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Resolution of the Phytanic Acid $\alpha$-Oxidation Pathway: Identification of Pristanal as Product of the Decarboxylation of 2-Hydroxyphytanoyl-CoA

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The structure and enzymology of the phytanic acid $\alpha$-oxidation pathway have long remained an enigma. Recent studies have shown that phytanic acid first undergoes activation to its coenzyme A ester, followed by hydroxylation to 2-hydroxyphytanoyl-CoA. In this paper we have studied the mechanism of decarboxylation of 2-hydroxyphytanoyl-CoA in human liver. To this end, human liver homogenates were incubated with 2-hydroxyphytanoyl-CoA in the presence or absence of NAD$^+$ Hereafter, the medium was analyzed for the presence of pristanal and pristanic acid by gas chromatography mass spectrometry. Our results show that pristanal is formed from 2-hydroxyphytanoyl-CoA. Pristanal is subsequently oxidized to pristanic acid in a NAD$^+$ dependent reaction. These results finally resolve the mechanism of the phytanic acid $\alpha$-oxidation process in human liver.

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is first metabolised by $\alpha$-oxidation, as $\beta$-oxidation is blocked by the methyl group in the 3-position. The product of $\alpha$-oxidation is pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can be further metabolised by peroxisomal $\beta$-oxidation. The elucidation of the mechanism and the subcellular localisation of the $\alpha$-oxidation process have taken many years of debate in literature already. However, recent data have shed new light on the pathway of phytanic acid $\alpha$-oxidation. It is now known that, after activation to phytanoyl-CoA, 2-hydroxyphytanoyl-CoA is formed in a reaction catalysed by phytanoyl-CoA hydroxylase [1,2,3]. This dioxygenase type of enzyme requires Fe$^{2+}$ and ascorbate as cofactors and $\alpha$-ketoglutarate as cosubstrate, as was shown in both rat and human liver [1,2,3]. Phytanoyl-CoA hydroxylation was found to be deficient in liver and fibroblasts from patients affected by Refsum disease, rhizomelic chondrodysplasia punctata and generalised peroxisomal disorders like Zellweger syndrome, providing the biochemical basis for the phytanic acid accumulation found in these disorders [1,4,5,6]. The mechanism whereby 2-hydroxyphytanoyl-CoA is decarboxylated to pristanic acid or pristanoyl-CoA, has remained unknown so far. A systematic study of the reaction mechanisms of enzymes suggested to us the possibility that the decarboxylation of 2-hydroxyphytanoyl-CoA might occur via a mechanism resembling the breakdown of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This metabolite is converted into acetoacetate and acetyl-CoA by means of HMG-CoA lyase [7]. If a similar enzyme would be operative in the breakdown of 2-hydroxyphytanoyl-CoA, one would expect formation of pristanal as intermediary metabolite next to formyl-CoA. Further NAD$^+$ dependent oxidation of pristanal to pristanic acid could occur by a similar mechanism as described for other fatty aldehydes [8].

In this paper we show that pristanal is produced from 2-hydroxyphytanoyl-CoA and undergoes further NAD$^+$ dependent oxidation to pristanic acid. Our data lead us to conclude that the long-sought structure of the phytanic acid $\alpha$-oxidation pathway is finally resolved, after more than 25 years of study.

MATERIALS AND METHODS

Materials. 2-Hydroxyphytanoyl acid, pristanic acid and [2-methyl-1$^3$H$_3$]pristanic acid were synthesized as described before [9]. 2-Hydroxyphytanoyl-CoA was synthesised from 2-hydroxyphytanoyl acid using the mixed anhydride method [10].

Pristanal ethoxime was synthesized in the Free University Hospital Amsterdam with a purity of >99%. Dodecylaldehyde (purity >98%) was obtained from Janssen Chimica (Belgium).

Methods. Pieces of human liver were stored at −70°C and thawed in a medium containing 250 mM mannitol, 5 mM MOPS-KOH, 0.1
mM EDTA at a final pH of 7.4. Protein concentrations were determined using an established method [11].

The incubations contained the following components: 1 mg of total liver homogenate, 150 mM KCl, 50 mM Hepes-NaOH, 2 mM potassium phosphate, 10 μM defatted bovine serum albumin and 0.02 mM 2-hydroxyphytanoyl-CoA. The volume was 1 ml and the final pH was 7.4. To some of the incubations, 1 mM NAD⁺ was added. 200 μl aliquots of the incubations were taken at the start of the reaction and after 10, 30, 60 and 120 minutes. For analysis of pristanal, to these aliquots, 100 μl ethoxylamine (0.1 g/ml) in water was added immediately and the pH was increased to >10 by addition of Na₂CO₃. 0.6 nmol dodecylaldehyde was added as internal standard. Hereafter, fatty ethoxime esters were extracted with 2 ml of diethylether by shaking the samples during 1 hour. The ether layers were separated from the aqueous layers and evaporated. The residues were redissolved in 20 μl of hexane and analyzed by gas chromatography (Hewlett Packard 5890 series II) using a CPsil19 CB capillary column (24 mx0.25 mm, film thickness 0.2 μM, Chrompack, Middelburg, The Netherlands). Detection was performed by positive chemical ionization mass spectrometry (Hewlett Packard Engine 5989B) using methane as reactant gas. For pristanal-ethoxime, m/z 326 (M⁺1) was monitored, for dodecylaldehyde-ethoxime m/z 228 (M⁺1) was monitored.

Pristanic acid in the incubations was measured as its pentafluorobenzyl derivative by stable isotope dilution gas chromatography mass spectrometry with negative chemical ionization, essentially as described before for plasma samples [12].

RESULTS

Figure 1 shows the mass fragmentograms of a standard solution containing pristanal-ethoxime (RF = 7.85 min.) and dodecylaldehyde-ethoxime (RF = 5.62 min.). Pristanal-ethoxime gives a double peak in its mass fragmentogram, due to separation between its cis- and trans isomers. No separation between the stereoisomers of dodecylaldehyde-ethoxime is observed.

In incubations of homogenised human liver with 2-hydroxyphytanoyl-CoA, formation of pristanal is observed, as is shown in fig. 1C. At t = 0 (fig. 1B), no pristanal was detectable, whereas at t = 100 min. (fig. 1C) a significant amount of pristanal was formed. In these incubations, only a small amount of pristanic acid (0.4 nmol/ml) was formed.

In figure 2 the time dependence of the formation of pristanal in incubations of 2-hydroxyphytanoyl-CoA is shown. A linear increase in pristanal concentrations, as calculated by dividing the peak area of pristanal-ethoxime by the peak area of the internal standard dodecylaldehyde-ethoxime, was found during the first 60 minutes of incubation. Hereafter, no further increase occurred. In these incubations, to which no NAD⁺ was added, only a small amount of pristanic acid (0.4 nmol/ml) was formed. When NAD⁺ was added to the incubation medium, there was no detectable formation of pristanal as shown in fig. 2. Under these condi-
dized to its corresponding fatty acid, in nature. Already in 1959 Martin and Stumpf described the α-oxidation of long chain fatty acids in peanuts [15]. A peroxidase enzyme shortens the chain of a fatty acid by one carbon atom, yielding a fatty aldehyde, which is further oxidized by a NAD⁺ specific dehydrogenase giving the corresponding fatty acid. This process, however, was then found to be independent of activation of the fatty acids to their coenzyme A derivatives.

The data described in this paper suggest that the pathway of phytanic acid α-oxidation is finally resolved now. Following earlier findings that phytanic acid first undergoes activation to phytanoyl-CoA succeeded by hydroxylation to 2-hydroxyphytanoyl-CoA, our data now...
suggest that the structure of the pathway is as outlined in fig. 3 with pristanal as obligatory intermediate. Current studies are directed towards identification of the enzymes involved, their subcellular localisation and their activity in tissues from patients suffering from peroxisomal disorders in which phytanic acid accumulates.

REFERENCES


