrates (24E)-3α, 7α, 12α, 24-tetrahydroxy-5β-cholestanoyl-CoA [(24E)-Δ\(^{24}\)-THCA-CoA] to (24R, 25R)-3α, 7α, 12α, 24-tetrahydroxy-5β-cholestanoyl-CoA [(24R, 25R)-Δ\(^{24}\)-OH-THCA-CoA], which has been previously identified to be a physiological intermediate in bile acid synthesis (Fig. 2 of paper II).
5.3. Site-directed mutagenesis of the 2-enoyl-CoA hydratase 2 (III)

To characterize the hydratase 2 reaction, two approaches were applied. One was complementation of S. cerevisiae fox-2 cells with a wild-type human MFE-2 (HsMFE-2) and its variants in vivo. The other was production of a recombinant protein in E. coli to study its properties in vitro.

Because amino acid sequences of mammalian hydratase 2(s) differ considerably from those of mammalian mitochondrial hydratase 1(s) and the E. coli β-oxidation multienzyme complex (Yang et al. 1991), the putative catalytic amino acid residues were identified by the sequence alignment of the hydratase 2 domain of HsMFE-2 with the other known hydratase 2 molecules (Fig. 2 of paper III). Each of the identified conserved protic residues was substituted to alanine by site-directed mutagenesis. When HsMFE-2 was expressed in S. cerevisiae fox-2 cells, it was found to complement fox-2 cells and restore the ability of the cells to grow on oleic acid as a carbon source. In this case, fox-2 cells could serve as a tool for examining different variants of HsMFE-2 in vivo. The expressed protein in soluble yeast extracts was recognized as 79 kDa by an antibody to the rat hydratase 2. Five variants (Tyr347Ala, Glu366Ala, Tyr505Ala, Asp510Ala and His515Ala) did not complement fox-2 as indicated by failure to produce zones of clearing on the oleic acid plates (Fig. 3 of paper III). The variants Tyr510Ala and His515Ala lost both their hydratase and dehydrogenase activities (Table 2 of paper III), suggesting that these substitutions probably influence the folding of the protein and were not studied further.

HsMFE-2(dhΔ) with a deleted dehydrogenase domain and its variants, HsMFE-2(dhΔE366A) and HsMFE-2(dhΔD510A), were expressed in E. coli and purified (Fig. 4B of paper III). Native molecular masses of HsMFE-2(dhΔ) and its variants were 83 kDa as determined by size-exclusion chromatography (Fig. 4A of paper III). Dynamic light scattering measurement indicated that the purified recombinant hydratase 2 samples were monodisperse and had hydrodynamic radii corresponding to a dimer. CD far UV-spectroscopy revealed that the molar ellipticities of the purified proteins were virtually identical (Fig. 5 of paper III). The $k_{cat}$ value of variant Glu366Ala is almost 100-fold lower than that of wild type at pH 5, but the $K_m$ value is not significantly affected by this substitution (Table 3 of Paper III). The pH-dependence curve of $k_{cat}/K_m$ is bell-shaped, giving pKa1 of 8.2 ± 0.1 and pKa2 of 9.7 ± 0.2, whereas the variant Glu366Ala has a lower pKa1 of 6.5 ± 0.1 (Fig. 6 of paper III). The variant Asp510Ala is completely inactive.

5.4. Analysis of the two catalytic domains of (3R)-hydroxyacyl-CoA dehydrogenase of yeast peroxisomal MFE-2 (IV)

The yeast peroxisomal MFE-2(s) has two (3R)-hydroxyacyl-CoA dehydrogenase domains, A and B, at the N-terminus, both of which consist of a NAD$^+$ binding site and a catalytic motif (Fig. 1 of paper IV, Jörnvall et al. 1995). Based on the Gly16Ser mutation identified in human MFE-2 deficiency (van Grunsven et al. 1998), a substitution of glycine to serine was introduced into the NAD$^+$ binding site of S. cerevisiae MFE-2 to dissect the two dehydrogenase domains (Fig. 2 of paper IV), resulting in ScMFE-2(aΔ), ScMFE-2(bΔ)
and ScMFE-2(aΔbΔ) variants. In order to reveal the physiological roles of these two dehydrogenase domains, *S. cerevisiae* fox-2 cells were taken as a model system and tested for complementation. The results show that *S. cerevisiae* fox-2 cells transformed with the expression plasmids containing ScMFE-2(aΔ) or ScMFE-2(bΔ) developed zones of clearing on oleic acid plates, whereas ScMFE-2(aΔbΔ) failed to grow (Fig. 3 of paper IV). In liquid oleic acid medium, the growth rates of fox-2 cells expressing ScMFE-2(aΔ) or ScMFE-2(bΔ) were about 50% of that found in wild type ScMFE-2 (Fig. 3 of paper IV). To verify that *S. cerevisiae* MFE-2 and its variants were expressed in fox-2, both 2-enoyl-CoA hydratase 2 and (3R)-hydroxyacyl-CoA dehydrogenase activities were measured in the soluble yeast extracts (Table 1 of paper IV). With the exception of the fox-2 cells, hydratase 2 activity was detected in all transformants. The dehydrogenase activity decreased in ScMFE-2(aΔ) and ScMFE-2(bΔ), but it was completely absent in the ScMFE-2(aΔbΔ) variant.

*C. tropicalis* MFE-2 with a deleted 2-enoyl-CoA hydratase 2 domain [CtMFE-2(h2Δ)] plus variants of domains A and B [CtMFE-2(h2ΔaΔ), CtMFE-2(h2ΔaΔbΔ)] were overexpressed in *E. coli*, purified and characterized (Table 2 of paper IV). Size-exclusion chromatography (Fig. 4 of paper IV) and CD spectroscopy (Fig. 5 of paper IV) showed that CtMFE-2(h2Δ) and variants were dimeric proteins with similar secondary structural elements. The CtMFE-2(h2Δ) showed the highest catalytic efficiency (kcat/Km) with the substrate (3R)-hydroxydecanoyl-CoA (C10). Interestingly, the dehydrogenase activity of CtMFE-2(h2Δ) broke into two different profiles when the variants were analyzed (Table 3). The catalytic constant (kcat) of CtMFE-2(h2ΔaΔ) for C4 was the same as that of CtMFE-2(h2Δ) (29 ± 1 s⁻¹ vs 31 ± 2 s⁻¹), whereas kcat of CtMFE-2(h2ΔaΔbΔ) was below the detection limit, indicating that domain B is solely responsible for the utilization of C4 substrate. The kcat values of CtMFE-2(h2ΔaΔ) for C10 and C16 were 17 ± 1 s⁻¹ and 12 ± 2 s⁻¹, respectively. Interestingly, the kcat values of CtMFE-2(h2ΔbΔ) for C10 and C16 were 33 ± 2 s⁻¹ and 36 ± 6 s⁻¹, respectively, suggesting that domain A contributes more than domain B to the metabolism of medium- and long-chain substrates. The activity of CtMFE-2(h2ΔaΔbΔ) toward the substrates tested was below the detection limits (<0.001 μmol x mg⁻¹ x min⁻¹) of the assay.
6. Discussion

6.1. A novel peroxisomal multifunctional enzyme type 2 (I)

Although mammalian 2-enoyl-CoA hydratase 2 was first found to be involved in the epimerization reaction (Hiltunen et al. 1989, Smeland et al. 1989) and purified from either microsomes or peroxisomes (Malila et al. 1993, Li et al. 1990), its physiological function in mammals was largely unknown. To investigate its role in fatty acid metabolism of mammalian cells, the microsomal 31.5 kDa hydratase 2 was cloned from rat liver in the present work.

Surprisingly, the results demonstrate that hydratase 2 is an integral part of a novel mammalian peroxisomal MFE-2. The presence of both (R)-specific dehydrogenase and hydratase 2 activities were confirmed by expression of the rat MFE-2 in P. pastoris (Table 2 of paper I). Mammalian MFE-2 has a SCP-2 like domain at the C-terminus. It has been reported that SCP-2 is not only a non-specific lipid transfer protein but also acts as a peroxisomal acyl-CoA binding protein (for a review, see Wirtz et al. 1998). The role of the SCP-2 homologue in MFE-2(s) requires further investigation. Mammalian MFE-2 shows a pronounced sequence similarity to yeast peroxisomal MFE-2, suggesting that both mammalian and yeast MFE-2(s) have evolved from a common ancestor and have been associated with β-oxidation of fatty acids via a (3R)-hydroxyacyl-CoA intermediate.

In mammalian cells, there is a conventional peroxisomal MFE-1 displaying 2-enoyl-CoA hydratase1/(3S)-hydroxyacyl-CoA dehydrogenase/Δ^-Δ^-enoyl-CoA isomerase. MFE-1 has a similar function as MFE-2 in β-oxidation of straight chain fatty acids but via a (3S)-hydroxyacyl-CoA intermediate. Even though MFE-1(s) have not been found in yeast and MFE-1 shares no sequence similarities with mammalian MFE-2(s), rat MFE-1 complements the S. cerevisiae fox-2 and restores the ability of fox-2 cells to grow on oleic acid (Filppula et al. 1995). This implies that the two types of peroxisomal MFEs are functionally related.

Mammalian MFE-2 is a protein that was previously identified as 17β HSD type IV (Leenders et al. 1994). As a multifunctional enzyme, MFE-2 was cloned and characterized simultaneously from mouse (Normand et al. 1995), rat (paper I, Dieuaide-Noubhani et al. 1996, Corton et al. 1996), and man (Adamski et al. 1995, Jiang et al. 1997) by five independent research groups.
The results suggested a new organization of the peroxisomal β-oxidation system in mammalian peroxisomes, which consists of two parallel pathways following reciprocal stereochemistry. The significance of the two alternative pathways in mammals for the β-oxidation of fatty acids requires further examination.

6.2. Recombinant 2-enoyl-CoA hydratase 2 derived from rat MFE-2 (II)

Mammalian peroxisomes are capable of β-oxidizing not only straight-chain fatty acids, but also isoprenoid-derived 2-methyl-branched fatty acids and carboxyl side-chains of the bile acid intermediates di- and trihydroxycoprostanic acids. Since rat MFE-2 slowly metabolizes 2-methyl-branched substrates pristenoyl-CoA and 2-methyltetradecenoyl-CoA esters (Table 2 of paper I), the question was raised whether this enzyme participates in the chain shortening of cholesterol during the synthesis of primary bile acids. The results demonstrate that the recombinant hydratase 2 hydrates (24E)-Δ24-THCA-CoA to (24R, 25R)-OH-THCA-CoA, which is a physiological isomer, subsequently dehydrogenated by the dehydrogenase domain of rat MFE-2 (Dieuaide-Noubhani et al. 1996). Bile acid profiles of a patient with MFE-2 deficiency show an accumulation of (24R, 25R)- and (24R, 25S)-THCA, as well as, small amounts of their (24S) counterparts (Une et al. 1997), implying that an alternative pathway for the formation of 24-keto-THCA-CoA would operate by a combination of MFE-1 and α-methylacyl-CoA racemase (Ikegawa et al. 1995) in the following steps: i) the hydration of (24E)-Δ24-THCA-CoA to (24S, 25S)-24-OH-THCA-CoA catalyzed by the hydratase 1 domain of MFE-1, ii) the hypothetical racemization of the α-methyl group to (24S, 25R)-24-OH-THCA-CoA by an α-methylacyl-CoA racemase, and iii) the dehydrogenation of (24S, 25R)-24-OH-THCA-CoA to 24-keto-THCA-CoA catalyzed by MFE-1 (Scheme 2 of paper II). Although α-methylacyl-CoA racemase was purified from rat (Schmitz et al. 1994) and human (Schmitz et al. 1995) livers and α-methyl-THCA-CoA racemase activity was found in peroxisomes (Ikegawa et al. 1995), direct evidence that this racemase is involved in the racemization of the α-methyl group of 24-OH-THCA-CoA diastereomers is still missing. Obviously, since hydratase 1 is commercially available and is widely applied in metabolic studies, no monofunctional hydratase 2 has been found in nature. Thus, this recombinant hydratase 2 is a useful tool for studying stereospecificities in the β-oxidation pathway.

6.3. Site-directed mutagenesis of the 2-enoyl-CoA hydratase 2 (III)

Hydratase 2 was first cloned from yeast S. cerevisiae as a domain of peroxisomal MFE-2 (Hiltunen et al. 1992) and subsequently cloned from pig (Leenders et al. 1994), mouse (Normand et al. 1995), humans (Adamski et al. 1995) and rat (paper I). The physiological role of the mammalian hydratase 2 was revealed to be associated with bile acid synthesis (paper II). However, the amino acid residues participating in catalysis were not yet known.

By complementing S. cerevisiae fox-2 with HsMFE-2 and its variants in vivo, three
amino acid residues Glu366, Tyr347 and Asp510 were identified to affect only hydratase 2 activity. \textit{HsMFE-2(dhΔ)}, \textit{HsMFE-2(dhΔE366A)} and \textit{HsMFE-2(dhΔD510A)} were expressed in \textit{E. coli} and purified (Fig. 4 of paper III). \textit{HsMFE-2(dhΔY347A)} could not be purified due to the changes in its chromatographic behavior although it was recognized in the soluble extract by the antibody to the rat hydratase 2. The pH-dependence curve of \( k_{cat}/K_m \) of \textit{HsMFE-2(dhΔ)} is bell-shaped (Fig. 6 of paper III), suggesting that two protic residues participate in catalysis. The \( K_m \) value of \textit{HsMFE-2(dhΔE366A)} is similar to that of \textit{HsMFE-2(dhΔ)}, indicating that the Glu366Ala substitution does not affect substrate binding of the enzyme. However, the pKa of \textit{HsMFE-2(dhΔE366A)} decreases from 8.2 to 6.5, showing that the substitution affects the catalytic efficiency of the enzyme at low pH (Table 3 of Paper III). This suggests that the negatively charged side-chain of Glu366 acts as a base in catalysis. \textit{HsMFE-2(dhΔD510A)} was enzymatically inactive. CD far UV-spectroscopy revealed that the molar ellipticities of \textit{HsMFE-2(dhΔ)} and \textit{HsMFE-2(dhΔE366A)} are identical (Fig. 5 of paper III), indicating that the substitution does not affect the composition of secondary structural elements. Alignment of the amino acid sequence of \textit{HsMFE-2(dhΔ)} with other MFE-2 proteins reveals a new fingerprint of hydratase 2, Ala-Ala-(Leu, Ile)-Tyr-Arg-Leu-(Xaa)-Ser-Gly-Asp\(^{510}\)-Xaa-Asn-Pro-Leu-His-(Ile, Val)-Asp-Pro-Xaa-(Phe, Leu)-Ala-(Xaa),Phe-(Xaa),Pro-Ile-Leu-His-Gly-(Leu, Met)-Cys-(Thr, Ser)-Xaa-Gly (Fig. 2 of Paper III). Asp510 is highly conserved in this motif from prokaryotes to eukaryotes and plays an important role in the hydratase 2 reaction, either acting as an acid in the catalysis or affecting the binding of substrate. Basically, the hydratase 2 reaction also follows a general acid-base catalysis, like the hydratase 1 reaction (Engel et al. 1996, Kiema et al. 1999). This implies that both reactions utilize a common mechanistic strategy for lowering the free energies of the rate-limiting transition states. Surprisingly, the R-specific dehydratase of \textit{S. cerevisiae} fatty acid synthase (Chirala et al. 1987) also contains this motif, which implies that the hydratase 2 and the dehydratase converged at a particular point during evolution.

The straight-chain fatty acids are substrates for rat MFE-2 \textit{in vitro} (Table 2 of paper I). In line with this observation, \textit{HsMFE-2} complements and restores the ability of \textit{fox-2} cells to utilize oleic acid (cis-C\(_{18:1}\)) as the carbon source, which provides the first evidence on the ability of MFE-2 to metabolize straight-chain acyl-CoA esters \textit{in vivo}. In line with this observation, the patients with MFE-2 deficiency were characterized by accumulation of very-long-chain fatty acids in plasma and fibroblasts (Suzuki et al. 1997, Van Grunsven et al. 1998). The available gene structure of \textit{HsMFE-2} (Leenders et al. 1998) will help the sequence analysis of such patients. One case of MFE-2 deficiency is caused by a deletion of the cDNA encoding amino acid residues 480-501 in the hydratase 2 domain, resulting in absence of the enzyme as shown by immunofluorescence staining (Van Grunsven et al. 1999). The identification of important amino acid residues (Glu366 and Asp510) will provide a deep insight into the functional analysis of MFE-2 deficiency in future.
6.4. Analysis of the two catalytic (3R)-hydroxyacyl-CoA dehydrogenase domains of yeast peroxisomal MFE-2 (IV)

Yeast peroxisomal MFE-2 provides a good example for studying gene duplication during evolution. The interesting question is raised of what the physiological functions of the two dehydrogenase domains are and whether they show enzymatic activities. By applying site-directed mutagenesis to inactivate either domain A or domain B, both in vivo and in vitro studies indicate that these two domains play important roles in the utilization of fatty acids as a sole carbon source in yeast cells. This conclusion is further supported by CD far UV spectroscopy of CtMFE-2(h2Δ) and its variants, which show that the composition of secondary structural elements are not affected. Conceivably, the differences in the kinetic data are due to the substitutions affecting catalytic sites of each domain rather than overall folding of the proteins.

It is also worth noting that the amino acid sequence of domain A of yeast MFE-2 is more homologous to that of HsMFE-2 (50% identity) than to domain B, suggesting that the dehydrogenase domain of HsMFE-2 may have evolved from the yeast dehydrogenase domain A.

It was previously suggested that gene duplication has led to the evolution of separate enzymatic activities, such as mammalian acyl-CoA dehydrogenases, which are presented as several paralogs (Tanaka et al. 1990). An acquisition of two domains with different chain-length specificities within a single polypeptide is a novel strategy for a multifunctional enzyme to overcome the problems related to the metabolism of a large variety of substrates.
7. Conclusions

Cloning of rat 2-enoyl-CoA hydratase 2 reveals that it is an integral domain of a novel mammalian MFE-2. The cDNA of rat MFE-2 has an open reading frame of 2205 bp encoding a 735 amino acid residues with a molecular mass of 79 kDa. Recombinant MFE-2 generated in yeast *P. pastoris* has 2-enoyl-CoA hydratase 2 and (3R)-hydroxyacyl-CoA dehydrogenase activities. The enzyme catalyzes reactions of several different substrates, including straight-chain (2E)-enoyl-CoA, branched 2-methyltetradecenoyl-CoA, pristenoyle-CoA esters and steroids. It was shown that in addition to the earlier described MFE-1, a novel MFE-2 exists in rat liver. Both MFE-1 and MFE-2 catalyze the sequential hydratase and dehydrogenase reactions of β-oxidation but through reciprocal stereochemical courses.

Recombinant hydratase 2 derived from rat MFE-2 was overexpressed in *E. coli* and the purified enzyme was found to hydrate (24E)-3α, 7α, 12α-trihydroxy-5β-cholest-24-enoyl-CoA to (24R, 25R)- 3α, 7α, 12α, 24-tetrahydroxy-5β-cholestanoyl-CoA, which is a physiological intermediate in bile acid synthesis. The result reveals that MFE-2, instead of MFE-1, is involved in the synthesis of bile acid.

Several amino acid residues in human MFE-2 were shown, by complementation in *vivo*, to be involved either in catalysis reactions of the hydratase by the hydratase domain or to influence folding of the protein. Based on the enzymatic properties, Glu366 and Asp510 were identified to be important amino acid residues for the catalysis reaction of hydratase 2 in *vivo*. The pH-dependence curve of $k_{cat}/K_m$ for the purified recombinant human hydratase 2 is bell-shaped, suggesting that the reaction utilize two protic amino acid residues.

Analysis of the two catalytic (3R)-hydroxyacyl-CoA dehydrogenase domains of yeast peroxisomal MFE-2 reveals that one of the domains is more active on medium- and long-chain substrates while the other domain is responsible for metabolism of the short-chain substrates. The results suggest that both domains are definitely required for propagation of yeast cells in an optimal manner on fatty acids as the carbon source.
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