Phytanoyl-CoA hydroxylase from rat liver: protein purification and cDNA cloning with implications for the subcellular localization of phytanic acid α-oxidation

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Abstract **Phytanoyl-CoA hydroxylase (PhyH) catalyzes the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA, which is the first step in the phytanic acid α-oxidation pathway. Recently, several studies have shown that in humans, phytanic acid α-oxidation is localized in peroxisomes. In rat, however, the α-oxidation pathway has been reported to be mitochondrial. In order to clarify this differential subcellular distribution, we have studied the rat PhyH protein. We have purified PhyH from rat liver to apparent homogeneity as judged by SDS-PAGE. Sequence analysis of two PhyH peptide fragments allowed cloning of the rat *PHYH* cDNA encoding a 38.6 kDa protein. The deduced amino acid sequence revealed strong homology to human PhyH including the presence of a peroxisome targeting signal type 2 (PTS2). Heterologous expression of rat *PHYH* in *Saccharomyces cerevisiae* yielded a 38.6 kDa protein whereas the PhyH purified from rat liver had a molecular mass of 35 kDa. This indicates that PhyH is probably processed in rat by proteolytic removal of a leader sequence containing the PTS2. This type of processing has been reported in several other peroxisomal proteins that contain a PTS2. Subcellular localization studies using equilibrium density centrifugation showed that PhyH is indeed a peroxisomal protein in rat. The finding that PhyH is peroxisomal in both rat and humans provides strong evidence against the concept of a differential subcellular localization of phytanic acid α-oxidation in rat and human.**—Jansen, G. A., R. Ofman, S. Denis, S. Ferdinandusse, E. M. Hogenhout, C. Jakobs, and R. J. A. Wanders. **Phytanoyl-CoA hydroxylase from rat liver: protein purification and cDNA cloning with implications for the subcellular localization of phytanic acid α-oxidation.** ***J. Lipid Res.*** **1999.** 40: **2244–2254.**

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Fatty acids containing a methyl group at the 3-position cannot be β-oxidized directly but first require the oxidative removal of the terminal carboxyl-group in a process called α-oxidation. The product of this pathway is a 2-methyl fatty acid which then can undergo β-oxidation.

Most studies on the mechanism of α-oxidation have been performed with phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) as this fatty acid accumulates in patients suffering from Refsum's disease (RD), a rare inborn error of metabolism. For a long time it was thought that α-oxidation of phytanic acid involved the free fatty acid and not the CoA-ester. Studies by Watkins, Howard, and Mihalik (1), however, revealed that phytanoyl-CoA is the true substrate for α-oxidation. Mihalik, Rainville, and Watkins (2) showed that the first step in the α-oxidation process, the conversion of phytanoyl-CoA into 2-hydroxyphytanoyl-CoA is catalyzed by phytanoyl-CoA hydroxylase (PhyH). This enzyme is a 2-oxoglutarate-dependent dioxygenase which requires Fe^{2+} and ascorbate as cofactors (Scheme 1). In humans, PhyH is localized in peroxisomes (3) and is directed to these organelles by means of a peroxisome targeting signal type 2 (PTS2) (4). We have found that PhyH is deficient in patients with RD (5) due to mutations in the corresponding gene (3).

The product of the hydroxylation reaction catalyzed by PhyH, 2-hydroxyphytanoyl-CoA, undergoes cleavage to yield pristanal and formyl-CoA (6–8). Pristanal is converted to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which after activation to its CoA-ester can undergo β-oxidation in peroxisomes (9, 10).

Although the individual enzymatic steps of the α-oxidation pathway have recently been resolved, there is still considerable debate about the subcellular localization

Abbreviations: PhyH, phytanoyl-CoA hydroxylase (phytanoyl-CoA, 2-oxoglutarate dioxygenase); *PHYH*, phytanoyl-CoA hydroxylase cDNA sequence; PTS, peroxisome targeting signal; RD, Refsum's disease; AGT, alanine glyoxylate aminotransferase.

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of phytanic acid α-oxidation. According to some authors, phytanic acid α-oxidation is mitochondrial (9, 11–15), whereas others reported a peroxisomal (16–18) or microsomal localization (19, 20). Moreover, involvement of cytosolic components has also been suggested (21).

Singh and coworkers (22) reported that α-oxidation is peroxisomal in humans and mitochondrial in rat. Such a differential localization of an enzyme between species is not unprecedented. One example is alanine glyoxylate aminotransferase (AGT) (23). In humans, baboons, macaques, rabbits, and guinea pigs, AGT is solely peroxisomal. In rats, marmosets, mice, hamsters, and opossums, AGT is approximately evenly distributed between peroxisomes and mitochondria, whereas in cats and dogs a primarily mitochondrial localization is observed (see ref. 23 for review).

As different targeting signals are required for directing proteins to organelles such as mitochondria and peroxisomes, one would expect the presence of a mitochondrial targeting signal in rat PhyH. In order to resolve this issue, we have purified PhyH from rat liver and cloned the *PHYH* cDNA. Expression of rat PhyH in *S. cerevisiae* resulted in high PhyH activity. The deduced amino acid sequence revealed strong homology with human PhyH including the presence of a PTS2 sequence. These findings strongly suggested that in rat PhyH is also localized in peroxisomes. Indeed, subsequent equilibrium density centrifugation experiments provided conclusive evidence for the peroxisomal localization of PhyH in rat.

MATERIALS AND METHODS

Materials

[1-^{14}C]2-oxoglutarate (50 Ci·mol^{-1}) was purchased from DuPont NEN, 's-Hertogenbosch, The Netherlands. Prior to use it was diluted with unlabeled 2-oxoglutarate to obtain a solution of 1 mm, 4.5 Ci·mol^{-1}. Nycodenz was from Nycomed Pharma AS, Oslo, Norway. Octyl Sepharose CL-4B, S-Sepharose FF, phenyl Sepharose HP, PBE 94, and Polybuffer 96 were from Pharmacia Biotech Benelux, Roosendaal, The Netherlands. The 5 ml CHT-II Econo-pac cartridge and goat anti-rabbit IgG coupled to alkaline phosphatase were from Bio-Rad Laboratories, Veenendaal, The Netherlands. Pefabloc was from Boehringer Mannheim NL, Almere, The Netherlands. Freund's complete adjuvant and Freund's incomplete adjuvant were from Gibco-Life Technologies, Breda, The Netherlands.

All other reagents were of analytical grade.

Phytanoyl-CoA synthesis

Phytanoyl-CoA was synthesized from phytanic acid as described (24). [1-^{14}C]phytanoyl-CoA was synthesized enzymatically from [1-^{14}C]phytanic acid as previously described (25).

PhyH activity measurement

PhyH activity measurements were performed using two different methods. Method 1: [1-^{14}C]phytanoyl-CoA was used as substrate and the amount of [1-^{14}C]2-hydroxyphytanoyl-CoA produced was determined using a radiochemical HPLC method as described elsewhere (25).

Method 2: PhyH activity measurement was based on the production of $^{14}CO_2$ from [1-^{14}C]2-oxoglutarate, which acts as cosubstrate in the enzymatic conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA (Scheme 1). After optimizing the assay for partially purified subcellular fractions (see Results section), PhyH activity measurements were performed as follows. A (partially) purified protein preparation was added to a reaction mixture containing 50 mm Tris-HCl, 0.2 mm dithiothreitol, 1.5 μm BSA, 10 mm ATP, 25 μm phytanoyl-CoA, 1 mm ascorbate, 1 mm ammonium iron[II]sulfate, 0.1 mm [1-^{14}C]2-oxoglutarate (4.5 Ci·mol^{-1}), final pH 7.5, total reaction volume 250 μl. The small glass reaction tubes, together with an 1.5-ml Eppendorf reaction tube containing 0.5 ml 2 m NaOH, were placed in a 20-ml glass vial which was closed air tight with a screw-cap equipped with a septum, and incubated at 37°C for 1 h. The reactions were terminated by adding 100 μl 1.3 m perchloric acid through the septum using a Hamilton syringe. $^{14}CO_2$ was trapped overnight at 4°C in the NaOH solution which was analyzed by liquid scintillation spectrometry.

Purification of PhyH from rat liver

All PhyH activity measurements were carried out according to method 2 as described above.

Step 1: isolation of peroxisomes from rat liver. Peroxisomes were isolated from 8 male Wistar rats that were fed a standard laboratory diet supplemented with 1.0% (w/w) diethylhexylphthalate (DEHP) for 9 days. Livers were minced and homogenized in an ice-cold medium containing 250 mm sucrose, 5 mm MOPS, 2.5 mm EDTA, pH 7.4. This homogenate was used to prepare a purified peroxisomal fraction using discontinuous Nycodenz centrifugation essentially as described previously (26) with slight modifications (27).

Step 2: octyl Sepharose CL-4B hydrophobic interaction chromatography. The purified peroxisomes obtained in step 1 were applied onto an octyl Sepharose CL-4B column (2.6 × 7 cm) equilibrated with a buffer containing 0.2% CHAPS, 5 mm dithiothreitol, 20 mm potassium phosphate, 1 m KCl, pH 7.4, at a flow rate of 0.8 ml·min^{-1}. The column was subsequently washed with the same buffer to obtain 1100 ml eluent containing unabsorbed proteins. PhyH activity measurement revealed that all activity was present in this wash-fraction. This fraction was concentrated using an Amicon YM10 cut-off filter (Millipore NL, Etten-Leur, The Netherlands) to obtain a volume of 400 ml, and dialyzed against 2 times 5 liters buffer solution containing 20 mm Tris-HCl plus 10% (v/v) glycerol, pH 7.6. The final volume was 510 ml.

Step 3: S-Sepharose FF cation exchange chromatography. Five hundred ten ml of the dialyzed fraction containing the unbound proteins obtained in step 2 was applied onto an S-Sepharose FF column (2.6 × 10 cm) equilibrated with a buffer containing 20 mm Tris-HCl, 10% (v/v) glycerol, 50 mm potassium chloride, pH 7.6. Absorbed proteins were eluted with a linear gradient of potassium chloride (50–250 mm) at a flow rate of 2.5 ml·min^{-1}. Fractions of 10 ml were collected and assayed for PhyH activity.

Step 4: hydroxy apatite chromatography. Fractions 18–22 of step 3 were pooled, diluted with 125 ml 10 mm sodium phosphate, 10% (v/v) glycerol, 5 mm dithiothreitol, pH 7.4, and loaded on top of a hydroxyapatite column equilibrated with a buffer containing 50 mm NaP_i, 10% (v/v) glycerol, 5 mm dithiothreitol, pH 7.4, and eluted with a linear sodium phosphate gradient (50–200 mM) at a flow rate of 0.75 ml·min^{-1}. Fractions of 3 ml were collected and assayed for PhyH activity.

Step 5: phenyl Sepharose HP hydrophobic interaction chromatography. Fractions 6–7 of step 4 were pooled and ammonium phosphate was added to a final concentration of 0.8 m, and applied to a phenyl Sepharose HP column (1.0 × 2.8 cm) equilibrated with a buffer containing 0.8 m ammonium phosphate, 10% (v/v) glycerol, 5 mm dithiothreitol, pH 7.6. Elution was carried out by a linear decrease of the ammonium phosphate concentration from 0.8–0.1 m at a flow rate of 0.85 ml·min^{-1}. Fractions of 1.7 ml were collected.

PBE 94 chromatofocusing

The pI of PhyH was determined by chromatofocusing using a 2 ml PBE 94 (1.0 × 5.0 cm) column, equilibrated with 25 mm 2-amino ethanol–acetate, 10% (v/v) glycerol, pH 9.4. One ml of the dialyzed fraction obtained in step 2 of the PhyH purification was applied onto the column, followed by 1 ml of 25 mm 2-amino ethanol–acetate, 10% (v/v) glycerol, pH 9.4. Elution was carried out using Polybuffer 96–acetate, 10% (v/v) glycerol, pH 6.0. Immediately after collection of each 1 ml fraction, the pH was determined and subsequently neutralized using 0.2 ml 0.5 m Tris-HCl, pH 7.5. PhyH activity was determined using method 2 as described above.

Protein sequencing

After purification of PhyH from rat liver, an aliquot of the protein solution was digested with Lys-C to obtain peptide fragments. The purified PhyH as well as the Lys-C digest were subjected to SDS-PAGE and blotted onto an Immobilon-P membrane (Millipore NL, Etten-Leur, The Netherlands) using a buffer containing 40 mm Tris-HCl, 40 mm boric acid, 20% (v/v) methanol, 0.1 $g \cdot L^{-1}$ (w/v) SDS, pH 8.6, for 1 h at 1.5 $mA \cdot cm^{-2}$. The immobilized polypeptides were visualized using Coomassie Blue R250 and subsequently subjected to N-terminal amino acid sequencing using a Procise 494 protein sequencer.

Cloning of rat *PHYH*

Using first strand cDNA from rat liver as template, the 5′-half of the rat *PHYH* cDNA was amplified by PCR using '-21M13 forward'- and 'M13 reverse'-tagged primers mPH-27f (sense 5′-TGT AAA ACG ACG GCC AGT GCA GAG TTT CGT CTG CTG GC-3′) and mPH643r (antisense 5′-CAG GAA ACA GCT ATG ACC CTT TGT GGG TAC CTG GAA GC-3′), based on the murine *PHYH* sequence (GenBank accession number AF023463). Subsequent sequence analysis of this fragment of both sense and antisense strands was performed using '-21M13 forward'- and 'M13 reverse' fluorescent primers, respectively, on an Applied Biosystems 377A automated DNA sequencer following the manufacturer's protocols. The 3′-end of the rat *PHYH* sequence was obtained using the Marathon cDNA 3′-RACE kit (Clontech Laboratories GmbH, Heidelberg, Germany). The '-21M13 forward'-tagged gene specific primers PH557f (sense 5′-TGT AAA ACG ACG GCC AGT GCA ACC TAA TTG TTT GTG C-3′) and PH649f (sense 5′-TGT AAA ACG ACG GCC AGT CAG ACT ATC ATC CCA GTA CC-3′) were used and the obtained fragment was subsequently sequenced.

Heterologous expression in *S. cerevisiae*

The complete coding sequence of rat *PHYH* was amplified from rat liver cDNA using *Xba*I and *Hind*III tagged primers (5′-AAA TCT AGA AAA ATG ACT ATA CGG CGC GC-3′ and 5′-AAA AAG CTT TCA AAG GTT TAT TCT TTC TCC-3′, respectively) and cloned into the plasmid pGEM-T (Promega, Leiden, The Netherlands) to obtain pPHYHr. The *PHYH* sequence was subsequently verified by sequence analysis using T7 and SP6 primers. The obtained *PHYH* clone was digested with *Xba*I and *Hind*III and subcloned into the *Xba*I and *Hind*III sites of the yeast expression plasmids pEL26 (high copy plasmid, containing 2 μ sequence) and pEL30 (low copy plasmid, containing CEN-sequence) (28), in both plasmids under the transcriptional control of the oleate inducible promoter p*CTA1*. The expression constructs and, as a control, the vectors pEL26 and pEL30 were transformed into *S. cerevisiae* strain BJ1991 (*MATα, leu2, trp1, ura3–52, prb1–1122, pep4–3*) (29). Transformants were grown in minimal essential medium containing 3 $g \cdot L^{-1}$ glucose and 6.7 $g \cdot L^{-1}$ yeast nitrogen base without amino acids (Difco Laboratories, Detroit MI) supplemented with appropriate amino acids at 30°C. In order to induce expression, cells were harvested by centrifugation and transferred into a medium containing 1.2 $g \cdot L^{-1}$ oleic acid, 1.2 $g \cdot L^{-1}$ Tween-40, 5 $g \cdot L^{-1}$ potassium phosphate (pH 6.0), 3 $g \cdot L^{-1}$ yeast extract, and 5 $g \cdot L^{-1}$ peptone. Cells were grown at 30°C and harvested by centrifugation after the culture reached a spectrophotometrical absorbance at 600 nm of about 0.5. The cells were resuspended in 250 μl buffer containing 20 mm Tris-HCl, pH 7.5, 5 mm dithiothreitol, 1 $\mu g \cdot ml^{-1}$ leupeptin, 2 $mg \cdot ml^{-1}$ Pefabloc and 10% (v/v) glycerol. After addition of 200 μl glass beads, the suspension was vortexed for 30 min at 4°C, centrifuged for 2 min at 12,000 *g* at 4°C, and the clear lysate containing rat PhyH was taken for PhyH activity measurements and further experiments.

Generation of anti-PhyH antiserum

Female New Zealand white rabbits were primed with 20 μg purified rat PhyH in 0.8 ml PBS mixed with an equal volume of Freund's complete adjuvant. After 2 weeks the immunization was followed by a boost injection of 20 μg of PhyH mixed with Freund's incomplete adjuvant. Two more boost injections were given at 4-week intervals. Eight days after each boost injection a 10-ml blood sample was taken, and serum was prepared.

SDS-PAGE and immunoblot analysis

Samples were subjected to SDS-PAGE on a 10% polyacrylamide gel and blotted onto a nitrocellulose filter. Nonspecific binding sites were blocked for 1 h using a PBS solution containing 1 $g \cdot L^{-1}$ Tween-20 (PBST), supplemented with 30 $g \cdot L^{-1}$ nonfat dried milk (NFDM). Primary (polyclonal anti-PhyH) and secondary (goat anti-rabbit IgG coupled to alkaline phosphatase) antibody incubations were performed in PBST. After each incubation, the blots were washed extensively in 3 $g \cdot L^{-1}$ NFDM/PBST. Antigen–antibody complexes were visualized using alkaline phosphatase staining in a buffer containing 0.1 m Tris-HCl (pH 9.5), 0.1 m NaCl, 5 mm $MgCl_2$, 0.33 $g \cdot L^{-1}$ 4-nitro blue tetrazolium chloride, 0.17 $g \cdot L^{-1}$ 5-bromo-4-chloro-3-indolyl-phosphate (disodium salt).

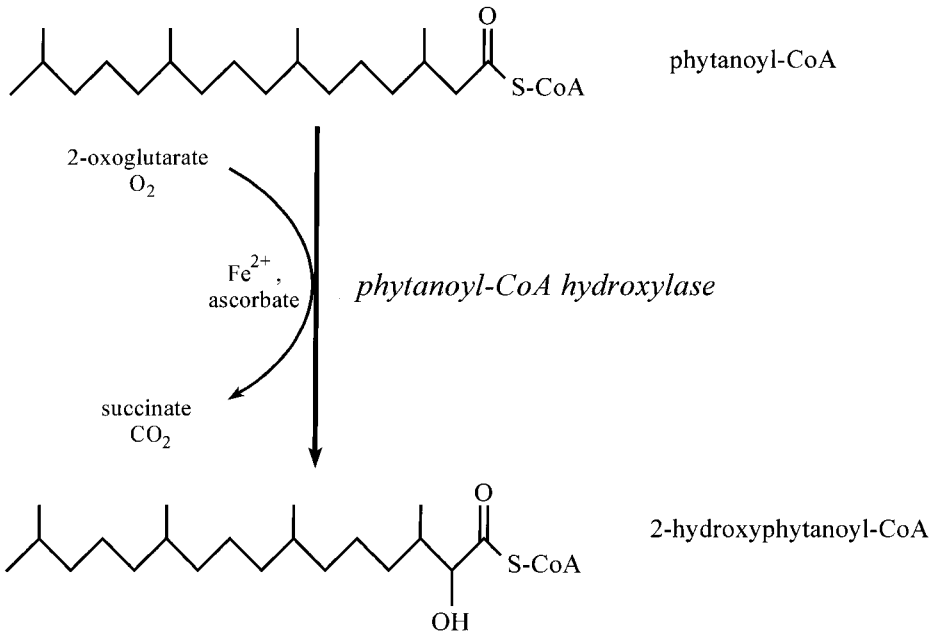
Subcellular fractionation of rat liver

For subcellular fractionation studies, fresh rat livers were minced, washed several times in ice-cold phosphate-buffered saline, and homogenized in a buffer containing 250 mm sucrose, 5 mm MOPS, and 0.1 mm EGTA, final pH 7.4. Homogenates were centrifuged 10 min at 600 *g* at 4°C to produce a postnuclear supernatant. This supernatant was subjected to differential centrifugation exactly as described before (30) to produce a light mitochondrial fraction. Subfractionation of this light mitochondrial fraction using equilibrium density gradient centrifugation was performed as described before (31). Catalase (peroxisomes) (32), glutamate dehydrogenase (mitochondria) (33), esterase (microsomes) (30), and lactate dehydrogenase (34) (cytosol) were used as marker enzymes, and were measured as described. Aliquots of the subcellular fractions were taken, protein was precipitated using 100 $g \cdot L^{-1}$ trichloroacetic acid and dissolved in Tris buffer, followed by SDS-PAGE/immunoblot analysis.

RESULTS

PhyH activity measurement using [1-^{14}C]2-oxoglutarate

In previous studies, measurement of PhyH activity was based on the conversion of radiolabeled phytanoyl-CoA into 2-hydroxyphytanoyl-CoA, using a radiochemical HPLC method allowing the detection of these CoA-esters (25).



Scheme 1. Phytanoyl-CoA hydroxylase catalyzes the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA. This enzyme is a 2-oxoglutarate-dependent dioxygenase which requires Fe^{2+} and ascorbate as cofactors.

As this assay is very time consuming and therefore not convenient for measurement of large series of samples, we have developed an assay based on the conversion of [1-^{14}C]2-oxoglutarate into succinate and $^{14}CO_2$. This conversion takes place stoichiometrically with the hydroxylation reaction (**Scheme 1**), and is carried out analogous to assay systems for other 2-oxoglutarate-dependent dioxygenases (35–37). PhyH activity measurements in crude liver homogenates, using this assay, showed a very high rate of $^{14}CO_2$ production, which was independent on the presence of phytanoyl-CoA, Fe^{2+}, or ascorbate, the substrate and obligatory cofactors of PhyH. Cell fractionation studies revealed that this $^{14}CO_2$ production occurred mainly in the mitochondrial fraction, and it is likely that decarboxylation of 2-oxoglutarate is caused by the α-ketoglutarate dehydrogenase complex. When purified peroxisomes were used for the assay, production of $^{14}CO_2$ was completely dependent on the presence of phytanoyl-CoA and Fe^{2+}, whereas omission of ascorbate decreased the production of $^{14}CO_2$ to about one-third of the activity observed in the presence of 1 mm ascorbate (**Table 1**). The optimal pH for PhyH activity was 7.5 and the reaction was linear with time for 1 h and linear with protein up to at least 0.5 mg·ml^{-1} (data not shown).

When this $^{14}CO_2$ release assay was compared to the assay based on the measurement of the product of the hydroxylation reaction, [1-^{14}C]2-hydroxyphytanoyl-CoA, similar activities were found, and cosubstrate and cofactor requirements were essentially the same (Table 1).

Purification of PhyH from rat liver

Stability of PhyH activity during the purification. Enzymatic activity of the PhyH protein in crude or partially purified fractions (liver homogenate or purified peroxisomes) was stable upon storage at 4°C. However, in more purified fractions, PhyH activity rapidly declined when stored at 4°C and even more at −20°C or −80°C, the latter probably also due to freeze-thawing effects on the preparation. Loss of enzymatic activity could only partially be prevented when 10% (v/v) glycerol and 5 mm dithiothreitol were added to all buffers used.

Purification of PhyH from rat liver peroxisomes. When rat liver peroxisomes were subjected to octyl-Sepharose CL-4B chromatography, all PhyH activity was present in the wash fraction (data not shown). S-Sepharose chromatography showed that all PhyH activity was bound to the column and eluted as a single peak upon increasing the concentration of potassium chloride (data not shown). SDS-PAGE

TABLE 1. Cofactor requirements for PhyH activity measurements in purified rat liver peroxisomes according to the newly developed method using $^{14}CO_2$ measurement and the original HPLC based method (see ref. 25)

Component Omitted from the Reaction Medium	PhyH Activity	
	$^{14}CO_2$	2-Hydroxyphytanoyl-CoA
	nmol·h^{-1}·mg^{-1}	
None	12.30	15.40
Phytanoyl-CoA	≤0.05	≤0.05
Fe^{2+}	≤0.05	≤0.05
Ascorbate	4.03	5.92

PhyH activity was measured according to the newly developed method (see Materials and Methods) using $^{14}CO_2$ production as a measure of activity (left column), or the method as previously described (25) using the formation of [1-^{14}C]2-hydroxyphytanoyl-CoA as a measure of PhyH activity (right column). Values represent the mean of two separate experiments.

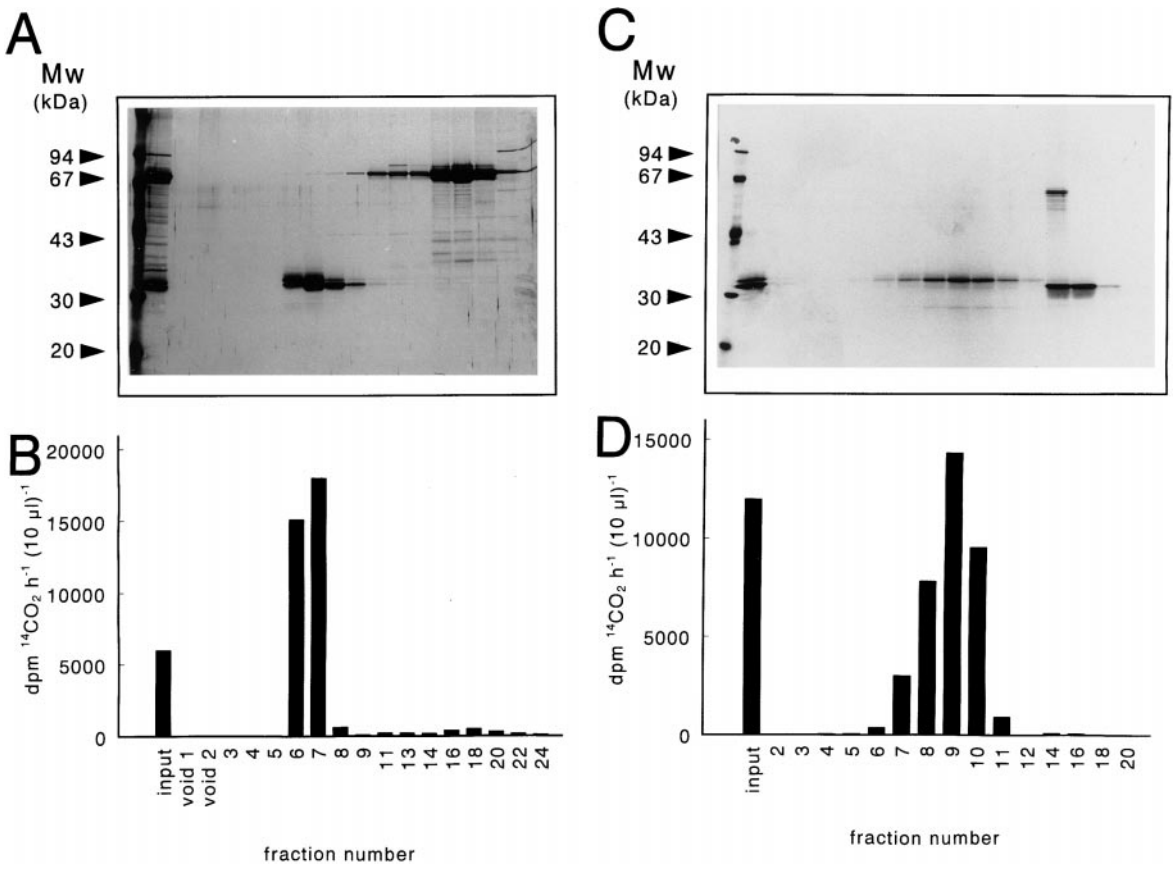


Fig. 1. Purification of PhyH from rat liver: step 4, hydroxy apatite chromatography (panels A, B), and step 5, phenyl Sepharose chromatography (panels C, D). Column fractions were separated by 10% SDS-PAGE followed by silver staining (A, C). PhyH activity measurements in the column fractions (B, D) were performed according to method 2 as described in Materials and Methods.

analysis of the fractions obtained by hydroxyapatite chromatography revealed that one protein band (apparent molecular mass 35 kDa) co-eluted exactly with PhyH activity, while a 33 kDa protein with a slightly different elution pattern was present in the fractions containing most PhyH activity (**Fig. 1A, B**). Using phenyl-Sepharose hydrophobic interaction chromatography, these two proteins could be separated. PhyH activity co-eluted again with the 35 kDa protein (Fig. 1C, D).

Cloning of the rat *PHYH* cDNA

After purification of the PhyH protein from rat liver, both the intact protein as well as a fragment obtained after Lys-C digestion were subjected to N-terminal protein sequencing. The amino-terminus of the purified protein had the following sequence: SGPASPANFXPEQFQYXLDN. This sequence was used to screen the EST database for cDNA clones homologous to this polypeptide, and previously led to the identification of the human *PHYH* cDNA (4). The amino acid sequence from the fragment obtained after Lys-C treatment (MYH(G/Q)IQDYD) showed very high homology to the deduced human PhyH sequence (70% identity, 100% similarity when conservative amino acid substitutions are considered). Using a different strategy, Mihalik and coworkers (38) identified both the human and murine *PHYH* cDNAs.

Searching the EST and genomic databases, no homologous sequences derived from rat tissues were found. In order to identify the rat *PHYH* cDNA sequence, primers based on the murine *PHYH* sequence were used to amplify the 5′-end of the *PHYH* sequence using first strand cDNA from rat liver as template. The 3′-end of the sequence was obtained using the 3′-RACE technique. The coding sequence of rat *PHYH* contained 1014 nucleotides, encoding a 338 amino acid protein (**Fig. 2**). The calculated mass of the rat PhyH was 38.6 kDa. The deduced amino acid sequence did not contain a mitochondrial targeting signal. However, a PTS2 sequence was found near the 5′-end of the open reading frame (Fig. 2). This PTS2 has already been found in both human and murine PhyH, strongly suggesting a peroxisomal localization. The deduced amino acid sequence of PhyH showed very high homology to both human and murine PhyH (**Fig. 3**).

Based on the observation that amino terminal sequencing of purified rat PhyH revealed a sequence identical to amino acids 31–50 rather than 1–20, we have suggested earlier that in vivo amino acids 1–30 are removed, probably by proteolytic cleavage (4). This type of post-translational modification which involves removal of the PTS2 containing leader sequence has been reported for several PTS2-containing peroxisomal proteins, including 3-keto-acyl-CoA thiolase (39, 40) and alkyl-dihydroxyacetonephosphate syn-

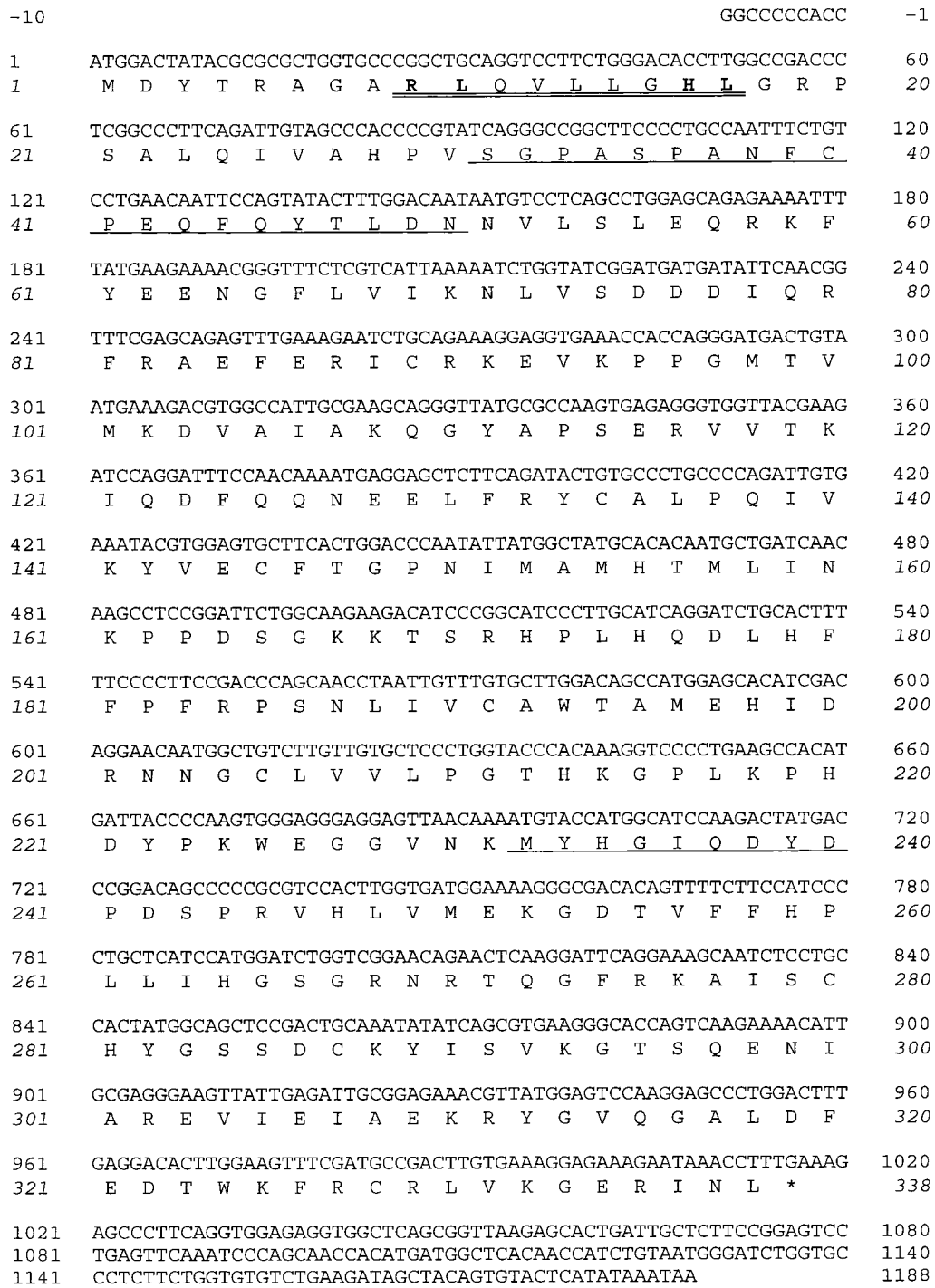


Fig. 2. Rat *PHYH* cDNA sequence and deduced amino acid sequence. The PTS2 sequence at amino acids 9–17 is double underlined. The peptide fragment identified by N-terminal amino acid sequencing of purified PhyH (underlined, at position 31–50) indicates the amino-terminus of the PhyH protein after removal of the PTS2 leader sequence. Amino acid sequencing of a peptide fragment obtained after Lys C treatment of PhyH revealed a sequence identical to amino acids 232–240 (underlined).

thase (41). Experimental support for this idea was obtained by chromatofocusing, which showed that the pI of rat liver PhyH is 8.5 (**Fig. 4**). The calculated pI based on the translation of the complete open reading frame was 8.8. However, when the presumed leader sequence was removed, the calculated pI was 8.5.

Heterologous expression of rat *PHYH* in *S. cerevisiae*

When lysates were prepared from the yeast *S. cerevisiae*, no PhyH activity could be detected in both wild-type and yeast transformed with the 'empty' pEL26 and pEL30 expression vectors. When the coding sequence of rat *PHYH* was expressed, a high PhyH activity was measured in the

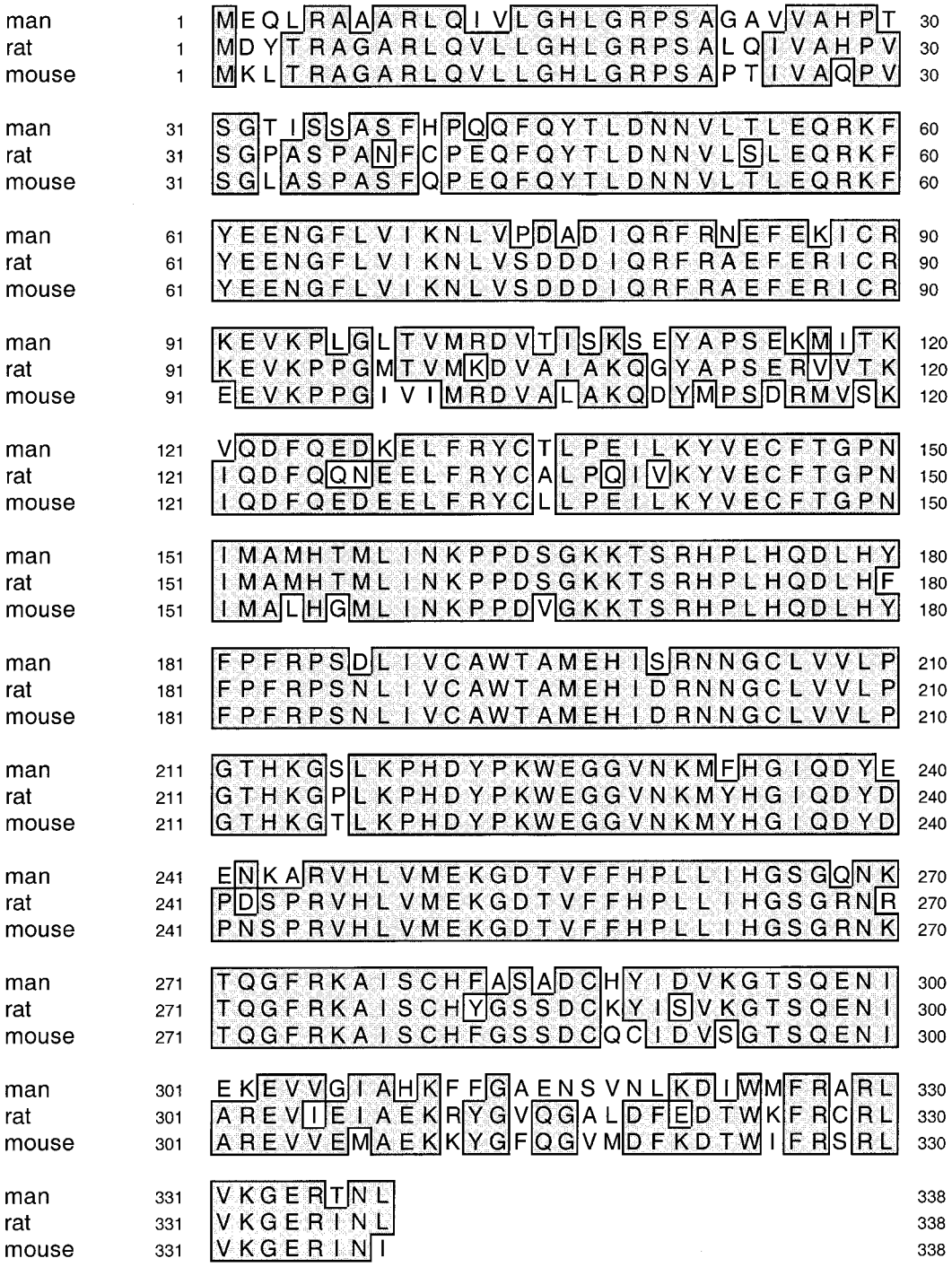


Fig. 3. Comparison of the deduced PhyH amino acid sequences from man, rat, and mouse. Identical amino acid residues are indicated in shaded boxes.

yeast lysate (6.1 and 35.1 pmol·h^{-1}·mg^{-1} in the low copy and high copy expression constructs, respectively). Immunoblot analysis showed that no PhyH could be detected in lysates from yeast transformed with the control vectors, but when PhyH was expressed, an immuno reactive protein of 38 kDa was detected. Analysis of rat liver homogenate or PhyH purified from rat liver showed a 35 kDa protein band (**Fig. 5**). These findings provided further evidence for the removal of the PTS2-containing leader sequence.

Subcellular localization of PhyH

When rat liver homogenate was subjected to differential centrifugation, almost all PhyH activity was present in the light mitochondrial (L) fraction, as was catalase, a peroxisomal marker enzyme (data not shown). In order to provide conclusive evidence for the peroxisomal localization of PhyH, a light mitochondrial fraction was subjected to equilibrium density centrifugation, and in all fractions marker enzyme activities were determined (**Fig. 6**). The

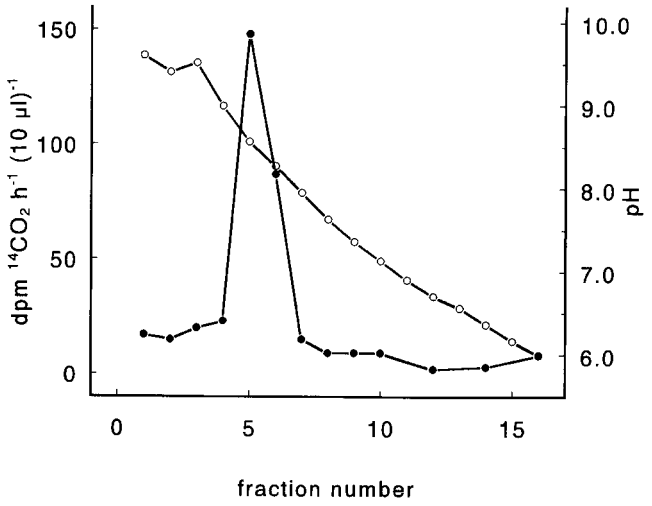


Fig. 4. PBE 94 Chromatofocusing of rat liver PhyH. An aliquot of the dialyzed wash-fraction obtained after octyl-Sepharose chromatography was subjected to chromatofocusing from pH 9–6 (○), and PhyH activity (●) was determined according to method 2 (see Materials and Methods).

distribution of PhyH was determined using SDS-PAGE/immunoblot analysis. Most PhyH was detected in the bottom fractions of the gradient, co-localizing with the peroxisomal marker enzyme catalase, indicating a peroxisomal localization of PhyH in rat liver.

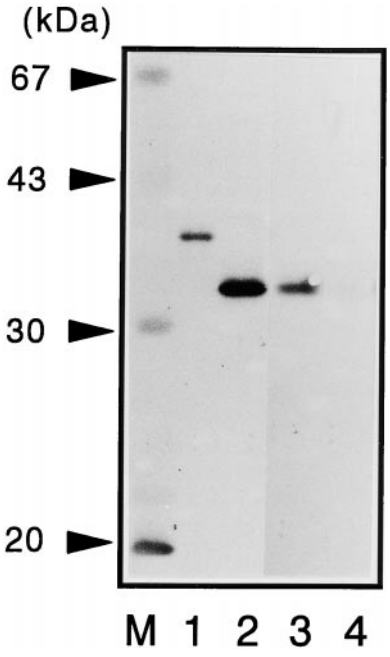


Fig. 5. Immunochemical detection of rat PhyH in liver homogenate and in lysates from transformed yeast expressing rat PhyH. Rat liver homogenate (lane 3), purified PhyH (lane 2), and lysates from yeast transformed with the PhyH expression vector (lane 1) or the 'empty' vector (lane 4) were separated by 10% SDS-PAGE and immunoblot analysis was performed as described in Materials and Methods. M, molecular weight marker.

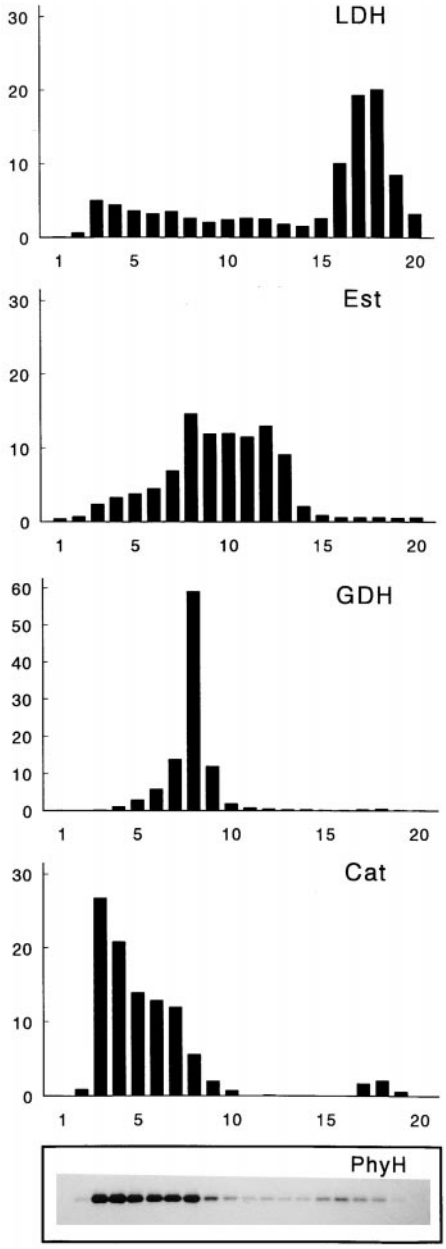


Fig. 6. Subcellular localization of rat PhyH. Rat liver was subjected to differential centrifugation to obtain a light mitochondrial fraction. This fraction was subfractionated using equilibrium density centrifugation and marker enzyme activities were measured in each fraction. Activities are expressed as percentage of the total activity present in the gradient. LDH, lactate dehydrogenase (cytosol); Est, esterase (microsomes); GDH, glutamate dehydrogenase (mitochondria); Cat, catalase (peroxisomes). The distribution of PhyH was determined by SDS-PAGE/immunoblot analysis.

Genbank accession number

The rat *PHYH* sequence was submitted to the Genbank database and was assigned the accession number AF121345.

DISCUSSION

For many years both the structure and the subcellular localization of the phytanic acid α-oxidation pathway were

obscure. However, recent studies have resolved the individual enzymatic steps involved in phytanic acid α-oxidation, including activation of phytanic acid to phytanoyl-CoA followed by *1*) hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA catalyzed by PhyH, *2*) cleavage of 2-hydroxyphytanoyl-CoA into pristanal and formyl-CoA, and *3*) oxidation of pristanal to pristanic acid.

With respect to the subcellular localization of the phytanic acid α-oxidation pathway, Tsai, Avigan, and Steinberg (11) were the first to suggest a mitochondrial localization. However, this work was done at a time of little awareness of the existence of peroxisomes. The finding that phytanic acid α-oxidation is deficient in cell lines from patients with Zellweger syndrome in which no functional peroxisomes are present led Poulos, Sharp, and Whiting (42) to suggest a peroxisomal localization. Subsequent studies in rat liver (9, 12, 14, 15) and human liver (9, 13) showed a mitochondrial localization. Huang and coworkers (19, 20) even proposed phytanic acid α-oxidation to be microsomal. Yet another view was held by Singh and coworkers (22), who suggested phytanic acid α-oxidation to be mitochondrial in rat but peroxisomal in humans. It is well known that the subcellular localization of an enzyme may differ among species. For instance, alanine glyoxylate aminotransferase (AGT) is equally divided between mitochondria and peroxisomes in rat, whereas in humans it is exclusively peroxisomal. This dual compartmentalization in rat is explained by the fact that the rat ATG gene has two transcription initiation sites producing two different mRNAs. The ORF of the shorter transcript encodes a protein with a C-terminal PTS1 (sequence: NKL), which directs this protein to peroxisomes. The longer transcript codes for a protein which contains an N-terminal extension including a mitochondrial targeting sequence, and this protein is directed to mitochondria. Apparently, the mitochondrial targeting signal present in the longer AGT protein overrules the PTS1. In humans, the transcription initiation site for the longer transcript has been lost during evolution. Thus, only the shorter transcript is produced, explaining the exclusive peroxisomal localization of AGT in humans.

In order to determine whether different targeting signals are present on the PhyH from rat and human, we have cloned the rat *PHYH* cDNA. Analysis of the deduced amino acid sequence of rat PhyH in comparison to the human PhyH revealed no known mitochondrial targeting signal, but instead a PTS2 located near the N-terminus was found in both species. Our subsequent activity measurements in subcellular fractions show a strict peroxisomal localization of PhyH in rat liver.

The question arises what the reason is for the different results obtained in studies performed during the last decades. An important point may be that all these studies were performed at times when the cofactor requirements for PhyH, the first step of phytanic acid α-oxidation, were unknown. Indeed, PhyH is completely dependent on the presence of the unusual components Fe^{2+} and 2-oxoglutarate. In addition, the absence of ascorbate severely decreases its activity (3). Careful inspection of the reaction conditions used in all studies performed before the discovery of PhyH in 1995, reveals that 2-oxoglutarate was not included in the reaction media used (1, 9, 11–19, 21, 22, 31, 43–53). This immediately explains the very low rates of [1-^{14}C]phytanic acid α-oxidation found in homogenates and purified organellar fractions when compared to intact hepatocytes. Indeed, in our earlier studies we found a high rate of phytanic acid α-oxidation (27.7 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein) in isolated rat hepatocytes, whereas the activity in homogenates prepared from the same livers a rate of only 1.1 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein was found. Similar low rates have been reported in all other studies performed before 1995 (1, 11–19, 21, 22, 31, 43–53).

The second implication of the lack of 2-oxoglutarate in the reaction medium is that the low α-oxidation rate observed is probably not mediated by PhyH. Yet another important issue to be mentioned is that in most studies the amount of $^{14}CO_2$ produced from [1-^{14}C]phytanic acid is taken as a measure of α-oxidation activity. However, CO_2 is not a direct product of the α-oxidation: in the second step of the α-oxidation pathway, 2-hydroxyphytanoyl-CoA is converted to pristanal and formyl-CoA. The latter product is converted to formic acid, and subsequently to CO_2 (45). The subcellular localization of the enzyme(s) involved in the conversion of formyl-CoA to CO_2 are still unknown, but a considerable amount of the formic acid produced is not converted to CO_2 (45). Therefore, the CO_2 produced can be regarded as an unreliable, indirect measure of the first two steps of phytanic acid α-oxidation.

Based on these arguments we conclude that all results from previous studies on phytanic acid α-oxidation in homogenates and subcellular factions need to be revised. The recent elucidation of the α-oxidation pathway now enables studies on the individual enzymes catalyzing the three steps of phytanic acid α-oxidation in various species. In this report we have provided conclusive evidence that PhyH, the enzyme catalyzing the first step of phytanic acid α-oxidation, is localized in peroxisomes in both rat and humans.

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