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Mitochondrial Metabolism of Valproic Acid†

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ABSTRACT: The β-oxidation of valproic acid (2-propylpentanoic acid), an anticonvulsant drug with hepatotoxic side effects, was studied with subcellular fractions of rat liver and with purified enzymes of β-oxidation. 2-Propyl-2-pentenoyl-CoA, a presumed intermediate in the β-oxidation of valproic acid, was chemically synthesized and used to demonstrate that enoyl-CoA hydratase or crotonase catalyzes its hydration to 3-hydroxy-2-propylpentanoyl-CoA. The latter compound was not acted upon by soluble L-3-hydroxyacyl-CoA dehydrogenases from mitochondria or peroxisomes but was dehydrogenated by an NAD^+-dependent dehydrogenase associated with a mitochondrial membrane fraction. The product of the dehydrogenation, presumably 3-keto-2-propylpentanoyl-CoA, was further characterized by fast bombardment mass spectrometry. 3-Keto-2-propylpentanoyl-CoA was not cleaved thiolytically by 3-ketoacyl-CoA thiolase or a mitochondrial extract but was slowly degraded, most likely by hydrolysis. The availability of 2-propylpentanoyl-CoA (valproyl-CoA) and its β-oxidation metabolites facilitated a study of valproate metabolism in coupled rat liver mitochondria. Mitochondrial metabolites identified by high-performance liquid chromatography were 2-propylpentanoyl-CoA, 3-keto-2-propylpentanoyl-CoA, 2-propyl-2-pentenoyl-CoA, and trace amounts of 3-hydroxy-2-propylpentanoyl-CoA. It is concluded that valproic acid enters mitochondria where it is converted to 2-propylpentanoyl-CoA, dehydrogenated to 2-propyl-2-pentenoyl-CoA by 2-methyl-branched chain acyl-CoA dehydrogenase, and hydrated by enoyl-CoA hydratase to 3-hydroxy-2-propylpentanoyl-CoA. The latter compound is dehydrogenated by a novel NAD^+-specific 3-hydroxyacyl-CoA dehydrogenase to 3-keto-2-propylpentanoyl-CoA which accumulates in the mitochondrial matrix and is slowly degraded, apparently by hydrolysis.

Valproic acid (2-*n*-propylpentanoic acid) is an effective anticonvulsant drug, which is widely used in the treatment of several forms of epilepsy (Browne, 1980). In a small number of patients treatment with valproic acid causes severe liver damage that can be fatal (Nau & Löscher, 1984). This situation has prompted many studies aimed at identifying the metabolites of valproic acid in vivo, at elucidating the metabolism of valproic acid in vitro, and at assessing the effects of valproic acid on hepatic metabolism.

Metabolites of valproic acid with oxygen functions at carbon atoms 5 and 4 including dehydration products thereof are formed by microsomal ω-oxidation and (ω − 1)-oxidation, respectively (Rettie et al., 1987, 1988). Metabolites with oxygen functions at carbon atom 3 and its dehydration product were thought to be formed by β-oxidation (Matsumoto et al., 1976). However, when it was reported that 3-hydroxy-2-propylpentanoic acid can be formed by microsomal (ω − 2)-oxidation (Prickett & Baillie, 1984), the metabolism of valproic acid by β-oxidation was left in doubt. But valproic acid affects mitochondrial functions as evidenced by valproate-induced inhibition of medium-chain and long-chain fatty acid β-oxidation (Becker & Harris, 1983; Turnbull et al., 1983; Coudé et al., 1983; Bjorge & Baillie, 1985). Since valproic acid is converted in mitochondria to 2-propylpentanoyl-CoA (Becker & Harris, 1983; Turnbull et al., 1983) which can be dehydrogenated to 2-propyl-2-pentenoyl-CoA (2-en-valproyl-CoA) by 2-methyl-branched chain acyl-CoA dehydrogenase (Ito et al., 1990), valproic can at least be partially degraded by β-oxidation. It remains unclear, however, how far valproic acid can be metabolized by this pathway and whether its β-oxidation occurs in mitochondria or peroxisomes or both organelles. Also, the possible accumulation of metabolites of valproate β-oxidation in mitochondria and their efflux from mitochondria have not yet been investigated.

This study was initiated with the aim of elucidating the metabolism of valproic acid by β-oxidation in rat liver mitochondria and/or peroxisomes.

Experimental Procedures

Materials. Sigma was the source of octanoyl-CoA, Nycodenz, pigeon breast muscle carnitine acetyltransferase (EC 2.3.1.7), pig heart 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and all standard biochemicals. 2-Octynoic acid and valproic acid (2-*n*-propylpentanoic acid) were obtained from

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Aldrich. 2-Octenoic acid was synthesized from hexanal and malonic acid in the presence of pyridine as described in principle by Linestead et al. (1933). Coenzyme A thioesters of 2-octenoic acid and 2-octynoic acid were synthesized by the mixed-anhydride method as described in principle by Goldman and Vagelos (1961). 3-Ketooctanoyl-CoA was prepared from 2-octynoyl-CoA as described by Thorpe (1986). Concentrations of all CoA derivatives were determined by the method of Ellman (1959) after cleavage of the thioester bond with hydroxylamine at pH 7. Pig heart 3-ketoacyl-CoA thiolase (EC 2.3.1.16) and bovine liver enoyl-CoA hydratase (EC 4.2.1.17) or crotonase were prepared as described by Staack et al. (1978) and Steinman and Hill (1975), respectively. 2-Propyl-2(*E*)-pentenoic acid was generously provided by Dr. H. Nau, Freie Universität Berlin, Berlin, FRG.

Syntheses of 2-Propyl-2-pentenoyl-CoA and 2-Propylpentanoyl-CoA. To 62 mg of dry 2-propyl-2-pentenoic acid (or 60 mg of valproic acid) was added 2 mL of $SOCl_2$. The mixture was kept to 60 °C for 20 h after which time excess $SOCl_2$ was evaporated under reduced pressure. The residue was dissolved in 4 mL of freshly distilled anhydrous tetrahydrofuran (THF) and added dropwise under a N_2 atmosphere to a solution of CoASH[1] (15 mg) and $KHCO_3$ (30 mg) in water (2 mL) and THF (3 mL). Additional $KHCO_3$ was added to maintain the pH at 8. The presence of unreacted CoASH was detected with Ellman's reagent (Ellman, 1959). After 25 min, when all CoASH had reacted, the reaction was terminated by adjusting the pH to 4 with concentrated HCl. THF was evaporated under reduced pressure. The resultant solution was extracted four times with ether and purged of dissolved ether by bubbling N_2 through the solution. Samples were further purified by HPLC. The concentration of 2-propylpentanoyl-CoA was determined spectrophotometrically with the extinction coefficient of 14600 cm^{-1} M^{-1} for CoASH at 260 nm. The concentration of 2-propyl-2-pentenoyl-CoA was also determined spectrophotometrically with the extinction coefficient of 21300 cm^{-1} M^{-1} for 2-*trans*-octenoyl-CoA at 260 nm.

Enzyme Assays. 3-Hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase were assayed as described by Olowe and Schulz (1982). Catalase and esterase were assayed as described by Bandhuuin et al. (1964) and Beaufay et al. (1974), respectively. Glutamate dehydrogenase was assayed as described by Leighton et al. (1968) except that the reaction was monitored at 350 nm with an extinction coefficient of 5308 M^{-1} cm^{-1} for NADH. All enzyme assays were performed at 25 °C on 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.

Isolation of Rat Liver Mitochondria and Preparation of a Soluble Extract and Membrane Fraction from Rat Liver Mitochondria. Rat heart mitochondria were isolated according to the procedure of Chappel and Hansford (1969). Rat liver mitochondria were isolated in a similar manner except that 0.25 M sucrose was used instead of 0.21 M mannitol plus 0.07 M sucrose and the treatment with Nagarse was omitted. Mitochondria suspended in isolation buffer were sonicated at 0 °C five times for 5 s each with a Branson sonifier (Model W-185) equipped with a microtip. The resulting mixture was centrifuged at a 105000*g* for 1 h. Protein concentrations of the mitochondrial suspension, mitochondrial membrane fraction, and the soluble mitochondrial extract were determined by the biuret method (Gornall et al., 1949).

Subcellular Distribution of 3-Hydroxy-2-propylpentanoyl-CoA Dehydrogenase. A 30% (w/v) solution of Nycodenz containing 1 mM EDTA, 5 mL of HEPES (pH 7.3), and 0.1% ethanol was prepared at 4 °C, and 7.6 mL of this solution was placed in a 10-mL ultracentrifuge tube on top of 0.5 mL of a 60% sucrose cushion. The gradient was generated by centrifugation at 28500 rpm (60400g_{av}) in a T865.1 small-angle rotor on a Du Pont RC70 ultracentrifuge at 5 °C for 24 h. Rat liver light mitochondria (3.8 mg of protein in 0.5 mL), prepared as described by Appelmans et al. (1955), were layered on top of the gradient. A solution containing 125 mM sucrose, 0.5 mM EDTA, and 0.05% ethanol was applied as a cover solution. The sample was centrifuged for 45 min at 32000 rpm (76200g_{av}) at 5 °C. Nine fractions were collected from the bottom of the tube. Peroxisomes, microsomes, and mitochondria were localized by assaying the marker enzymes catalase, esterase, and glutamate dehydrogenase, respectively, and were also assayed for 3-hydroxy-2-propylpentanoyl-CoA dehydrogenas as described below.

Degradation of 2-Propyl-2-pentenoyl-CoA by Purified β-Oxidation Enzymes and Solubilized Rat Liver Mitochondria. The hydration of 2-propyl-2-pentenoyl-CoA was studied by adding either purified enoyl-CoA hydratase (crotonase) or rat liver mitochondria solubilized with 0.07% Triton to a solution of 30 μM 2-propyl-2-pentenoyl-CoA in 0.2 M Tris-HCl (pH 10) and monitoring the progress of the reaction spectrophotometrically at 263 nm. For assaying the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA, 30 μM 2-propyl-2-pentenoyl-CoA and 29 units of crotonase were dissolved in 0.2 M Tris-HCl (pH 10), containing 0.07% Triton X-100 and 1.6 mM NAD^+ or 1.6 mM $NADP^+$. The dehydrogenation reaction was started by the addition of either rat liver mitochondria or L-3-hyroxyacyl-CoA dehydrogenase and monitored spectrophotometrically at 340 nm. The rate of synthesis of 2-propylpentanoyl-CoA from valproic acid (2-propylpentanoic acid) by mitochondria solubilized with Triton X-100 was assayed according to a procedure described by Cuebas et al. (1985). To 0.2 M Tris-HCl (pH 8) buffer were added 0.5 mM 2-propylpentanoic acid, 10 mM $MgCl_2$, 4 mM ATP, and 0.5 mM CoASH. The reaction was started by the addition of rat liver mitochondria. At indicated times, the reaction was stopped by acidifying the mixture to pH 1 with HCl. After 1 min, samples were filtered and adjusted to pH 5 with 4 M KOH. They were then centrifuged at 9220g_{av} for 5 min at 4 °C and filtered through a 0.22-μm filter before being analyzed by HPLC.

Metabolism of Valproic Acid in Intact Rat Liver Mitochondria. For the initial characterization of valproate metabolites, coupled rat liver mitochondria (40 mg) were suspended in 20 mL of basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM potassium phosphate, 4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added bovine serum albumin (0.5 mg/mL), 0.5 mM L-malate, 1 mM ADP, 4 mM ATP, and 2 mM L-carnitine. The reaction was started by the addition of 0.5 mM valproic acid. After incubation for 10 min at 25 °C, the

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CoASH, coenzyme A; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form, $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

suspension was cooled to 4 °C and centrifuged at 9200*g* for 5 min. The mitochondrial pellet was resuspended in 0.6 mL of water, and the pH was adjusted to 1 with concentrated HCl. After this suspension had been kept for 1 min at 4 °C, the pH was adjusted to 5, and the sample was centrifuged for 5 min at 9200*g*. The resultant supernatant was analyzed by HPLC as described below. For studying the mitochondrial metabolism of valproic acid as a function of time, rat liver mitochondria (4 mg) in 1 mL of buffer containing the same components as listed above were incubated at 25 °C. Reactions were terminated by the addition of 20 μL of concentrated HCl to bring the pH to 1. After removal of the precipitate by centrifugation at 13000*g* for 5 min, the pH was adjusted to 5 with 4 N KOH, and the samples were centrifuged again at 13000*g* for 5 min. The resultant supernatants were filtered through 0.22-μm filters before being analyzed by HPLC as described below.

HPLC Analysis of Reaction Products. HPLC analyses were performed on a μBondapak C_{18} reverse-phase column (30 cm × 3.9 mm) attached to a Waters gradient HPLC system. Separation was achieved by linearly increasing the acetonitrile content of the 50 mM ammonium phosphate (pH 5.5) elution buffer from 10% to 45% in 30 min at a flow rate of 2 mL/min.

Mass Spectrometry of Acyl-CoA's. Fast atom bombardment (FAB) mass spectra of acyl-CoA thioesters were obtained on a VG 70S double-focusing instrument (VG Analytical Ltd., Manchester, U.K.) operating at a resolving power of 1000. The FAB source incorporated a saddle-field ion gun (Ion Tech Ltd., Teddington, U.K.) which generated an 8-keV xenon atom beam at 1-mA current. Sample introduction was via direct insertion probe. Coenzyme A thioesters in aqueous solution were first desalted by the addition of small quantities of Dowex-50 cation exchange resin (H form), and small aliquots (1–2 μL) were mixed with a small quantity of glycerol on the probe tip. The mass spectrometer employed an exponential scan function from *m/z* 1000 to *m/z* 100 in 3 s with a 0.1-s delay. Tandem (MS/MS) spectra were acquired on a VG Trio-3 triple-stage quadrupole instrument (VG Masslab Ltd., Altrincham, U.K.) operating in the daughter ion mode. The instrument incorporated a FAB source and ion gun similar to the VG 70S, and sample introduction was accomplished as described above. Daughter ion spectra were obtained by setting the first quadrupole to transmit a parent ion from the CoA thioester and collisionally activating (10-eV collision energy) it with 0.4-μbar of argon in the second quadrupole. The third quadrupole was then scanned linearly over the appropriate mass range to transmit daughter ions to the detector.

Results

Activation of Valproic Acid. 2-Propylpentanoyl-CoA has been detected in hepatocytes and liver mitochondria isolated from rats treated with valproic acid (Becker & Harris, 1983). Since 2-propylpentanoic acid is a fatty acid with eight carbon atoms, it is assumed to pass freely through the mitochondrial membrane in a carnitine-independent manner and to be converted to its CoA thioester in the mitochondrial matrix. The conversion of 2-valproic acid to its CoA derivative and the rate of this reaction were investigated with rat liver motochondria solubilized with Triton X-100. In the presence of ATP, CoASH, and Mg^{2+}, the conversion of valproic acid to 2-propylpentanoyl-CoA was confirmed by analyzing the reaction mixture on HPLC (data not shown). The rate of formation of 2-propylpentanoyl-CoA plus its metabolites was found to be 0.25 nmol min^{-1} (mg of protein)$^{-1}$. Thus as least one enzyme exists in mitochondria which can convert valproic acid to its CoA derivative.

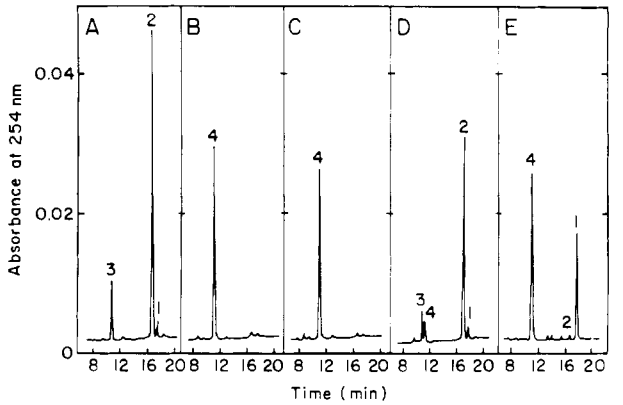

FIGURE 1: HPLC analysis of products formed during the β-oxidation of valproic acid. Reactions are described under Experimental Procedures. (A) Incubation of 2-propyl-2-pentenoyl-CoA with purified bovine liver crotonase. (B) Incubation of 2-propyl-2-pentenoyl-CoA with broken rat liver mitochondria in the presence of NAD^+ and excess crotonase. (C) Same as (B) with additional 3-ketoacyl-CoA thiolase and 0.3 mM coenzyme A. (D) Same as (B) but in the absence of NAD^+. (E) Incubation of 2-propylpentanoic acid with intact rat liver mitochondria for 2.5 min. Peaks identified by use of authentic samples were (1) 2-propylpentanoyl-CoA, (2) 2-propyl-2-pentenoyl-CoA, (3) 3-hydroxy-2-propylpentanoyl-CoA, and (4) 3-keto-2-propylpentanoyl-CoA.

Hydration of 2-Propyl-2-pentenoyl-CoA. It has been reported by Ito et al. (1990) that 2-propylpentanoyl-CoA can be dehydrogenated to 2-propyl-2-pentenoyl-CoA. However, the configuration of the dehydrogenation product has not been determined. Since a compound with the 2*E* configuration was predominant among the unsaturated metabolites of 2-propylpentanoic acid in urine and plasma (Nau & Wittfoht, 1981), 2-propyl-2(*E*)-pentenoic acid was chosen as a substrate for this study. The successful synthesis of 2-propyl-2-pentenoyl-CoA made it possible to study the enzymatic hydration of 2-propyl-2-pentenoyl-CoA. The results prove that purified crotonase from bovine liver causes a decrease in the absorbance at 263 nm (data not shown), presumably as a result of the hydration of 2-propyl-2-pentenoyl-CoA. When the reaction mixture was analyzed by HPLC (see Figure 1A), a product (peak 3) more polar than the substrate (peak 2) was observed. This observation supports the assumed hydration of 2-propyl-2-pentenoyl-CoA by crotonase. The small amount of material corresponding to peak 1 is 2-propylpentanoyl-CoA, reflecting the presence of valproic acid in the preparation of 2-propyl-2-pentenoic acid used in this study. It is well established that the hydration product of 2-*trans*-enoyl-CoA catalyzed by crotonase is 3-hydroxyacyl-CoA with a 3(*S*)-hydroxy group and a 2-*pro-R* hydrogen atom (Willadsen & Eggerer, 1975). Thus, the hydration product of 2-propyl-2-(*E*)-pentenoyl-CoA formed by crotonase is expected to be 3(*S*)-hydroxy-2(*S*)-propylpentanoyl-CoA. The ratio of 2-propyl-2-pentenoyl-CoA to 3-hydroxy-2-propylpentanoyl-CoA at equilibrium is 3.1 as determined by HPLC. When Triton X-100 solubilized rat liver mitochondria were used as an enzyme source, the same reaction product was observed (see Figure 1D). Peak 4 in Figure 1D is a further metabolite which is most likely formed because of the presence of NAD^+ in mitochondria and will be discussed in the next section. The rate of hydration of 2-propyl-2-pentenoyl-CoA catalyzed by mitochondria solubilized with Triton X-100 was determined to be 26 nmol min^{-1} (mg of mitochondrial protein)$^{-1}$. This result proves that 2-propyl-2-pentenoyl-CoA can be hydrated in rat liver mitochondria by crotonase.

Dehydrogenation of 3-Hydroxy-2-propylpentanoyl-CoA and Characterization of 3-Keto-2-propylpentanoyl-CoA by Mass

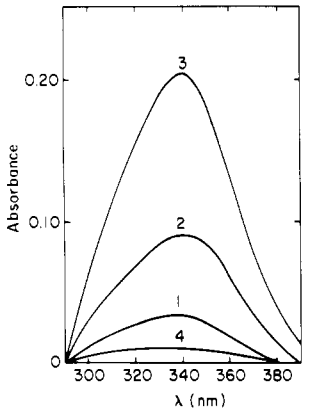

FIGURE 2: Formation of NADH during the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA. Details are given under Experimental Procedures. Incubation of 2-propyl-2-pentenoyl-CoA with broken rat liver mitochondria in the presence of 1.6 mM NAD^+ and excess crotonase. Spectra were taken (1) after 1 min, (2) after 4 min, (3) after 20 min, and (4) after the addition of pyruvate to the sample corresponding to spectrum 3.

Spectrometry. When 2-propyl-2-pentenoyl-CoA was incubated with solubilized rat liver mitochondria plus purified crotonase in the presence of NAD^+, an increase in absorbance at 340 nm was observed (Figure 2, curves 1–3). Upon addition of pyruvate, which is the substrate for lactate dehydrogenase present in the reaction mixture, the peak at 340 nm disappeared (Figure 2, curve 4). This observation proves that NADH was formed during the reaction. However, upon addition of $MgCl_2$, no absorbance change at 303 nm was detected which is characteristic of the Mg^{2+}–enolate complex of 3-ketoacyl-CoA compounds. For this reason the reaction mixture was further analyzed by HPLC as shown in Figure 1B. All 2-propyl-2-pentenoyl-CoA was converted to product (peak 4), which emerged in a region where normally 3-ketoacyl-CoA's are eluted. This conversion was dependent on the presence of NAD^+. In the absence of externally added NAD^+, only trace amounts of material corresponding to peak 4 were formed while 3-hydroxy-2-propylpentanoyl-CoA accumulated (see Figure 1D). Together, these observations suggest that the compound corresponding to peak 4 is 3-keto-2-propylpentanoyl-CoA which is formed by NAD^+-dependent dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA. The rate of its formation was 30 nmol min^{-1} (mg of mitochondrial protein)$^{-1}$.

When $NADP^+$ was substituted for NAD^+, the rate of dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was less than 0.2 nmol min^{-1} (mg of mitochondrial protein)$^{-1}$ as detected by monitoring the reaction spectrophotometrically at 340 nm. Interestingly, no dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was observed with purified pig heart 3-hydroxyacyl-CoA dehydrogenase or peroxisomal trifunctional enzyme. When mitochondria solubilized with Triton X-100 were used as a source of dehydrogenase, the ratio of 3-hydroxy-2-propylpentanoyl-CoA dehydrogenation to 3-hydroxyoctanoyl-CoA dehydrogenation was 0.35. When a mitochondrial membrane fraction was used, the ratio increased to 0.59, whereas with a soluble mitochondrial extract no dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was observed. A further study of the subcellular location of this unknwon dehydrogenase was performed by Nycodenz gradient centrifugation (see Figure 3). The novel dehydrogenase comigrated with the mitochondrial marker enzyme, glutamate dehydrogenase, but was absent from microsomal and peroxisomal fractions. These results suggest that a mitochondrial membrane-associated dehydrogenase rather than L-3-hydroxyacyl-CoA dehydrogenase of the β-oxidation pathway is responsible for the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA.

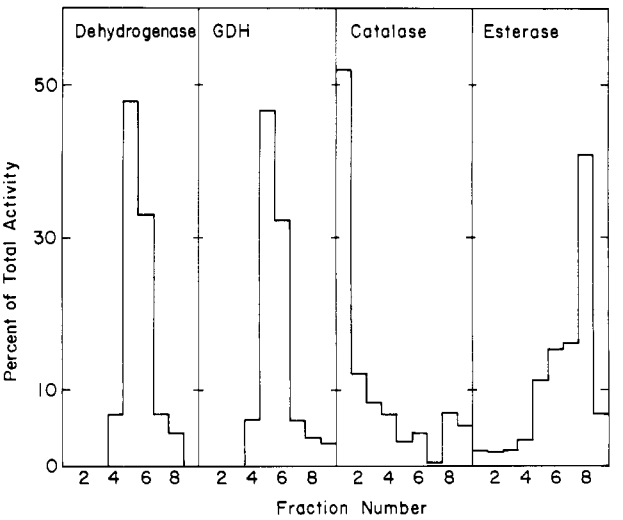

FIGURE 3: Separation of a rat liver L-fraction by centrifugation on a Nycodenz density gradient. Fractions collected from the bottom of the tube were assayed for 3-hydroxy-2-propylpentanoyl-CoA dehydrogenase in addition to catalase, esterase, and glutamate dehydrogenase (GDH) as marker enzymes for peroxisomes, microsomes, and mitochondria, respectively.

To further analyze the structure of the compound assumed to be 3-keto-2-propylpentanoyl-CoA, the material corresponding to peak 4 (see Figure 1B) was collected and analyzed by mass spectrometry. As shown in Figure 4A, the partial FAB mass spectrum of the compound corresponding to peak 4 exhibits a protonated molecular ion $(M + H)^+$ at m/z 908.4. The molecular weight of the corresponding acyl-CoA thioester is therefore 907.4, which is the molecular weight of 3-keto-2-propylpentanoyl-CoA. The fragmentation pattern (not shown) and general spectral appearance were consistent with those of other acyl-CoA's studied, namely, 2-propylpentanoyl-CoA, *n*-octanoyl-CoA, and 3-ketooctanoyl-CoA. Each of these acyl-CoA compounds produced the common fragment ion $[(M + H)^+ - 507]^+$ in the FAB source of the mass spectrometer (Norwood et al., 1990). The FABMS/MS daughter ion spectrum of m/z 401 derived from 3-keto-2-propylpentanoyl-CoA (Figure 4B) is similar to that obtained from 3-ketooctanoyl-CoA. Although this similarity indicates that these two structures are isomeric, the data do not permit the unambiguous assignment of the keto group position. However, the presence of ion m/z 57, consistent with a propionyl residue, in the FABMS/MS daughter ion spectrum of 3-keto-2-propylpentanoyl-CoA (see Figure 4B) and its absence from the corresponding spectrum of 3-ketooctanoyl-CoA (not shown) lend further support to assigning the keto function to carbon atom 3.

Mitochondrial Metabolism of 3-Keto-2-propylpentanoyl-CoA. The last step in the β-oxidation of valproic acid would be the thiolase-catalyzed cleavage of 3-keto-2-propylpentanoyl-CoA to *n*-pentanoyl-CoA and *n*-propanoyl-CoA. When 3-keto-2-propylpentanoyl-CoA was incubated with CoASH in the presence of a soluble extract from rat liver mitochondria fortified by purified pig heart 3-ketoacyl-CoA thiolase, analysis of substrates and products by HPLC revealed no evidence for the thiolytical cleavage of this 3-ketoacyl-CoA compound (see Figure 1C). The rate of disappearance of 3-keto-2-propylpentanoyl-CoA was less than 0.01 nmol min^{-1} (mg of mitochondrial protein)$^{-1}$. When 3-keto-2-propylpentanoyl-CoA was incubated with disrupted rat liver mito-

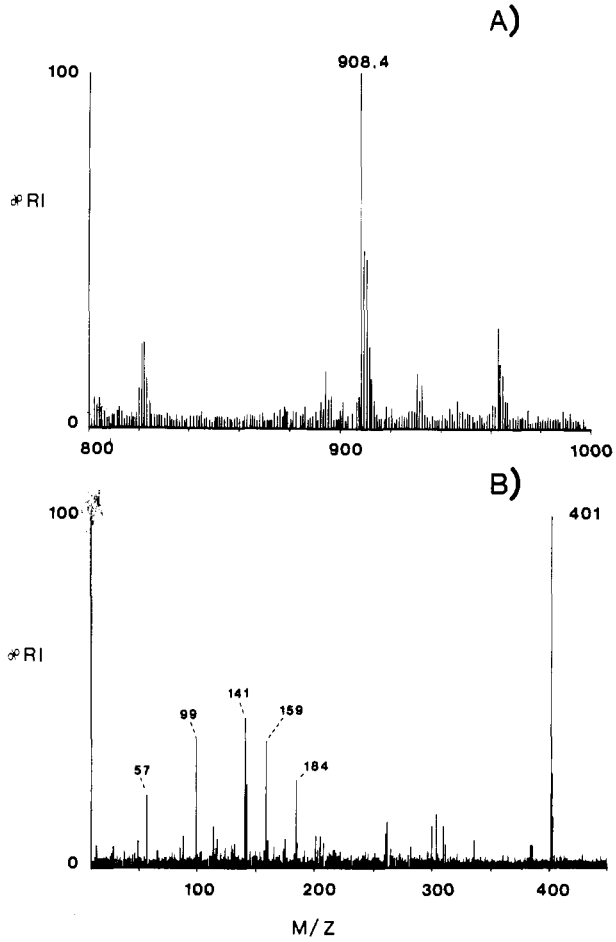

FIGURE 4: Mass spectra of 3-keto-2-propylpentanoyl-CoA. (A) Fast atom bombardment (FAB) mass spectrum showing the molecular ion region; $(M + H)^+ = 908.4$. (B) FAB MS/MS daughter ion spectrum from the parent m/z 401; $[(M + H)^+ - 507]^+$.

chondria, it disappeared at a rate of 0.07 nmol min^{-1} (mg of mitochondrial protein)$^{-1}$ (data not shown). Since the rate of disappearance was the same in the presence and absence of L-carnitine, the breakdown of 3-keto-2-propylpentanoyl-CoA was not due to the transfer of the acyl group to carnitine but most likely was a consequence of thioester hydrolysis.

Metabolism of Valproic Acid in Coupled Rat Liver Mitochondria. An experiment was performed to determine if valproic acid is metabolized in coupled mitochondria as suggested by the above experiments with broken mitochondria and purified enzymes. When valproic acid was incubated with rat liver mitochondria in the presence of ATP, ADP, Mg^{2+}, L-malate, and L-carnitine, two major CoA-containing metabolites were identified in mitochondria, if mitochondria, before being disrupted, were separated from the incubation mixture. One compound was 2-propylpentanoyl-CoA (valproyl-CoA) and the other was 3-keto-2-propylpentanoyl-CoA (see Figure 1E). Trace amounts of 2-propyl-2-pentenoyl-CoA were also detected (see Figure 1E). The same result was obtained when L-carnitine was omitted (data not shown). The time course of valproate β-oxidation in coupled rat liver mitochondria was analzyed by HPLC after terminating the incubation by acidification without first separating mitochondria from the incubation medium. As shown in Figure 5, the major metabolite under this condition was valproyl-CoA; other metabolites were 3-keto-2-propylpentanoyl-CoA, 2-propyl-2-pentenoyl-CoA, and trace amounts of 3-hydroxy-2-propylpentanoyl-CoA. The amounts of all metabolites increased with time. However, 2-propylpentanoyl-CoA, the major metabolite,

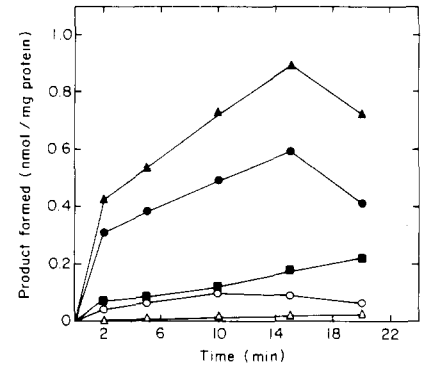

FIGURE 5: Degradation of valproic acid by intact mitochondria. Valproic acid was incubated with intact rat liver mitochondria in the presence of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM potassium phosphate, 4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added bovine serum albumin (0.5 mg/mL), 0.5 mM L-malate, 1 mM ADP, 4 mM ATP, and 2 mM carnitine. The reaction was started by the addition of 0.5 mM 2-propylpentanoic acid. Products were identified and quantitated by HPLC. 2-Propylpentanoyl-CoA (●); 2-propyl-2-pentenoyl-CoA (○); 3-keto-2-propylpentanoyl-CoA (■); 3-hydroxy-2-propylpentanoyl-CoA (△); sum of the above metabolites (▲).

reached a maximum at 15 min of incubation and then began to decline, whereas 3-keto-2-propylpentanoyl-CoA continued to accumulate (see Figure 5). Another prominent peak was due to an unknown compound which was present only in the incubation medium. Since CoASH was not present in the incubation medium, the unknown compound cannot be a CoA derivative of valproic acid or of one of its metabolites. The nature and origin of this compound are currently being investigated.

Discussion

The results of this study demonstrate that valproic acid can be metabolized by mitochondrial β-oxidation as outlined in Figure 6. The main impediment to its complete β-oxidation appears to be the 2-propyl branch which prevents the thiolytic cleavage of 2-propylpentanoyl-CoA to *n*-propionyl-CoA and *n*-pentanoyl-CoA which are known to be oxidized in mitochondria. All other β-oxidation reactions do proceed, albeit slowly, although at least two of them are catalyzed by nonstandard β-oxidation enzymes.

The uptake of valproic acid and its activation to a CoA thioester may proceed by the same steps and involve the same enzymes that facilitate the β-oxidation of medium-chain fatty acids in mitochondria (Schulz, 1985). If so, medium-chain acyl-CoA synthetase (Mahler et al., 1953) would catalyze the activation of valproic acid as shown in the first reaction of the pathway summarized in Figure 6. The dehydrogenation of 2-propylpentanoyl-CoA (**2**) to 2-propyl-2-pentenoyl-CoA (**3**) is, however, catalyzed by 2-methyl-branched chain acyl-CoA dehydrogenase (Ito et al., 1990) which functions in the oxidative degradation of isoleucine and valine (Ikeda & Tanaka, 1983) but not in fatty acid β-oxidation. The hydration of 2-propyl-2-pentenoyl-CoA (**3**) to 3-hydroxy-2-propylpentanoyl-CoA (**4**) is catalyzed by enoyl-CoA hydratase or crotonase, an enzyme of the β-oxidation cycle. The dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA (**4**) by an unknown NAD^+-specific dehydrogenase, which is associated with mitochondria and which appears to be membrane bound, was expected to yield 3-keto-2-propylpentanoyl-CoA (**5**). The assigned structure of this compound agrees with its behavior on HPLC and with its mass spectrum. 3-Keto-2-propylpentanoyl-CoA (**5**) is not acted upon by mitochondrial thiolases but appears to be slowly hydrolyzed.

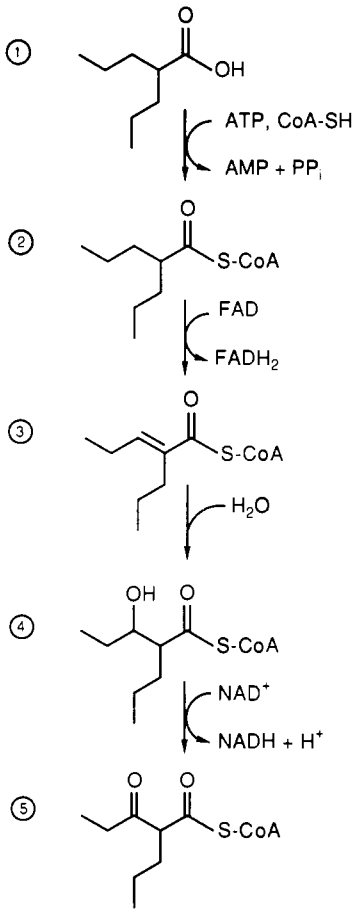

FIGURE 6: Proposed pathway of valproate β-oxidation in rat liver mitochondria. (**1**) Valproic acid or 2-propylpentanoic acid; (**2**) 2-propylpentanoyl-CoA; (**3**) 2-propyl-2(*E*)-pentenoyl-CoA; (**4**) 3-hydroxy-2-propylpentanoyl-CoA; (**5**) 3-keto-2-propylpentanoyl-CoA.

The proposed pathway of valproate β-oxidation shown in Figure 6 was initially based only on the study of the individual reactions. However, the results obtained when the metabolism of valproic acid was studied with coupled rat liver mitochondria support the proposed pathway. All metabolites shown in Figure 6 were identified in soluble extracts of rat liver mitochondria incubated with valproic acid. The time course of valproate metabolism in coupled mitochondria (see Figure 5) additionally revealed that the levels of all metabolites increased initially. After 15 min of incubation, the concentration of 2-propylpentanoyl-CoA declined, whereas that of 3-keto-2-propylpentanoyl-CoA continued to increase. Since 3-keto-2-propylpentanoyl-CoA apparently is not cleaved thiolytically or transferred to carnitine, its slow hydrolysis may be the only mechanism by which free CoA is regenerated with the concomitant release of 3-keto-2-propylpentanoic acid (3-keto-valproic acid) which is a major extracellular metabolite of valproic acid (Browne, 1980). The most abundant derivative of valproic acid in mitochondria was 2-propylpentanoyl-CoA which upon hydrolysis would be converted back to valproic acid and which as a result of acyl transfer to carnitine would yield (2-propylpentanoyl)carnitine (valproylcarnitine). The latter compound has been identified in the plasma and urine of patients treated with valproic acid (Millington et al., 1985). Other plasma or urinary metabolites of valproic acids, like 3-hydroxy-2-propylpentanoic acid (3-hydroxyvalproic acid) and 2-propyl-2-pentenoic acid (2-en-valproic acid) could be formed by hydrolysis of 3-hydroxy-2-propylpentanoyl-CoA and 2-propyl-2-pentenoyl-CoA, respectively. However, because the latter CoA derivatives are present at very low concentrations, it remains to be demonstrated if they are the precursors of the urinary metabolites 3-hydroxyvalproic acid and 2-en-valproic acid.

The question of whether valproic acid is β-oxidized in mitochondria, peroxisomes, or both organelles is answered in favor of mitochondria. Since acyl-CoA oxidase of rat liver peroxisomes is active only with substrates having acyl groups with eight carbon atoms or more (Osumi & Hashimoto, 1978), 2-propylpentanoyl-CoA was not expected to be dehydrogenated by this enzyme. Moreover, the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA is only catalyzed by an enzyme which is membrane bound and associated with mitochondria. Whether this dehydrogenase is identical with the membrane-bound long-chain 3-hydroxyacyl-CoA dehydrogenase described by El Fakhri and Middleton (1982) or is a so far unknown 3-hydroxyacyl-CoA dehydrogenase, perhaps involved in isoleucine metabolism, remains to be established.

With an understanding of valproate β-oxidation at hand, it is possible to study the mechanism(s) by which valproic acid inhibits fatty acid β-oxidation (Becker & Harris, 1983; Turbull et al., 1983; Condé et al., 1983; Bjorge & Baillie, 1985). Trapping of a finite pool of mitochondrial CoA as valproyl-CoA, 3-keto-2-propylpentanoyl-CoA, and other valproic acid metabolites may deplete CoASH necessary for the conversion of acylcarnitine to acyl-CoA and for the thiolytic cleavage of 3-ketoacyl-CoA's and thereby inhibit fatty acid β-oxidation. Another possible mechanism of inhibition may be inhibition of one or several of the enzymes of β-oxidation by 3-keto-2-propylpentanoyl-CoA, which as substrate or product analogue may bind to 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, respectively, without being acted upon by these enzymes and thereby function as inhibitor. Since also acyl-CoA dehydrogenases (Davidson & Schulz, 1982) and enoyl-CoA hydratase (Waterson & Hill, 1972) tightly bind 3-ketoacyl-CoA compounds, the inhibition could affect any of the β-oxidation enzymes.

ACKNOWLEDGMENTS

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Registry No. Valproic acid, 99-66-1; 2-propyl-2-pentenoyl-CoA, 130469-10-2; 2-propylpentanoyl-CoA, 86029-72-3; 2-propyl-2-pentenoic acid, 60218-41-9; L-3-hydroxyacyl-CoA dehydrogenase, 9028-40-4; enoyl-CoA hydratase, 9027-13-8; 3-keto-2-propylpentanoyl-CoA, 130469-11-3; 3-hydroxy-2-propylpentanoyl-CoA, 130469-12-4; 3-ketoacyl-CoA thiolase, 9029-97-4.

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Conformational Motion in Bacteriorhodopsin: The K to L Transition†

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ABSTRACT: By comparison of the time dependence of linear dichroism and transient absorption in light-adapted bacteriorhodopsin over the first 10 μs following excitation, conformational motion in the protein has been detected. Time-resolved linear dichroism and transient absorption scans are reported for several wavelengths that probe the K_{610} and L_{550} intermediates in the bacteriorhodopsin photocycle. The transient absorption scans are insensitive to conformational motion and yield the lifetimes of the K_{610} and L_{550} intermediates. In contrast, the time-resolved linear dichroism scans demonstrate orientational motion of the chromophore with a 1.7-μs rotational time. The wavelength dependence of the least-squares fitting parameters establishes that this motion is associated with L_{550}. This motion is discussed in relation to a protein conformational change in the course of the bacteriorhodopsin photocycle. No evidence is observed for orientational motion on the time scale of the $L_{550} \rightarrow M_{410}$ transition.

Bacteriorhodopsin (bR),[1] the photoactive protein incorporated in the purple membrane of the halophilic bacterium *Halobacterium halobium*, functions to transduce light energy into chemical potential by pumping protons across the cell membrane. The functional form of the protein, light-adapted bR, undergoes a fascinating sequence of photophysical and photochemical events, that includes trans–cis isomerization of the retinylidene chromophore, relaxation of strained intermediates, and proton transfers [see Smith et al. (1985) and Braiman et al. (1988a) and references cited therein].

A series of transient intermediates constituting the bR photocycle (Figure 1) have been identified and their lifetimes

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[1] Abbreviations: bR, bacteriorhodopsin; TRLD, time-resolved linear dichroism.

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