Subcellular distribution and characteristics of trihydroxycoprostanoyl-CoA synthetase in rat liver

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The subcellular distribution and characteristics of trihydroxycoprostanoyl-CoA synthetase were studied in rat liver and were compared with those of palmitoyl-CoA synthetase and choloyl-CoA synthetase. Trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase were localized almost completely in the endoplasmic reticulum. A quantitatively insignificant part of trihydroxycoprostanoyl-CoA synthetase was perhaps present in mitochondria. Peroxisomes, which convert trihydroxycoprostanoyl-CoA into choloyl-CoA, were devoid of trihydroxycoprostanoyl-CoA synthetase. As already known, palmitoyl-CoA synthetase was distributed among mitochondria, peroxisomes and endoplasmic reticulum. Substrate- and cofactor- (ATP, CoASH) dependence of the three synthesis activities were also studied. Cholic acid and trihydroxycoprostanic acid did not inhibit palmitoyl-CoA synthetase; palmitate inhibited the other synthetases non-competitively. Likewise, cholic acid inhibited trihydroxycoprostanic acid activation non-competitively and vice versa. The pH curves of the synthetases did not coincide. Triton X-100 affected the activity of each of the synthetases differently. Trihydroxycoprostanoyl-CoA synthetase was less sensitive towards inhibition by pyrophosphate than choloyl-CoA synthetase. The synthetases could not be solubilized from microsomal membranes by treatment with 1 M-NaCl, but could be solubilized with Triton X-100 or Triton X-100 plus NaCl. The detergent-solubilized trihydroxycoprostanoyl-CoA synthetase could be separated from the solubilized choloyl-CoA synthetase and palmitoyl-CoA synthetase by affinity chromatography on Sepharose to which trihydroxycoprostanic acid was bound. Choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase could not be detected in homogenates from kidney or intestinal mucosa. The results indicate that long-chain fatty acids, cholic acid and trihydroxycoprostanic acid are activated by three separate enzymes.

INTRODUCTION

In the liver, cholesterol is converted into di- and trihydroxycoprostanic acid via a series of enzymic steps that involve reduction of the double bond, hydroxylations of the steroid nucleus and oxidation of one of the terminal methyl groups of the aliphatic side chain. The enzymes that catalyse these reactions are distributed among the mitochondria, endoplasmic reticulum and cytosol [1]. Di- and tri-hydroxycoprostanic acid are then activated to their CoA esters, whose acyl side chains are subsequently shortened via β -oxidation in peroxisomes, resulting in the formation of propionyl-CoA and of chenodeoxycholoyl-CoA and choloyl-CoA respectively [2]. These latter CoA-esters serve as substrates for the conjugation reactions with taurine or glycine. Conjugating activity is found in endoplasmic reticulum as well as in peroxisomes [3].

The activation of di- and tri-hydroxycoprostanic acid has never been studied in detail, and it is not known by which enzyme the reaction is catalysed. Numerous compounds that contain an aliphatic chain with a terminal carboxy group are activated to CoA esters before they are further metabolized via β -oxidation, esterification or conjugation with amino acids. These compounds include short-, medium-, long- and very-long-chain fatty acids,

dicarboxylic fatty acids, prostaglandins, retinoic acid [4], xenobiotics with a carboxy side chain [5], di- and trihydroxycoprostanic acid and C_{24} bile acids, such as chenodeoxycholic and cholic acid.

Short- and medium-chain fatty acids are activated mainly in the mitochondrial matrix [6–8]. Long-chain acyl-CoA synthetase activity is found in the mitochondrial outer membrane, the peroxisomal membrane and the endoplasmic reticulum [9–11]. The enzymes in the three organelles are of similar molecular mass and amino acid composition, show immunological cross-reactivity and have the same kinetic characteristics, indicating that the activities reside in identical, or closely related, proteins [12–14]. It is not clear to what extent very-long-chain fatty acids can be activated by long-chain acyl-CoA synthetase. Evidence is emerging that a separate specific enzyme exists, at least in liver peroxisomes [15–17] and in microsomes (microsomal fractions) from liver [15,17], brain [18,19] and platelets [20], and that very-long-chain acyl-CoA synthetase activity may be absent from mitochondria [15,17]. Prostaglandins [21], dicarboxylic fatty acids [22] and the C_{24} bile acids [23] are activated solely in the endoplasmic reticulum. The enzymes that catalyse the activation of dicarboxylic fatty acids and of C_{24} bile acids appear to be different from the long-chain fatty acyl-CoA synthetase [22,24].

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In the present work we studied the subcellular distribution of trihydroxycoprostanoyl-CoA synthetase and compared the characteristics of the enzyme with those of long-chain fatty acyl-CoA synthetase and choloyl-CoA synthetase. The latter enzyme is responsible for the activation of C₂₄ bile acids re-entering the liver via enterohepatic recirculation and is considered to catalyse the rate-limiting reaction in the conjugation of these recirculating bile acids [25,26]. Our results show that, like choloyl-CoA synthetase, trihydroxycoprostanoyl-CoA synthetase is present predominantly in the endoplasmic reticulum. Its properties differ in several respects from those of long-chain acyl-CoA synthetase and choloyl-CoA synthetase, and the three synthetases can be separated from each other by affinity chromatography, indicating the occurrence of three separate enzymes. The further characterization and identification of acyl-CoA synthetases is not of purely academic interest, since a deficiency of a peroxisomal very-long-chain acyl-CoA synthetase has recently been proposed as the basic defect in X-chromosome-linked adrenoleucodystrophy [16,17,27,28].

EXPERIMENTAL

Materials

CoA and dithiothreitol were from P-L Biochemicals, Milwaukee, WI, U.S.A. ATP and Triton X-100 were obtained from Boehringer-Mannheim, Mannheim, Germany. Bovine serum albumin (fraction V, powder) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The albumin was defatted by the method of Chen [29], dialysed in the cold for 48 h against water, then freeze-dried. [1-¹⁴C]Palmitate (sp. radioactivity 58 mCi/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. [2,4-³H]Cholic acid (sp. radioactivity 25 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A. Trihydroxycoprostanic acid was purified from alligator bile [30]. Radiolabelled [3,7,12-³H]trihydroxycoprostanic acid (sp. radioactivity 26.3 mCi/mmol) was prepared as described previously [31]. Epoxy-activated Sepharose 6B was from Pharmacia, Uppsala, Sweden.

Animals

Male Wistar rats weighing 120–150 g were used. They were maintained on a standard laboratory chow diet.

Preparation of subcellular fractions

Liver homogenates (20%, w/v) were prepared in 0.25 M-sucrose containing 0.1% (v/v) ethanol and 5 mM-dithiothreitol, and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and soluble (S) fractions as described by de Duve *et al.* [32]. The L-fraction was subfractionated in a self-generating Percoll gradient [10].

Determination of marker enzymes and protein

Marker enzymes [glutamate dehydrogenase (mitochondrial matrix); catalase (peroxisomal matrix); urate oxidase (peroxisomal core) and glucose-6-phosphatase (endoplasmic reticulum)] were determined as described

previously [33]. Protein was measured by the method of Peterson [34].

Assay of palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

Trihydroxycoprostanoyl-CoA synthetase was measured as described recently [31]. The reaction mixture consisted of a modified Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 5 mM-ATP, 0.5 mM-CoA, 2 mM-dithiothreitol and 125 μ M-[³H]trihydroxycoprostanic acid (sp. radioactivity 3.4 mCi/mmol). The reaction was started by adding 0.05 ml of homogenate or subcellular fraction, appropriately diluted in 0.25 M-sucrose containing 1 mM-dithiothreitol, to 0.2 ml of reaction mixture. The mixtures were incubated at 37 °C, and the reactions were terminated by the addition of 2 ml of ethyl acetate and 1.75 ml of 0.1 M-acetate buffer, pH 4. Phases were separated by centrifugation, and the aqueous phase was extracted a second time with ethyl acetate. Essentially all unchanged trihydroxycoprostanic acid and no trihydroxycoprostanoyl-CoA partitioned in the ethyl acetate phase. Depending on the presence or not of albumin in the reaction mixtures, recoveries of trihydroxycoprostanoyl-CoA in the final aqueous phase ranged from 30 to 65%. The remainder of the radioactive CoA ester was found at the water/ethyl acetate interface. Care was taken, therefore, not to remove the interface after ethyl acetate extraction. The protein concentrated on the interface was dissolved in the underlying aqueous phase by adding 0.3 ml of 10 M-KOH. The alkali-treated mixtures were kept at room temperature for 60 min, and a portion of the mixtures was counted for radioactivity. This procedure resulted in a recovery of 85% for (the hydrolysed) trihydroxycoprostanoyl-CoA. Choloyl-CoA synthetase and palmitoyl-CoA synthetase were measured under the same conditions, except that trihydroxycoprostanic acid was replaced in the reaction mixtures by 125 μ M-[³H]cholic acid (sp. radioactivity 2.85 mCi/mmol) and by 62.5 μ M-[¹⁴C]palmitate (sp. radioactivity 0.9 mCi/mmol) respectively. Cholic acid activation and palmitic acid activation were terminated and the reaction mixtures extracted as described by Polokoff & Bell [24] and by Roughan & Slack [35] respectively. The activation of trihydroxycoprostanic acid and cholic acid was linear with time for up to 4 min in whole liver homogenates and for up to 20 min in subcellular fractions. The chosen reaction times were 3 min (homogenates) or 10 min (subcellular fractions). Palmitoyl-CoA synthetase activity was linear with time for 5 min in whole homogenates and in subcellular fractions. The chosen reaction time was 3 min.

Solubilization of the synthetase activities

Isolated microsomes were treated successively with 1 M-NaCl, 1% (w/v) Triton X-100 and 1% (w/v) Triton X-100 plus 1 M-NaCl. The salt and detergent solutions were buffered with 10 mM-Mops, pH 7.2, and contained 1 mM-dithiothreitol, 0.5 mM-*p*-toluenesulphonyl fluoride and 5% (v/v) dimethyl sulphoxide. The initial microsomal protein concentration was approx. 20 mg/ml. Contact time with salt or detergent was 30 min at 4 °C. After each treatment, the proteins that were not released were sedimented by centrifugation at 100 000 *g* for 40 min. The supernatants were saved for protein and enzyme determinations and the pellets subjected to the subsequent treatment.

Affinity chromatography

The 3-hydroxy group of trihydroxycoprostanic acid was linked to Sepharose by means of a Lewis acid-catalysed coupling of 3 α -hydroxy-7 α ,12 α -diformyloxycoprostanic acid to epoxy-activated Sepharose, followed by hydrolysis of the protective formyl ester groups. The 7 α ,12 α -diformyl derivative of trihydroxycoprostanic acid was prepared by formylation of trihydroxycoprostanic acid, followed by partial deformylation in NH_3 -saturated methanol as described by Tserng & Klein [36]. A trace amount of [3,7,12- ^3H]trihydroxycoprostanic acid was added at the start of the formylation reaction. A portion (2 g) of freeze-dried epoxy-activated Sepharose 6B was allowed to swell in water and washed with, successively, water, anhydrous ethanol, acetone and dry methylene chloride. The washed gel was resuspended in 8 ml of dry methylene chloride containing 60 mg of the diformylated ligand. The slurry was swirled at room temperature, and 0.5 ml of boron fluoride ethyl ether was added in five equal portions at intervals of 15 min. After another 30 min, the gel was washed with, successively, methylene chloride, acetone, water, 0.1 M-borate buffer, pH 8, 0.1 M-acetate buffer, pH 4, and water. Excess reactive groups on the Sepharose were blocked with 1 M-ethanolamine overnight at 40 °C. The protective formyl ester groups of the diformylated ligand were hydrolysed in this step. After having been washed with water, the derivatized gel was stored at 4 °C in water containing 0.02% (w/v) NaN_3 . Approx. 9 μmol of trihydroxycoprostanic acid were coupled/g of Sepharose, as estimated from radioactivity measurements on portions of the gel.

Isolated microsomes were solubilized with Triton X-100 plus NaCl as described above, except that the initial protein concentration was approx. 4 mg/ml. The solubilized proteins were dialysed overnight at 4 °C against 10 mM-Mops, pH 7.2, containing 5 mM- β -mercaptoethanol and 1% (w/v) Triton X-100 to remove most of the salt. ATP and MgCl_2 were added to the dialysed protein solution to final concentrations of 5 mM and 3 mM respectively. A 2 ml portion of the solution, containing approx. 4 mg of protein, was loaded on a trihydroxycoprostanic acid-Sepharose column (bed volume 3.1 ml), previously equilibrated with 10 mM-Mops, pH 7.2, containing 5 mM- β -mercaptoethanol and 0.1% (w/v) Triton X-100. The column was washed with 3 column vol. of the equilibration buffer containing 5 mM-ATP and 3 mM- MgCl_2 and eluted successively with 20 mM-potassium phosphate buffer, pH 7.2, 20 mM-acetate buffer, pH 4, and 20 mM-acetate buffer containing 0.5 M-NaCl, as indicated in the legend to Fig. 8 (below). All buffers contained 5 mM- β -mercaptoethanol and 0.1% (w/v) Triton X-100. The column was eluted at 4 °C at a flow rate of 4 ml/h.

RESULTS AND DISCUSSION

Subcellular distribution

In order to investigate the subcellular distribution of trihydroxycoprostanoyl-CoA synthetase, we fractionated a liver homogenate by differential centrifugation and measured the activation of trihydroxycoprostanic acid, cholic acid and palmitic acid along with marker enzymes for the mitochondria, peroxisomes and endoplasmic reticulum. The results of the experiments are shown in

Fig. 1. In agreement with its known localization in mitochondria, peroxisomes and endoplasmic reticulum, palmitoyl-CoA synthetase activity was distributed among all particulate fractions. Like choloyl-CoA synthetase, trihydroxycoprostanoyl-CoA synthetase closely followed the distribution of glucose-6-phosphatase, indicating that it is localized predominantly in the endoplasmic reticulum. Since different amounts of acyl-CoA hydrolase activity in the various subcellular fractions might affect the apparent subcellular distribution of the synthetase activities, we also measured the activation of cholic acid and of trihydroxycoprostanic acid in the subcellular fractions in the presence of albumin at a substrate/albumin molar ratio of 1:1. The presence of albumin does not inhibit trihydroxycoprostanoyl-CoA synthetase or choloyl-CoA synthetase (see below), but partially suppresses the hydrolase activities [21,31]. The subcellular distributions of the synthetases were identical when measured in the presence or absence of albumin (results not shown).

In a subsequent experiment, the light mitochondrial fraction (L-fraction), prepared by differential centrifugation, was subfractionated by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient. As estimated from the activities of the marker enzymes, this L-fraction contained 25% of the mitochondria, 30% (catalase)–54% (urate oxidase) of the peroxisomes and only 7% of the endoplasmic reticulum. The discrepancy between the percentages of catalase and urate oxidase present in the L-fraction is due to the fact that part of the catalase leaks from the peroxisomes during homogenization of the liver. Fig. 2 shows the gradient distribution of marker enzymes and of trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase. Choloyl-CoA synthetase and especially trihydroxycoprostanoyl-CoA synthetase displayed a bimodal distribution: the main part of the activity was present in the gradient fractions containing the endoplasmic reticulum fragments, but activity was also found in the fractions enriched in mitochondria. Peroxisomes clearly did not show activity. In the interpretation of the results it should be borne in mind that, on a relative basis, endoplasmic reticulum was 3–4 times less abundant than mitochondria in the L-fraction loaded on the gradient. Assuming that all synthetase activity present in the peak mitochondrial and microsomal (endoplasmic reticulum) fractions, was localized exclusively in mitochondria and endoplasmic reticulum respectively, we could calculate from the activities of the respective marker enzymes in these peak fractions that mitochondria would be responsible for at most 6.5 and 2% of the total cellular activity of trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase respectively. Synthetase activities were also measured in the presence of optimum concentrations of Triton X-100 (see below). The presence of the detergent did not affect the subcellular distribution pattern of the synthetases.

Substrate- and cofactor-dependence

The subsequent experiments were carried out with microsomal fractions prepared by differential centrifugation. Fig. 3 shows the substrate-dependence of the synthetases measured with palmitic, cholic and trihydroxycoprostanic acid. Palmitoyl-CoA synthetase (Fig. 3a) followed Michaelis-Menten kinetics. The apparent K_m of 4 μM and the apparent V_{max} of 84 nmol/min

Fig 1. Subcellular distribution of palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

A rat liver homogenate was fractionated by differential centrifugation into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P) and a soluble (S) fraction, and marker enzymes were measured in each fraction. (a) Catalase (peroxisomal matrix); (b) urate oxidase (peroxisomal core); (c) glutamate dehydrogenase (mitochondrial matrix); (d) glucose-6-phosphatase (endoplasmic reticulum). Synthetase activities were measured with palmitate (e), trihydroxycoprostanic acid (f) and cholic acid (g) as substrates. Results are expressed as relative specific activities versus percentage of total protein. Relative specific activity is defined as the percentage of total recovered activity present in a particular fraction divided by the corresponding percentage of total protein. Recoveries were between 85 and 145%.

per mg of protein, calculated from the double-reciprocal plot (Fig. 3a, inset), are of the order of magnitude of previously published values [36,37]. With cholic acid as the substrate (Fig. 3b), half-maximal activity was reached at 7 μ M-cholic acid and the maximal activity was approx. 1.7 nmol/min per mg of protein. These values are in agreement with those previously reported [24,38]. The double-reciprocal plot (Fig. 3b, inset) displayed a breakpoint, which is probably the consequence of an underestimation of the rate of activation at the lowest substrate concentration. Reasons for this underestimation may be substrate consumption (approx. 40% of the substrate consumed at the end of the incubation with 5 μ M-cholic acid) or cholic acid binding to some microsomal component, which would perhaps lower the unbound cholic acid concentration to a relatively larger extent at low, than at high, cholic acid concentrations. When the activity at the lowest substrate concentration is not taken into account, an apparent K_m of 6 μ M can be calculated. Trihydroxycoprostanoyl-CoA synthetase reached an activity of approx. 2.6 nmol/min per mg of protein at a trihydroxycoprostanic acid concentration of 200 μ M. Half of this activity was reached at 20 μ M-trihydroxycoprostanic acid (Fig. 3c). The double-reciprocal plot revealed a breakpoint compatible with the occurrence of a high- and a low-affinity component. The

apparent K_m of the high-affinity component was 10 μ M. The breakpoint in the double-reciprocal plot might suggest that trihydroxycoprostanic acid can be activated by two enzymes: an enzyme with high substrate affinity and one with low substrate affinity. If so, the low-affinity enzyme probably does not play a major role at physiological trihydroxycoprostanic acid concentrations. Its activity may be a side activity of one of the acyl-CoA synthetases, normally not involved in trihydroxycoprostanic acid metabolism. However, other explanations are possible. Similar and unexplained breakpoints in linearly transformed plots have been observed for other membrane-bound enzymes such as peroxisomal dihydroxyacetone-phosphate acyltransferase [33,40]. Finally, trihydroxycoprostanic acid is by itself a detergent, and the breakpoint might correspond to micelle formation. The critical micelle concentration of trihydroxycoprostanic acid is not known. That of cholic acid is 13–15 mM [41]. The breakpoint in the plot corresponds to a trihydroxycoprostanic acid concentration of 25–50 μ M. We do not know whether a difference of three carbon atoms in the side chain (between cholic acid and trihydroxycoprostanic acid) might produce such a dramatic decrease in critical micelle concentration.

Apparent K_m values were also determined for CoA and ATP (curves not shown). The values for CoA were

Fig 2. Subfractionation of a light mitochondrial fraction

A light mitochondrial fraction, prepared by differential centrifugation, was subfractionated by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient. The different fractions were analysed for glucose-6-phosphatase (a), catalase (b), glutamate dehydrogenase (c) and protein (d). Synthetase activities were measured with cholic acid (e) and trihydroxycoprostanic acid (f) as substrates. Results are expressed as percentage of total gradient activity or content present in each fraction numbered on the abscissa. Fractions 1 and 15 represent the fractions of highest and lowest density respectively. Recoveries were between 100 and 117%.

Fig 3. Substrate concentration dependence of microsomal palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

Synthetase activities were measured in rat liver microsomes with different concentrations of palmitate (a), cholic acid (b) and trihydroxycoprostanic acid (THCA, c) as substrates. Results are shown as rates plotted against substrate concentration and are means for three experiments. Linear transformation of the curves by the method of Lineweaver-Burk [39] is shown in the insets. Units (not shown to avoid complicating the Figure) for $1/v$ and $1/s$ are $(\text{nmol/min per mg of protein})^{-1}$ and μM^{-1} respectively.

Fig 4. Effect of pyrophosphate on palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

Microsomal palmitoyl-CoA synthetase (●), choloyl-CoA synthetase (○) and trihydroxycoprostanoyl-CoA synthetase (△) activities were measured in the absence and presence of increasing pyrophosphate concentrations. Results are expressed as percentages of the synthetase activities measured in the absence of pyrophosphate and are means for two experiments.

16, 38 and 13 μM for palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase respectively ($n = 2$). The values for ATP were 1.5, 3.5 and 4 mM respectively ($n = 2$).

Pyrophosphate, a product of the reaction, inhibited all three synthetases. There was a clear distinction between the inhibition curves for palmitoyl-CoA synthetase and choloyl-CoA synthetase on the one hand and for trihydroxycoprostanoyl-CoA synthetase on the other (Fig. 4), suggesting that choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase may be distinct enzymes.

Substrate-competition experiments

Cholic acid and trihydroxycoprostanic acid used over the concentration range 0–200 μM did not inhibit palmitoyl-CoA synthetase (results not shown). As already reported by our laboratory [31], palmitate potently inhibited trihydroxycoprostanic acid activation in a non-competitive way. Likewise, cholic acid activation was potently and non-competitively inhibited by palmitate (approx. 50% inhibition at 10 μM -palmitate; results not shown). We also studied the effect of cholic acid on trihydroxycoprostanic acid activation and vice versa. Each substrate inhibited the activation of the other substrate, trihydroxycoprostanic acid being the more potent inhibitor (Fig. 5). The pattern of the curves as well as the double-reciprocal plots agree with the occurrence of non-competitive inhibition. We emphasize, however, that because of the complex nature of the kinetics observed in Fig. 3, we do not want to rely too heavily on this interpretation of the results.

Fig 5. Effect of cholic acid on trihydroxycoprostanoyl-CoA synthetase and of trihydroxycoprostanic acid on choloyl-CoA synthetase

The activation of trihydroxycoprostanic acid and of cholic acid was measured in isolated microsomes at increasing substrate concentrations in the absence and presence of increasing concentrations of the other substrate. (a) Trihydroxycoprostanic acid (THCA) activation in the absence (●) and in the presence of 10 μM (○)-, 50 μM (■)- or 200 μM (□)-cholic acid. (b) Cholic acid activation in the absence (●) and in the presence of 10 μM (○)-, 50 μM (■)- or 100 μM (□)-trihydroxycoprostanic acid. Results are plotted as rates against concentration. Linear transformation of the curves by the method of Lineweaver-Burk [39] is shown in the insets. Units for $1/s$ and $1/v$ are as for Fig. 3.

Fig. 6. pH-dependence of microsomal palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

Synthetase activities were measured with palmitate (●), cholic acid (○) and trihydroxycoprostanic acid (Δ) in a pH region from 6 to 9. The incubation mixtures were buffered with 80 mM-Tris/HCl. Results are expressed as percentages of optimum activity versus pH. Essentially the same results were obtained when this experiment was repeated.

pH-dependence

pH-dependence was studied over the pH range 6–9. Trihydroxycoprostanoyl-CoA synthetase showed an optimum at pH 7.5, whereas the two other synthetases reached their highest activities at pH 9 (Fig. 6). These results again suggest that trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase may be separate enzymes.

Effect of albumin and Triton X-100

Albumin, added to the standard assay mixtures, stimulated all three synthetase activities. The highest activities were reached at a substrate/albumin molar ratio of 2:1 for palmitoyl-CoA synthetase and at a molar ratio of 1:1 for choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase. The respective percentages of stimulation were 105, 102 and 80. The stimulatory effect of albumin remains unexplained, but (part of) the explanation might be a suppression of acyl-CoA hydrolase activities (see above). Triton X-100, added at low concentrations to the standard assay mixtures, also stimulated the synthetase activities (Fig. 7). The optimum stimulatory concentration of detergent differed for each of the three activities. At a high concentration of 0.5% (w/v), the detergent remained stimulatory for palmitoyl-CoA synthetase, it inhibited

Fig. 7. Effect of Triton X-100 on microsomal synthetase activities

Synthetase activities were measured with palmitate (●), cholic acid (○) and trihydroxycoprostanic acid (Δ) in the absence and presence of increasing concentrations of Triton X-100. Results are expressed as percentages of the activities obtained in the absence of detergent, versus detergent concentration. Essentially the same results were obtained when this experiment was repeated.

slightly trihydroxycoprostanoyl-CoA synthetase and inhibited markedly choloyl-CoA synthetase. Since Triton X-100 was used above its critical micelle concentration, the detergent may have formed mixed micelles with each of the substrates. It is therefore difficult to know whether the differential effect of the detergent on the different enzyme activities was due to a direct effect on the enzyme or to an effect on the physical state of the respective substrate.

Solubilization

The synthetases could not be solubilized by treatment with high salt concentration (Table 1). The major part of palmitoyl-CoA synthetase was readily solubilized with Triton X-100. Choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase appeared to be more resistant, and a substantial part of their activities required Triton X-100 plus salt for solubilization. The results indicate that each of the synthetases is firmly embedded in the lipid phase of the membrane.

Separation of trihydroxycoprostanoyl-CoA synthetase from choloyl-CoA synthetase and acyl-CoA synthetase

Microsomal proteins were solubilized with Triton X-100 plus NaCl and subsequently dialysed to remove the salt. A portion of the dialysed proteins was loaded on an affinity column, which consisted of Sepharose to which trihydroxycoprostanic acid had been bound (Fig. 8). The column was then washed with 3 column volumes of buffer. Loading and washing were performed in the presence of ATP, since preliminary experiments had

Table 1. Solubilization of palmitoyl-CoA synthetase, choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase

Microsomes were treated successively with 1 M-NaCl, 1 % (w/v) Triton X-100 and 1 % (w/v) Triton X-100 plus 1 M-NaCl as described in the Experimental section. After each treatment the released protein and synthetase activities were measured. The Triton X-100 concentration in the assays was 0.2 % (w/v). The NaCl concentrations in the assays were not adjusted, since NaCl did not affect the synthetase activities. Results are expressed as percentages of the total recovered activity or amount and are means for two experiments. Essentially the same release pattern was observed when the synthetase activities were measured in the presence of 0.05 % (w/v) Triton X-100 (results not shown).

Treatment	Released (% of total)			
	Protein	Palmitoyl-CoA synthetase	Choloyl-CoA synthetase	Trihydroxycoprostanoyl-CoA synthetase
1 M-NaCl	34	13	2	9
1 % (w/v) Triton X-100	41	68	53	51
1 % (w/v) Triton X-100 plus 1 M-NaCl	20	18	42	39
Final pellet:				
Not released (% of total)...	5	1	3	1
Recovery (%)...	116	110	144	77

Fig 8. Affinity chromatography of solubilized microsomal proteins on trihydroxycoprostanic acid-Sepharese

Solubilized microsomal proteins were loaded on a trihydroxycoprostanic acid-Sepharese column as described in the Experimental section. The column was washed with equilibration buffer containing ATP (a) and eluted successively with potassium phosphate buffer, pH 7.2 (b), acetate buffer pH 4 (c), and acetate buffer containing NaCl (d). Protein (○), palmitoyl-CoA synthetase (●), trihydroxycoprostanoyl-CoA synthetase (□) and choloyl-CoA synthetase (■) were measured on the eluate. The volume of each eluate fraction was 4.7 ml, except for fraction 12, the volume of which was 8 ml. The Triton X-100 concentrations in the assays were 0.05 % (w/v) for trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase and 0.025 % (w/v) for palmitoyl-CoA synthetase. Results are expressed as percentages of total recovered activity, or amount, present per fraction. Total recoveries from the column were 75 % (protein), 118 % (palmitoyl-CoA synthetase), 65 % (trihydroxycoprostanoyl-CoA synthetase) and 77 % (choloyl-CoA synthetase).

shown that trihydroxycoprostanoyl-CoA synthetase was better retained on the column in the presence of this nucleotide. The washing removed more than 99 % of the recovered protein and of the recovered palmitoyl-CoA synthetase from the column, but also approximately half of the recovered trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase. Elution of the column with buffer in the absence of ATP resulted in a substantial peak of trihydroxycoprostanoyl-CoA synthetase (Fig. 8, fraction 4). Very little choloyl-CoA synthetase activity was eluted with this peak, clearly showing that trihydroxycoprostanoyl-CoA synthetase and choloyl-CoA synthetase are separate enzymes. Subsequent elution at acidic pH resulted in the appearance of smaller peaks of trihydroxycoprostanoyl-CoA synthetase or choloyl-CoA synthetase activity. It is not surprising that choloyl-CoA synthetase was also partly retained on the column, since trihydroxycoprostanic acid is a potent inhibitor of choloyl-CoA synthetase (Fig. 5b). The specific activity of trihydroxycoprostanoyl-CoA synthetase in fraction 4 of the eluate was 113 munits/mg of protein, a 124-fold purification in comparison with the starting material loaded on the column.

Tissue distribution

Synthetase activities were also measured in homogenates from kidney and intestinal mucosa. Palmitoyl-CoA synthetase was found, but choloyl-CoA synthetase and trihydroxycoprostanoyl-CoA synthetase could not be detected. Polokoff & Bell [24] also failed to detect choloyl-CoA synthetase activity in extrahepatic tissues. Palmitoyl-CoA synthetase amounted to approx. 0.5 and 1 μ mol/min per g of tissue in kidney and intestinal mucosa respectively. This is 20 and 10 times less respectively than the activity found per g of liver.

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