Formation of a 2-methyl-branched fatty aldehyde during peroxisomal α-oxidation

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Abstract In the final reaction of peroxisomal α-oxidation of 3-methyl-branched fatty acids a 2-hydroxy-3-methylacyl-CoA intermediate is cleaved to formyl-CoA and a hitherto unidentified product. The release of formyl-CoA suggests that the unidentified product may be a fatty aldehyde. When purified rat liver peroxisomes were incubated with 2-hydroxy-3-methylhexadecanoyl-CoA 2-methylpentadecanal was indeed formed. The production rates of formyl-CoA (measured as formate) and of the aldehyde were in the same range. While the production of formate remained unaltered in the presence of NAD^+, the amount of 2-methylpentadecanal was decreased, which was accompanied by the formation of 2-methylpentadecanoic acid. These data indicate that (1) during α-oxidation the 2-hydroxy-3-methylacyl-CoA is cleaved to a 2-methyl-branched aldehyde and formyl-CoA and (2) liver peroxisomes are capable of converting this aldehyde to a 2-methyl-branched fatty acid.

Key words: Aldehyde; α-Oxidation; Formate; Peroxisome; Phytanic acid; Refsum's disease

1. Introduction

3-Methyl-substituted fatty acids such as the naturally occurring phytanic acid or the synthetic 3-mehylhexadecanoic acid cannot undergo β-oxidation due to the presence of a methyl group in the 3-position. They first have to be shortened by one carbon atom via α-oxidation, a process until recently thought to yield CO_2 and a 2-methyl-substituted fatty acid as the primary end products [1]. The 2-methyl-branched fatty acid is then degraded via β-oxidation predominantly in the peroxisome [2–6].

The subcellular localization of α-oxidation and the reactions involved have remained unclear until recently, when it was demonstrated in the rat [7,8] and the human [9,10] that α-oxidation is localized to peroxisomes and that it consists of an activation step leading to a 3-methylacyl-CoA, followed by a 2-hydroxylation resulting in the formation of a 2-hydroxy-3-methylacyl-CoA intermediate. In the final reaction formyl-CoA is released, which is then enzymatically hydrolyzed to formate [11]. The release of formyl-CoA suggests that the second — hitherto unidentified — end product of α-oxidation may be a 2-methyl-branched fatty aldehyde [11] rather than a 2-methyl-branched fatty acid, as was generally believed [1]. We, therefore, investigated whether 2-methylpentadecanal is formed in purified rat liver peroxisomes incubated with the synthetic α-oxidation intermediate 2-hydroxy-3-methylhexadecanoyl-CoA.

2. Materials and methods

2.1. Synthesis of substrates

2.1.1. 2-Methylpentadecanal. For the synthesis of 2-methylpentadecanal, 2-pentadecanone (7 mmol; Fluka) was converted to 2-hydroxy-2-methylpentadecanyl ethyl ether according to a procedure described by Normant and Crisan [12]. The ethyl ether was subsequently refluxed in formic acid [12] and the produced 2-methylpentadecanal extracted into diethylether. The ether extracts were dried, the residue dissolved in hexane/diethylether (95:5, v/v), applied on a silica column and eluted with an increasing percentage of diethylether in hexane. Purity was checked by thin layer chromatography (TLC: Silicagel 60 F_{254}; hexane/ether 60:40; $R_f = 0.89$) and by gas chromatography (GC). Identity of the 2-methylpentadecanal was confirmed by liquid secondary ion mass spectrometry (positive mode; matrix thioglycerol) after derivatisation to a hydrazone with Girard Reagent T (carboxymethyl trimethylammonium chloride hydrazide; Fluka).

2.1.2. 2-Hydroxy-3-methylhexadecanoyl-CoA. In a first step, 3-methylhexadec-2-enoic acid ethyl ester was formed by a Wittig synthesis of 2-pentadecanone (10 mmol) with (carboethoxymethylene)triphenylphosphorane (20 mmol) refluxed in diglyme for 72 h at 140°C. After addition of water (200 ml), the crude product was extracted into hexane. The hexane was evaporated in vacuo and the residue purified over a silica column by using hexane/diethylether (80:20, v/v; yield 50%). Subsequently, a portion of the obtained 3-methylhexadec-2-enoic acid ethyl ester (3.4 mmol) was dissolved in ethanol and hydrogenated in a Parr-apparatus with palladium hydroxide on carbon as a catalyst (room temperature; 3 kg H_2/cm^2; 18 h). After retaining the catalyst on Blauband® filters, the solvent was evaporated in vacuo and the produced 3-methylhexadecanoic ethyl ester was saponified.

The obtained 3-methylhexadecanoic acid (3.3 mmol, yield 97%) was 2-hydroxylated with lithium diisopropylamide and oxygen, essentially as described in reference [13]. The crude product was purified over a silica column eluted with hexane/diethylether/acetic acid (70:30:1, v/v; yield 25%). Purity of the reaction intermediates and end-product was checked by TLC (silica gel 60 F_{254}; hexane/diethylether/acetic acid 60:40:1, v/v) and identity was confirmed by liquid secondary ion mass spectrometry.

To obtain the CoA-ester, 2-hydroxy-3-methylhexadecanoic acid was first converted to its thiophenyl ester followed by transesterification, analogously to the synthesis of 2-methyl-3-hydroxyhexadecanoyl-CoA described before [14]. Purity was checked by TLC and HPLC.

2.1.3. 2-Hydroxy-3-methyl[1-^{14}C]hexadecanoyl-CoA. To obtain 2-hydroxy-3-methyl[1-^{14}C]hexadecanoic acid, 3-methylhexadecanoic acid (1 mmol) was used as precursor. The acid was converted into 2-methylpentadecyl iodide by photochemical iododecarboxylation according to a procedure described in [15] with some modifications (yield 93%). Identity of the alkylhalide was confirmed by liquid secondary ion mass spectrometry after derivatisation with triethylamine. A portion of the iodide (38 μmol) was converted into 3-methyl-pentadecyl[1-^{14}C]nitrile in a phase-transfer catalyzed $K^{14}CN$ incorporation [16] similarly to the previously described synthesis of 2-methyl-[1-^{14}C]palmitic acid [3] and 3-methyl[1-^{14}C]heptadecanoic acid [17].

The labelled nitrile was refluxed under alkaline conditions (4 M KOH in 80% ethanol) in the presence of 3% H_2O_2. When enough

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amide was converted to acid, as monitored by radio-TLC developed in hexane/diethylether/acetic acid (60:40:1, v/v), the ethanol was evaporated in vacuo and the mixture extracted with diethylether (to recover the labelled amide). After acidification of the aqueous layer, the 3-methyl[1-^{14}C]hexadecanoic acid was extracted into diethylether.

The subsequent 2-hydroxylation was done as described above for the unlabelled compound, and the 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoic acid was purified via preparative TLC in hexane/diethylether/acetic acid (60:40:1, v/v).

Labelled and unlabelled 2-hydroxy acids were mixed to obtain the required specific activity and the acid was converted to the CoA ester as described above.

2.2. Preparation and incubation of purified peroxisomes

Peroxisomes were purified from livers of overnight-fasted male Wistar rats by a combination of differential and isopycnic centrifugation as described before [11], and were stored at −20°C until use.

Incubations (37°C) were started by adding 100 μl of the purified peroxisomes (50–100 μg protein) to 400 μl of reaction medium. Final concentrations were 100 mM KCl, 50 mM Tris-HCl (pH 7.5) and 0.010 mM defatted bovine serum albumin. Under these conditions, referred to in the text as standard conditions, formation of formate proceeded optimally [Croes K., Casteels M., Mannaerts G.P., Van Veldhoven P.P., unpublished data]. When added, concentrations of NAD^+, *N*-ethylmaleimide and semicarbazide were 2 mM, 1 mM and 0.8 mM, respectively. Substrate concentrations were 0.050 mM unlabelled (measurement of aldehydes and fatty acids) or ^{14}C-labelled (measurement of [^{14}C]formate) 2-hydroxy-3-methylhexadecanoyl-CoA (specific radioactivity: 0.9 Ci/mol) or 0.025 mM 2-methylpentadecanal.

For measurement of aldehydes and fatty acids, reactions were terminated by the addition of 500 μl of methanol containing 2% (v/v) acetic acid. To measure [^{14}C]formate production, reactions were stopped with 250 μl of 6% (w/v) $HClO_4$ and processed as described before [18]. Using this method, any [^{14}C]formyl-CoA present in the reaction mixture is measured as [^{14}C]formate [11].

2.3. Analysis of the reaction medium for aldehydes and fatty acids

The acidified samples were kept on ice for the rest of the procedure except when mentioned otherwise. After addition of the internal standards, tetradecanal (≈15 nmol based on purity given by the company (Aldrich)) and heptadecanoic acid (5 nmol; Fluka), the samples were extracted with 2 ml of hexane. After centrifugation at 4°C for 10 min, the hexane layers were collected and evaporated to dryness under N_2 at room temperature. The residue was reconstituted in 40 μl of hexane, 1 μl of which was subjected to GC analysis for the detection of aldehydes (Pye Unicam PU 4550 gas chromatograph; on-column injection; capillary column: 30 m×0.32 mm i.d. Econocap EC-1 (SE-30), film thickness 0.25 μm (Alltech); column temperature: 80–230°C at a rate of 10°C/min; flame ionization detection, detector temperature: 300°C).

For the analysis of the fatty acids, 20 μl of the reconstituted residue was transferred to a screw-cap tube and methylated using acidic methanol. The methyl esters were extracted into hexane and the solvent was evaporated. The residue was reconstituted in 20 μl of hexane, 1 μl of which was subjected to GC analysis (system as described above except for the column temperature: 80–180°C at a rate of 20°C/min, 180–228°C at a rate of 4°C/min).

3. Results and discussion

When purified peroxisomes were incubated under standard conditions with 2-hydroxy-3-methylhexadecanoyl-CoA, GC analysis of the hexane extract for aldehydes revealed a peak at approximately 11.3 min, coincident with a 2-methylpentadecanal standard (Fig. 1). Although exact quantification of the amount of 2-methylpentadecanal was difficult due to the lack of an internal standard of known purity, the estimated amount formed was of the same order of magnitude as the amount of formate produced (4.2 and 2.6 nmol/min/mg protein for 2-methylpentadecanal and formate, respectively). As for formate, the production of the 2-methylpentadecanal was time dependent. *N*-Ethylmaleimide decreased the formate production and the formation of 2-methylpentadecanal from 2-hydroxy-3-methylhexadecanoyl-CoA to approximately the same extent (results not shown), suggesting that formate and 2-methylpentadecanal are formed in the same reaction.

While addition of semicarbazide, an aldehyde trapping agent, to the reaction mixture did not alter the formate production, it markedly decreased the recoveries of both the tetradecanal internal standard and the 2-methylpentadecanal, confirming the aldehyde nature of both compounds (results not shown). As a membrane-bound clofibrate-inducible peroxisomal aldehyde dehydrogenase using NAD^+ (or $NADP^+$)

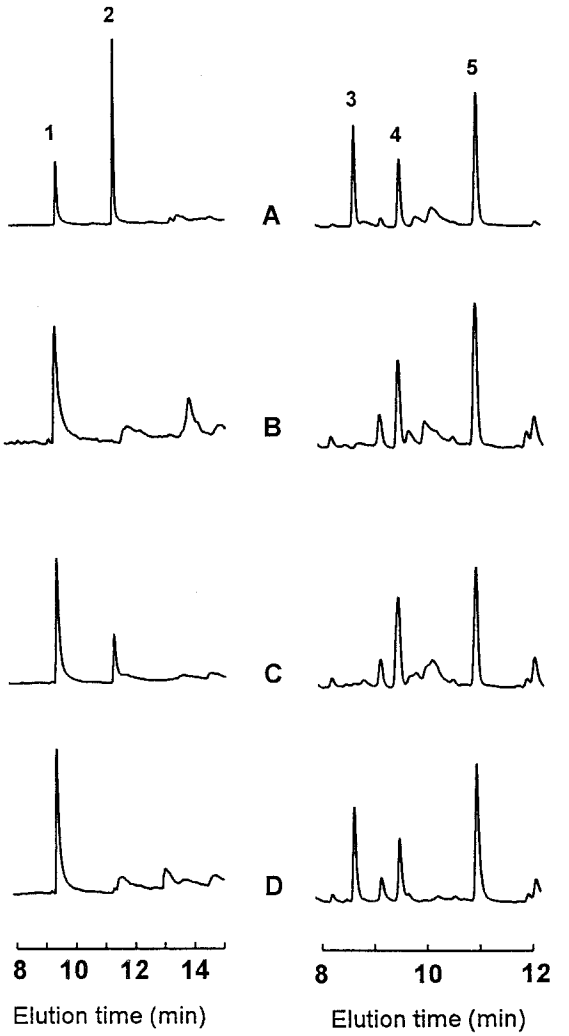

Fig. 1. GC analysis of aldehydes and fatty acid methyl esters formed during the peroxisomal oxidation of 2-hydroxy-3-methylhexadecanoyl-CoA. Purified peroxisomes (100 μg) were incubated for 10 min with (C,D) and without (B) 2-hydroxy-3-methylhexadecanoyl-CoA (0.050 mM) in the absence (B,C) and presence (D) of NAD^+ (2 mM). After termination of the reaction with acidic methanol and addition of the internal standards (tetradecanal and heptadecanoic acid), the reaction medium was extracted with hexane. The solvent was evaporated, the residue reconstituted in hexane and an aliquot analysed by GC for aldehydes (left panel). Another aliquot was analysed by GC after methylation for the presence of fatty acid methyl esters (right panel). Elution of standards is shown in (A). The numbered peaks indicate the following compounds: 1, tetradecanal; 2, 2-methylpentadecanal; 3, 2-methylpentadecanoyl methyl ester; 4, hexadecanoyl methyl ester and 5, heptadecanoyl methyl ester.

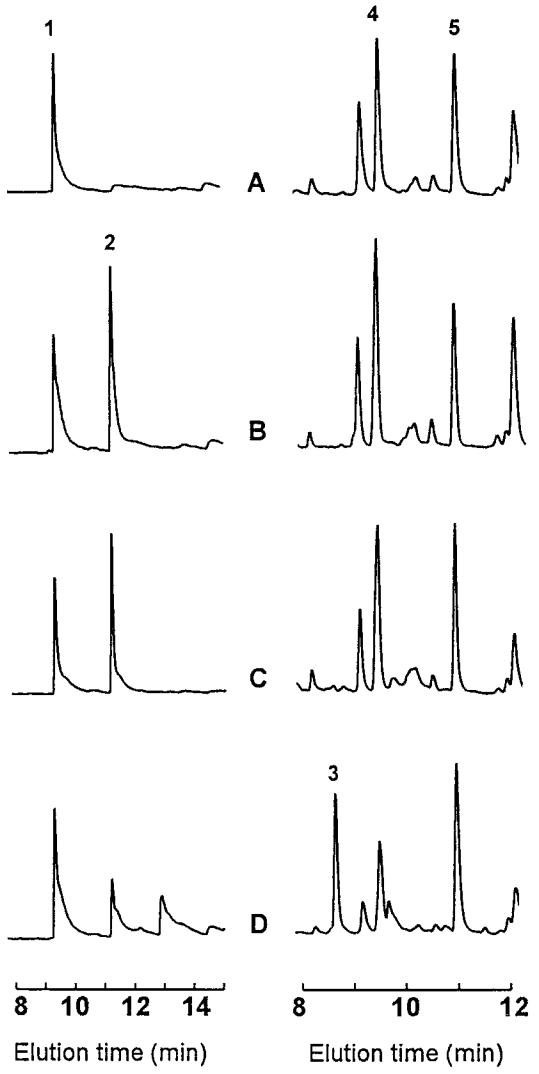

Fig. 2. GC analysis of aldehydes and fatty acid methyl esters formed during the peroxisomal oxidation of 2-methylpentadecanal. Purified peroxisomes (50 μg) were incubated for 0 (B) and 10 min (A,C,D) with (B,C,D) and without (A) 2-methylpentadecanal (0.025 mM) in the absence (A,B,C) and presence (D) of NAD^+ (2 mM). After termination of the reaction, reaction mixtures were mixed with internal standards, extracted and analysed by GC for aldehydes (left panel) and fatty acid methyl esters (right panel) as described in the legend to Fig. 1. Peaks are numbered as for Fig. 1.

as a coenzyme has been described [19], peroxisomes were also incubated with 2-hydroxy-3-methylhexadecanoyl-CoA in the presence of NAD^+. The formate production remained unaltered (data not shown), but a selective decrease in the amount of 2-methylpentadecanal was seen. When the hexane extracts were methylated and analysed for the presence of fatty acid methyl esters by GC, a peak eluted at approximately 8.6 min, only when the peroxisomes were incubated with 2-hydroxy-3-methylhexadecanoyl-CoA in the presence of NAD^+ (Fig. 1). The unknown compound coeluted with a 2-methylpentadecanoyl methyl ester standard.

As a control, purified peroxisomes were incubated with synthetic 2-methylpentadecanal as the substrate. Fig. 2 shows that the substrate is converted to 2-methylpentadecanoic acid on condition that NAD^+ is present.

In conclusion, our results show that rat liver peroxisomes cleave 2-hydroxy-3-methylhexadecanoyl-CoA to 2-methylpentadecanal and formate (formyl-CoA), probably due to the presence of a specific lyase. Since 2-hydroxy-3-methylacyl-CoA is a proven intermediate of the α-oxidation process, our findings imply that, besides formyl-CoA, the second end-product of α-oxidation is a 2-methyl-branched fatty aldehyde and not a fatty acid. The generated aldehyde is then most probably oxidized to the corresponding fatty acid by a peroxisomal long chain aldehyde dehydrogenase [19].

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