Purification and further characterization of peroxisomal trihydroxycoprostanoyl-CoA oxidase from rat liver

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The acyl-CoA oxidase, catalysing the peroxisomal desaturation of the CoA-ester of trihydroxycoprostanic acid, a bile acid intermediate, has been purified to homogeneity from rat liver. Its native molecular mass, as determined by gel filtration and native gel electrophoresis, was 120 and 175 kDa respectively, suggesting a homodimeric protein consisting of 68.6 kDa subunits. If isolated in the presence of FAD, the enzyme showed a typical flavoprotein spectrum and contained most likely 2 mol of FAD per mol of enzyme. The cofactor, however, was loosely bound. The enzyme acted exclusively on 2-methyl-branched compounds, including pristanoyl-CoA and 2-methylhexanoyl-CoA if albumin was present. Important parameters to obtain a pure and active enzyme were the following: (1) using chromatographic separations like hydrophobic interaction and metal affinity, which allow the presence of high salt concentrations, conditions which stabilize the oxidase; (2) avoiding dialysis and $(NH_4)_2SO_4$ precipitation; (3) including, when appropriate, FAD, dithiothreitol and a diol-compound in the solvents; and (4) carefully monitoring the removal of other acyl-CoA oxidases which possess the same native molecular mass and subunit size.

INTRODUCTION

Cholesterol breakdown proceeds via a number of steps that involve changes of the steroid nucleus and ω-oxidation of aliphatic side chains, resulting in the formation of the bile acid intermediates dihydroxycoprostanic acid (DHCA) and trihydroxycoprostanic acid (THCA) (see [1]). After activation to their CoA-esters, which in the rat occurs in the endoplasmic reticulum [2,3], the side chains of DHCA and THCA are shortened via peroxisomal β-oxidation in rodents as well as in man (see [4]). The first reaction of peroxisomal β-oxidation is catalysed by an acyl-CoA oxidase, which desaturates an acyl-CoA to a 2-*trans*-enoyl-CoA and thereby forms hydrogen peroxide. In rat liver, the desaturation of trihydroxycoprostanoyl-CoA (THC-CoA) is catalysed by trihydroxycoprostanoyl-CoA oxidase (THCCox) [5,6], an acyl-CoA oxidase which is different from the recently characterized pristanoyl-CoA oxidase (PCox) [7–9] and the well-known palmitoyl-CoA oxidase [10,11]. The gene of the latter enzyme gives rise, via alternative splicing, to two mRNAs but only one of the oxidases, corresponding to mRNA type I, has been isolated so far [12]. We refer to this enzyme as palmitoyl-CoA oxidase type I (ACox-I).

In man, THC-CoA is recognized by an acyl-CoA oxidase, which acts on bile acid intermediates and on pristanic acid (both 2-methyl-substituted compounds). The human enzyme has been named branched-chain acyl-CoA oxidase (BRCACox) [13].

Since its discovery in 1988 [5], followed by its partial purification [6], considerable efforts have been made to purify rat THCCox to homogeneity by our laboratory and others, without great success. We now report on our final strategy to isolate this enzyme in an active state and in sufficient amounts to prepare antibodies and to obtain internal amino acid sequences.

MATERIALS AND METHODS

Materials

Butyl-Sepharose 4B, Phenyl-Sepharose Fast Flow (low sub), a Hiload 16/60 Superdex-200 gel-filtration column, HiTrap chelating affinity columns (5 ml) and palmitoyl-CoA were purchased from Pharmacia Belga, Brussels, Belgium. Hydroxyapatite columns (Econo-Pac HTP cartridge; 5 ml) were from Bio-Rad, Richmond, CA, U.S.A. THC-CoA [6], (±)-2-methylpalmitoyl-CoA [7], pristanoyl-CoA [7] and (±)-2-methylhexanoyl-CoA [8] were prepared as described before. Ultrafiltration devices (Centriprep-10 concentrators) were obtained from Grace-Amicon, Beverly, MA, U.S.A.

Partially purified oxidase preparations

Initially, the acyl-CoA oxidases were partially purified from hypotonic rat liver extracts by means of heat treatment in the presence of FAD and $(NH_4)_2SO_4$ fractionation exactly as described before [7]. In later experiments, the extracts were prepared in a slightly different way. Livers were gently homogenized in 4 vol. of 0.25 M sucrose, 1 mM EDTA, pH 7.2, 0.1% (v/v) ethanol and centrifuged at 100000 *g* for 1 h. The pellet was resuspended by means of a Polytron homogenizer in 10 mM pyrophosphate buffer, pH 9.0, 1 mM EDTA, 5 mM benzamidine, 0.5 mM dithiothreitol (DTT), 5 μM FAD, 0.2 mM phenylmethanesulphonyl fluoride, 0.2 mM tosylphenylalanylchloromethane (5 ml per g of starting liver) and centrifuged again at 100000 *g*. The supernatant was then subjected to heat treatment and $(NH_4)_2SO_4$ fractionation as described [7], except that all solutions contained in addition 0.5 mM DTT (see below). The partially purified oxidase preparations were stored at −20 °C until use. The three acyl-CoA oxidases (ACox-I, PCox, THCCox), which were enriched 35- to 40-fold, appeared to be stable under these conditions.

Purification of THCCox

In a first chromatographic step, THCCox and ACox-I were separated from PCox by hydrophobic interaction chromatography on butyl-Sepharose at 4 °C as recently described [9],

Abbreviations used: ACox-I, palmitoyl-CoA oxidase type I; BRCACox, branched-chain acyl-CoA oxidase; DHCA, dihydroxycoprostanic acid; DTT, dithiothreitol; PCox, pristanoyl-CoA oxidase; THCA, trihydroxycoprostanic acid; THC-CoA, trihydroxycoprostanoyl-CoA; THCCox, trihydroxycoprostanoyl-CoA oxidase.

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except that the buffers were supplemented with 0.5 mM DTT and that larger samples (derived from 200 to 240 g of liver) were loaded on larger columns (5.0 cm × 8.0 cm; flow rate of 8 ml/min). THCCox and ACox-I are eluted closely together from this matrix upon lowering the $(NH_4)_2SO_4$ concentration, whereas PCox requires high concentrations of ethylene glycol for elution [9]. Fractions which were eluted between 0.65 and 0.35 M $(NH_4)_2SO_4$ and contained the THCCox and ACox-I activities were combined and directly applied to a phenyl-Sepharose column (2.6 cm × 10 cm), equilibrated in 20 mM potassium phosphate, pH 7.5, 0.4 M $(NH_4)_2SO_4$, 0.5 mM DTT, 5 μM FAD (buffer Ph-A) at 4 °C and at a flow rate of 3 ml/min. After washing the column with buffer Ph-A until the absorbance at 280 nm had almost decreased to baseline, adsorbed proteins were eluted (5 ml fractions) at a flow rate of 1.5 ml/min (1) by means of a decreasing salt gradient for 60 min (100% buffer Ph-A to 100% buffer Ph-B), (2) isocratically for 10 min with buffer Ph-B, and (3) by an increasing gradient of 1,2-propanediol for 80 min (100% buffer Ph-B to 100% buffer Ph-C). Buffer Ph-B consisted of 20 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 5 μM FAD; buffer Ph-C of 20 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 5 μM FAD, 60% (v/v) 1,2-propanediol.

The fractions that were eluted between 35 and 85% buffer Ph-C and which contained the THCCox activity, were pooled, diluted 2-fold with 0.5 mM DTT, 5 μM FAD and loaded on to a hydroxyapatite column (Econo-Pac HTP cartridge; 5 ml), equilibrated in 10 mM potassium phosphate, 0.5 mM DTT, 5 μM FAD, 20% (w/v) ethylene glycol (buffer HA-A), at 4 °C and at a flow rate of 1.5 ml/min. The adsorbed proteins were eluted (3 ml fractions) at 1 ml/min by increasing the potassium phosphate concentration in a step-wise manner (8% buffer HA-B for 10 min, followed by 24% buffer HA-B for 10 min and finally 100% buffer HA-B). Buffer HA-B consisted of 200 mM potassium phosphate, 0.5 mM DTT, 5 μM FAD, 20% (w/v) ethylene glycol.

Fractions that were eluted at 24% buffer HA-B and which contained the THCCox activity, were concentrated to approx. 1 ml by means of ultrafiltration at 4 °C and subjected to metal-affinity chromatography on a Zn-Sepharose column at 4 °C. Before application on to the column, the yellow enzyme solution was diluted 2-fold with the equilibration buffer of the Zn-column [20 mM potassium phosphate, pH 7.2, 0.5 M sodium acetate, 20% (w/v) ethylene glycol] and then loaded, after removal of any particulate material by centrifugation, on to the column at a flow rate of 1 ml/min. Adsorbed proteins were eluted at 2 ml/min for 10 min by an increasing gradient of glycine (up to 1.2 M) in equilibration buffer. Fractions of 5 ml were collected in tubes containing 2.5 μl of 1 mM FAD and 2.5 μl of 1 M DTT.

The active fractions were again concentrated by means of ultrafiltration, freed of particulate material by centrifugation and subjected to gel filtration (Superdex-200; 16 mm × 60 mm) at 1 ml/min in 0.2 M potassium phosphate, pH 7.5, 0.5 mM DTT, 5 μM FAD, 20% (w/v) ethylene glycol. Fractions of 2 ml were collected.

Oxidase measurements

Acyl-CoA oxidases were measured as described previously [8] except that assay volumes were reduced to 125 μl in order to save substrate. Final substrate concentrations, which were based on our previous work [8], were 100 μM palmitoyl-CoA bound to 0.06% (w/v) defatted albumin for ACox-I, 100 μM 2-methyl-palmitoyl-CoA without albumin for PCox and 50 μM THC-CoA bound to 0.24% (w/v) defatted albumin for THCCox.

Gel electrophoresis and immunoblotting

Analysis of the rat liver extracts and column eluates by SDS/gel electrophoresis and/or immunoblotting was done as described before [9]. Antibodies against the purified THCCox were generated as described before [9], using the 68.6 kDa subunit excised from SDS/polyacrylamide gels.

RESULTS AND DISCUSSION

In previous experiments in our laboratory [6] THCCox was partially purified from purified peroxisomes by means of heat treatment in the presence of FAD and $(NH_4)_2SO_4$ fractionation, followed by chromatography on a chromatofocusing gel and on hydroxyapatite and gel filtration. The apparent lability of the enzyme prevented the investigation of further chromatographic steps, while the use of purified peroxisomes as enzyme source imposed limits on the amount of starting material. Therefore, total rat liver was chosen as the enzyme source. As reported before [7,8], a 30-fold-enriched preparation could be obtained from total rat liver with a yield of approx. 30% in the following way. Liver was homogenized with a Polytron homogenizer in hypotonic alkaline medium in order to release the peroxisomal matrix enzymes [14] in the presence of FAD and some protease inhibitors. After removal of the membranes, the soluble proteins that had not been precipitated by 10% $(NH_4)_2SO_4$, were heated to 50 °C in the presence of FAD. Acyl-CoA oxidases are stable under these conditions. A peculiar feature of THCCox was the requirement for a high salt (sodium acetate, NaCl, $(NH_4)_2SO_4$, potassium phosphate) concentration during the heat treatment ([7]; P. P. Van Veldhoven and G. P. Mannaerts, unpublished work). With the partially purified preparations, different attempts were undertaken to purify THCCox. However, in most trials we faced the sudden, apparently unpredictable loss (or inactivation) of the enzyme during the purification process. $(NH_4)_2SO_4$ precipitation and dialysis appeared to contribute to the loss of enzyme activity. Therefore, in the final procedure the sequence of steps was such that column eluates could be applied directly to the next column and when concentration was necessary, ultrafiltration devices were used.

Since the enzyme appeared to be stabilized by high ionic strength (see above) we invested a lot of effort into optimizing the hydrophobic interaction chromatography. After the removal of PCox on butyl-Sepharose [9], the closely eluting ACox-I and THCCox were applied to phenyl-Sepharose. As shown in Figure 1(a), the major portion of the palmitoyl-CoA oxidase activity (due to ACox-I) eluted from the phenyl matrix upon decreasing the salt concentration; THCCox was more firmly bound and required the addition of a polarity-reducing organic solvent such as a detergent or a diol. In contrast with butyl-Sepharose [9], the use of phenyl-Sepharose resulted in variable and low recoveries of THCCox activity. In general, recoveries were higher when high-pressure columns were used (shorter elution time) or when detergents were used to elute the bound oxidase. However, the use of detergents was abandoned because of a possible interference with subsequent chromatographic steps and the troublesome column regeneration procedure. Depending on the supplier, the diol compounds employed appeared to contain a considerable amount of hydrogen peroxide which inactivated the enzyme. Inactivation of this enzyme by hydrogen peroxide is not surprising since THCCox is sensitive towards *N*-ethylmaleimide [6], pointing to a sulphydryl group in or near the catalytic site. In order to keep the enzyme in a reduced state, reducing compounds such as mercaptoethanol or DTT, were added to the chromatographic buffers. However, these compounds, which also act as hydrogen peroxide scavengers, seriously interfere with the

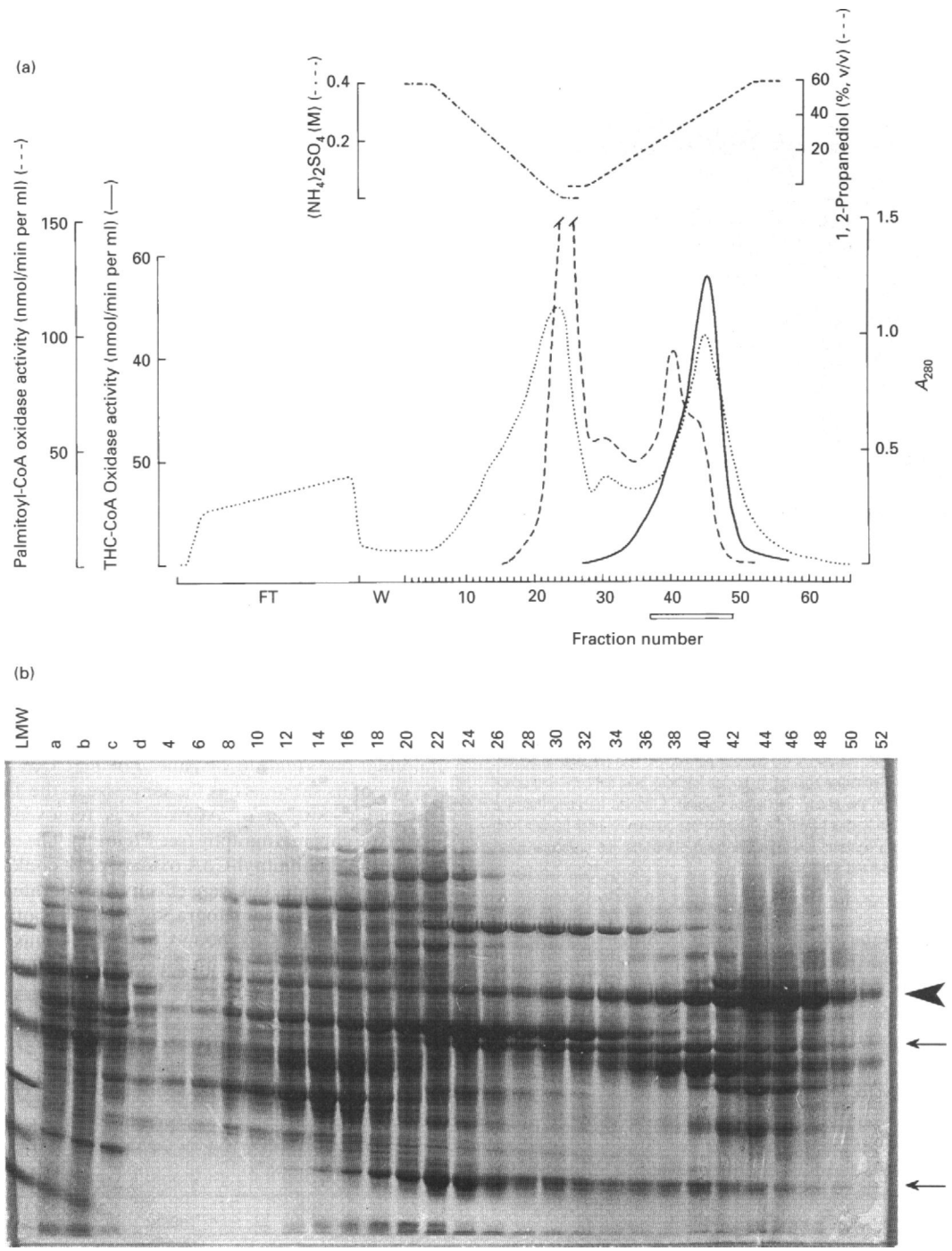

Figure 1 Separation of ACox-I and THCCox by hydrophobic interaction chromatography

(**a**) Acyl-CoA oxidases, eluted from the butyl-Sepharose column, were separated on phenyl-Sepharose as described in the Materials and methods section. Eluted fractions (5 ml) were analysed for protein content (absorbance at 280 nm; . . .) and, every second fraction, for palmitoyl-CoA oxidase (– – –) and THCCox (—) activities. Gradient A to B (see Materials and methods) was monitored by measuring the conductance; B to C (see Materials and methods) by measuring the refraction. The open bar denotes fractions that were pooled and loaded on to a hydroxyapatite column. Abbreviations: FT, flow-through fraction; W, wash. (**b**) Proteins, present in 18.75 μl of the indicated fraction, were analysed by SDS/gel electrophoresis and stained with Coomassie Blue. Arrows indicate the position of the subunits of the inducible ACox-I, while the arrowhead points to the position of the 68.6 kDa subunit of THCCox. [LMW, low-molecular-mass standards corresponding to 94, 67, 43, 30, 20.1 and 14.4 kDa (from top to bottom)]. Lanes a–d: (a), proteins present in 3.75 μl of the partially purified oxidase preparation; (b), in 7.5 μl of the partially purified oxidase preparation after dialysis against the equilibration buffer of the butyl-Sepharose column; (c), in 15 μl of pooled fractions of the butyl-Sepharose column; (d), in 45 μl of the flow-through fraction of the phenyl-Sepharose column.

determination of the oxidase activity which is based on the measurement of hydrogen peroxide production rates [15]. Therefore, after determining the maximum amount of thiol protector which could be tolerated in the assay, 0.5 mM DTT was added to all buffers just before use (except for the Zn-column, since DTT forms an adduct with bivalent cations [16]), while the

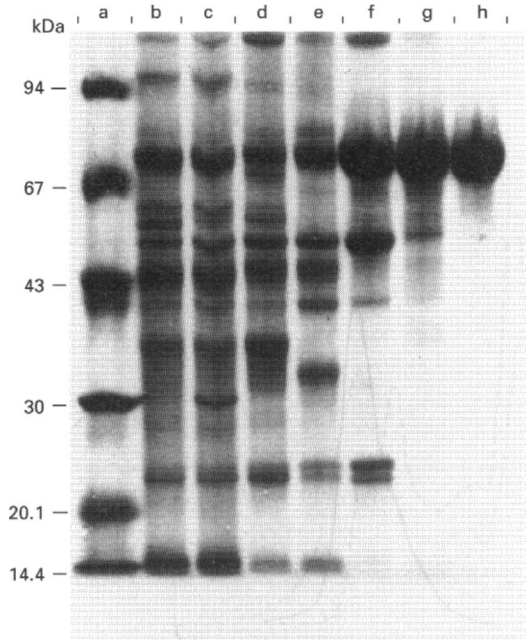

Figure 2 Electropherogram of preparations of each purification step of rat liver THCCox

Lanes b to h illustrate the enrichment of THCCox during the different chromatographic steps, each lane contains 5 μg of protein (based on Lowry quantification with serum albumin as standard), as stained by Coomassie Blue. Key to lanes: b, partially purified oxidase preparation of rat liver; c, soluble proteins after dialysis of the partially purified oxidase preparation against equilibration buffer of the butyl-Sepharose column; d, pooled fractions from the butyl-Sepharose column; e, pooled fractions from the phenyl-Sepharose column; f, pooled fractions from the hydroxyapatite column; g, pooled fractions from the Zn-Sepharose column; h, peak fraction from the gel-filtration column. Lane a contains low-molecular-mass standards, the molecular mass of which is indicated at the left-hand side.

amount of starting material was increased in order to allow for a 20-fold dilution of the column eluates before running the assay. In the presence of DTT, THCCox was very stable in high concentrations of ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 2,3-butanediol and 1,3-butanediol (40–60 %, v/v). As observed before with ethylene glycol [6], these diols, with the exception of 1,3-butanediol, also activated the enzyme with a maximum effect at a concentration around 40 % (v/v). The longer diol compound 1,2-pentanediol caused inactivation. Among the different diol-compounds tested, 1,2-propanediol turned out to be optimal with regard to the stabilization and elution of THCCox from phenyl-Sepharose columns. A second peak of palmitoyl-CoA oxidase was eluted just ahead of THCCox (Figure 1a). It is tempting to speculate that this activity is due to type-II acyl-CoA oxidase, since the column fractions contained the 50 and 23 kDa subunits of palmitoyl-CoA oxidase, as revealed by SDS/gel electrophoresis (Figure 1b) and immunoblotting (results not shown). The apparent higher hydrophobicity of the oxidase would also be consistent with the deduced amino acid sequence of the type-II acyl-CoA oxidase [12]. So far the presence of this oxidase has not been demonstrated in liver or other tissues.

The following step consisted of hydroxyapatite chromatography, being the easiest way to concentrate the enzyme and, at the same time, to remove the organic modifier. In order to prevent contamination of the final product with a 45 kDa polypeptide, which also bound to the Zn-column and eluted only slightly later than THCCox on gel filtration, a step gradient was employed to elute the hydroxyapatite column (results not shown).

A serious obstacle was the total removal of ACox-I (and/or other palmitoyl-CoA oxidases, see above). This enzyme is more abundant than THCCox, is very stable and seems to possess a higher catalytic activity. Moreover, it has almost the same native molecular mass (eluting just ahead of THCCox on gel filtration), and one of its subunits has the same size as the THCCox subunit. The major portion of ACox-I was removed during phenyl-Sepharose chromatography (see Figure 1). The enzyme(s) present in the second palmitoyl-CoA oxidase peak could not be removed by hydroxyapatite, but were effectively separated from THCCox by metal-affinity chromatography. Whereas both palmitoyl-CoA oxidase and THCCox bound to Cu columns, palmitoyl-CoA oxidase was not retained on Zn columns (compare lanes 5 and 6 of Figure 2).

Table 1 Purification of THCCox from rat liver

Starting from 240 g of rat liver, THCCox was purified to homogeneity as described in the Materials and methods section. At each step, a small aliquot was removed and analysed for protein and oxidase activities with palmitoyl-CoA and THC-CoA as substrates. Oxidase activities are expressed in nmol/min (m-unit). In order to lower the interference of DTT (present in all solutions) with the oxidase assays, fractions were diluted at least 20-fold before analysis. Abbreviation: N.D., not detectable.

Purification stage	Volume (ml)	Protein (mg)	Palmitoyl-CoA oxidase activity (m-units)	THCCox activity (m-units)	THCCox specific activity (m-units/mg of protein)	Yield (%)
Homogenate	1200	51242	192000	27800	0.54	100
100000 *g* pellet	600	34757	148350	21680	0.62	78
Partially purified preparation	240	269	51200	5084	18.9	26.8
Start of butyl column	211	168	33400	3496	20.8	12.6
Start of phenyl column	247	60.4	10240	2247	37.2	8.1
Start of hydroxyapatite column	100	15.0	2070	1835	46.0	6.6
Start of Zn-affinity column	1.4	6.2	868*	762*	123*	2.7
Start of gel-filtration column	0.75	1.8	N.D.	384	211	1.4
Peak of gel-filtration column	2.0	0.53	N.D.	125	236	0.45

* Although the recovery of the acyl-CoA oxidase from the hydroxyapatite cartridge was good (80–90%), considerable activity was lost during the subsequent concentration step before loading on the Zn-column.

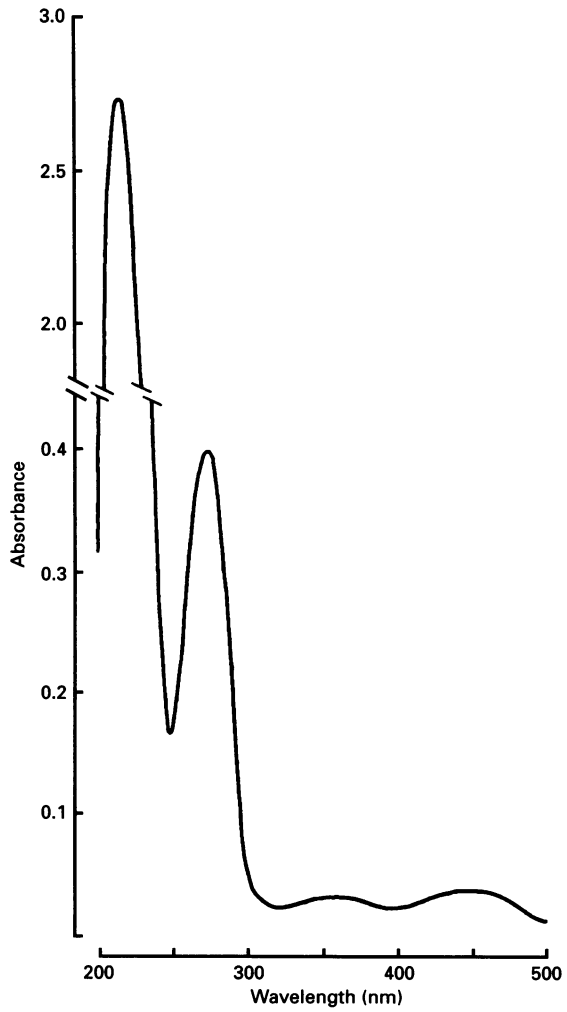

Figure 3 Spectrum of purified THCCox

The absorbance spectrum of purified THCCox (267 μg Lowry protein/ml), dissolved in 0.2 M potassium phosphate buffer, pH 7.5, 0.5 mM DTT, 5 μM FAD (gel-filtration buffer), was determined at the indicated wavelengths in a Kontron 860 u.v.-spectrophotometer and corrected for the absorbance due to the buffer (added to the reference cell). Between 210 and 240 nm, the spectrum was recalculated after scanning a 5-fold diluted enzyme preparation.

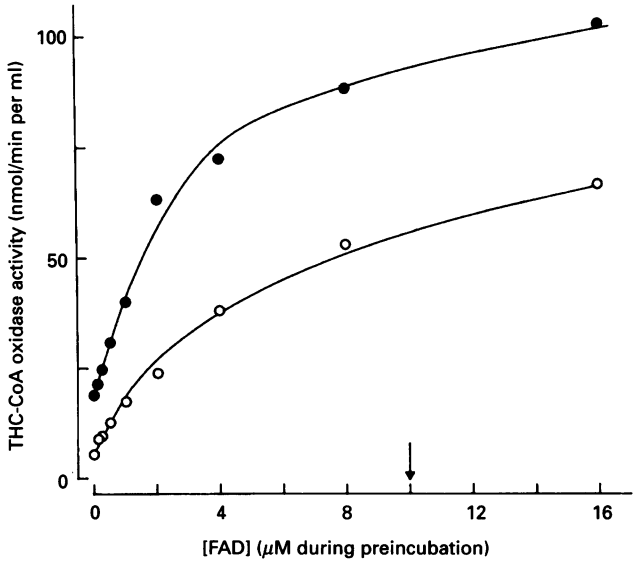

Figure 4 Dependency of THCCox on FAD

Partially purified THCCox was dialysed for 3 days against 200 vol. of 10 mM potassium phosphate buffer, pH 7.5, 0.5 M $(NH_4)_2SO_4$ (○) or 1 M potassium phosphate buffer, pH 7.5 (●). Buffers were changed daily. After dialysis, the enzyme solutions were diluted 50-fold in 10 mM potassium phosphate, pH 7.5, 20% (w/v) glycerol, and an aliquot of 20 μl was mixed with 5 μl of an FAD solution, resulting in the indicated concentrations. After incubation on ice for 10 min, reactions were started by adding 100 μl of reaction mixture. The arrow in the abscissa indicates the concentration of FAD routinely used. Although not resulting in maximal activity, we adhere to this concentration since higher amounts of FAD result in higher background fluorescence values.

In a final step, THCCox which was eluted from the Zn column was submitted to gel filtration (Superdex 200 matrix). The absorbance at 280 nm of the eluate showed only one major homogenous peak coinciding with the enzyme activity and eluting as a 120 kDa protein. This is consistent with a previous value of 139 kDa determined on a Ultrogel AcA 44 matrix [6]. Analysis of the most active fractions by SDS/gel electrophoresis revealed only one major band, the molecular mass of which was calculated to be 68590 ± 620 Da (mean ± S.E.M.; $n = 5$) (Figure 2). After subjecting purified THCCox to native gel electrophoresis, a single band with a mass of 174.8 ± 5.2 kDa (mean ± S.E.M.; $n = 4$) was obtained (results not shown). Starting from 240 g of rat liver, approx. 500 μg of pure protein could be recovered in the peak fraction of the gel filtration (yield 0.45%; Table 1). The interference of DTT with the enzyme assay (see above), the activation of the enzyme by high concentrations of polyols, and the lability of the enzyme prevent an accurate calculation of the degree of purification, but the purification factor must exceed 500 (Table 1).

The enzyme, purified in the presence of FAD, showed a typical flavoprotein spectrum (Figure 3). Absorbance maxima were seen at 447 and 360 nm and absorbance ratios (normalized to the absorbance at 447 nm) were 11.7, 0.85 and 1.0 at 277, 360 and 447 nm respectively. Assuming an absorption coefficient of 11 300 at 450 nm, we estimated the FAD content at 11.27 nmol/mg of protein (based on Lowry quantification with serum albumin as standard). This would result in 0.77 nmol of FAD/68.6 kDa subunit. Tightly protein-bound FAD possesses a higher absorption coefficient (see [9]), but in contrast with PCox, the cofactor was very loosely bound, as demonstrated by dialysis experiments (see Figure 4). Therefore, THCCox most likely contains two FAD equivalents per dimer.

As described before [8], the enzyme acts not only on THC-CoA, but also on 2-methyl-branched fatty acyl-CoA esters like pristanoyl-CoA, 2-methylpalmitoyl-CoA and 2-methylhexanoyl-CoA. A peculiar feature of the enzyme is its striking dependency on albumin with all these substrates. The recognition of the length and the position of the branch on the CoA-ester appeared to be tightly controlled. Only one of the isomeric forms of 2-methylhexanoyl-CoA was recognized, while the CoA-esters of the straight short- and long-chain acids (hexanoic acid and palmitic acid) or of other branched-chain acids such as 3-methylheptadecanoic acid, 2-ethylhexanoic acid, and 2-propylpentanoic acid were not desaturated (P. P. Van Veldhoven, S. Asselberghs, H. J. Eyssen and G. P. Mannaerts, unpublished work).

Immunologically, rat liver THCCox resembles most the human

BRCACox as revealed by cross-reactivity of the respective antibodies with the purified oxidases. Antibodies against the 70 kDa subunit of rat PCox and the 23 kDa subunit of rat ACox did not appear to recognize THCCox. A weak response was observed with the antibody against the 50 kDa subunit of rat ACox. An antiserum against the 68.6 kDa subunit of THCCox did not react with rat PCox and the 23 kDa subunit of ACox, while a weak cross-reactivity was observed with the 50 kDa and the non-cleaved 70 kDa subunit of rat ACox (results not shown).

In addition to rat and human liver, THCCox activity was also found in liver of other mammals (mouse, golden hamster, guinea pig, dog, rabbit), of chicken and of the frog *Rana esculenta*. Activities were in the same range as in the rat, except for rabbit, which displayed an almost 3-fold higher activity. Whether THC-CoA is desaturated in the other species by a specific oxidase, as is the case in rat, or by a BRCACox, as is the case in man, is not known. The fact that an antibody directed against rat liver PCox does not reveal immunoreactivity in these livers [9], points to the latter possibility. Since anti-(rat THCCox) cross-reacts with the human BRCACox, the definitive answer awaits the purification of these oxidases in the other species.

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