Δ3,5-Δ2,4-Dienoyl-CoA Isomerase from Rat Liver

MOLECULAR CHARACTERIZATION*

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rECH1, a recently identified rat cDNA (FitzPatrick, D. R., Germain-Lee, E., and Valle, D. (1995) Genomics 27, 457–466) encodes a polypeptide belonging to the hydratase/isomerase superfamily. We modeled the structure of rECH1 based on rat mitochondrial 2-enoyl-CoA hydratase 1. The model predicts that rECH1p has the hydratase fold in the core domain and two domains for interaction with other subunits. When we incubated 3,5,8,11,14-eicosaapentanoyl-CoA with purified rECH1p, the spectral data suggested a switching of the double bonds from the Δ3-ΔΔ to the Δ3-ΔΔ positions. This was confirmed by demonstrating that the product was a valid substrate for 2,4-dienoyl-CoA reductase. These results indicate that rECH1p is Δ3,5-Δ2,4-dienoyl-CoA isomerase. Subcellular fractionation and immunoelectron microscopy using antibodies to a synthetic polypeptide derived from the C terminus of rECH1p showed that rECH1p is located in the matrix of both mitochondria and peroxisomes in rat liver. Consistent with these observations, the 36,000-Da rECH1p has a potential N-terminal mitochondrial targeting signal as well as a C-terminal peroxisomal targeting signal type 1. Transport of the protein into the mitochondria with cleavage of the targeting signal results in a mature mitochondrial form with a molecular mass of 32,000 Da; transport to peroxisomes yields a protein of 36,000 Da.

An increasing body of evidence indicates that the metabolism of unsaturated fatty acids can occur in both mitochondria and in peroxisomes (1). Unsaturated fatty acids, which have only a few double bonds and not very long carbon chains, are effectively degraded by mitochondrial β-oxidation. Conversely, very long chain (poly)unsaturated fatty acids and their derivatives, some of which are inhibitors of mitochondrial β-oxidation, undergo rapid chain shortening in mammalian peroxisomes with the probable exception of fatty enoyl-CoA esters that have Δ5 double bonds (2).

Since trans-2-enoyl-CoA is the only unsaturated interme-

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1 The abbreviations used are: MFE1, multifunctional hydratase/dehydrogenase enzyme type 1; rECH1, rat enoyl-CoA hydratase; rECH1p, rECH1 protein; PBS, phosphate-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid.
formed using information from multiple sequence alignments and secondary structural information from the three-dimensional structures. The model was built with the program Insight II (Biosym Technologies, Inc., San Diego, CA). A side chain rotamer library was used to model amino acid substitutions. Deletions and insertions were modeled by search of the Protein Data Bank (PDB). The model was energy minimized with the program Discover using Amber force field in a step-wise manner. First, hydrogen atoms were relaxed, then the borders of insertions and deletions were harmonically restrained, and the rest of the molecule was fixed. In the next step, the borders of insertions and deletions and the Cα atoms of the conserved regions and finally only the Cα atoms of the conserved regions were harmonically constrained.

**Expression of Truncated rECH1 in E. coli**—We utilized rECH1 cDNA in Bluescript KS+ (15) as a template for polymerase chain reaction amplification. The forward primer (5’-GACATATG- GCATAGGAGTCTATACGATTACGTCG) corresponds to nucleotides 170–196 with an additional 11 5’ nucleotides providing an NdeI restriction site, an ATG initiation methionine codon, and a GCA (ala-nine) codon. 3’ of this, the sequence continues with TAC encoding a glycine residue. The protein was expressed in the E. coli BL21 (DE3) (Novagen, Madison, WI) according to manufacturer instructions, except that the tissues were infiltrated from 70% ethanol instead of completion of the dehydration protocol. The resin was washed in 500-μl fractions obtained were evaluated on the basis of three criteria: root mean square deviation from the anchor points, sequence similarity, and interference with the core region. There were three insertions and no deletions with the base of chromatographic homology. Two rounds of energy minimization were performed for fragments of the required length and end point separation by using two residues at each end of the loop as anchor points. The fragments obtained were evaluated on the basis of three criteria: root mean square deviation from the anchor points, sequence similarity, and interference with the core region. There were three insertions and no deletions with the base of chromatographic homology. Two rounds of energy minimization were performed for fragments of the required length and end point separation by using two residues at each end of the loop as anchor points. The fragments obtained were evaluated on the basis of three criteria: root mean square deviation from the anchor points, sequence similarity, and interference with the core region. There were three insertions and no deletions with the base of chromatographic homology. 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at Gly-30) (39) shows 48 additional N-terminal amino acid residues, three insertions, and three amino acid residues in the C terminus (SKL). The C-terminal extension could not be modeled. Regions containing a single amino acid residue insertion appear in the beginning and in the end of the trimerization domain 1, but the insertions do not affect the general scaffolding of the protein. There is a large insertion in the helical portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2). This section was modeled as an extended portion of the second part of the hydratase spiral (Fig. 2).
of 2,5,8,11,14-eicosapentenoyl-CoA, isomerization results in the formation of a conjugated Δ⁵-Δ⁶ double bond, which is known to absorb at 240 nm (13, 14). The addition of rECH1p into this reaction mixture caused a rapid decrease of the absorbance at 240 nm and a concomitant increase of the absorbance at 300 nm. This absorption spectrum is typical of Δ²-Δ⁴-enoyl-CoA esters (35, 42), which suggests that the conjugated Δ⁵-Δ⁶ double bond has been transferred to the Δ²-Δ⁴ position of
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strate. When we incubated rECH1p with 60 mM 3,5,8,11,14-eicosapentenoyl-CoA as a substrate for 2,4-dienoyl-CoA reductase was produced only in the initial incubation with arachidonoyl-CoA. These results show that rECH1p transfers the conjugated double bonds from trans-3-enoyl-CoA to trans-2-enoyl-CoA isomerase.

In a separate experiment, the Δ3,5-Δ2,4-dienoyl-CoA isomerase activity of rECH1p was 0.12 μmol/min × mg of protein−1 when using 60 μM 3,5,8,11,14-eicosapentenoyl-CoA as a substrate. When we incubated rECH1p with 60 μM trans-2-decenoyl-CoA, 1,3-hydroxydecanoyl-CoA was generated at the rate of 5 nmol/min × mg of protein−1, indicating that rECH1p also had a low 2-enoyl-CoA hydratase 1 (enoyl-CoA hydratase) activity. However, the Δ3-Δ2-enoyl-CoA isomerase (2-enoyl-CoA hydratase 2) activity with trans-3-decenoyl-CoA (trans-2-decenoyl-CoA) was below the detection limits of the assay systems used (<1 nmol/min × mg of protein−1).

Subcellular Localization—Two bands corresponding to molecular masses of 36,000 and 32,000 Da were detected by immunoblot analysis with anti-rECH1p of rat liver homogenate subjected to SDS-polyacrylamide gel electrophoresis (Fig. 5). When purified peroxisomes and mitochondria were immunoblotted, the 36,000-Da protein was associated with peroxisomes, whereas the 32,000-Da protein was enriched in the mitochondrial preparation. These data suggest that rECH1p in rat liver has dual subcellular location. To study this further, we performed immunoelectron microscopy utilizing the protein A-collloid gold-labeling technique. Gold particles localized the rECH1p to peroxisomes and mitochondria in both control and clofibrate-treated rats. To determine the relative amount of antigen in both organelles, we calculated the gold/area ratio (number of gold particles/μm², mean ± S.D. in electron microscopy frames measured) for both organelles. The area outside the organelles was taken as background. In control (clofibrate-treated) rats, the labeling density was 4.17 ± 1.47 (31.2 ± 13.0; p < 0.003 control versus clofibrate-treated) in mitochondria, 7.0 ± 4.29 (25.7 ± 9.2; p < 0.006) in peroxisomes, and 1.75 ± 0.96 (0.75 ± 0.96; difference not significant) in background (Fig. 6).

As controls, we used antibodies against peroxisomal MFE1 (peroxisomes) and short-chain Δ3-Δ2-enoyl-CoA isomerase (mitochondria). In both cases, the antibodies recognized the ex-
Sapentenoyl-CoA to the reductase. Identification of rECH1p as a the near UV region and by demonstrating that the product of controls. These observations agree with previous results (29, 43).

incorporated in clofibrate-treated animals as compared with controls. These observations agree with previous results (29, 43).

Published studies on the distribution of the Δ^{3,5,4}-dienoyl-CoA isomerase in the rat including subcellular fractionation, immunoblotting, immunoelectron microscopy, enzyme activity measurements, and protein purification provide evidence that the dienoyl-CoA isomerase activity in liver is associated with both peroxisomes and mitochondria (11–14, 44). However, the amino acid sequences or molecular characteristics of the enzyme(s) are not known. The results of our present experiments indicate unambiguously that rECH1 encodes dienoyl-CoA isomerase. Overexpression of rECH1 in E. coli gives an enzymatically active protein, and purified recombinant protein transfers the conjugated Δ^{1,5,5} double bond of 3,5,8,11,14-eicosenoyl-CoA to the Δ^{5,5} position. We verified this reaction spectrophotometrically by following the changes of absorbance in the near UV region and by demonstrating that the product of the double-bond transfer was a substrate for 2,4-dienoyl-CoA reductase. Identification of rECH1p as a Δ^{3,5,4}-dienoyl-CoA isomerase adds a new activity among the reactions catalyzed by the members of the hydratase/isomerase family.

At present, the only members of the hydratase/isomerase enzyme family with known structures are 2-enoyl-CoA hydratase 1 (25) and 4-chlorobenzoyl dehalogenase (26). The modeled subunit of rECH1 shows the presence of both trimerization domains similar to hydratase 1 and 4-chlorobenzoyl dehalogenase, suggesting that the enzyme forms a trimer. In line with this proposal, size exclusion chromatography gave a molecular mass of 170,000 Da. This indicates that in the native state rECH1p may be a hexamer similar to the rat mitochondrial 2-enoyl-CoA hydratase 1, which is a dimer of two trimers (25).

Prediction of the secondary structure elements of the rECH1p indicates the presence of the hydratase spiral (25) forming the CoA binding site and the pocket where catalysis takes place. It is also logical to assume that the basic folding of rECH1 would be similar to the other members of the same family. Interestingly, in the hydratase 1, E144 (25), which acts as the catalytic base for activation of a water molecule in the hydratase, is replaced with Asp-127 in rECH1.

The origin of the two immunodetected bands of molecular masses of 36,000 and 32,000 Da in the liver homogenates is intriguing. A logical explanation is provided by the results of immunoblot experiments with peroxisomes and mitochondria from rat liver. Because the band of 36,000 Da was detected in isolated peroxisomes and rECH1p has the known peroxisomal targeting signal type 1 (SKL) in its C terminus, it is possible that the product encoded by the entire open reading frame is targeted to the peroxisomal matrix and remains uncleaved. However, the first 40 amino acid residues of rECH1p resemble a mitochondrial matrix targeting signal as shown by Mitoprot II analysis (45). Thus, the band of 32,000 Da detected in tissue homogenates and in isolated mitochondria likely represents a processed mitochondrial isofrom. The size difference between the 36,000 and 32,000 Da bands is consistent with the length of the predicted mitochondrial targeting signal. Thus, rECH1p is a new example of dual-organelle distribution of the same protein in mammalian cells. The immunoelectron microscopy of liver sections also provides further support for the dual distribution of rECH1p. An earlier example of this kind of intracellular targeting is the mammalian hydroxymethylglutaryl-CoA lyase (46), which also has a single mRNA translated into a polypeptide with both targeting signals.

As a consequence, in mammalian tissues, a larger peroxisomal and a smaller mitochondrial hydroxymethylglutaryl-CoA lyase isoform is found. Similarly, in some species like rat and mouse, alanine:glyoxylate aminotransferase is located both in mitochondria and peroxisomes, whereas in humans it is mostly peroxisomal. In some patients with inherited deficiency of alanine:glyoxylate aminotransferase (primary hyperoxaluria type 1), mutations alter the targeting of the protein so that most of it localizes to mitochondria rather than to peroxisomes (47). Interestingly, expression in Pichia pastoris of full-length rECH1 gave two bands with molecular weights corresponding to those observed in rat liver homogenates (data not shown).

The 32,000-Da size of the form of rECH1p that we localized to mitochondria is consistent with the subunit sizes previously reported for mitochondrial dienoyl-CoA isomerase (13). Furthermore, our observation that mitochondrial and peroxisomal dienoyl-CoA isomerases are actually isoforms of the same protein explains their similar kinetic properties (12). The relationship of rECH1p to the rat liver dienoyl-CoA isomerase activities of 55,000 Da (11) and 66,000 Da (12) is uncertain.

Recently Tserng et al. (48) carried out experiments applying a stable isotope-labeled substrate technique to assess the contribution of dienoyl-CoA isomerase/dienoyl-CoA reductase-dependent pathway versus direct β-oxidation of cis-5-enoyl-CoA esters. C_{10} substrate was metabolized completely via dienoyl-CoA isomerase-reductase pathway in both rat liver and heart mitochondria. When the chain length of the substrate was extended to C_{14}, the isomerase/reductase pathway remained the major metabolic route, although its overall contribution to the metabolism decreased to 86 and 65% in liver and heart mitochondria, respectively. This experiment verified the role of isomerase/reductase pathway in rat mitochondria, and the authors also suggest that 2,4-dienoyl-CoA reductase is the rate-limiting enzyme in this pathway.

Thus far only one inborn error of the auxiliary enzymes of β-oxidation has been identified in humans, namely a single...
patient with 2,4-dienoyl-CoA reductase deficiency (49). This paucity of clinical material might simply reflect the fact that until recently this enzyme system has been poorly appreciated, or it may be that affected individuals are not recognized.

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