Hepatic β-Oxidation of 3-Phenylpropionic Acid and the Stereospecific Dehydration of (R)- and (S)-3-Hydroxy-3-phenylpropionyl-CoA by Different Enoyl-CoA Hydratases†

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ABSTRACT: The hepatic β-oxidation of 3-phenylpropionic acid (PPA) was studied by the use of subcellular fractions and purified enzymes with the aim of characterizing intermediates and the subcellular location of this pathway. Respiration measurements with coupled rat liver mitochondria indicate that PPA is efficiently metabolized by mitochondrial β-oxidation. In contrast, the peroxisomal β-oxidation of this compound is at best a very slow process, as evidenced by the low activity of peroxisomal acyl-CoA oxidase toward 3-phenylpropionyl-CoA. In mitochondria, 3-phenylpropionyl-CoA is effectively dehydrogenated to cinnamoyl-CoA, which is only slowly converted to benzoylacetyl-CoA due to the unfavorable equilibrium of the hydration of cinnamoyl-CoA to 3-hydroxy-3-phenylpropionyl-CoA. Benzoylacetyl-CoA is a substrate of 3-ketoacyl-CoA thiolase. The dehydration of 3-hydroxy-3-phenylpropionyl-CoA to cinnamoyl-CoA forms the basis for a sensitive and stereospecific assay of enoyl-CoA hydratases. The progress of this reaction, which proceeds to near completion, can be measured spectrophotometrically at 308 nm. Soluble mitochondrial and peroxisomal enoyl-CoA hydratases only act on the (R,L) isomer, whereas the peroxisomal D-3-hydroxyacyl-CoA dehydratase is specific for the (S,D) isomer. Both substrates can be easily prepared from the commercially available enantiomeric acids. It is concluded that PPA, a key compound in Knoop's classical study that led him to formulate the principle of β-oxidation, is overwhelmingly, if not completely, degraded by mitochondrial β-oxidation.

3-Phenylpropionic acid (PPA[1]) was one of the ω-phenyl fatty acids used by Knoop (1904) in his classical studies that led him to propose the mechanism of β-oxidation for the degradation of fatty acids. Knoop observed that dogs that had been fed PPA excreted hippuric acid in their urine, the glycine conjugate of benzoic acid. Subsequently, Dakin (1909) succeeded in similar experiments to identify the glycine conjugates of cinnamic acid, 3-hydroxy-3-phenylpropionic acid, and benzoylacetic acid in addition to hippuric acid. The recognition that animal cells contain two β-oxidation systems, one in mitochondria and one in peroxisomes (Lazarow & de Duve, 1976), raised the question as to which of the two systems might be responsible for the degradation of ω-phenyl fatty acids. This question was addressed by Yamada et al. (1987), who studied the hepatic β-oxidation of 4-phenylbutyric acid (PBA) and its longer chain homologues. They concluded that peroxisomes make a major contribution to the hepatic β-oxidation of ω-phenyl fatty acids. Not included in their study was PPA, which recently was found to accumulate in patients with a deficiency of medium-chain acyl-CoA dehydrogenase (MCAD) (Rumsberg et al., 1986; Duran et al., 1986). This observation would suggest that PPA may be metabolized in mitochondria where MCAD is located (Schulz, 1991). The dehydrogenation of PP-CoA by MCAD has been demonstrated (Rinaldo et al., 1990) and has been used to design a specific assay of this enzyme on the basis of the spectrophotometric quantitation of the reaction product, cinnamoyl-CoA (Yao & Schulz, 1993).

This study was undertaken with the aim of providing a detailed understanding of the hepatic β-oxidation of PPA. An additional reason for this study was the availability of (R)- and (S)-3-hydroxy-3-phenylpropionic acids, which were used to study the stereospecificities of several known enoyl-CoA hydratases and to develop specific assays for the D- and L-specific isoforms of these enzymes.

EXPERIMENTAL PROCEDURES

Materials. (R)-L- and (S)-D-3-hydroxy-3-phenylpropionic acids were purchased from Eastman Kodak. Sigma was the source of CoASH, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, dodecanoyl-CoA, hexadecanoyl-CoA, Nycodenz, defatted BSA, pig heart L-3-hydroxyacyl-CoA dehydrogenase (HAD) (EC 1.1.1.35), L-lactate dehydrogenase (LDH) (EC 1.1.1.27), and all standard biochemicals. Acyl-CoA oxidase from *Arthrobacter* species was purchased from Boehringer Mannheim. Hydrocinnamic acid (PPA), *trans*-cinnamic acid, and 4-phenylbutyric acid (PBA) were bought from Aldrich. Bio-Rad was the supplier of the dye reagent for protein assays. Coenzyme A thioesters of D- and L-3-hydroxy-3-phenylpropionic acids, *trans*-cinnamic acid, PPA, and PBA were synthesized by the mixed anhydride procedure as detailed by Fong and Schulz (1981). LOPP-CoA, DOPP-CoA, and

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; CoASH, coenzyme A; DEHP, bis(2-ethylhexyl)phthalate; DOPP-CoA, D-3-hydroxy-3-phenylpropionyl-CoA; EDTA, ethylenediaminetetraacetate; FAD, flavin adenine dinucleotide, oxidized form; HAD, L-3-hydroxyacyl-CoA dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, L-lactate dehydrogenase; LOPP-CoA, L-3-hydroxy-3-phenylpropionyl-CoA; MCAD, medium-chain acyl-CoA dehydrogenase; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; PBA, 4-phenylbutyric acid; PB-CoA, 4-phenylbutyryl-CoA; PMS, phenazine methosulfate; PP-CoA, 3-phenylpropionyl-CoA; PPA, 3-phenylpropionic acid; SD, standard deviation; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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2-*trans*-cinnamoyl-CoA were further purified by HPLC. The concentrations of all acyl-CoA solutions were determined by quantifying free CoASH by the Ellman procedure (Ellman, 1959) after completely cleaving the thioester bond with 1 M hydroxylamine at pH 7 (Fong & Schulz, 1981). The following enzymes were purified by the indicated procedures: enoyl-CoA hydratase or crotonase (EC 4.2.1.17) from bovine liver (Steinman & Hill, 1975); 3-ketoacyl-CoA thiolase (EC 2.3.1.16) from pig heart (Schulz & Staack, 1981); D-3-hydroxyacyl-CoA dehydratase from rat liver (Li et al., 1990); fatty acid oxidation complex from *Escherichia coli* (Yang et al., 1988); peroxisomal trifunctional enzyme, which exhibits enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and Δ^3, Δ^2-enoyl-CoA isomerase activities, from rat liver (Osumi & Hashimoto, 1979); and acyl-CoA oxidase from rat liver (Osumi et al., 1980). Male Sprague Dawley rats (250 g) were obtained from Taconic Farms (Germantown, NY) and kept on a diet of either normal rodent chow or rodent chow containing 2% (w/w) DEHP.

Preparation of Mitochondria, Peroxisomes, and a Soluble Extract of Mitochondria. Liver mitochondria were prepared according to the general procedure outlined by Nedergard and Cannon (1979). This fraction contained 14.6% of the protein present in a liver homogenate and 37% of the mitochondrial marker enzyme L-malate dehydrogenase. A rat liver light mitochondrial fraction enriched with respect to peroxisomes was isolated as described by Appelmans et al. (1955). This fraction contained 10% of the protein present in a liver homogenate and 54% of the peroxisomal marker enzyme catalase. Purified rat liver peroxisomes were obtained by centrifugation of a light mitochondrial fraction on a Nycodenz density gradient as described (Li et al., 1990). A soluble extract of rat liver mitochondria was prepared by sonicating mitochondria suspended in 0.1 M HEPES (pH 7.4) and 0.1 M KCl eight times for 5 s each at 0 °C with a Heat Systems sonifier (Model W-385) equipped with a microtip. The resulting mixture was centrifuged at 105000*g* for 1 h. The supernatant, after passage through Sephadex G-25, served as a source of soluble mitochondrial enzymes.

Oxygen Uptake Measurements. Oxygen uptake by rat liver mitochondria (RCR ≥ 5) was measured polarographically at 25 °C with a Clark oxygen electrode attached to a Gilson oxygraph. The incubation mixture contained mitochondria (2 mg of protein/2 mL) in 20 mM Tris-HCl (pH 7.4), 4 mM KP_i, 0.1 M KCl, 4 mM $MgCl_2$, BSA (1 mg/mL), 0.5 mM L-malate, 13 mM L-carnitine, 1 mM ATP, 0.2 mM CoASH, and 1 mM ADP when either 0.1 mM PPA, 0.1 mM PBA, or 0.1 mM octanoic acid served as substrate. In separate experiments, where either 0.1 mM PPA, 0.1 mM PBA, 0.1 mM octanoic acid, 50 μM PP-CoA, 50 μM PB-CoA, or 50 μM octanoyl-CoA served as substrate, the incubation mixture contained mitochondria (2 mg of protein in 2 mL) in 20 mM Tris-HCl (pH 7.4), 4 mM KP_i, 0.1 M KCl, 4 mM $MgCl_2$, BSA (1 mg/mL), 0.5 mM L-malate, and 1 mM ADP. Substrate-dependent respiration of mitochondria was initiated by the addition of ADP and substrate.

Protein and Enzyme Assays. Protein concentrations were determined by the dye binding assay as described by Bradford (1976) with BSA as standard. Enoyl-CoA hydratase was assayed by measuring either the decrease in absorbance at 263 nm due to the hydration of crotonyl-CoA (Fong & Schulz, 1981) or the increase in absorbance at 308 nm due to the dehydration of LOPP-CoA to cinnamoyl-CoA. When D-3-hydroxyacyl-CoA dehydratase was assayed, DOPP-CoA served as a substrate. A standard assay mixture contained 0.2 M KP_i (pH 8), 70 μM LOPP-CoA or 70 μM DOPP-CoA, and enzyme to give an absorbance change of at least 0.04 min^{-1}. An extinction coefficient of 26 500 M^{-1} cm^{-1} was used to calculate rates of cinnamoyl-CoA formation (Yao & Schulz, 1993). Acyl-CoA oxidase was assayed by the method of Small et al. (1985) or by the method used to assay MCAD (Yao & Schulz, 1993) except that PMS was deleted. All enzyme assays were performed at 25 °C with a Gilford recording spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute.

Metabolic Studies. The β-oxidation of cinnamoyl-CoA or 4-phenylcrotonyl-CoA was measured spectrophotometrically by determining the formation of NADH at 360 nm. An assay mixture contained 0.2 M KP_i (pH 8), 50 μM cinnamoyl-CoA, 1 mM NAD^+, 0.3 mM CoASH, BSA (0.2 mg/mL), 3-ketoacyl-CoA thiolase (0.2 unit), and either rat liver mitochondria, rat liver peroxisomes, peroxisomal trifunctional enzyme, or a 1:1 (w/w) mixture of enoyl-CoA hydratase plus HAD. The amount of enzyme was adjusted to obtain a ΔA/min value of approximately 0.04. When mitochondria were used as an enzyme source, Triton X-100 (0.1%, w/v) was included in the assay mixture. 4-Phenylcrotonyl-CoA was generated *in situ* from PB-CoA by including acyl-CoA oxidase (1 unit) and 0.1 mM FAD in the assay mixture. When the dehydrogenation of LOPP-CoA was measured, the assay mixture contained 0.2 M KP_i (pH 8.0), 50 μM LOPP-CoA, 1 mM NAD^+, 0.3 mM CoASH, 3-ketoacyl-CoA thiolase (0.2 unit), and HAD, to obtain an absorbance change of approximately 0.04 min^{-1} at 360 nm. Rates of NADH formation were calculated by using an extinction coefficient of 4087 M^{-1} cm^{-1}.

Purification and Analysis of Acyl-CoA's by HPLC. Prior to HPLC, the pH of the sample was first brought to 1 with concentrated HCl and then adjusted to 5 with 4 N KOH. Precipitated protein was removed by centrifugation at 13000*g* for 3 min, and the resultant supernatant was cleared by passage through a 0.22 μm (pore size) filter before being applied to a μBondapak C_{18} reverse-phase column (30 cm × 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H_2O (9:1, v/v) content of the 25 mM ammonium phosphate (pH 5.5) elution buffer from either 10% or 20% to 50% in 15–20 min at a flow rate of 2 mL/min. When acyl-CoA's were purified, acetonitrile was removed by evaporation under reduced pressure, and the resultant solution was applied to a Waters C_{18} Sep-pak column. Bound acyl-CoA's were eluted with methanol/water (10:1, v/v) and further concentrated by evaporating methanol under a stream of N_2.

RESULTS

Mitochondrial Metabolism of PPA. The capacity of rat liver mitochondria to oxidize PPA was evaluated by measuring rates of respiration supported by PPA. For comparison, rates with octanoic acid and PBA as substrates were determined. The results shown in Figure 1 demonstrate that PPA is a respiratory substrate in rat liver mitochondria, as are octanoic acid and PBA. The rates obtained with only the acids as substrates were higher than the rates observed either with their CoA derivatives or with the acids incubated in the presence of CoASH, ATP, and L-carnitine. In the latter situation, the acids are most likely converted to their CoA derivatives extramitochondrially. The presence of L-carnitine had a slight stimulatory effect only when octanoic acid served

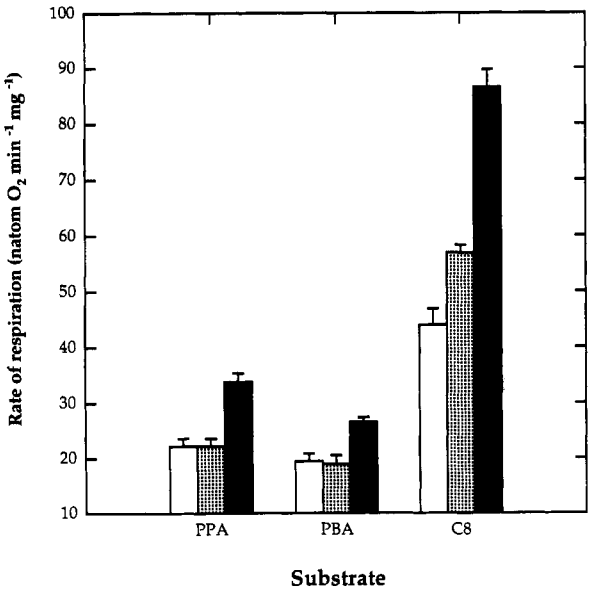

FIGURE 1: Respiration measurements with coupled rat liver mitochondria. Substrates were PPA, PBA, or octanoic acid (C_8) in the form of their CoA thioesters (open bars), free acids plus ATP, CoASH, $MgCl_2$, and L-carnitine (dotted bars), or free acids alone (solid bars). Experimental details are given under Experimental Procedures. Values are means ± SD based on three measurements.

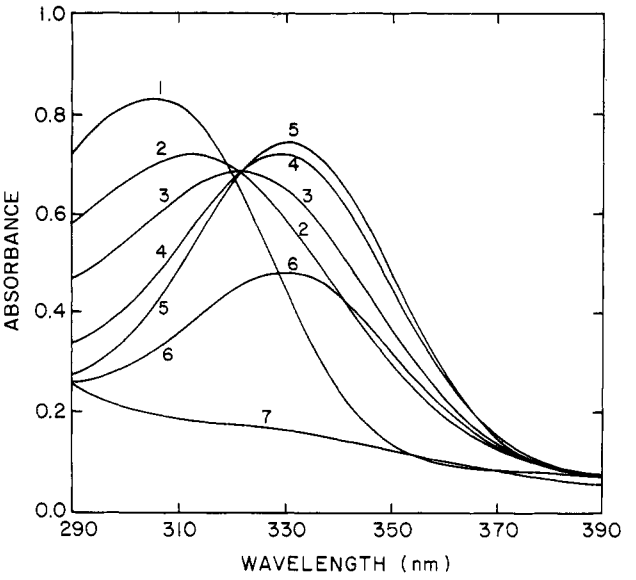

FIGURE 2: Spectral changes associated with the hydration of *trans*-cinnamoyl-CoA by enoyl-CoA hydratase (crotonase) and dehydrogenation of the resultant reaction product by HAD: spectrum 1, before the addition of enzymes; spectra 2–5, 30 s, 3 min, 10 min, and 20 min, respectively, after the addition of crotonase plus HAD; spectrum 6, after the addition of EDTA in excess of $MgCl_2$ to the reaction mixture corresponding to spectrum 5; spectrum 7, after the addition of 3-ketoacyl-CoA thiolase and CoASH to the sample corresponding to spectrum 6. For experimental details, see the Experimental Procedures.

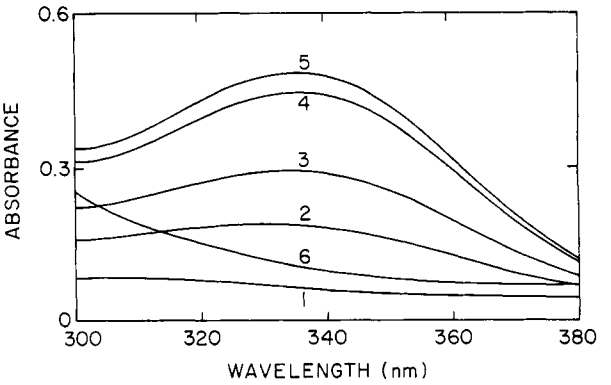

FIGURE 3: Formation of NADH associated with the dehydrogenation of LOPP-CoA by HAD: spectrum 1, before the addition of HAD; spectra 2–5, 1, 3, 8, and 15 min after the addition of HAD, respectively; spectrum 6, after the addition of LDH plus pyruvate to the reaction mixture corresponding to spectrum 5. For experimental details, see the Experimental Procedures.

as a substrate. Since the highest rates of respiration were observed with the free acids under conditions where they could not be activated extramitochondrially, PPA and PBA, like octanoic acid, seem to be converted to their CoA thioesters in the mitochondrial matrix.

The degradation of PP-CoA by β-oxidation was investigated by use of purified enzymes. Since the dehydrogenation of PP-CoA to cinnamoyl-CoA was previously shown to be catalyzed by MCAD (Rinaldo et al., 1990; Yao & Schulz, 1993), the metabolism of cinnamoyl-CoA was studied. When cinnamoyl-CoA was incubated with crotonase, its absorbance at 308 nm remained virtually unchanged (data not shown). This observation suggests that either cinnamoyl-CoA is not acted upon by crotonase or the equilibrium of the hydration reaction is far to the side of cinnamoyl-CoA. The latter reason is correct, as demonstrated by the disappearance of the chromophore at 308 nm when the hydration of cinnamoyl-CoA was coupled to the dehydrogenation of the hydration product (see Figure 2). In this experiment, cinnamoyl-CoA was incubated with crotonase and HAD in the presence of NAD^+, $MgCl_2$, and LDH plus pyruvate to reoxidize NADH. The time-dependent disappearance of the chromophore of cinnamoyl-CoA at 308 nm and the simultaneous emergence of a new chromophore centered around 330 nm are shown in Figure 2, spectra 1–5. The new chromophore at 330 nm was attributed to the Mg^{2+}–enolate complex of benzoylacetyl-CoA, which would be formed if cinnamoyl-CoA is hydrated by enoyl-CoA hydratase and the resultant 3-hydroxy-3-phenylpropionyl-CoA is dehydrogenated by HAD.

In agreement with this structural assignment is the observed 50% decrease in absorbance at 330 nm upon the addition of EDTA in excess of $MgCl_2$ to the incubation mixture (see Figure 2, curves 5 and 6). When 3-ketoacyl-CoA thiolase and CoASH also were added, the absorbance at 330 nm declined to the base-line level (see Figure 2, curves 6 and 7). These spectral changes support the conclusions that benzoylacetyl-CoA was formed from cinnamoyl-CoA by enoyl-CoA hydratase and HAD in the presence of NAD^+ and that benzoylacetyl-CoA is cleaved by 3-ketoacyl-CoA thiolase in the presence of CoASH to benzoyl-CoA and acetyl-CoA. The formation of acetyl-CoA was demonstrated by a further reduction of NAD^+ upon the addition of L-malate, citrate synthase, and malate dehydrogenase to the incubation mixture (data not shown).

Finally, the reduction of NAD^+ was demonstrated when LOPP-CoA, the presumed intermediate in the β-oxidation of cinnamoyl-CoA, was incubated with HAD and NAD^+ in the presence of 3-ketoacyl-CoA thiolase and CoASH to cleave benzoylacetyl-CoA and to drive the reaction to completion (see Figure 3). Together these experiments establish that cinnamoyl-CoA is degraded by β-oxidation to benzoyl-CoA and acetyl-CoA. Rates of cinnamoyl-CoA β-oxidation were determined with isolated rat liver mitochondria and purified enzymes. When mitochondria served as an enzyme source, a rate of 31 nmol min^{-1} (mg of protein)$^{-1}$ was observed with 50 μM cinnamoyl-CoA. At the same substrate concentration, a rate of 2 μmol min^{-1} (mg of protein)$^{-1}$ was measured with

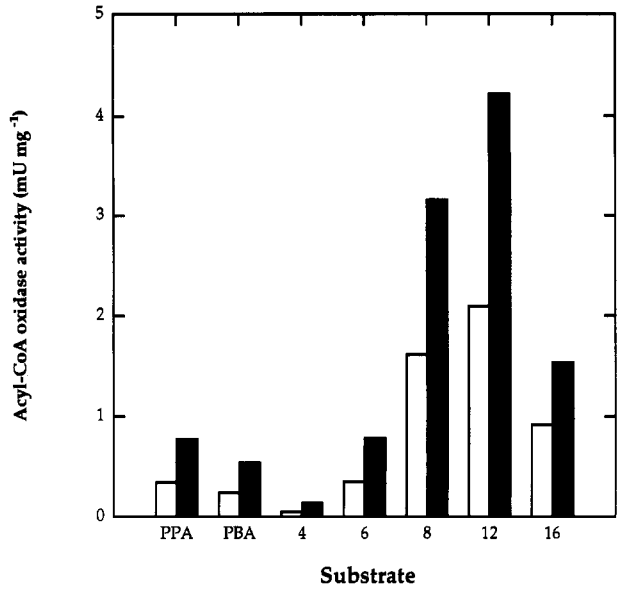

FIGURE 4: Acyl-CoA oxidase activity measured with various substrates in rat liver L-fractions from normal rats (open bars) and from rats fed DEHP (solid bars). Abbreviations: 4–16, butyryl-CoA through palmitoyl-CoA. For experimental details, see the Experimental Procedures.

a 1:1 (w/w) mixture of purified crotonase and HAD. A 1:1 (w/w) mixture of the two enzymes was chosen, because this ratio is similar to the relative abundance of these enzymes in the mitochondrial matrix. For comparison, the mitochondrial β-oxidation of 4-phenylcrotonyl-CoA, generated *in situ* from 50 μM PB-CoA by acyl-CoA oxidase, was measured and found to proceed at a rate of 114 nmol min^{-1} (mg of protein)$^{-1}$.

Peroxisomal Metabolism of PP-CoA. The degradation of PP-CoA by peroxisomal β-oxidation was studied by first measuring its dehydrogenation by acyl-CoA oxidase present in a rat liver fraction enriched with respect to peroxisomes (L-fraction). For comparison, rates with straight-chain acyl-CoA's and PB-CoA were determined (see Figure 4). The specific activity observed with PP-CoA as a substrate was almost equal to the activity detected with hexanoyl-CoA, which is a poor substrate of rat acyl-CoA oxidase (Osumi & Hashimoto, 1978). However, PP-CoA was a better substrate of this enzyme than either PB-CoA or butyryl-CoA. When an L-fraction from rats fed DEHP was used as a source of acyl-CoA oxidase, rates with all substrates, including PP-CoA, were elevated. This observation suggests that PP-CoA is acted upon by the inducible acyl-CoA oxidase (Scheppers et al., 1990). The product of PP-CoA oxidation by acyl-CoA oxidase was identified by HPLC as cinnamoyl-CoA (data not shown). Accurate rates of PP-CoA oxidation by acyl-CoA oxidase were determined by measuring the formation of cinnamoyl-CoA at 308 nm (Yao & Schulz, 1993). With an L-fraction from rat liver and 50 μM PP-CoA, a rate close to 1 nmol min^{-1} (mg of protein)$^{-1}$ was observed. The peroxisomal metabolism of cinnamoyl-CoA was evaluated by measuring the formation of NADH in a system that contained 50 μM cinnamoyl-CoA, NAD^+, CoASH, 3-ketoacyl-CoA thiolase, and either purified peroxisomes or peroxisomal trifunctional enzyme. Rates of 22 and 120 nmol min^{-1} (mg of protein)$^{-1}$ were obtained with peroxisomes and purified trifunctional enzyme, respectively. For comparison, 50 μM 4-phenylcrotonyl-CoA, generated *in situ* from PB-CoA and acyl-CoA oxidase, was metabolized almost 10 times more rapidly, as evidenced by a rate of NADH formation of 205 nmol min^{-1} (mg of protein)$^{-1}$.

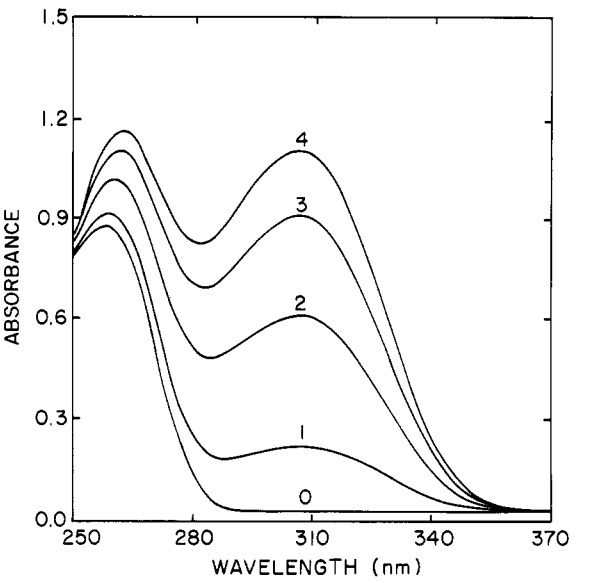

FIGURE 5: Spectral changes associated with the dehydration of 3-hydroxy-3-phenylpropionyl-CoA to *trans*-cinnamoyl-CoA. Substrates were LOPP-CoA and DOPP-CoA with enoyl-CoA hydratase and D-3-hydroxyacyl-CoA dehydratase, respectively. Spectrum 0 was recorded before the addition of enzyme. Spectra 1–4 were recorded 1, 5, 10, and 20 min after the addition of enzyme, respectively. For experimental details, see the Experimental Procedures.

Dehydration of DOPP-CoA and LOPP-CoA by Various Enoyl-CoA Hydratases. Since 3-hydroxy-3-phenylpropionyl-CoA is an intermediate in the β-oxidation of PPA, and since both of its enantiomers are commercially available, the dehydration of DOPP-CoA and LOPP-CoA by several purified enoyl-CoA hydratases was studied. Shown in Figure 5 is the time-dependent change in the UV absorbance that was observed when either LOPP-CoA was incubated with enoyl-CoA hydratase (crotonase) or DOPP-CoA was reacted with D-3-hydroxyacyl-CoA dehydratase. The emergence of an absorbance maximum centered around 308 nm is indicative of the formation of cinnamoyl-CoA, the expected product. The formation of cinnamoyl-CoA was demonstrated by HPLC (Figure 6). When LOPP-CoA (see Figure 6A) was incubated with crotonase or DOPP-CoA (see Figure 6A) was reacted with D-3-hydroxyacyl-CoA dehydratase, cinnamoyl-CoA was formed (see Figure 6B,C). The equilibrium of these reactions was far to the side of the dehydration product cinnamoyl-CoA. At equilibrium, 97% of LOPP-CoA or DOPP-CoA was converted to cinnamoyl-CoA. Surprisingly, crotonase did not act on DOPP-CoA, and D-3-hydroxyacyl-CoA dehydratase was not active with LOPP-CoA (see Figure 6D). Enoyl-CoA hydratases associated either with the trifunctional enzyme from rat liver peroxisomes or with the fatty acid oxidation complex from *E. coli* were found to act only on LOPP-CoA (data not shown).

Kinetic parameters (K_m, V_{max}) were determined for the dehydration of LOPP-CoA and DOPP-CoA catalyzed by several purified enoyl-CoA hydratases and compared with the kinetic parameters obtained with crotonyl-CoA as substrate (see Table 1). The K_m values determined for LOPP-CoA were equal to or lower than the K_m values obtained with crotonyl-CoA. With bovine liver crotonase and enoyl-CoA hydratase of the fatty acid oxidation complex from *E. coli*, maximal activities with LOPP-CoA were lower than those with crotonyl-CoA. However, enoyl-CoA hydratase of the peroxisomal trifunctional enzyme from rat liver was more active toward LOPP-CoA than crotonyl-CoA. Also, with D-3-hydroxyacyl-CoA dehydratase from rat liver peroxisomes,

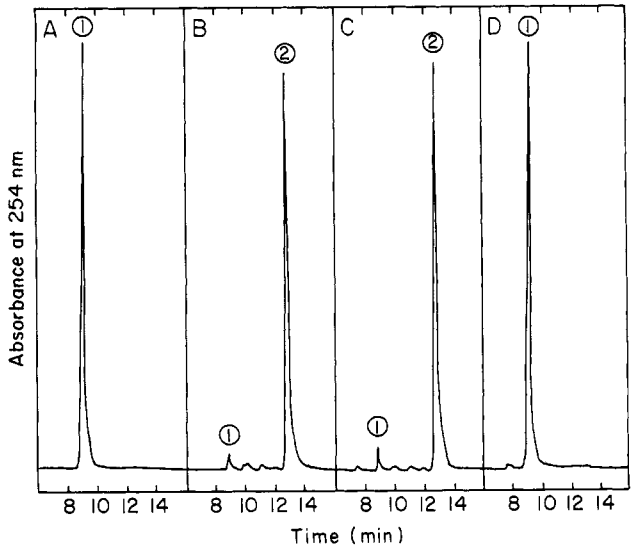

FIGURE 6: HPLC analysis of products formed by dehydration of LOPP-CoA or DOPP-CoA: (A) LOPP-CoA or DOPP-CoA; (B) LOPP plus crotonase; (C) DOPP-CoA plus D-3-hydroxyacyl-CoA dehydratase; (D) LOPP-CoA plus D-3-hydroxyacyl-CoA dehydratase or DOPP-CoA plus crotonase. Peaks were identified by use of authentic compounds: 1, LOPP-CoA or DOPP-CoA; 2, *trans*-cinnamoyl-CoA. For experimental details, see the Experimental Procedures.

Table 1: Kinetic Parameters of Several Enoyl-CoA Hydratases Determined with Crotonyl-CoA, DOPP-CoA, and LOPP-CoA

	crotonyl-CoA		LOPP-CoA		DOPP-CoA	
enzyme[a]	V_{max}[b]	K_m[b]	V_{max}[b]	K_m[b]	V_{max}[b]	K_m[b]
crotonase	4320	30	1210	30	NA[c]	
TE	367	84	569	16	NA	
FAOC	252	65	157	36	NA	
DHAD	NA		NA		142	70

[a] Crotonase, mitochondrial enoyl-CoA hydratase from bovine liver; TE, peroxisomal trifunctional enzyme from rat liver; FAOC, fatty acid oxidation complex from *E. coli*; DHAD, peroxisomal D-3-hydroxyacyl-CoA dehydratase from rat liver. [b] Units of V_{max} are μmol min^{-1} (mg of protein)$^{-1}$; units of K_m are μM. [c] NA, no activity.

DOPP-CoA was the better substrate (V_{max} = 142 units mg^{-1}; K_m = 70 μM) than was D-3-hydroxyoctanoyl-CoA (V_{max} = 67 units mg^{-1}; K_m = 71 μM), which until now was the best substrate of this enzyme (Li et al., 1990). Altogether, this kinetic evaluation revealed that LOPP-CoA and DOPP-CoA are good substrates of enoyl-CoA hydratases and D-3-hydroxyacyl-CoA dehydratase, respectively. Moreover, all hydratases and the dehydratase listed in Table 1 only dehydrate one of the two enantiomeric 3-hydroxy-3-phenylpropionyl-CoA's.

DISCUSSION

3-Phenylpropionic acid is metabolized by rat liver mitochondria, as evidenced by its support of mitochondrial respiration. The observation that PPA alone is a better respiratory substrate than is either the acid in the presence of ATP, CoASH, and L-carnitine or PP-CoA suggests that PPA is activated in the mitochondrial matrix. The conversion of PPA to its CoA thioester and the subsequent β-oxidation of PP-CoA to benzoyl-CoA and acetyl-CoA are outlined in Figure 7. The dehydrogenation of PP-CoA (Figure 7, compd 2) to cinnamoyl-CoA by MCAD (Figure 7, compd 3) has previously been demonstrated (Rinaldo et al., 1990; Yao & Schulz, 1993). This study shows that cinnamoyl-CoA can be degraded to benzoyl-CoA plus acetyl-CoA by the combined

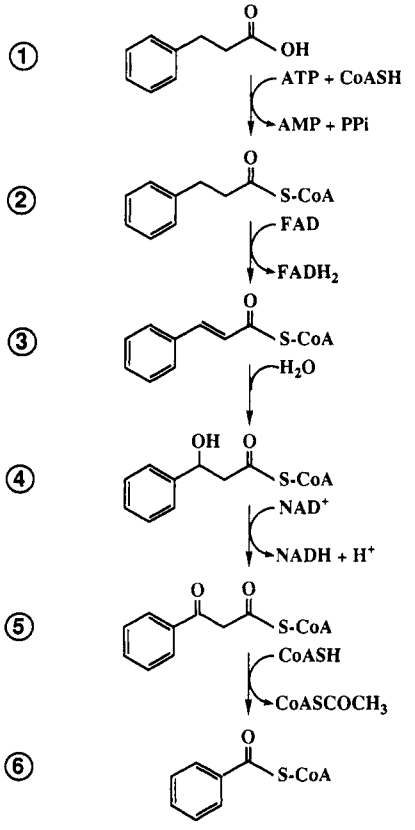

FIGURE 7: Proposed pathway of 3-phenylpropionate β-oxidation: 1, 3-phenylpropionic acid; 2, 3-phenylpropionyl-CoA; 3, *trans*-cinnamoyl-CoA; 4, 3-hydroxy-3-phenylpropionyl-CoA; 5, benzoylacetyl-CoA; 6, benzoyl-CoA.

actions of mitochondrial enoyl-CoA hydratase, HAD, and 3-ketoacyl-CoA thiolase in the presence of NAD$^+$ and CoASH (see Figure 7, compds 3–6). Since PP-CoA passes through the β-oxidation spiral once, its degradation yields 1 mol each of $FADH_2$, NADH, and acetyl-CoA. The complete oxidation of these products requires 6 oxygen atoms, and hence an observed respiration rate of 32 natom of oxygen min^{-1} (mg of protein)$^{-1}$ corresponds to a rate of substrate β-oxidation of at least 5 nmol min^{-1} (mg of protein)$^{-1}$. The rate is most likely higher, because acetyl-CoA is only oxidized to a limited extent by isolated mitochondria in the presence of added L-malate. Thus, the rate of PPA β-oxidation by rat liver mitochondria may be between 5 and 15 nmol min^{-1} (mg of protein)$^{-1}$. Rates of 68 and 31 munits mg^{-1} obtained with rat liver mitochondria for the dehydrogenation of PP-CoA to cinnamoyl-CoA and for the β-oxidation of cinnamoyl-CoA to benzoyl-CoA and acetyl-CoA, respectively, are sufficient to support a rate for PPA β-oxidation of 15 nmol min^{-1} (mg of protein)$^{-1}$. The relatively low rate of cinnamoyl-CoA β-oxidation is a consequence of the unfavorable equilibrium of the hydration of cinnamoyl-CoA, which only sustains low levels of LOPP-CoA and thereby restricts the rate of LOPP-CoA dehydrogenation.

Peroxisomes seem to have a very limited capacity for β-oxidizing PPA, as evidenced by an acyl-CoA oxidase activity of only 1 munit (mg of protein)$^{-1}$ measured with PP-CoA and a subcellular rat liver fraction enriched with respect to the peroxisomes (L-fraction). Since acyl-CoA oxidase seems to catalyze the rate-limiting step in peroxisomal β-oxidation (Reubsaet, 1988), its activity is a measure of the maximal rate of β-oxidation in peroxisomes. The peroxisomal β-oxidation of cinnamoyl-CoA is slow, but a rate of 22 nmol min^{-1} (mg of protein)$^{-1}$ for peroxisomes is faster than the oxidase-

catalyzed dehydrogenation of PP-CoA. It is interestingto note that the specific activity of purified trifunctional enzyme from rat liver peroxisomes with cinnamoyl-CoA as a substrate is only 0.12 unit mg^{-1}, compared to 2 units mg^{-1} observed with a 1:1 mixture of mitochondrial enoyl-CoA hydratase and HAD. This difference between the mitochondrial and peroxisomal enzymes in catalyzing the coupled hydration–dehydrogenation of cinnamoyl-CoA possibly reflects different rates of dehydrogenation at the very low equilibrium concentration of LOPP-CoA.

For the purpose of estimating the contributions mitochondria and peroxisomes make to the β-oxidation of PPA in rat liver, the mitochondrial rate of PPA β-oxidation based on respiration measurements was compared with the acyl-CoA oxidase activity determined with PP-CoA as a substrate. The rate of mitochondrial β-oxidation of PPA was estimated to be at least 5 and maximally 15 nmol min^{-1} (mg of protein)$^{-1}$ for a mitochondrial fraction (M-fraction) obtained by differential centrifugation of a rat liver homogenate. The acyl-CoA oxidase activity with PP-CoA was 1 nmol min^{-1} (mg of protein)$^{-1}$ for a light mitochondrial fraction (L-fraction) that is enriched with respect to peroxisomes. Since the M-fraction contains approximately 50% more protein than the L-fraction and only 37% of the mitochondrial marker enzyme malate dehydrogenase is in the M-fraction, in contrast with 54% of the peroxisomal marker enzyme catalase being present in the L-fraction, peroxisomal β-oxidation may account for at best 10% of PPA degradation, but may be only 3%. These estimates assume that mitochondria and peroxisomes have equal access to the cellular pool of PPA and that the activation of PPA does not restrict its peroxisomal β-oxidation. The conclusion of this evaluation is that peroxisomes make only a very small contribution, if any, to the hepatic β-oxidation of PPA.

The novel spectophotometric method for assaying enoyl-CoA hydratases and D-3-hydroxyacyl-CoA dehydratase with either LOPP-CoA or DOPP-CoA as substrate has several important advantages over the traditional spectrophotometric assay based on the hydration of crotonyl-CoA or other 2-enoyl-CoAs. With the new assay, the formation of product is measured at 308 nm, whereas the traditional assay is based on the disppearance of substrate at 263 nm. It is advantageous to measure product formation as compared to substrate disappearance, because substrates can also be consumed by other reactions as, for example, by hydrolysis of the thioester. The shift of the absorbance measurement from 263 to 308 nm is significant, as it avoids high background absorbances due to nucleotides, including CoA, and proteins which complicate measurements at 263 nm. A further advantage of the novel assay method is a very favorable reaction equilibrium, which is the reason that the reaction proceeds longer at a linear rate than the hydration of 2-enoyl-CoA's which reaches equilibrium when only 75% of substrate is converted to product. The sensitivity of the novel assay is greater than that of the traditional method due to the high extinction coefficient of 26 500 M^{-1} cm^{-1} at 308 nm for cinnamoyl-CoA *vs* 6700 M^{-1} cm^{-1} at 263 nm or 5100 M^{-1} cm^{-1} at 280 nm (He et al., 1992) for 2-enoyl-CoAs (Fong & Schulz, 1981).

However, none of the above arguments would be convincing if LOPP-CoA and DOPP-CoA were poor substrates of enoyl-CoA hydratases and D-3-hydroxyacyl-CoA dehydratase, respectively. Fortunately, they are good substrates, with K_m values equal to or lower than those of the preferred substrates. The dehydration of LOPP-CoA and DOPP-CoA proceeds at least at one-fourth the rate of the hydration of preferred substrates and in some instances exceeds the maximal velocities obtained with the best substrates. Finally, the assays of the enoyl-CoA hydratases with the new substrates are stereospecific. Whereas enoyl-CoA hydratases from mammalian mitochondria, peroxisomes, and *E. coli* act on both L- and D-3-hydroxyacyl-CoA's, these enzymes are only active with LOPP-CoA. Preliminary evidence indicates that the long-chain enoyl-CoA hydratase of the trifunctional β-oxidation complex from pig heart mitochondria is most active with LOPP-CoA, but exhibits some activity with DOPP-CoA.[2] However, since this enzyme is membrane-bound (Luo et al., 1993), soluble extracts from mammalian tissues only contain hydratases and dehydratases that act on either LOPP-CoA or DOPP-CoA. Hence, an assay with DOPP-CoA can be used to determine the peroxisomal D-3-hydroxyacyl-CoA dehydratase activity in the presence of both mitochondrial and peroxisomal enoyl-CoA hydratases.

In the absence of information about the binding of substrate to the active site of enoyl-CoA hydratases, it is not clear what prevents the dehydration of DOPP-CoA by enoyl-CoA hydratases. It is possible that DOPP-CoA, due to its large phenyl residue, cannot bind to the active site in a syn conformation, which is necessary to facilitate the syn elimination of the *pro-R* hydrogen of carbon atom 3 and the 3-hydroxyl group (Willadsen & Eggerer, 1975). A definite explanation of the stereochemical course of the dehydration reaction catalyzed by enoyl-CoA hydratases has to await the identification of the functional groups involved in catalysis and the resolution of the three-dimensional structure of enoyl-CoA hydratase.

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