



# Inhibition of UDP-Glucuronosyltransferase Activity by Fatty Acyl-CoA

## KINETIC STUDIES AND STRUCTURE–ACTIVITY RELATIONSHIP

Atsushi Yamashita,\* Tomonari Nagatsuka, Masanobu Watanabe, Hironori Kondo,  
Takayuki Sugiura and Keizo Waku

FACULTY OF PHARMACEUTICAL SCIENCES, TEIKYO UNIVERSITY, SAGAMIKO, KANAGAWA 199-01, JAPAN

**ABSTRACT.** We previously identified and purified UDP-glucuronosyltransferase (UGT) isoforms as targets of protein acylation from rat liver microsomes (Yamashita *et al.*, *Biochem J* 312: 301–308, 1995). The acylation of UGT isoforms occurred upon incubation with acyl-CoA without another protein acyltransferase, suggesting that it was autoacylation. The study revealed the interaction of UGT isoforms with acyl-CoA. In the present study, the effects of fatty acyl-CoA on UGT activities were examined thoroughly, using a rat liver microsomal and purified enzyme fractions. The UGT activities of both fractions were inhibited by acyl-CoA in a concentration-dependent manner. The effect of acyl-CoA was observed on the activities toward various substrates, suggesting that the effect shows the wide spectrum of the isoforms of UGT. To assess the mechanism underlying the inhibition of UGT activity by acyl-CoA, the relationship of the inhibition, acyl-CoA binding to the proteins, and changes in the tertiary structure of the enzyme were examined. The kinetics of these phenomena were related closely with each other. Furthermore, the inhibition of UGT activity was specified for acyl-CoA, though a structurally related compound, acyl-3'-dephosphoCoA, had no inhibitory effect. The results suggested that the specific binding of acyl-CoA to UGT isoforms induced conformational changes of the enzymes and resultant inhibition of UGT activity. *BIOCHEM PHARMACOL* 53;4:561–570, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** acyl-CoA; UDP-glucuronosyltransferase; inhibitor; rat liver

Drug-metabolizing enzymes in animals are responsible for the modification of a large number of endogenous substrates, as well as innumerable foreign chemicals. Many foreign compounds, such as drugs and environmental pollutants, are nonpolar and readily dissolve in lipids. The principal role of drug-metabolizing enzymes is the conversion of these chemicals into highly water-soluble products that can be excreted. Drug metabolism has classically been divided into Phase I (functionalization) and Phase II (conjugation) reactions. Phase I enzymes introduce a functional group, such as OH, into the substrates; Phase II enzymes then use this functional group as a handle for conjugation with such moieties as glucuronic acid, sulfate, and glutathione [1–4]. Glucuronide formation is known to be catalyzed by the UGT† (EC 2.4.1.17) system. Evidence for the existence of several isoforms has been provided by purification and cDNA cloning [3, 4]. These facts indicate that UGT constitutes a multigene family, as reported for cytochrome P450 isoenzymes [5]. Recently, a nomenclature system and

a systematic classification of the UGT superfamily were proposed [4].

We previously purified UGT isoforms as targets of protein acylation from rat liver microsomes [6]. These isoforms were identified as UGT2B3, UGT2B2, and UGT2B6, which conjugate steroid hormones, by amino acid sequencing analysis and UGT assay. UGT2B3, UGT2B2, and UGT2B6 are very closely related enzymes, and are members of the UGT 2B gene subfamily [4]. UGT2B3 and UGT2B6 have very similar structures (93% similar in amino acid sequence) and substrate specificities [7, 8]. UGT2B6 and UGT2B2 also have very similar amino acid sequences (83%) but have distinct substrate specificities [9]. We also found that these isoforms have acyl-CoA binding activity, and that the interaction of UGT proteins with acyl-CoA suppresses the UGT activities toward testosterone and androsterone. Our results suggested that acyl-CoA was a modulator of some members of the UGT 2B subfamily.

To date, there is little information concerning inhibitors of glucuronidation [3, 10, 11], and results regarding the regulation of UGT activity are also extremely limited. The details of the inhibition by acyl-CoA have not been fully elucidated. In particular, the specificity and mechanism of the inhibition by acyl-CoA have not been examined. Thus, several questions arise, i.e. whether the effect of acyl-CoA is a specific action of the compound, and whether acyl-CoA

† Corresponding author. Tel. 81-426-85-3747; FAX 81-426-85-1345.

† Abbreviations: used CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TLC, DTT, dithiothreitol; and UGT; UDP-glucuronosyltransferase. Fatty acids are designated in terms of the number of carbon atoms:the number of double bonds, e.g. 20:4 denotes arachidonic acid.

Received 27 June 1996; accepted 22 August 1996.

can act on any isoforms of UGT. To solve these problems, in the present study, (i) the effect of acyl-CoA on UGT activity in purified enzyme and microsomal fractions was studied, (ii) the kinetics of the inhibition of the UGT activity by acyl-CoA and the mode of inhibition were characterized, and (iii) the structure-activity relationship of acyl-CoA as to the inhibition was investigated, and the screening of the modulator of enzyme activity from related compounds was performed. Furthermore, the relationship of the inhibition, acyl-CoA binding, and the change in the tertiary structure were examined to assess the mechanism of the inhibition. Our systematic study revealed that acyl-CoA is the most potent inhibitor of UGT activity. Finally, the physiological significance of the inhibition of UGT activity is discussed.

## MATERIALS AND METHODS

### Chemicals

[1-<sup>14</sup>C]Arachidonoyl-CoA (1.7 GBq/mmol), UDP-[<sup>14</sup>C]glucuronic acid (10.55 GBq/mmol), and EN<sup>3</sup>HANCE were purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Fatty acids (C14:0, C16:0, C18:0, C18:1, C18:2, and C20:4), short and medium chain acyl-CoAs (C2:0-, C3:0-, C4:0-, C6:0-, C8:0-, C10:0-, and C12:0-CoA), UDP-glucuronic acid, and azolectin (crude soybean lecithin) were obtained from Sigma (St. Louis, MO, U.S.A.). CoA was obtained from Kyowa Hakko, Ltd. (Tokyo, Japan). CHAPS was purchased from Dojindo Laboratories (Kumamoto, Japan). Testosterone, androsterone, bilirubin, 4-nitrophenol, and Triton X-100 were purchased from Wako Pure Chemical Industries (Osaka, Japan). TSK-gel AF Red-, Blue- and heparin-Toyopearl 650 ML were purchased from Tohso (Tokyo, Japan). Bio-gel HTP was from Bio-Rad (Richmond, CA, U.S.A.). Sephacryl S300 HR was from Pharmacia LKB (Uppsala, Sweden). TLC plates (silica gel 60, 5721) were from Merck (Darmstadt, Germany). Long chain acyl-CoA esters having various fatty acid moieties were synthesized as described previously [12]. Palmitoyl-3'-dephosphoCoA was prepared by nuclease P1 treatment of palmitoyl-CoA, and was purified by TLC developed with *n*-butanol:acetic acid:water (5:2:3) [13]. [<sup>14</sup>C]Arachidonoyl-3'-dephosphoCoA was also prepared by the same procedure. Adenosine 5'-alkylphosphates were donated by Drs. Takayuki Shiraishi and Yutaka Uda (Niigata College of Pharmacy) [14]. All other reagents were of reagent or analytical grade.

### Preparation of a Microsomal Fraction from Rat Liver

Male rats of the Wistar strain, about 300 g body weight (Sankyo Lab. Service Co., Tokyo, Japan), were used. All animals were exposed to an alternating light-dark cycle (light from 8:00 a.m. to 6:00 p.m.), and were fed *ad lib.* a control diet. Their livers were isolated rapidly, and microsomes were prepared by differential centrifugation as described previously [6, 12]. Briefly, the organs were homog-

enized with a Potter-Elvehjem glass Teflon homogenizer in 0.25 M sucrose/0.1 M Tris-HCl/1 mM EDTA (pH 7.4) (STE buffer) containing 0.2 mM phenylmethylsulfonyl fluoride, and then the homogenate was centrifuged at 700 g for 10 min to remove cell debris and nuclear materials. The supernatant was centrifuged at 10,000 g for 15 min, and the resultant supernatant was further centrifuged at 105,000 g for 60 min. The pellet was washed twice with STE buffer by the same centrifugation, and then was suspended in STE buffer and stored at -80°C until used.

### Measurement of UGT Activity using a Radiolabeled Substrate

UGT activity was measured according to the method of Bansal and Gessner [15] with minor modifications. The microsomal fraction (0.2 mg) was incubated with 0.8 mM UDP-[<sup>14</sup>C]glucuronic acid (3.7 kBq) and 5 mM MgCl<sub>2</sub> in the presence of a 200 μM concentration of various substrates. In some cases, the activity was measured in the presence of palmitoyl-CoA (20 μM). Incubation was carried out at 30° C for 30 min, and the reaction was terminated by the addition of 2 vol. of ethanol. The ethanolic mixtures were evaporated and applied to silica gel TLC plates, and then the chromatograms were developed with *n*-butanol:acetone:acetic acid:ammonia:water (70:50:18:1.5:60, by vol.). After development, the plates were subjected to fluorography. The areas corresponding to [<sup>14</sup>C]glucuronides were scraped off into counting vials, and then the radioactivity was measured.

### Spectrophotometrical Measurement of UGT Activity

UGT activity toward 4-nitrophenol was also determined by measurement of the absorbance of 4-nitrophenol at 405 nm [16] using a spectrophotometer model UV-1600 (Shimadzu Corp., Kyoto, Japan). The microsomal fraction was incubated in a cuvette with 0.4 mM 4-nitrophenol and 4 mM UDP-glucuronic acid in the presence of 5 mM MgCl<sub>2</sub>, the decrease in absorbance being monitored continuously. The activity was calculated from the slopes of the decrease of the absorbance versus incubation time ( $18.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ). To determine the effects of various compounds, including acyl-CoA, the activity was measured in the presence of various reagents.

Alternatively the UGT activity toward 4-nitrophenol was also determined in the presence of various amounts of 4-nitrophenol (0.1 to 1.5 mM) and UDP-glucuronic acid (1 to 8 mM). Incubation was carried out at 30° for 15 min, and the reaction was terminated by the addition of the same volume of 1% trichloroacetic acid. Each incubate was diluted and adjusted to pH > 10 with 1 N NaOH, and then the absorbance at 405 nm was measured.

### [<sup>14</sup>C]Acyl-CoA Labeling (Autoacylation) Assay [6]

The fractions to be tested (2.5 μg protein unless otherwise indicated) were incubated with [<sup>14</sup>C]arachidonoyl-CoA

(20  $\mu$ M) for various periods at 30°. The reaction was terminated by the addition of SDS (3% final concentration) and bromphenol blue, and the products were analyzed by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, destained, and then subjected to fluorography using EN<sup>3</sup>HANCE at -80° with an intensifying screen. In some cases, the bands of interest on the gels were also quantified with a liquid scintillation spectrometer.

#### **Purification of UGT Isoforms from Rat Liver Microsomes**

UGT isoforms were purified as described previously [6] with [<sup>14</sup>C]acyl-CoA labeling. Briefly, the purification procedure consisted of the solubilization of microsomes with CHAPS and sequential chromatography. The microsomal fraction was solubilized with 1% CHAPS, 20% ethyleneglycol and 50 mM Tris-HCl (pH 7.4) (detergent/protein ratio, approximately 2), and the resultant clear solution was centrifuged at 105,000 g for 60 min. The resultant supernatant was subjected to sequential chromatography on Red-Toyopearl, hydroxylapatite, heparin-Toyopearl, and Blue-Toyopearl. The elution pattern on each column was checked by SDS-PAGE, with staining with Coomassie Brilliant Blue and [<sup>14</sup>C]acyl-CoA labeling.

#### **[<sup>14</sup>C]Acyl-CoA Binding Assay**

UGT isoforms (Blue-Toyopearl fraction, 2.5  $\mu$ g) were incubated with various concentrations of [<sup>14</sup>C]arachidonoyl-CoA for 20 min at 30°. Protein-bound and free [<sup>14</sup>C]arachidonoyl-CoA were separated by gel filtration. The mixture was loaded onto Sephacryl S300 HR ( $V_t$  = 20 mL), pre-equilibrated with 25 mM Tris-HCl (pH 7.4), 0.1% CHAPS and 1 mM DTT, and eluted with the same buffer, 0.5-mL fractions being collected. The radioactivity of each fraction was determined with a liquid scintillation spectrometer. In some cases, UGT proteins were incubated with 20  $\mu$ M [<sup>14</sup>C]arachidonoyl-CoA for various periods, and then protein-bound [<sup>14</sup>C]arachidonoyl-CoA was measured after bound/free separation by gel filtration. Binding of [<sup>14</sup>C]arachidonic acid and [<sup>14</sup>C]arachidonoyl-3'-dephosphoCoA to the proteins was measured by the same procedure.

#### **Fluorescence Spectroscopy**

Fluorescence studies were performed with a JASCO model FP777 spectrofluorometer according to the principles described by Ward [17]. The excitation and emission bandpasses were both 4 nm for all experiments. A solvent blank was measured immediately before each spectrum. Emission spectra, from 290 to 400 nm, were recorded at room temperature in a cuvette containing purified proteins (0.12  $\mu$ M protein in 20 mM Tris-HCl, pH 7.4, 0.05% CHAPS, 1% ethyleneglycol, 50 mM DTT, 5  $\mu$ M EDTA, and 5  $\mu$ g/mL azolectin) with excitation at 285 nm. To determine the

effect of acyl-CoA, we measured the relative decrease in the intrinsic fluorescence of the purified UGT upon the addition of a long chain acyl-CoA. In some experiments, the purified proteins were treated with CoA or palmitoyl-3'-dephosphoCoA instead of acyl-CoA, and then spectra were recorded.

#### **Protein Assay**

Protein contents were determined by the method of Lowry *et al.* [18] with bovine serum albumin as a standard.

#### **Statistical Analysis**

Data are given as means  $\pm$  SD, the results obtained with the Student's *t*-test being presented where appropriate.

### **RESULTS**

#### **Effect of acyl-CoA on UGT Activity in the Purified Enzyme and Microsomal Fractions**

First, we examined the effect of acyl-CoA on the UGT activity of the purified enzyme. As shown in Fig. 1A, arachidonoyl-CoA reduced the enzyme activity that glucuronidated testosterone in a concentration-dependent manner. The concentration of arachidonoyl-CoA required for half-maximal inhibition was found to be 11  $\mu$ M. The inhibition occurred immediately because the effect of pre-incubation with arachidonoyl-CoA was not prominent (Fig. 1B). Several types of long chain acyl-CoA with different chain lengths and different degrees of unsaturation also inhibited the enzyme activity (Fig. 1C).

Then, to determine whether acyl-CoA affects the activity toward other substrates, the effects of acyl-CoA on microsomal UGT activities toward various substrates were examined. As shown in Table 1, glucuronidation of the indicated endogenous lipophilic substances and foreign compounds, such as drugs and environmental pollutants, occurred. The results demonstrated the presence of UGT activities toward various substrates in the microsomal fraction of rat liver. The addition of 20  $\mu$ M palmitoyl-CoA reduced the UGT activities in microsomes toward these substrates to 30–60% (Table 1). An inhibitory effect of acyl-CoA was observed not only toward steroids but also other substrates, such as bilirubin, vitamin A, and 1-naphthol. The results suggested that acyl-CoA affected other subfamilies as well as the isoforms that conjugated steroids. The inhibition by palmitoyl-CoA of each activity was dependent on the concentration of acyl-CoA. The concentration required for half-maximal inhibition was approximately 20–30  $\mu$ M for various substrates (data not shown; similar results are shown in Fig. 2).

#### **Mode of Inhibition of the UGT Activity Toward 4-Nitrophenol by acyl-CoA**

To assess the mode of inhibition by acyl-CoA, kinetic studies were performed. We analyzed the activity toward 4-ni-

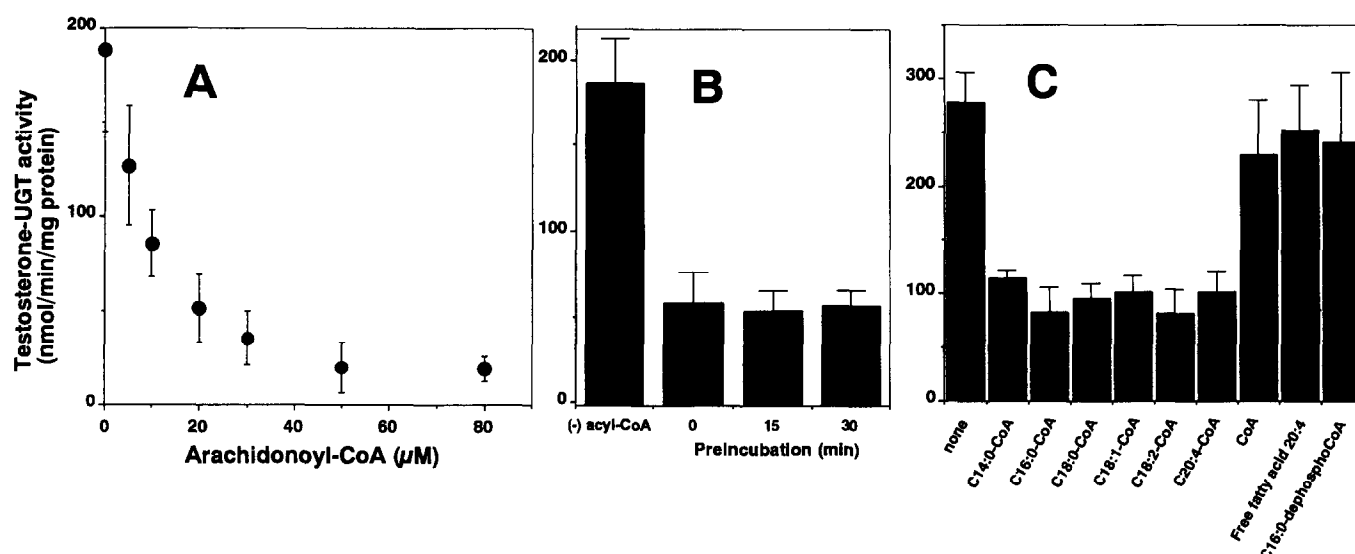


FIG. 1. Inhibition of testosterone-UGT activity by acyl-CoA. (A) Testosterone-UGT activity of the purified enzyme (2.5 μg protein) was measured in the presence of the indicated amounts of arachidonoyl-CoA. (B) The purified enzyme (2.5 μg protein) was preincubated with 20 μM arachidonoyl-CoA for 0, 15, and 30 min, and then assayed for testosterone-UGT activity (30 min of incubation). (C) UGT activity of the purified enzyme (2.5 μg protein) was measured in the presence of a 20 μM concentration of the indicated reagents. Data represent means  $\pm$  SD of triplicate determinations.

trophol in the microsomal fraction, because 4-nitrophenol is relatively water-soluble, and its activity can be measured easily by the spectrophotometrical method. UGT catalyzes a bisubstrate reaction, and the kinetic mechanism of UGT comprises rapid equilibrium, random order [19]. For this reason, the initial rates of the reaction in the absence or presence of the inhibitor, palmitoyl-CoA (20 and 40 μM), were studied as a function of the concentration of UDP-glucuronic acid at a fixed concentration (1.5 mM) of

4-nitrophenol, and as a function of the concentration of 4-nitrophenol at a fixed concentration (4 mM) of UDP-glucuronic acid. As shown in panels A and B of Fig. 2, the data, when plotted in double-reciprocal form, indicated that the intercept on the 1/S axis for either substrate was  $-1/K_m$ . The  $K_m$  values for UDP-glucuronic acid and 4-nitrophenol were 1.9 and 0.3 mM, respectively. The values did not change in the presence of acyl-CoA, suggesting that acyl-CoA acted on the enzyme non-competitively for each

TABLE 1. Effect of palmitoyl-CoA on UGT activities toward various endogenous (A) and exogenous (B) substrates

Substrates	UGT activity (nmol/min/mg protein)		
	Control	Palmitoyl-CoA	% of Control
(A) Endogenous substrates			
Bilirubin	0.24 $\pm$ 0.04	0.12 $\pm$ 0.05*	50.0
Vitamin A	0.52 $\pm$ 0.08	0.16 $\pm$ 0.09†	30.8
Vitamin D	0.32 $\pm$ 0.03	0.19 $\pm$ 0.03†	59.4
Serotonin	0.12 $\pm$ 0.03	0.05 $\pm$ 0.01*	41.7
Triiodothyronine	0.36 $\pm$ 0.10	0.13 $\pm$ 0.03*	36.1
Thyroxine	0.27 $\pm$ 0.05	0.14 $\pm$ 0.02*	51.9
Testosterone	1.87 $\pm$ 0.10	0.98 $\pm$ 0.05‡	52.4
Androsterone	2.01 $\pm$ 0.19	1.04 $\pm$ 0.11‡	51.7
(B) Exogenous substrates (xenobiotics)			
p-Aminobenzoic acid	0.58 $\pm$ 0.11	0.23 $\pm$ 0.04†	39.7
1-Naphthol	6.43 $\pm$ 0.84	3.77 $\pm$ 0.50†	58.6
4-Methylumbelliferone	1.73 $\pm$ 0.36	0.49 $\pm$ 0.21†	28.3
Phenolphthalein	1.17 $\pm$ 0.16	0.66 $\pm$ 0.07†	56.4
Aniline	0.17 $\pm$ 0.04	0.09 $\pm$ 0.02*	52.9

Rat liver microsomes were incubated with various substrates in the presence or absence of 20 μM palmitoyl-CoA. The standard assay mixtures consisted of 50 mM Tris-HCl (pH 7.4), 200 μM various substrates, 800 μM UDP-[ $^{14}$ C]glucuronic acid, 5mM MgCl<sub>2</sub>, and 0.05% Triton X-100. Values means  $\pm$  SD (N = 3 or 4).

\*  $P < 0.05$ .

†  $P < 0.01$ .

‡  $P < 0.001$ .

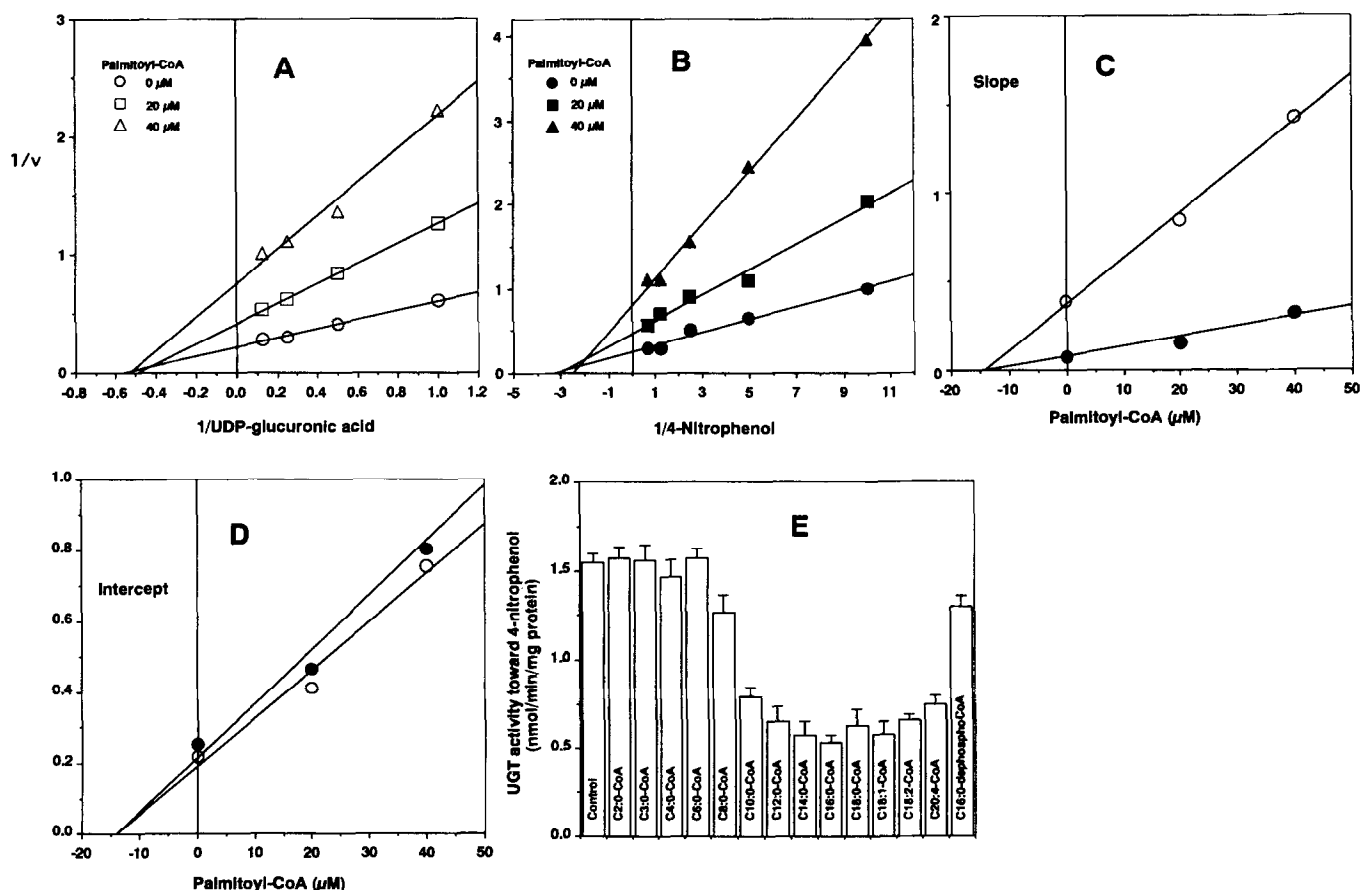


FIG. 2. Kinetics and specificity of the inhibition of 4-nitrophenol-UGT activity by palmitoyl-CoA. (A) Double-reciprocal plots of 4-nitrophenol-UGT activity as a function of the concentration of UDP-glucuronic acid at a fixed concentration (1.5 mM) of 4-nitrophenol in the absence (open circles), and presence of 20  $\mu\text{M}$  (open squares) and 40  $\mu\text{M}$  (open triangles) palmitoyl-CoA. (B) Double-reciprocal plots of the 4-nitrophenol-UGT activity as a function of the concentration of 4-nitrophenol at a fixed concentration (4 mM) of UDP-glucuronic acid in the absence (closed circles) and presence of 20  $\mu\text{M}$  (closed squares) and 40  $\mu\text{M}$  (closed triangles) palmitoyl-CoA. Data in panels A and B represent means of triplicate determinations. (C and D) The results in panels A and B were replotted [the slope (C) and the intercept (D)] as a function of the concentration of palmitoyl-CoA. (E) The structure-activity relationship of 4-nitrophenol-UGT activity. 4-Nitrophenol-UGT activity was measured in the presence of a 20  $\mu\text{M}$  concentration of various acyl-CoAs and C16:0-3'-dephosphoCoA. Data represent means  $\pm$  SD of triplicate determinations.

substrate. The  $K_i$  value of palmitoyl-CoA was found to be 15  $\mu\text{M}$  on secondary plotting of the slope of each line in Fig. 2, A and B, versus the concentration of the inhibitor (Fig. 2C; the intercept on the palmitoyl-CoA axis gives the  $K_i$  value). The  $K_i$  value ( $K_i$  for the enzyme already saturated with substrate) was the same value (15  $\mu\text{M}$ ), as judged on secondary plotting of the intercept ( $1/v$  axis) versus palmitoyl-CoA (Fig. 2D; the intercept on the palmitoyl-CoA axis gives the  $K_i'$  value). This evidence also suggested that the mode of inhibition by acyl-CoA was non-competitive.

#### Acyl-CoA Structure-Activity Relationship for Inhibition of the UGT Activity

We further examined the structure-activity relationship of acyl-CoA as to inhibition of the testosterone-UGT activity of the purified enzyme (Fig. 1C) and 4-nitrophenol-UGT activity in the microsomal fraction (Fig. 2E). Both enzyme

activities were inhibited by all long chain acyl-CoAs but not by short chain acyl-CoAs. The inhibition was observed upon the addition of acyl-CoAs having a fatty acyl moiety of a carbon chain length of 10 or more (C10:0-, C12:0-, C14:0-, C16:0-, C18:0-, C18:1-, C18:2-, and C20:4-CoA) to the assay system. Free CoA and fatty acid failed to inhibit the enzyme activity. Furthermore, palmitoyl-3'-dephosphoCoA also failed to inhibit them, suggesting that the 3'-phosphate moiety was important for the inhibition. These results also suggested that inhibition of UGT activity was specific for acyl-CoA and that the enzyme strictly recognizes the structure of acyl-CoA.

We searched for other inhibitors of UGT activity. All tested compounds, such as CoA, ATP, fatty acids, fatty acid-containing compounds [lysophosphatidylcholine, lysophosphatidylinositol, acyl-carnitine, and anandamide (*N*-arachidonylethanolamine)], Triacsin C, a fatty acid analogue that was discovered as an inhibitor of acyl-CoA syn-

thetase [20], and adenosine 5'-alkylphosphates (hexadecyl-, docosyl- and tetracosyl-AMP), which are also known as inhibitors of acyl-CoA synthetase [14], failed to inhibit the enzyme activity (data not shown). These results again suggested that the inhibition of UGT activity was specific for acyl-CoA.

#### Arachidonoyl-CoA Binding Activity of UGT Isoforms

To assess the mechanism underlying the inhibition, we examined the relationship of the inhibition and the arachidonoyl-CoA binding of the purified UGT isoforms. Arachidonoyl-CoA binding to the UGT isoforms was examined by bound/free separation by gel filtration. UGT isoforms were incubated with [ $^{14}$ C]arachidonoyl-CoA, and then the incubates were loaded onto a Sephacryl S300 HR column, eluted, and fractionated. As shown in Fig. 3A (closed circles), two peaks of  $^{14}$ C-label were observed in the elution profile; the first (fractions 14–20) was the protein-bound form and the second (fractions 26–36) was the free form of [ $^{14}$ C]arachidonoyl-CoA. When [ $^{14}$ C]arachidonoyl-CoA was incubated with the buffer without the purified protein, the first peak was not observed, suggesting that only the free form of acyl-CoA was observed (Fig. 3A, open circles). When [ $^{14}$ C]arachidonic acid (Fig. 3A, open squares) and [ $^{14}$ C]arachidonoyl-3'-dephosphoCoA (Fig. 3A, open triangles) were incubated with the UGT isoforms, the first peak was also not observed, suggesting that fatty acid and acyl-3'-dephosphoCoA failed to bind to the UGT isoforms. The results indicated that the binding is specific for acyl-CoA. The specificity of the binding was similar to that of the inhibition of UGT activity, as shown in Fig. 1C. Furthermore, the experiments measuring [ $^{14}$ C]arachidonoyl-CoA binding to purified UGT enzyme, using gel filtration, were performed in the presence of excess amounts of substrates, UDP-glucuronic acid or testosterone. We confirmed that [ $^{14}$ C]arachidonoyl-CoA binding (5  $\mu$ M) to purified enzyme was not inhibited by the addition of 800  $\mu$ M UDP-glucuronic acid or 200  $\mu$ M testosterone (data not shown). The results suggested that acyl-CoA did not act on the binding sites of both substrates and corresponded to the results of the kinetic studies (Fig. 2, A–D), i.e. the non-competitive inhibition of UGT activity by acyl-CoA.

Using the assay system, several characteristics of acyl-CoA binding to the UGT isoforms were examined. UGT isoforms were incubated with various concentrations of [ $^{14}$ C]arachidonoyl-CoA. [ $^{14}$ C]Arachidonoyl-CoA binding increased with increasing concentrations of [ $^{14}$ C]arachidonoyl-CoA, and saturation of binding was observed (Fig. 3B). The  $K_d$  and  $B_{max}$  values were 14.1  $\mu$ M and 0.94 mol/mol protein, respectively, as determined by Scatchard plot analysis (Fig. 3C). Then, the time course of binding was examined (Fig. 3D). [ $^{14}$ C]Arachidonoyl-CoA binding was dependent on the incubation period, and saturated binding was observed during 5–10 min of incubation. The apparent first order rate constant was 0.277  $\text{min}^{-1}$ , and the time for half-maximal binding was 2.5 min. The kinetic coefficients

were closely related to the nature of the inhibition of UGT activity.

#### Quenching of Tryptophan Fluorescence of UGT Isoforms by acyl-CoA

To further examine the relevance of the inhibition of UGT activity and acyl-CoA binding, we explored the changes in the tertiary structure of UGT, since a change in the tertiary structure seemed to occur in the inhibited state of the enzyme. Tryptophan residues are useful intrinsic probes for detecting alterations in a protein tertiary structure because their fluorescence is sensitive to changes in the environment [17, 21, 22]. We examined the intrinsic fluorescence of purified proteins and the effect of acyl-CoA treatment. The purified proteins were excited at 285 nm, and emission spectra were recorded from 290 to 400 nm. The spectra showed typical tryptophan fluorescence, which exhibited maximal emission at 340 nm (Fig. 4A). Treatment of UGT with 40  $\mu$ M arachidonoyl-CoA quenched the intrinsic fluorescence of the proteins. The changes in intrinsic fluorescence are considered to be due to exposure of tryptophan residues to a more polar environment. These results suggested that acyl-CoA induced conformational changes in the UGT isoforms.

The kinetics of the changes in intrinsic fluorescence were examined. First, the concentration-dependency of the effect of acyl-CoA was examined. UGT isoforms were incubated with various concentrations of arachidonoyl-CoA and palmitoyl-CoA for 10 min, and then the intrinsic fluorescence was measured. The decrease in fluorescence was dependent on the concentration of each acyl-CoA (Fig. 4B), a half-maximal effect being observed at 11.0  $\mu$ M (arachidonoyl-CoA) and 6.1  $\mu$ M (palmitoyl-CoA), as determined by Scatchard plot analysis (Fig. 4C). Palmitoyl-3'-dephosphoCoA and CoA did not affect the intrinsic fluorescence of the proteins at low concentrations. Next, the time course of the changes in intrinsic fluorescence was examined. UGT isoforms were incubated with 40  $\mu$ M arachidonoyl-CoA for various periods, and then the intrinsic fluorescence was measured. The decrease in intrinsic fluorescence caused by arachidonoyl-CoA was also dependent on the incubation period (Fig. 4D). Quenching of the fluorescence reached a maximum after 10–20 min of incubation. When the saturation curve was reanalyzed as a first order plot (Fig. 4D), the apparent first order rate constant obtained from the negative slope was 0.139  $\text{min}^{-1}$ . The time for induction of the half-maximal change was 5.0 min. Since the kinetics and specificities of the above three phenomena (inhibition, acyl-CoA binding, and tertiary structure of the enzyme) were closely related, the acyl-CoA binding to UGT isoforms induced the conformational changes of the enzymes and the resultant inhibition of UGT activity.

#### DISCUSSION

Previously, we purified 50 kDa/53 kDa proteins as targets of protein acylation from rat liver microsomes [6]. Interest-

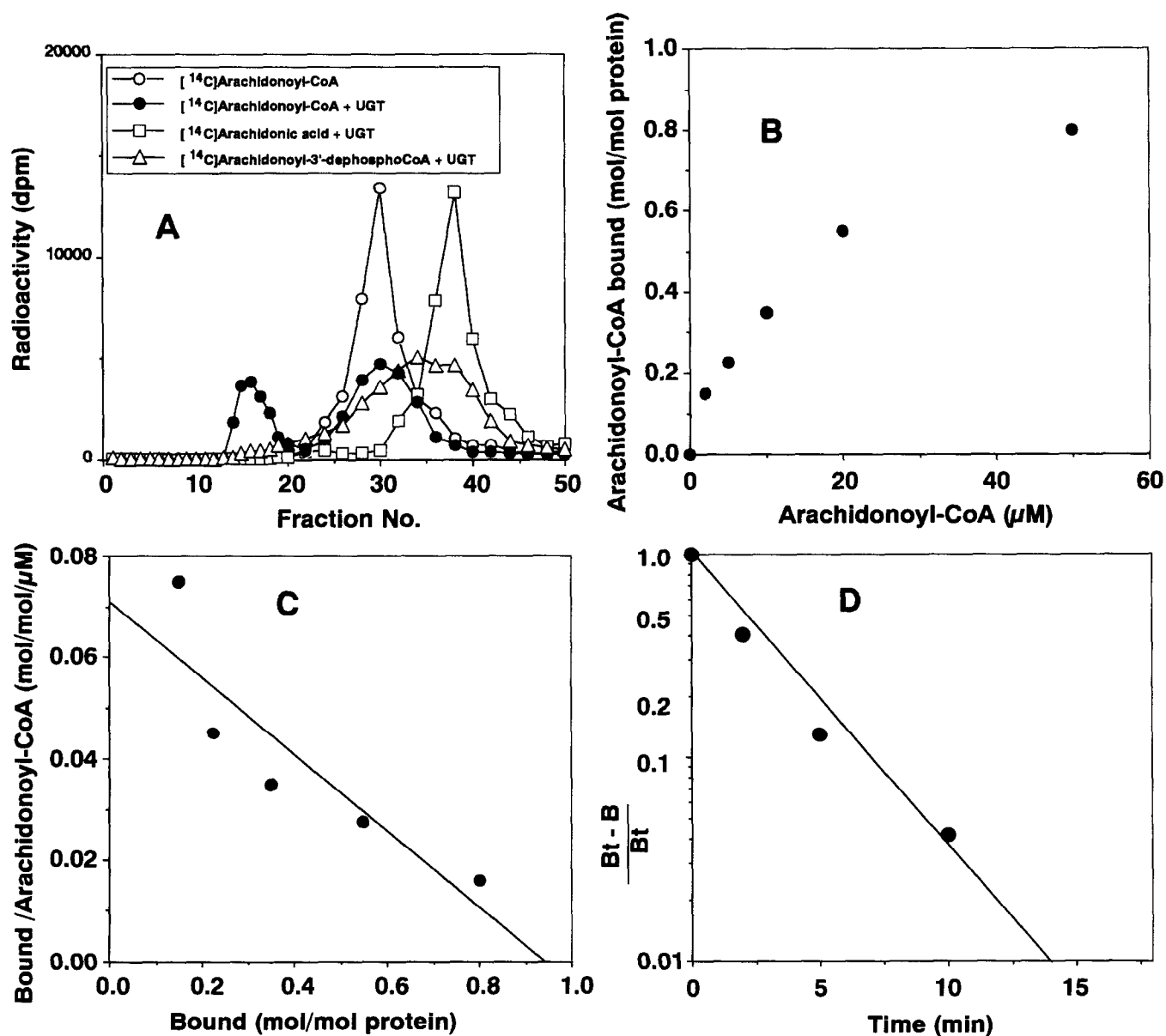


FIG. 3. Kinetics and specificity of acyl-CoA binding to UGT isoforms. (A) Elution profile of [ $^{14}$ C]arachidonoyl-CoA on Sephacryl S300 HR. UGT isoforms (Blue-Toyopearl fraction, 2.5  $\mu$ g) incubated with 5  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA (closed circles), [ $^{14}$ C]arachidonic acid (open squares), or [ $^{14}$ C]arachidonoyl-3'-dephosphoCoA (open triangles), and then applied to Sephacryl S300 HR ( $V_t$  = 20 mL). Buffer without a purified protein was incubated with 5  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA (open circles) and analyzed by the same procedures. Fractions of 0.5 mL were collected, and the radioactivity in an aliquot of each fraction was measured. (B) Concentration dependency of the acyl-CoA binding to UGT isoforms. UGT isoforms (Blue-Toyopearl fraction, 2.5  $\mu$ g) were incubated with various concentrations of [ $^{14}$ C]arachidonoyl-CoA for 30 min. The incubates were applied to Sephacryl S300 HR for separation of the bound and free forms, and the radioactivity of the bound form was measured. Data represent means of duplicate determinations. (C) Scatchard plot analysis of the data in panel B. (D) Time course of acyl-CoA binding to UGT isoforms. UGT isoforms were incubated with 20  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA for various periods, and then the data were analyzed as to first order kinetics. Data represent means of duplicate determinations.

ingly, these proteins were acylated in the absence of other proteins, suggesting that they have novel autoacylation activity. We also found that the proteins have acyl-CoA binding activity. We identified the proteins as UGT isoforms, UGT2B3, UGT2B2, and UGT2B6, which glucuronidated steroids. The physiological significance of the interaction of the UGT isoforms with acyl-CoA remains to be established. The most plausible explanation for the role

of the interaction of the UGT isoforms with acyl-CoA is that the interaction affects the UGT activity. In fact, acyl-CoA treatment inhibited the UGT activity of the purified protein and microsomal fractions in the *in vitro* assay (Figs. 1 and 2). We first considered that the inhibition of UGT activity by acyl-CoA was due to autoacylation of the proteins, as protein acylation is known to modulate protein function [23, 24]. However, our preliminary observation of

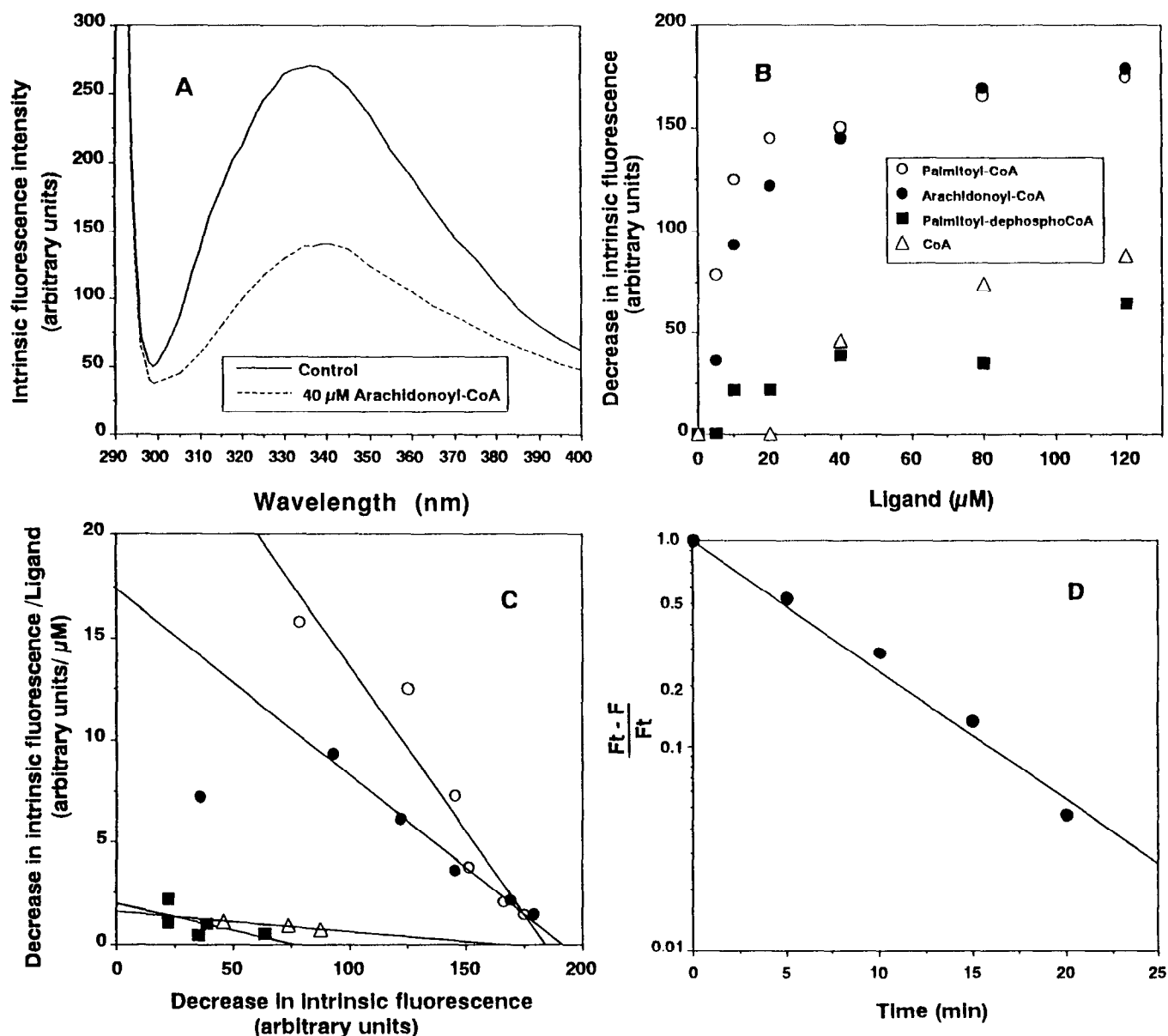


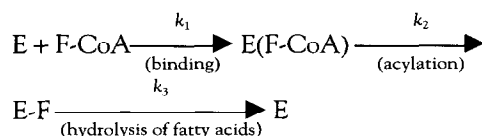
FIG. 4. Effect of acyl-CoA on intrinsic fluorescence of UGT. (A) Quenching of the intrinsic fluorescence of UGT by acyl-CoA. UGT isoforms (Blue-Toyopearl fraction, 0.12  $\mu$ M proteins in 20 mM Tris-HCl (pH 7.4), 0.05% CHAPS, 1% ethleneglycol, 50  $\mu$ M DTT, 5  $\mu$ M EDTA, and 5  $\mu$ g/mL azolectin) were treated with (dashed line) or without (solid line) 40  $\mu$ M arachidonoyl-CoA for 15 min. Each was then excited at 285 nm, and emission spectra were recorded from 290 to 400 nm. (B) Concentration-dependency of the effects of acyl-CoA, CoA, and acyl-3'-dephosphoCoA on quenching of the fluorescence of UGT. UGT isoforms were incubated with various concentrations of palmitoyl-CoA (open circles), arachidonoyl-CoA (closed circles), palmitoyl-3'-dephosphoCoA (closed squares) and CoA (open triangles) for 10 min. Each was then excited at 285 nm, and emission at 340 nm was recorded. Data represent means of duplicate determinations. (C) Scatchard plot analysis of the data from panel B. (D) Time courses of the decrease in intrinsic fluorescence caused by acyl-CoA. UGT isoforms were treated with 40  $\mu$ M arachidonoyl-CoA for various periods, and then the data were analyzed as to first order kinetics. Data represent means of duplicate determinations.

a relationship between the autoacylation reaction and the inhibition of UGT activity revealed that autoacylation alone could not account for the inhibition of UGT activity [6]. This observation prompted us to examine other mechanisms for the inhibition of UGT activity by acyl-CoA. In the present study, we examined whether the binding of acyl-CoA to UGT isoforms could induce inhibition of the enzyme activity, as an alternative mechanism is the induc-

tion of enzyme inhibition by non-covalent acyl-CoA binding. To examine this possibility, the relevance of the enzyme inhibition and the acyl-CoA binding to the proteins was systematically examined. The specificities and kinetic properties of these phenomena were closely related (Figs. 1–3). The change in the tertiary structure of the enzyme, as monitored by tryptophan fluorescence, was also concomitant (Fig. 4). In particular, the time course of the inhibi-



tion, acyl-CoA binding, and the change in the tertiary structure of the enzyme were correlated (Figs. 1B, 3D, and 4D). UGT isoforms were considered to catalyze the sequential reactions of acyl-CoA binding, autoacylation, and hydrolysis of ester bonds, according to the following scheme [6].



where E represents UGT isoforms, F-CoA is acyl-CoA, and E (F-CoA) and E-F are bound and acylated forms of UGT;  $k_1$ ,  $k_2$ , and  $k_3$  are the rate constants of each reaction.

The time course of the decreases in intrinsic fluorescence was more significantly related to that of [ $^{14}\text{C}$ ]arachidonoyl-CoA binding than that of autoacylation or hydrolysis of fatty acids (the rate constant for the decrease in intrinsic fluorescence,  $0.139 \text{ min}^{-1}$ ;  $k_1 = 0.277 \text{ min}^{-1}$  (acyl-CoA binding);  $k_2 = 0.014 \text{ min}^{-1}$  (autoacylation);  $k_3 = 0.006 \text{ min}^{-1}$  (hydrolysis) [6]. Furthermore, inhibition of the enzyme activity also occurred immediately upon the addition of acyl-CoA. From these observations, we concluded that the inhibition of UGT activity by acyl-CoA is due mainly to binding [non-covalent, form E (F-CoA)] of acyl-CoA rather than acylation (covalent fatty acid binding, form E-F), i.e. acyl-CoA binding to UGT isoforms induces conformational changes in the enzymes and the resulting enzyme inhibition.

In this report, the structure-activity relationship for the inhibition was examined extensively. Acyl-CoA is the most potent inhibitor of UGT activity. Since UGT isoforms are membrane integral proteins, they have hydrophobic domains, and these domains seem to interact with hydrophobic molecules in a non-specific manner. Since acyl-CoA is a detergent and a hydrophobic reagent, it is very difficult to determine precisely whether the effect of the compound is specific for membrane proteins. In fact, UGT activity is known to be regulated by detergents and phospholipids [15, 19, 25, 26]. It has been reported that the UGT isoform for 4-nitrophenol is a phospholipid-binding protein and that its activity is dependent on the bound phospholipids [25]. Conformational changes in the enzyme as a result of phospholipid binding were also studied by circular dichroism spectroscopy [26]. In the present study, conformational changes in UGT evoked by phospholipids were confirmed by changes in intrinsic fluorescence. The intrinsic fluorescence was increased by phospholipids (data not shown), whereas it was decreased by acyl-CoA. Microsomal enzymes were already activated by microsomal phospholipids. Acyl-CoA inhibited the enzyme activity even in the presence of microsomal phospholipids, suggesting that acyl-CoA acts at a site different from the phospholipids (Figs. 1 and 2). Other fatty acid-containing compounds did not inhibit it. Since even palmitoyl-3'-dephosphoCoA was ineffective, the inhibition was very specific for acyl-CoA. A

study of the structure-activity relationship revealed that UGT precisely recognized the structure of the acyl-CoA molecule.

The cDNA sequences of more than twenty forms of UGT are known for humans and rodents. From the similarities of their predicted amino acid sequences, they can be grouped into two families, UGT1 and UGT2. The isoforms of the UGT1 family catalyze the glucuronidation of bilirubin and small planar phenolic compounds, whereas those of the UGT2 family are responsible for glucuronidation of steroids [3, 4]. Recent analysis of genomic DNAs of the enzymes revealed that the genes of the UGT1 family consist of isoform-specific exons encoding the N-terminal half of the enzymes, and commonly used exons encoding the C-terminal half. The commonly used exons encode the binding site of a common substrate, UDP-glucuronic acid, whereas the isoform-specific exons encode the binding sites of various substrates [27, 28]. From the UGT1 gene complex, through alternative utilization of multiple first exons in combination with the commonly used exons, a variety of distinct forms may be synthesized, similar to the case of the immunoglobulin gene. In contrast to UGT1, the genes of the UGT2 subfamily, while very similar, are different. Since the effect of acyl-CoA was observed on various substrates, the acyl-CoA binding site is located not in the variable domain for each substrate but in the commonly used domain, i.e. the C-terminal half of the proteins. However, this site was not the binding site of UDP-glucuronic acid itself since acyl-CoA acts non-competitively toward UDP-glucuronic acid (Fig. 2).

The physiological significance of the inhibition of UGT activity by acyl-CoA remains to be determined. Indeed, information concerning modulators, including inhibitors, of UGT is extremely limited despite the importance of UGT in the detoxification of hydrophobic compounds [3, 4]. Long chain fatty acyl-CoA is a metabolically active form of fatty acid and plays an important role in fatty acid metabolism; there are various modes of enzymatic conversion of fatty acids, including desaturation, chain elongation, and oxidation, mainly in the form of fatty acyl-CoA in mammalian tissues, and another important role of acyl-CoA is an acyl donor in the formation of various types of simple and complex lipid molecules [29]. It is noticeable that UGT activity is regulated by the key compound in fatty acid metabolism, long chain acyl-CoA. The level of acyl-CoA and the activities of acyl-CoA-metabolizing enzymes are known to be altered under some conditions, such as between fed and fasted states, and following the administration of hormones and drugs [30–32]. It is possible that UGT activity is regulated by intracellular long chain acyl-CoA under these physiological and non-physiological (drugs) conditions.

In conclusion, long chain fatty acyl-CoA is a modulator of glucuronidation, which is mediated via specific binding to UGT protein. Further studies of the key compound, acyl-CoA, should lead to a precise understanding of the rel-

evance/cross-talk of fatty acid metabolism and glucuronidation.

*We are grateful to Mr. Hideo Tanihata, Ms. Yuko Hosono, and Mr. Satoshi Tsujimoto for their technical assistance. This study was supported, in part, by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.*

## References

- Dutton GJ (Ed.) *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL, 1980.
- Kasper CB and Henton D, Glucuronidation. In: *Enzymatic Basis of Detoxication* (Ed. Jakoby WB) Vol. II, pp. 3–34. Academic Press, New York, 1980.
- Mulder GJ, Glucuronidation and its role in regulation of biological activity of drugs. *Annu Rev Pharmacol Toxicol* **32**: 25–49, 1992.
- Burchell B, Nebert DW, Nelson DR, Bock KW, Iyanagi T, Jansen PLM, Lancet D, Mulder GJ, Chowdhury JR, Siest G, Tephly TR and Mackenzie PI, The UDP glucuronosyltransferase gene superfamily: Suggested nomenclature based on evolutionary divergence. *DNA Cell Biol*. **10**: 487–494, 1991.
- Mizukami Y, Fujii-Kuriyama Y and Muramatsu M, Multiplicity of deoxyribonucleic acid sequences with homology to a cloned complementary deoxyribonucleic acid coding for rat phenobarbital-inducible cytochrome P-450. *Biochemistry* **22**: 1223–1229, 1983.
- Yamashita A, Watanabe M, Tonegawa T, Sugiura T and Waku K, Acyl-CoA binding and acylation of UDP-glucuronosyltransferase isoforms of rat liver: Their effect on enzyme activity. *Biochem. J.* **312**: 301–308, 1995.
- Mackenzie PI, Rat liver UDP-glucuronosyltransferase. Identification of cDNAs encoding two enzymes which glucuronidate testosterone, dihydrotestosterone, and  $\beta$ -estradiol. *J. Biol. Chem.* **262**: 9744–9749, 1987.
- Mackenzie PI, The cDNA sequence and expression of a variant 17 $\beta$ -hydroxysteroid UDP-glucuronosyltransferase. *J. Biol. Chem.* **265**: 8699–8703, 1990.
- Mackenzie PI, Rat liver UDP-glucuronosyltransferase. cDNA sequence and expression of a form glucuronidating 3-hydroxy-androgens. *J Biol Chem* **261**: 14112–14117, 1986.
- Zhong Z, Kauffman FC and Thurman RG, Inhibition of glucuronidation of benzo(a)pyrene phenols by long-chain fatty acids. *Cancer Res* **51**: 4511–4515, 1991.
- Krcmery M and Zakim D, Effects of oleoyl-CoA on the activity and functional state of UDP-glucuronosyltransferase. *Biochem Pharmacol* **46**: 897–904, 1993.
- Sugiura T, Masuzawa Y and Waku K, Coenzyme A-dependent transacylation system in rabbit liver microsomes. *J Biol Chem* **263**: 17490–17498, 1988.
- Rajasekharan R, Mariani RC, Shockey JM and Kemp JD, Photoaffinity labeling of acyl-CoA oxidase with 12-azidooleoyl-CoA and 12-[(4-azidosalicyl)amino]dodecanoyl-CoA. *Biochemistry* **32**: 12386–12391, 1993.
- Shiraishi T, Tezuka K and Uda Y, Selective inhibition of lignoceroyl-CoA synthetase by adenosine 5'-alkylphosphates. *FEBS Lett* **352**: 353–355, 1994.
- Bansal SK and Gessner T, A unified method for the assay of uridine diphosphoglucuronyl-transferase activities toward various aglycones using uridine diphospho[U- $^{14}$ C]glucuronic acid. *Anal Biochem* **109**: 321–329, 1980.
- Vessey DA and Zakim D, Regulation of microsomal enzymes by phospholipids. II. Activation of hepatic uridine diphosphate-glucuronyltransferase. *J Biol Chem* **247**: 3023–3028, 1972.
- Ward LD, Measurement of ligand binding to proteins by fluorescence spectroscopy. *Methods Enzymol* **117**: 400–414, 1985.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
- Hochman Y, Zakim D and Vessey DA, A kinetic mechanism for modulation of the activity of microsomal UDP-glucuronyltransferase by phospholipids. Effects of lysophosphatidylcholines. *J Biol Chem* **256**: 4783–4788, 1981.
- Omura S, Tomoda H, Xu QM, Takahashi Y and Iwai Y, Triacins, new inhibitors of acyl-CoA synthetase produced by *Streptomyces* sp. *J Antibiot (Tokyo)* **39**: 1211–1218, 1986.
- Higashijima T, Ferguson KM, Sternweis PC, Ross EM, Smigel MD and Gilman AG, The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide-binding regulatory proteins. *J Biol Chem* **262**: 752–756, 1987.
- Higashijima T, Ferguson KM, Smigel MD and Gilman AG, The effect of GTP and  $Mg^{2+}$  on the GTPase activity and the fluorescent properties of  $G_{\alpha}$ . *J Biol Chem* **262**: 757–761, 1987.
- Towler DA, Gordon JL, Adams SP and Glaser L, The biology and enzymology of eukaryotic protein acylation. *Annu Rev Biochem* **57**: 69–99, 1988.
- Casy P, Protein lipidation in cell signaling. *Science* **268**: 221–225, 1995.
- Singh OMP, Graham AB and Wood GC, The phospholipid dependence of UDP glucuronosyltransferase. Purification, delipidation and reconstitution of microsomal enzyme from guinea-pig liver. *Eur J Biochem.* **116**: 311–316, 1981.
- Singh OMP, Graham AB and Wood GC, The phospholipid dependence of UDP-glucuronyltransferase: Conformation/reactivity studies with purified enzyme. *Biochem Biophys Res Commun* **107**: 345–349, 1982.
- Iyanagi T, Molecular basis of multiple UDP-glucuronosyltransferase isozyme deficiencies in the hyperbilirubinemic rat (Gunn rat). *J Biol Chem* **266**: 24048–24052, 1991.
- Emi Y, Ikushiro S and Iyanagi T, Drug-responsive and tissue-specific alternative expression of multiple first exons in rat UDP-glucuronosyltransferase family 1 (UGT1) gene complex. *J Biochem (Tokyo)* **117**: 392–399, 1995.
- Van Golde LMG and Van den Bergh SG, Liver. In: *Lipid Metabolism in Mammals* (Snyder F), Vol. 1, pp. 35–149. Plenum Press, New York, 1977.
- Yamashita A, Sato K, Watanabe M, Tokudome Y, Sugiura T and Waku K, Induction of coenzyme A-dependent transacylation activity in rat liver microsomes by administration of clofibrate. *Biochim. Biophys. Acta* **1211**: 263–269, 1994.
- Corkey BE, Analysis of acyl-coenzyme A esters in biological samples. *Methods Enzymol* **166**: 55–70, 1988.
- Deeney JT, Tornheim K, Korchak HM, Prentki M and Corkey BE, Acyl-CoA esters modulate intracellular  $Ca^{2+}$  handling by permeabilized clonal pancreatic  $\beta$ -cells. *J Biol Chem* **267**: 19840–19845, 1992.