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Isolation and Characterization of Rat Liver Microsomal R-Ibuprofenoyl-CoA Synthetase

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ABSTRACT. Microsomal long-chain acyl-CoA synthetase (EC 6.1.2.3.) has been suggested to be involved in the stereoselective formation of the CoA thioester of ibuprofen. In this study, we demonstrated that the microsomal enzyme from rat liver responsible for palmitoyl-CoA synthesis also catalyzes the formation of R-ibuprofenoyl-CoA in a Mg^{2+}- and ATP-dependent process. Long-chain acyl-CoA synthetase from rat liver microsomes was purified to homogeneity as evidenced by SDS-gel electrophoresis. Simultaneous measurements of palmitoyl-CoA and R-ibuprofenoyl-CoA formation with HPLC in various fractions and purification steps during protein isolation revealed a high correlation between both activities. The purification procedure included solubilization of the microsomes obtained from rat livers with Triton X-100 and subsequent chromatography of the 100,000 × g supernatant on blue-sepharose, hydroxyapatite, and phosphocellulose. The purified enzyme exhibited an apparent molecular weight of 72 kDa as estimated by SDS gel electrophoresis, with specific activities of 71 nmol · min^{-1} · mg^{-1} protein and 901 nmol · min^{-1} · mg^{-1} protein for formation of R-ibuprofenoyl-CoA and palmitoyl-CoA, respectively. Palmitoyl-CoA formation catalyzed by the purified enzyme exhibited biphasic kinetics indicative of two isoforms, a high-affinity (K_M 0.13 ± 0.11 μM), low-capacity form and a low-affinity (K_M 81 ± 11.5 μM), high-capacity form. In contrast, measurement of R-ibuprofenoyl-CoA synthesis over a concentration range from 5 to 3000 μM showed the participation of a single CoA ligase with a K_M of 184 ± 19 μM, corresponding to the low-affinity isoform of palmitoyl-CoA synthesis with a marked enantioselectivity towards the R-form of ibuprofen. R-ibuprofenoyl-CoA formation of the enzyme preparation was inhibited by palmitic acid (K_i 13.5 ± 0.5 μM) and S-ibuprofen (K_i 405 ± 10 μM). In summary, these data give strong evidence for the identity of R-ibuprofenoyl-CoA and long-chain acyl-CoA synthetase. BIOCHEM PHARMACOL 52;7:1007–1013, 1996.

KEY WORDS. ibuprofen; long-chain acyl-CoA synthetase (EC 6.1.2.3.); palmitoyl-CoA; R-ibuprofenoyl-CoA; R/S-inversion; 2-arylpropionic acids

Ibuprofen, like other 2-arylpropionic acids, undergoes a metabolic chiral inversion in various species in which the inactive (in terms of cyclooxygenase inhibition) R-enantiomer is inverted to the active cyclooxygenase inhibitor S-ibuprofen [1]. It is now well established that the mechanism of chiral inversion [2] involves three metabolic steps: After thioesterification of R-ibuprofen with CoA[§] [3, 4] via an adenylate intermediate [5] catalyzed by a stereospecific microsomal and mitochondrial localized synthetase, the R-ibuprofenoyl-CoA thioester epimerizes [6, 7] via a cytosolic and mitochondrial enzyme [8, 9]. Hydrolysis of the CoA thioesters occurs in a nonstereoselective manner [6, 10].

Tissue location of 2-arylpropionic acid inversion may have important therapeutic and toxicological implications. Chiral inversion has been investigated in cellular homogenates [3, 10], whole isolated cells [11, 12], and whole perfused organs [13] including the lung [14], kidney [15], and intestine [16]. However, the liver remains the only tissue proven to be of significant quantitative importance in the *in vivo* inversion of R-ibuprofen after thioesterification.

In the present study, we focused on a rat long-chain acyl-CoA synthetase [17] suggested to be the key enzyme catalyzing the enantiospecific activation of R-ibuprofen to the R-ibuprofenoyl-CoA thioester and, therefore, the R- to S-inversion of ibuprofen [18]. This hypothesis is supported by numerous studies of CoA thioester formation by ibuprofen and other 2-arylpropionic acids in rat and human liver tissue preparations supplemented with CoA, ATP, and Mg^{2+} [19–22]. Moreover, thioesterification is the first step

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§ *Abbreviations:* CoA, coenzyme A: ATP, adenosine triphosphate; GC/MS gaschromatography/masspectrometry; DTT, dithiothreitol.

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in the formation of amino acid conjugates [23], and is also necessary for the incorporation of arylpropionic acids into triacylglycerols [24].

The relative contribution of microsomal long chain acyl-CoA synthetases to the formation of R-ibuprofenoyl-CoA has not been fully elucidated. Therefore, long-chain acyl-CoA synthetase from rat liver microsomes was purified [25] to determine whether or not this acyl-CoA synthetase, as well as a mitochondrial form, catalyzes the thioester formation of both palmitic acid and R-ibuprofen.

MATERIALS AND METHODS

Chemicals

ATP Grade I, coenzyme A, dithiothreitol, palmitoyl-CoA (as chromatographic standard), palmitic acid, and Triton X-100 were purchased from Sigma (Deisenhofen, Germany). R- and S-ibuprofen were kindly supported by Pharma Trans Sanaq AG (Basel, Switzerland). Acetonitrile and other chemicals were obtained from Merck (Darmstadt, Germany).

Chromatography Materials

Blue-Sepharose CL-6B fast flow and Sephadex G-25 were obtained from Pharmacia LKB (Freiburg, Germany), hydroxyapatite from Biorad (Munich, Germany), phosphocellulose (P-11) from Whatman (Biometra, Göttingen, Germany) and C_8-reverse-phase column (Nucleosil 120-3C_8) from Macherey-Nagel, (Düren, Germany). Centriplus-50 concentrators were purchased from Amicon (Witten, Germany).

Synthesis of R-ibuprofenoyl-CoA

R-ibuprofenoyl-CoA thioester (as chromatographic standard) was prepared as described by Porubek et al. [26] for flurbiprofenoyl-CoA, with some modifications. Briefly, R-ibuprofen (1 g) was mixed with thionylchloride (1 mL) in a 10-mL flask and 3 drops of dimethylformamide were added. After 1 hr, the reaction mixture was heated (50°C) and the excess thionylchloride removed under reduced pressure. The oily reaction product was gassed with nitrogen (30 min). $NaHCO_3$ (10 mL, 0.1 M, pH 7.5) was flushed with nitrogen (1 hr). Coenzyme A (50 mg) was dissolved in the buffer and the oily solution added in drops under controlled pH. The reaction mixture was allowed to react for at least 2 hr. The production of the thioester was monitored by HPLC analysis. The reaction was stopped by adjusting the pH to 3 (HCl) followed by extraction of unreacted ibuprofen with diethylether. Quantification of the thioester was performed after hydrolysis with NaOH solution (2N) and GC/MS determination of R-ibuprofen according to Scheuerer *et al.* [27]. The yield of the reaction was approximately 20%.

Structure Determination of Chemically Synthesized R-ibuprofenoyl-CoA

The electron impact mass spectrometry (EIMS) did not show any ion above m/z 500. Highest observable mass was m/z 448, corresponding to a fragment formed by cleavage of the pantothenic acid-adenosine bond. Intensive peaks at m/z 135 and m/z 161 were attributable to adenine and decarboxy-ibuprofen cations, respectively. In the negative ion fast atom-bombardment mass spectrometry (FABMS), ions at m/z 954 $[M\text{-}H]^-$, 976 $[M\text{-}H + Na]^-$, 992 $[M\text{-}H + K]^-$, 1014 $[M\text{-}2H + Na + K]^-$ and 1030 $[M\text{-}2H + 2K]^-$ were observed. The ^1H nuclear magnetic resonance spectrum (^1H-NMR) in D_2O showed all the expected proton resonances, as follows: ibuprofen: δ = 7.16 and 7.12 (4 × ar-H), 3.96 (H-2), 2.31 (CH_2 at C-1″), 1.70 (H-2″), 1.38 (CH_3 at C-2) and 0.75 (2 × CH_3 at C-2″); adenosine: δ = 8.56 and 8.17 (H-2 and H-8), 6.12 (H-1′), 4.78, 4.55, 4.17 and 3.94 (H-2′ – H-5′); pantothenic acid: δ = 3.82 and 3.51 (2 × H-8), 3.25 (H-6, 2 × H-3), 2.21 (2 × H-2), 0.66 and 0.82 (2 × CH_3 at C-7); β-mercaptoethylamin: δ = 3.25 (CH_2 at N), 2.99 and 2.86 (CH_2 at S). The structure was further confirmed by proton-detected heteronuclear correlation spectroscopy (HMQC, HMBC). The purity of R-ibuprofenoyl-CoA was found to be >95%.

HPLC Analysis of R-ibuprofenoyl-CoA and Palmitoyl-CoA

Thioester production was assayed using an HPLC system consisting of a solvent-delivery module (Beckman system gold 110B, Camino Ramon, CA), a programmable detector (Beckman module 166), an analog interface (Beckman module 406), a sample injector (Gilson Abimed model 232, Langenfeld, Germany), a dilutor (Gilson Abimed module 401), and an integrator (Perkin Elmer LCI-100, Weiterstadt, Germany). The thioesters were separated on a C_8-reverse phase column by eluting with K_2HPO_4 solution (10 mM, pH 6.0) and 21% acetonitrile for ibuprofenoyl-CoA and 38% acetonitrile for the assay of palmitoyl-CoA. The flow rate was 1 mL/min and the UV detection was set at 254 nm. The retention times observed for ibuprofenoyl-CoA and palmitoyl-CoA were 5.3 and 3.8 min, respectively.

Purification of Acyl-CoA Synthetase

The isolation of the R-ibuprofenoyl-CoA synthetase was performed according to the previously reported protocol of Tanaka *et al.* [25] for the purification of long-chain acyl-CoA synthetase from rat liver, with some modifications. Briefly, rat liver microsomes were prepared from livers of rats (N = 10, male, Sprague Dawley, 250–300 g body weight from Savo Ivanovas, Kisslegg, Germany) by homogenization in a buffer containing sucrose (0.25 M), Tris-base (5 mM), EDTA (1 mM, pH 7.4) and subsequent differential ultracentrifugation according to standard procedures. The microsomes were resuspended in buffer (50 mL) containing K_2HPO_4 (20 mM), DTT (2 mM), and EDTA (1 mM, pH 7.4). Solubilization of the microsomes was achieved by add-

ing in drops Triton X-100 (20 mL, 0.6% v/v, final concentration 0.3%, protein concentration approximately 5 mg/mL), and stirring the protein suspension (1 hr) on ice. Then the mixture was centrifuged (100,000 × g, 1 hr 15 min). The resulting supernatant (60 mL) was collected and diluted with 5 volumes of a solution containing K_2HPO_4 (20 mM), DTT (2 mM), and Triton X-100 (0.15% v/v, pH 7.4; buffer A). The diluted solution (300 mL) was applied to a Blue-Sepharose column (50 mL gel, fast-flow quality, XK 26/20 Pharmacia, Freiburg, Germany) equilibrated with buffer A. The column was washed with 3 volumes of buffer A and 3 volumes of buffer A containing ATP (10 mM). Ibuprofenoyl-CoA synthetase was eluted with buffer A containing ATP (10 mM) and NaCl (1.2 M) at a flow rate of 1.5 mL/min and a fraction size of 5 mL. Fractions showing a high activity for thioester formation were pooled (approximately 300 mL), and concentrated (10 mL) by ultrafiltration with a Diaflo membrane filter PM-10 (Amicon, Witten, Germany). For desalting, the concentrate was passed through a Sephadex G-25 column (3 × 20 cm) equilibrated with buffer A for desalting. The protein-containing fractions (15 mL) were combined and diluted with one volume of buffer A and applied on a hydroxyapatite column (XK 16/20 Pharmacia, Freiburg, Germany). The column was washed with buffer A (3 volumes) and then eluted with a linear concentration gradient established between buffer A (90 mL) and the same volume of buffer A containing potassium phosphate (200 mM). The flow rate was 0.5 mL/min and fractions of 2.5 mL were collected. The enzyme with the ibuprofenoyl and palmitoyl-thioester activity appeared in a single peak that coincided with the main protein peak. Fractions 36–48, exhibiting high activities and a protein band at 72 kDa in the SDS gel, were pooled (30 mL) and concentrated to 2mL with Centriplus 50 (Amicon, Witten, Germany). The concentrate was diluted with buffer A (4 volumes) and applied on a phosphocellulose (P-11) column (Whatman, Biometra, Göttingen, Germany). The column was washed with buffer A (3 volumes) and then eluted with a linear concentration gradient made between buffer A (90 mL) and buffer A (90 mL) containing K_2HPO_4 (200 mM) at a flow rate of 0.7 mL/min, and fractions of 2.5 mL were collected. The enzyme with both thioester-formation activities appeared in a single peak. The Bio-Rad protein assay (Bio-Rad, Munich, Germany), based on the Bradford dye-binding procedure, was used to measure total protein concentration with bovine serum albumin as standard. SDS polyacrylamide gel electrophoresis with minigels (10 × 9 cm) containing polyacrylamide (10%) was performed with a system from GIBCO BRL (Mini-V 8.10) according to the procedure of Laemmli [28]. After electrophoresis, the gels were silver stained.

Formation of Ibuprofenoyl- and Palmitoyl-CoA Thioester

An aliquot (25 μL) of each protein fraction was preincubated (1 min at 37°C) and a standard reaction mixture (25 μL) was added to give a final concentration of 3 mM ibuprofen or 100 μM palmitic acid, respectively. The final concentrations of the other cofactors were: 15 mM Mg^{2+}, 150 mM KCl, 1.8 mM ATP, and 0.33 mM CoA in 100 mM Tris-HCl pH 7.4. Incubations were performed at 37°C (2 min for palmitic acid and 5 min for ibuprofen), and the reaction was stopped by adding acetonitrile (50 μL). After

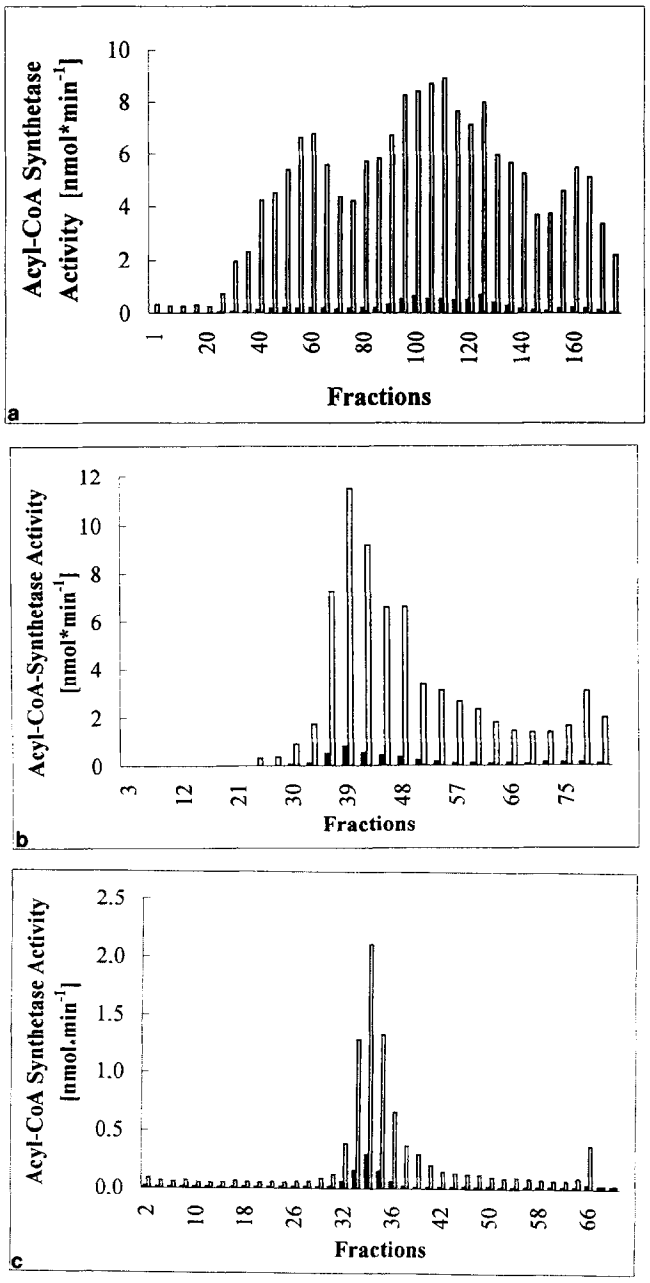

FIG. 1. Blue-Sepharose (1A), hydroxyapatite (1B), and phosphocellulose chromatography (1C) of R-ibuprofenoyl-CoA synthetase from rat liver microsomes. Elution profiles of R-ibuprofenoyl- (black columns) and palmitoyl-CoA (white columns) activities were determined for each purification step using a 25 μL probe of each fraction, following incubation with the standard reaction mixture of R-ibuprofen (5 min) and palmitic acid (2 min), respectively. For details see Materials and Methods.

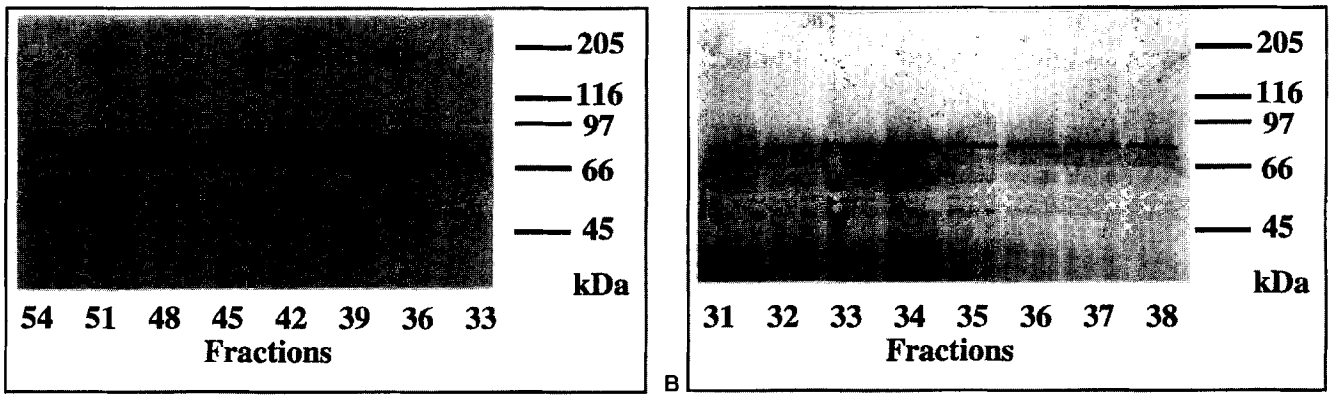

FIG. 2. SDS gel electrophoresis of various fractions of hydroxyapatite (2A) and phosphocellulose chromatography (2B) exhibiting a high activity for R-ibuprofenoyl- and corresponding palmitoyl-CoA formation, assayed by the HPLC method with the standard reaction mixture.

centrifugation, the precipitated protein was found in the pellet. An aliquot (80 μL) of the supernatant was diluted with H_2O (80 μL) to reach a final acetonitrile concentration of 25%; 50 μL of this probe was assayed by HPLC. The increasing salt concentration in the protein fractions led to a slight shift towards lower values in the retention times of the enzymatically-produced thioesters compared with the standards.

Data Analysis

K_M and V_{max} were estimated from Eadie-Hofstee plots of the data [29]. Analysis of the data from the inhibition studies was carried out using the method of Dixon [30].

RESULTS

Isolation of R-ibuprofenoyl-CoA Synthetase

SDS gel electrophoresis of the protein preparation (phosphocellulose fraction) and silver staining of the gel showed a single band at a molecular weight of 72 kDa. The pooled fractions of phosphocellulose chromatography (fractions 33–36) exhibited specific activities of 71 nmol · min^{-1} · mg^{-1} protein and 901 nmol · min^{-1} · mg^{-1} protein for the formation of R-ibuprofenoyl-CoA and palmitoyl-CoA, respectively. After each chromatographic fractionation, enzyme activity for palmitoyl-CoA and R-ibuprofenoyl-CoA formation was determined by HPLC. Both palmitoyl-CoA synthetase and R-ibuprofenoyl-CoA synthetase activities eluted together in the various protein peaks and fractions measured (Fig. 1), and with approximately the same ratio of activities in all peaks (data not shown). Gel electrophoresis of fractions with higher activities (Fig. 2) showed a strong correlation between the amount of enzyme at 72 kDa and the determined activities. A further attempt to separate R-ibuprofenoyl- and palmitoyl-CoA synthetases using gel filtration was unsuccessful.

Characterization of Acyl-CoA Synthetase

The activity of microsomal R-ibuprofenoyl-CoA synthetase was assessed over a concentration range of 5–3000 μM R-ibuprofen. An Eadie-Hofstee plot showed the involvement of a single enzyme in the formation of R-ibuprofenoyl-CoA (inlay of Fig. 3). The K_M and V_{max} were determined on the data from 3 incubations, giving a mean K_M of 184 ± 19 μM and a V_{max} of 71 nmol · min^{-1} · mg^{-1} protein. The determination of R-ibuprofenoyl-CoA activities with 0.1–5 μM R-ibuprofen as substrate resulted in no measurable thioester concentrations.

The measurement of palmitoyl-CoA activity of the purified enzyme over a concentration range of 0.1–500 μM revealed biphasic kinetics (Fig. 3), corresponding to high-affinity (K_M 0.13 ± 0.011 μM), low-capacity and low-affinity (K_M 81 ± 11.5 μM), high-capacity isoforms.

Investigations of the inhibition kinetics of R-

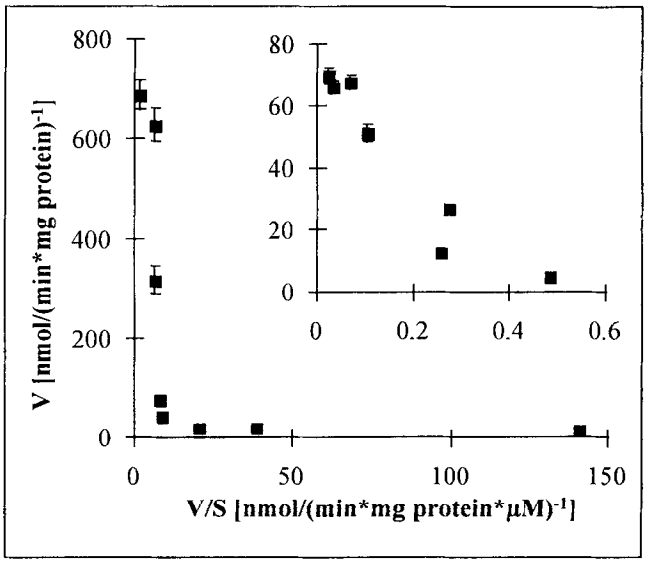

FIG. 3. Representative Eadie-Hofstee plot for palmitoyl-CoA formation by rat liver microsomal long-chain acyl-CoA synthetase. Palmitic acid concentrations were 0.1, 0.5, 1, 5, 10, 50, 100, and 500 μM (mean ± SD, N = 4). Inlay of Fig. 3 shows a representative Eadie-Hofstee plot for R-ibuprofenoyl-CoA formation by rat liver microsomal long-chain acyl-CoA synthetase. R-ibuprofen concentrations were 10, 50, 100, 500 μM and 1, 2, 3 mM (mean ± SD, N = 4).

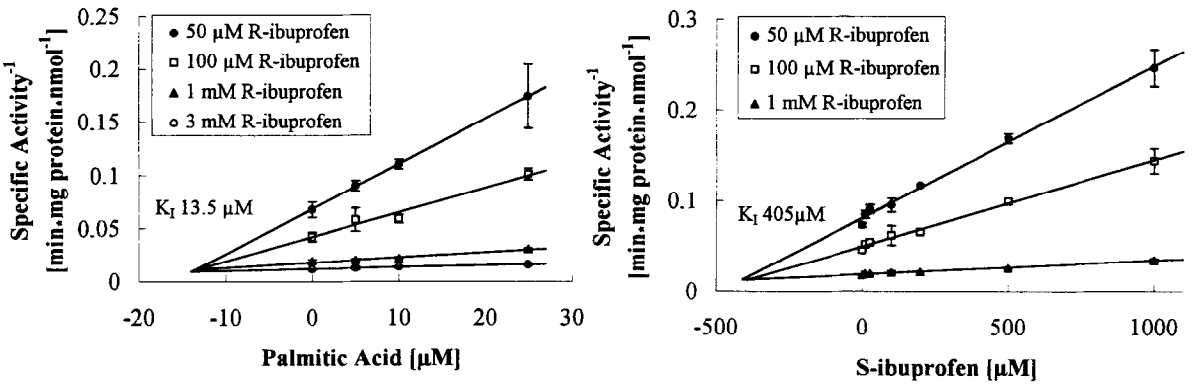

FIG. 4. A. Dixon plot for the inhibition of R-ibuprofenoyl-CoA formation by palmitic acid (5–25 µM, mean ± SD, N = 3). B. Dixon plot for the inhibition of R-ibuprofenoyl-CoA formation by S-ibuprofen (10–1000 µM, mean ± SD, N = 3).

ibuprofenoyl-CoA synthetase were performed using 50, 100 µM, 1 and 3 mM R-ibuprofen. Plots of reciprocal velocity vs inhibitor concentration are shown in Fig. 4A for palmitic acid and Fig. 4B for S-ibuprofen. An intercept above the baseline is consistent with competitive inhibition kinetics. The K_I values for the inhibition of R-ibuprofenoyl-CoA synthesis by palmitic acid and S-ibuprofen were 13.5 ± 0.5 µM and 405 ± 10 µM, respectively.

The heat stability of purified R-ibuprofenoyl-CoA synthetase was investigated between 30 and 60°C (data not shown). Comparable to results reported for purified palmitoyl-CoA synthetase from rat lung [31], the enzyme remained fully active at 40°C. Inactivation began at 45°C and was complete at 55°C. Loss of enzyme activity was accompanied by observable precipitation of protein.

The dependence of R-ibuprofenoyl-CoA formation on pH is shown in Fig. 5. A broad optimum was observed between pH 5.5 and 8, comparable to previous results reported for long chain acyl-CoA synthetase from rat liver microsomes and mitochondria [32].

DISCUSSION

Microsomal and mitochondrial long-chain acyl-CoA synthetase [EC 6.2.1.3] catalyzes the formation of acyl-CoA from fatty acids, ATP, and CoA [25]. Up to now, two different long chain acyl-CoA synthetases expressed in the liver and the brain of rats have been characterized by molecular biology [33]. Nevertheless, the multiplicity of isoforms and their ability to activate various xenobiotic compounds to their thioesters are of pharmacological interest and require further investigation.

In the present work, we report on the purification to homogeneity and enzymatic characterization of a microsomal long-chain acyl-CoA synthetase from rat liver that is responsible for the enantioselective activation of R-ibuprofen to its CoA-thioester. The protein isolation procedure resulted in the enrichment of a 72 kDa protein (Fig. 2) containing activity for the formation of R-ibuprofenoyl- and palmitoyl-CoA. The small difference in the molecular mass of our isolated protein compared with the 76 kDa protein reported by Tanaka *et al.* [25] could be explained by our use of minigels and a different gel electrophoresis system. However, according to the enzymatic activity, there was no doubt that the purified 72kDa protein was a long-chain acyl-CoA synthetase able to form palmitoyl-CoA. The high correlation of R-ibuprofenoyl- and palmitoyl-CoA activities in each purification step (Fig. 1) and every measured fraction gave strong evidence that the same enzyme is responsible for both activities in rat liver microsomes. It is worth noting that palmitoyl-CoA formation is more than 10 times faster than that of R-ibuprofenoyl (Fig. 1), illustrating the preference of the enzyme for fatty acids as physiological substrates compared with xenobiotics.

The enzymatic characterization of the purified protein exhibited biphasic kinetics for the activation of palmitic acid to palmityol-CoA, as previously reported for microsomal and peroxisomal preparations [34, 35]. The K_M values of the high-affinity (0.13 ± 0.011 µM), low-capacity and low-affinity (81 ± 11.5 µM), high-capacity isoforms markedly differ from the values reported in preparation from organelles (peroxisomes 2.3 µM/831 µM, microsomes 1.6 µM/506 µM). The difference in kinetic behaviour could result from the interaction between lipids and palmitic acid in the crude enzyme preparations. The require-

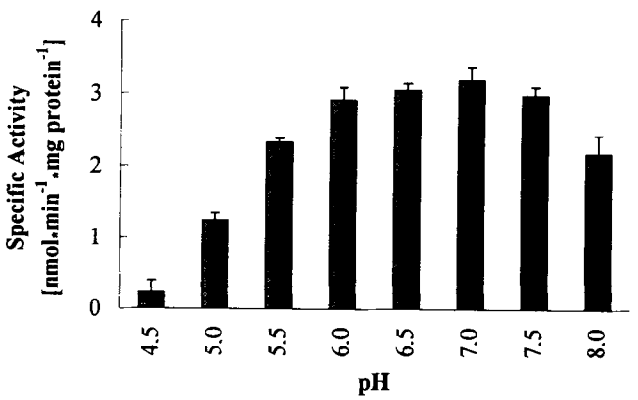

FIG. 5. Dependence of R-ibuprofenoyl-CoA formation on pH. Activity was measured with the standard reaction mixture as described in Methods.

ment of higher concentrations of palmitate with the microsomal enzyme may indicate that some palmitate interacts nonspecifically with the enzyme as a detergent. In addition, the delipidation step that is necessary to purify the membrane protein and that changes the protein environment could have a significant influence on the apparent K_M values. Recently, Benoit et al. [21] investigated the influence of Triton X-100 on the affinity of fenoprofen for long-chain acyl-CoA synthetase in rat liver microsomes, and also observed a reduction in the apparent K_M value. Like Benoit *et al.* [21], we used a direct quantification method for the assay of thioester formation, which may explain the Michaelis-Menten parameters obtained in the present study.

Measurement of the thioesterification of R-ibuprofen over the concentration range 5–3000 μM showed that a single protein participated in the formation of R-ibuprofenoyl-CoA. In contrast to palmitic acid as substrate, it was not possible to determine Michaelis-Menten parameters for R-ibuprofen in the range 0.1–5 μM because product formation fell below the detection limit of the assay. Consequently, the existence of a second isoform of R-ibuprofenoyl-CoA synthetase, as previously described by Knights *et al.* [36] in rat liver microsomes, cannot be excluded.

Under the conditions chosen, we could not observe the activation of S-ibuprofen to S-ibuprofenoyl-CoA by purified R-ibuprofenoyl-CoA synthetase. On the contrary, the thioesterification of R-ibuprofen was inhibited (K_M 405 ± 10 μM) competitively by S-ibuprofen (Fig. 3).

The inhibition of R-ibuprofenoyl-CoA synthesis by palmitic acid (K_I 13.5 ± 0.5 μM), the pH-optimum (Fig. 4), and the heat stability of the purified enzyme lend further support to the conclusion that R-ibuprofenoyl- and palmitoyl-CoA synthesis are catalyzed by the same enzyme.

Further investigations are required to determine whether or not different isoforms of long-chain acyl-CoA synthetase are responsible for the thioesterification of R-ibuprofen and 2-arylpropionic acids, in general.

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