

# Structural and functional comparison of two human liver dihydrodiol dehydrogenases associated with 3 $\alpha$ -hydroxysteroid dehydrogenase activity

Yoshihiro DEYASHIKI, Hiroyuki TANIGUCHI, Tetsuya AMANO, Toshihiro NAKAYAMA, Akira HARA and Hideo SAWADA\*

Department of Biochemistry, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502, Japan

Two monomeric dihydrodiol dehydrogenases with pI values of 5.4 and 7.6 were co-purified with androsterone dehydrogenase activity to homogeneity from human liver. The two enzymes differed from each other on peptide mapping and in their heat-stabilities; with respect to the latter the dihydrodiol dehydrogenase and 3 $\alpha$ -hydroxysteroid dehydrogenase activities of the respective enzymes were similarly inactivated. The pI 5.4 enzyme was equally active towards *trans*- and *cis*-benzene dihydrodiols, and towards (*S*)- and (*R*)-forms of indan-1-ol and 1,2,3,4-tetrahydronaphth-1-ol and oxidized the 3 $\alpha$ -hydroxy group of C<sub>19</sub>-, C<sub>21</sub>- and C<sub>24</sub>-steroids, whereas the pI 7.6 enzyme showed high specificity for *trans*-benzene dihydrodiol, (*S*)-forms of the alicyclic alcohols and C<sub>19</sub>- and C<sub>21</sub>-steroids. Although the two enzymes reduced various xenobiotic carbonyl compounds and the 3-oxo group of C<sub>19</sub>- and C<sub>21</sub>-steroids, and were A-specific in the hydrogen transfer from NADPH, only the pI 5.4 enzyme showed reductase activity towards 7 $\alpha$ -hydroxy-5 $\beta$ -cholestan-3-one and dehydrolithocholic acid. The affinity of the two enzymes for the steroidal substrates was higher than that for the xenobiotic substrates. The two enzymes also showed different susceptibilities to the inhibition by anti-inflammatory drugs and bile acids. Whereas the pI-5.4 enzyme was highly sensitive to anti-inflammatory steroids, showing mixed-type inhibitions with respect to indan-1-ol and androsterone, the pI 7.6 enzyme was inhibited more potently by non-steroidal anti-inflammatory drugs and bile acids than by the steroidal drugs, and the inhibitions were all competitive. These structural and functional differences suggest that the two enzymes are 3 $\alpha$ -hydroxysteroid dehydrogenase isoenzymes.

## INTRODUCTION

3 $\alpha$ -Hydroxysteroid dehydrogenase (3HSD), which plays an important role in regulating steroid-hormone levels, is widely distributed in the microsomal and cytoplasmic fractions of various animal tissues [1]. The cytoplasmic enzyme from rat liver has been characterized most extensively (see [2] for review). It is a monomeric protein with an  $M_r$  of 34000, and catalyses the reversible oxidation of the 3 $\alpha$ -hydroxy group of C<sub>19</sub>-, C<sub>21</sub>- and C<sub>24</sub>-steroids [3–5] and has been also shown to be the same enzyme as 7 $\alpha$ ,12 $\alpha$ -dihydroxy-5 $\beta$ -cholestan-3-one 3 $\alpha$ -reductase, which is implicated in bile-acid synthesis from cholesterol [6]. In addition, the rat liver enzyme has been reported to exhibit dihydrodiol dehydrogenase [7–9] and carbonyl reductase activities [8–10], which indicates another role of the enzyme in drug metabolism. Similar broad substrate specificity for steroids and xenobiotics has been described with 3HSDs purified from mouse [11], hamster [12,13] and monkey [14,15] livers. On the other hand, Kudo *et al.* [16] have recently isolated three bile-acid 3-oxoreductases with similar  $M_r$  values of 32000 from human liver, and have shown that all the enzyme forms were not active towards 5 $\beta$ -pregnane-3,17-dione and 7 $\alpha$ -hydroxy-5 $\beta$ -cholestan-3-one. However, the specificity of the human liver enzymes for xenobiotics has not been investigated.

We previously purified five dihydrodiol dehydrogenases with  $M_r$  values of about 35000 from human liver, two of which oxidized some 3 $\alpha$ -hydroxyandrostanes and 3 $\alpha$ -hydroxypregnanes [17]. In the present study we further examined the properties of the two human liver dihydrodiol dehydrogenases associated with

3HSD activity to establish structural and functional relationships among the two dihydrodiol dehydrogenases, animal liver 3HSDs and human liver bile-acid 3-oxoreductase. Since 3HSDs from animal livers have been shown to be strongly inhibited by anti-inflammatory drugs [8,11,12,14] and bile acids [18], the effects of the drugs and bile acids on the two enzymes were also compared.

## EXPERIMENTAL

### Materials

7 $\alpha$ -Hydroxy-5 $\beta$ -cholestan-3-one was kindly given by Dr. K. Uchida (Shionogi Research Laboratory, Osaka, Japan). Other steroids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and pyridine nucleotides and pI markers were from Oriental Yeast Co. (Tokyo, Japan). The stereoisomers of indan-1-ol and tetralol (1,2,3,4-tetrahydronaphth-1-ol) were purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.) and their purities were as follows: (*R*)-(-)-indan-1-ol, 99% [ $\alpha$ ]<sub>D</sub><sup>20</sup> -29° (c 2.0 in chloroform); (*S*)-(+)-indan-1-ol, 99% [ $\alpha$ ]<sub>D</sub><sup>20</sup> +30° (c 2.0 in chloroform); (*R*)-(-)-tetralol, 99% [ $\alpha$ ]<sub>D</sub><sup>17</sup> -32° (c 2.5 in chloroform); and (*S*)-(+)-tetralol, 99% [ $\alpha$ ]<sub>D</sub><sup>17</sup> +32° (c 2.5 in chloroform). *Staphylococcus aureus* V8 proteinase was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and *cis*-benzene dihydrodiol (1,2-dihydroxycyclohexa-3,5-diene) was from Fluka Chemie AB (Buchs, Switzerland). *trans*-Benzene dihydrodiol and naphthalene dihydrodiol (*trans*-1,2-dihydroxy-1,2-dihydronaphthalene) were synthesized as described by Platt & Oesch [19,20].

Abbreviations and trivial names: 3HSD, 3 $\alpha$ -hydroxysteroid dehydrogenase; androsterone, 3 $\alpha$ -hydroxy-5 $\alpha$ -androstan-17-one; lithocholic acid, 3 $\alpha$ -hydroxy-5 $\beta$ -cholan-24-oic acid; dehydrolithocholic acid, 3-oxo-5 $\beta$ -cholan-24-oic acid; deoxycholic acid, 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholan-24-oic acid; ursodeoxycholic acid, 3 $\alpha$ ,7 $\beta$ -dihydroxy-5 $\beta$ -cholan-24-oic acid; chenodeoxycholic acid, 3 $\alpha$ ,12 $\alpha$ -dihydroxy-5 $\beta$ -cholan-24-oic acid; cholic acid, 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholan-24-oic acid; glycochenodeoxycholic acid, *N*-(3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholan-24-oyl)glycine.

\* To whom correspondence should be addressed.

**Table 1. Co-purification of dihydrodiol dehydrogenase and 3HSD activities from human liver**

The activities of dihydrodiol dehydrogenase (DD) and 3HSD were determined using 1.8 mM-naphthalene dihydrodiol or 5  $\mu$ M-androsterone respectively.

Step	Total protein (mg)	DD activity		3HSD total activity (units)	DD/3HSD ratio
		Total (units)	Specific (units/mg)		
Crude supernatant	10840	90.2	0.008	23.5	3.8
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction	6392	80.0	0.013	20.9	3.8
Sephadex G-100	1093	72.1	0.066	16.5	4.6
DD2					
Q-Sepharose	40.2	20.7	0.51	0.20	103
Matrex Red A	11.8	18.7	1.58	0.15	125
HA-Ultrogel	10.2	17.0	1.67	0.14	121
Matrex Red A	7.2	15.7	2.18	0.13	121
DD4					
Q-Sepharose	75.8	15.0	0.20	13.8	5.5
Matrex Red A	9.8	10.3	1.05	8.37	1.2
HA-Ultrogel	6.2	7.30	1.18	5.40	1.4
DEAE-Sephacel	2.3	3.85	1.67	2.70	1.4

### Enzyme assay

The dehydrogenase activity was measured at 25 °C by monitoring NADPH fluorescence at 450 nm (excitation at 340 nm). Each cuvette contained 2.0 ml of the reaction mixture, which consisted of 0.1 M-glycine/NaOH, pH 10.0, 0.25 mM-NADP<sup>+</sup>, 5  $\mu$ M-androsterone or 1.8 mM-naphthalene dihydrodiol and enzyme. Substrate was omitted from the blank. The reaction was initiated by the addition of enzyme. Kinetic assays were performed similarly in at least duplicate with various amounts of substrate added as specified in the text, except that 0.1 M-potassium phosphate buffer, pH 7.4, was used instead of the glycine/NaOH buffer. The substrates were dissolved in methanol and 50  $\mu$ l portions were added to the assay mixture. Reductase activity was assayed by recording the decrease of NADPH absorbance at 340 nm in 2.0 ml of 100 mM-potassium phosphate, pH 7.4, containing 0.1 mM-NADPH, carbonyl substrate and enzyme at 25 °C. Carbonyl reductase activity was determined as described [21]. One unit of enzyme was defined as the amount that catalyses oxidation or reduction of 1  $\mu$ mol of NADPH/min. In a separate experiment the assay was done at various temperatures (17–50 °C) with 1 mM-(S)-indan-1-ol as the substrate, and the activation energy for the reaction was calculated from the Arrhenius plot [22]. For determining thermal stability of the enzyme, the enzyme solution (0.05 mg/ml), in 20 mM-potassium phosphate, pH 7.5, containing 5 mM-2-mercaptoethanol, 0.5 mM-EDTA, 0.14 M-KCl and 0.1 % BSA, was incubated at 44 °C, and then 50  $\mu$ l aliquots were removed for assay at specific times.

Protein concentration was determined as described by Bradford [23].

### Enzyme purification

A human liver was obtained, with informed consent of the next-of-kin, from a 37-year-old Japanese man undergoing legal medical autopsy whose cause of death was a traffic accident. The tissue was frozen 12 h after death and stored at –75 °C until used. The two dihydrodiol dehydrogenases associated with 3HSD activity (DD2 and DD4) were purified from the human liver as previously described [17]. In the final step of purification of DD4 with a DEAE-Sephacel column (1.2 cm  $\times$  15 cm), the enzyme activity was resolved into one minor and one major peak, which were eluted at NaCl concentrations of 30 mM and 60 mM re-

spectively. The existence of two forms (pI 5.4 and pI 5.9) of DD4 had already been reported [17], and the minor and major enzyme peaks were found to contain the pI 5.9 form and pI 5.4 form respectively when they were analysed by isoelectric focusing [24]. The pI 5.4 enzyme and the purified DD2 enzyme with a pI of 7.6 showed single bands on SDS/PAGE [25].

### Product identification

The products in the oxidation of 3 $\alpha$ -hydroxy-5 $\beta$ -androstan-17-one and 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol or in the reduction of 5 $\beta$ -androstane-3,17-dione and 20 $\alpha$ -hydroxy-5 $\beta$ -pregnan-3-one were analysed by t.l.c. The reaction mixture (4.0 ml) for the oxidation contained 0.1 M-glycine/NaOH, pH 10.0, 0.5 mM-NADP<sup>+</sup>, one of the steroids (25  $\mu$ M) and enzyme, and that for the reduction consisted of 0.1 M-potassium phosphate, pH 6.0, 0.1 mM-NADPH, 25  $\mu$ M-oxosteroid and enzyme. After incubation for 1 h at 30 °C, the products were extracted with 10 ml of ethyl acetate. The organic phase was concentrated by evaporating the solvent and then analysed by t.l.c. on silica-gel plates using as solvent system benzene/acetone (1:4, v/v) as described previously [26]. The  $R_f$  values of 3 $\alpha$ -hydroxy-5 $\beta$ -androstan-17-one, 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol, 5 $\beta$ -androstane-3,17-dione and 20 $\alpha$ -hydroxy-5 $\beta$ -pregnan-3-one were 0.38, 0.24, 0.55 and 0.43 respectively. The  $R_f$  values for the products were also distinguished from those of the corresponding isomers.

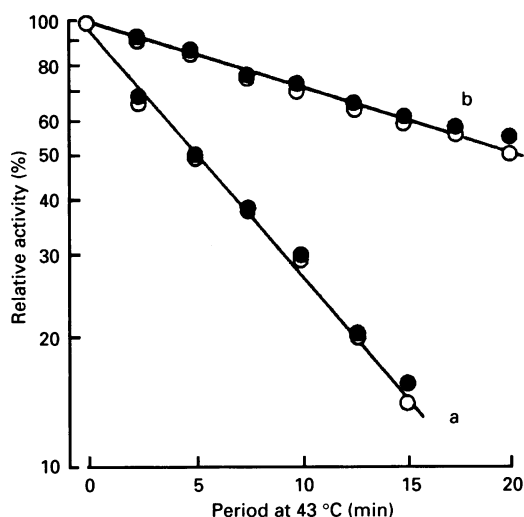
### Peptide mapping

The enzyme solution (0.3 mg/ml), in 125 mM-Tris/HCl, pH 6.8, containing 1 mM-EDTA, 0.002 % Bromophenol Blue, 0.1 % SDS and 10 % glycerol was heated at 100 °C for 5 min, and *Staphylococcus aureus* V8 proteinase (0.33  $\mu$ g/ml) was then added to the enzyme solution. Slab PAGE of the mixture (20  $\mu$ l) and protein staining were performed as described previously [13].

## RESULTS

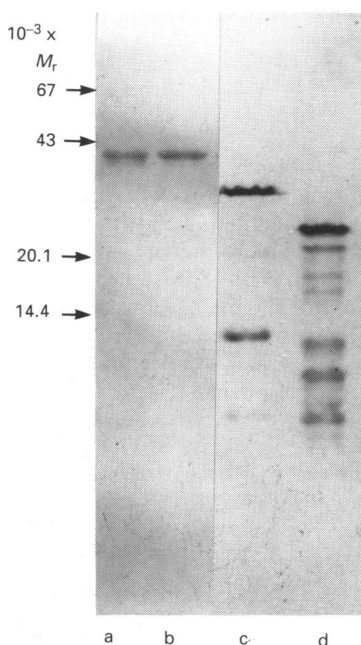
### Purification and purity

The results of purification of the two dihydrodiol dehydrogenases associated with 3HSD activity from human liver are summarized in Table 1. The dihydrodiol dehydrogenase and 3HSD activities of the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction appeared at the same



**Fig. 1. Thermal stability of naphthalene dihydrodiol and androsterone dehydrogenase activities of DD2 and DD4**

The enzyme activities of DD2 (a) and DD4 (b) were assayed with 1.8 mM-naphthalene dihydrodiol (○) and 5  $\mu$ M-androsterone (●).



**Fig. 2. Peptide mapping of human liver dihydrodiol dehydrogenases**

The peptides of DD2 (c) and DD4 (d), after enzymic digestion, were resolved on a 17.5–20% gradient polyacrylamide gel containing SDS and stained using a silver procedure. Details of the digestion were described in the Experimental section. Lanes a and b are the controls of DD2 and DD4 respectively, which were run without the enzymic digestion. The arrows indicated the positions of  $M_r$  standards (top to bottom: BSA, ovalbumin, soybean trypsin inhibitor and  $\alpha$ -lactalbumin).

position ( $M_r$  35000) on Sephadex G-100 filtration. At the next Q-Sepharose step, the dihydrodiol dehydrogenase activity was resolved into four peaks, which correspond to DD1, DD2, DD3 and DD4 described previously [17], whereas 3HSD activity was detected in the fractions of DD2, DD3 and DD4, but not in that of DD1. Since DD3 has been identified as aldehyde reductase [17], the low 3HSD activity (24 munits/mg of protein) in the fraction of DD3 is probably due to contamination by DD4 and

was not further purified. The dihydrodiol dehydrogenase and 3HSD activities of DD2 and DD4 were not separated in the subsequent purification steps, and the ratios of dihydrodiol dehydrogenase activity and 3HSD activity of the respective enzymes were also essentially constant. It should be noted that carbonyl reductase lacking dihydrodiol dehydrogenase activity was separated from DD2 and DD4 in the Q-Sepharose step and the Matrex Red A chromatography of DD2, which suggests that DD2 and DD4 are distinct from carbonyl reductase [21].

#### Comparison of structural properties

The preparations of DD2 and DD4 showed single protein bands with  $M_r$  values of 37000 and 39000 respectively on SDS/PAGE, and the respective pI values determined by isoelectric focusing were 7.6 and 5.4. The two enzymes also showed different thermostabilities: the times required for 50% inactivation at 44 °C were 5.2 min and 20 min for DD2 and DD4 respectively, and the dehydrogenase activities for naphthalene dihydrodiol and androsterone of the respective enzymes were similarly inactivated (Fig. 1). Activation energy for (*S*)-(+)-indan-1-ol oxidation by DD2 at pH 7.0 was 52.7 kJ/mol, whereas that by DD4 was 57.7 kJ/mol. Corroborative evidence of a primary difference in the two enzymes was obtained from peptide mapping, which revealed polypeptide fragments of distinct number and size for each enzyme (Fig. 2).

#### Comparison of catalytic properties

Although androsterone dehydrogenase activities of DD2 and DD4 were optimal at pH 10.0 and their pH optima in the reduction of 5 $\beta$ -androstane-3,17-dione were observed around 5.5, we determined their kinetic constants for the xenobiotics and steroids at pH 7.4 to compare reactivity of the two enzymes to the substrates under physiological conditions (Table 2). There was a clear difference in stereospecificity of the two enzymes for alicyclic alcohols. Whereas DD2 showed higher dehydrogenase activity towards *trans*-benzene dihydrodiol, (*S*)-(+)-indan-1-ol and (*S*)-(+)-tetralol than towards their stereoisomers, DD4 was equally active towards *trans*- and *cis*-benzene dihydrodiols, and (*S*)- and (*R*)-forms of the alicyclic alcohols. The steroid specificity of DD2 was also different from that of DD4: DD4 oxidized various 3 $\alpha$ -hydroxysteroids, including bile acids, whereas DD2 was inactive towards bile acids. The oxidized products of 3 $\alpha$ -hydroxy-5 $\beta$ -androstane-17-one and 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol by the two enzymes were identified with 5 $\beta$ -androstane-3,17-dione and 5 $\beta$ -pregnan-20 $\alpha$ -ol-3-one respectively on t.l.c.

In the reverse reaction with NADPH as a cofactor, DD2 and DD4 reduced a variety of carbonyl compounds, including 3-oxosteroids (Table 3). The reduced products of 5 $\beta$ -androstane-3,17-dione and 20 $\alpha$ -hydroxy-5 $\beta$ -pregnan-3-one formed by the two enzymes were identified with 3 $\alpha$ -hydroxy-5 $\beta$ -androstane-17-one and 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol respectively. However, the  $k_{cat}$  and  $K_m$  values for the substrates were different between the two enzymes, and a marked difference was observed in the reactivity to dehydrolithocholic acid and 7 $\alpha$ -hydroxy-5 $\beta$ -cholestan-3-one which were reduced by DD4 but not by DD2.

Stereospecificity of hydrogen transfer of NADPH was examined with (*4R*)- or (*4S*)-[4- $^3$ H]NADPH as a cofactor and with 2-nitrobenzaldehyde, which was the best model substrate (showing the highest  $k_{cat}$  value). When DD2 and DD4 were incubated in the presence of (*4R*)-[4- $^3$ H]NADPH, the respective recoveries of radioactivity in the reduced product, 2-nitrobenzyl alcohol, were 36  $\pm$  5% and 38  $\pm$  7%, although we have no explanation for the low incorporation of  $^3$ H. On the other hand, less than 2% of the label was transferred to the product by the two enzymes when (*4S*)-[4- $^3$ H]NADPH was used as the cofactor. This result indicates that the two enzymes are A-stereospecific.

**Table 2. Stereospecificity in the oxidation of xenobiotic alcohols and steroids by the two human liver dehydrogenases**

Kinetic constants were determined from Lineweaver–Burk analyses of assays performed in 0.1 M-potassium phosphate, pH 7.4, containing 0.25 mM-NADP<sup>+</sup> and substrates. The catalytic constants were calculated by assuming  $M_r$  values for DD2 and DD4 of 37000 and 39000 respectively. Values in parentheses were calculated from the activities with 0.5 mM xenobiotic alcohols and 5  $\mu$ M-steroids.

Substrate	DD2		DD4	
	$k_{cat.}$ (min <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat.}$ (min <sup>-1</sup> )	$K_m$ ( $\mu$ M)
<b>Alcohols</b>				
(S)-(+)-Tetralol	16	260	35	350
(S)-(+)-Indan-1-ol	9.5	520	19	450
<i>trans</i> -Benzene dihydrodiol	1.9	5000	2.2	29
Naphthalene dihydrodiol	1.7	220	5.4	420
(R)-(-)-Indan-1-ol	(0.30)	–	16	300
(R)-(-)-Tetralol	(0.17)	–	18	190
<i>cis</i> -Benzene dihydrodiol	(0.02)	–	19	1090
<b>Steroids</b>				
Androsterone	0.71	7.8	5.4	1.0
5 $\beta$ -Pregnane-3 $\alpha$ ,20 $\alpha$ -diol	0.22	1.0	2.3	0.2
3 $\alpha$ -Hydroxy-5 $\beta$ -androstan-17-one	(0.09)	–	4.7	1.4
5 $\beta$ -Androstane-3 $\alpha$ ,17 $\beta$ -diol	(0.09)	–	7.6	1.3
3 $\alpha$ -Hydroxy-5 $\beta$ -pregnan-20-one	(0.08)	–	2.0	1.6
5 $\alpha$ -Androstane-3 $\alpha$ ,17 $\beta$ -diol	(0.04)	–	6.8	1.8
Cholic acid	(0)	–	15	24
Deoxycholic acid	(0)	–	3.1	6.3
Chenodeoxycholic acid	(0)	–	1.9	1.1
Lithocholic acid	(0)	–	1.7	0.7
Ursodeoxycholic acid	(0)	–	(0.5)	–

**Table 3. Substrate specificity for carbonyl compounds**

Kinetic constants were determined as described in Table 2. The activity was assayed in 0.1 M-potassium phosphate, pH 7.4, containing 0.1 mM-NADPH as a cofactor. Values in parentheses were calculated from the activities with 0.1 mM-menadione, 5  $\mu$ M-steroids and 0.5 mM other substrates.

Substrate	DD2		DD4	
	$k_{cat.}$ (min <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat.}$ (min <sup>-1</sup> )	$K_m$ ( $\mu$ M)
<b>Other substrates</b>				
2-Nitrobenzaldehyde	3.2	0.6	11	1.1
Butane-2,3-dione	2.1	3.3	4.0	6.5
Pyridine-3-aldehyde	1.9	57	10	330
Menadione	1.5	13	(3.1)	–
Camphorquinone	1.4	0.5	8.1	0.6
3-Nitrobenzaldehyde	0.93	2.0	2.1	17
4-Benzoylpyridine	(0.66)	–	(1.8)	–
Indan-1-one	(0.22)	–	(1.1)	–
<b>Steroids</b>				
5 $\beta$ -Androstane-3,17-dione	0.62	0.3	4.8	0.6
5 $\beta$ -Pregnane-3,20-dione	0.59	0.2	3.0	0.8
20 $\alpha$ -Hydroxy-5 $\beta$ -pregnan-3-one	(0.44)	–	1.7	0.6
5 $\alpha$ -Androstane-3,17-dione	(0.22)	–	(3.4)	–
17 $\beta$ -Hydroxy-5 $\beta$ -androstan-3-one	(0.01)	–	(1.0)	–
7 $\alpha$ -Hydroxy-5 $\beta$ -cholestan-3-one	(0)	–	(1.4)	–
Dehydrolithocholic acid	(0)	–	0.87	0.1

#### Comparison of inhibitor-sensitivities

Since DD2 and DD4 have been reported to be inhibited by indomethacin [17], we first compared the effects of various anti-inflammatory agents on the (S)-(+)-indan-1-ol dehydrogenase activities of the two enzymes. The non-steroidal anti-inflam-

matory drugs, except indomethacin, were more inhibitory to DD2 than to DD4 (the inhibitions by flufenamic acid and naproxen being most especially potent), whereas anti-inflammatory steroids inhibited DD4 more potently than DD2. In addition, the patterns of inhibition shown by the drugs with respect to (S)-(+)-indan-1-ol differed between the two enzymes. All the anti-inflammatory drugs were competitive inhibitors of DD2, whereas they, except flufenamic acid, showed mixed-type inhibitions for DD4.

When we examined the sensitivity of DD2 and DD4 to bile acids and thiol-group-reacting reagents which inhibit rat liver 3HSD [18] or human liver bile acid 3-oxoreductase [16], we found that bile acids were potent and competitive inhibitors with respect to the substrate for DD2. The inhibitor constants for lithocholic acid, ursodeoxycholic acid and dehydrolithocholic acid were extremely low, whereas glycochenodeoxycholic acid and 5 $\beta$ -cholestan-3 $\alpha$ -ol, which do not have the carboxy group at C-24, were much less potent inhibitors. Although DD4 was moderately inhibited by several bile acids, the inhibition patterns could not be determined, except that the inhibition by dehydrolithocholic acid was competitive. Since DD4 oxidized bile acids, the inhibition of DD4 by the bile acids is probably due to a mixed-substrate phenomenon. DD2 and DD4 were essentially insensitive to thiol-group-reacting reagents: *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulphonate did not inhibit the activities of the enzymes when the reagents (0.5 mM) were added to the assay mixture, and about 20% of the enzyme activity was inactivated even when the enzymes were incubated with 0.5 mM-*p*-chloromercuribenzenesulphonate for 10 min before the assay of the activity.

To confirm that a single protein in the preparations of DD2 and DD4 catalyses the oxidation of xenobiotic alcohols and 3 $\alpha$ -hydroxysteroids, we examined the inhibition patterns and inhibitor constants of the representative inhibitors in respect of androsterone dehydrogenase activity. The 3HSD activity

**Table 4. Effects of anti-inflammatory drugs on dehydrogenase activity of human liver dihydrodiol dehydrogenases**

The enzyme activity was assayed at pH 7.4. The drugs and bile acids were dissolved in 50% (v/v) methanol, and 50  $\mu$ l portions were added to the assay mixture just before the reaction was initiated by the addition of enzyme. The inhibition percentages (*I*, means  $\pm$  S.D., *n* = 3) by 0.1 mM-drugs and 5  $\mu$ M-bile acids were determined with 1 mM-(*S*)-(+)-indan-1-ol as a substrate, and inhibitor constants,  $K_{is}$  and  $K_{ii}$ , were estimated from Dixon plot and/or Cornish-Bowden plot of the velocities obtained in the substrate range 0.2–1.0 mM, with or without four concentrations of the inhibitor. Abbreviation: nd, not determined.

Inhibitor	DD2		DD4		
	<i>I</i> (%)	$K_{is}$ ( $\mu$ M)	<i>I</i> (%)	$K_{is}$ ( $\mu$ M)	$K_{ii}$ ( $\mu$ M)
<b>Drugs</b>					
Flufenamic acid	99 $\pm$ 2	0.3	55 $\pm$ 4	20	–
Naproxen	96 $\pm$ 5	0.6	24 $\pm$ 3	nd	nd
Ibuprofen	86 $\pm$ 6	11	2 $\pm$ 2	nd	nd
Indomethacin	53 $\pm$ 5	43	60 $\pm$ 5	21	58
Aspirin	35 $\pm$ 4	113	16 $\pm$ 2	nd	nd
6-Methylprednisolone	29 $\pm$ 3	nd	97 $\pm$ 3	2.2	1.1
Dexamethasone	14 $\pm$ 2	1079	91 $\pm$ 4	2.9	1.5
Betamethasone	12 $\pm$ 3	nd	90 $\pm$ 3	9.0	3.4
<b>Bile acids</b>					
Lithocholic acid	98 $\pm$ 3	0.02	55 $\pm$ 4	nd	nd
Ursodeoxycholic acid	98 $\pm$ 2	0.03	10 $\pm$ 5	nd	nd
Dehydrolithocholic acid	94 $\pm$ 4	0.04	98 $\pm$ 3	0.11	–
Chenodeoxycholic acid	92 $\pm$ 3	0.11	18 $\pm$ 2	nd	nd
Deoxycholic acid	41 $\pm$ 3	3.9	11 $\pm$ 2	nd	nd
Cholic acid	31 $\pm$ 5	13	2 $\pm$ 2	nd	nd
Glycochenodeoxycholic acid	9 $\pm$ 3	nd	3 $\pm$ 2	nd	nd
5 $\beta$ -Cholestan-3 $\alpha$ -ol	5 $\pm$ 2	nd	4 $\pm$ 2	nd	nd
Cholesterol	3 $\pm$ 2	nd	2 $\pm$ 2	nd	nd

of DD4 was non-competitively inhibited by indomethacin [ $K_{is}$ (slope effect) = 36  $\mu$ M and  $K_{ii}$ (intercept effect) = 315  $\mu$ M] and dexamethasone ( $K_{is}$  = 1.6  $\mu$ M and  $K_{ii}$  = 5.9  $\mu$ M). In the case of DD2, the 3HSD activity was competitively inhibited by the following inhibitors (the value in parentheses is the  $K_{is}$  value): indomethacin (32  $\mu$ M), dexamethasone (1070  $\mu$ M), lithocholic acid (0.03  $\mu$ M), dehydrolithocholic acid (0.05  $\mu$ M), chenodeoxycholic acid (0.11  $\mu$ M) and deoxycholic acid (2.6  $\mu$ M). These inhibitor constants were comparable with the values obtained with (*S*)-(+)-indan-1-ol as the substrate (Table 4).

## DISCUSSION

In the present purification of human liver dihydrodiol dehydrogenases, we confirmed the previous report that human liver contained at least four multiple forms of the enzyme [17], but the amount of DD2 in the liver specimen used was higher than the previous results of purification with *trans*-benzene dihydrodiol as the substrate. This discrepancy may be due in part to the use of naphthalene dihydrodiol as the substrate; this is a better substrate for DD2 than *trans*-benzene dihydrodiol, but suggests inter-individual differences in the contents of the respective multiple forms. Since a marked inter-individual difference in the dihydrodiol dehydrogenase activity of human liver has been recently reported [27], further studies are necessary to clarify whether inter-individual differences in specific enzyme activities in liver are related to different contents of the multiple forms of the enzyme.

The two dihydrodiol dehydrogenases, DD2 and DD4, have been shown to differ in their  $M_r$  values, pI values and specificity in the oxidation of hydroxysteroids [17]. The present study further showed clear differences between the two enzymes in respect of their stereospecificity in the oxidation of xenobiotic alcohols, specificity for bile acids and susceptibility to the inhibition by anti-inflammatory drugs and bile acids. In addition,

the heat-stabilities and peptide maps of the two enzymes were fairly distinctive. These differences, together with the identification of DD2 and DD4 as major dihydrodiol dehydrogenases in this and previous studies [17] with different liver specimens, rules out the possibility that DD2, with an  $M_r$  slightly lower than that of DD4, is a product of proteolytic cleavage of DD4. Therefore DD2 and DD4 may be structurally and functionally distinct proteins.

The results of co-purification and heat stability of dihydrodiol dehydrogenase and 3HSD activities revealed that both DD2 and DD4 possess 3HSD activity. Since the inhibition patterns and inhibitor constants of the respective inhibitors on (*S*)-(+)-indan-1-ol and androsterone dehydrogenase activities of DD2 or DD4 were almost identical, the dehydrogenation of the xenobiotic alcohols and 3 $\alpha$ -hydroxysteroids may take place at the same catalytic site on the respective enzymes. The lower  $K_m$  values for the 3 $\alpha$ -hydroxysteroids and 3-oxosteroids than for most xenobiotic substrates suggest that the steroids are endogenous substrates for DD2 and DD4. Since the two enzymes showed A-stereospecificity in hydrogen transfer from NADPH, they may be 3HSD (EC 1.1.1.213) isoenzymes. On the basis of their steroid specificities, DD4 may be important for the metabolism of steroid hormones and bile acids, and DD2 may be involved in the catabolism of androgens and progesterone.

Although the broad substrate specificity of DD4 for xenobiotics and steroids is similar to those of 3HSDs from animal livers [4–15], DD2 differs from these animal liver 3HSDs in its inability to oxidize bile acids. The substrate specificities of the present two enzymes are distinct from those of three bile-acid 3-oxoreductases purified from human liver [16]. The resistance of DD2 and DD4 to the thiol-group-reacting reagents is an additional difference from thiol-group-reacting-reagent-sensitive bile acid 3-oxoreductases of human liver [16]. The differences between the present enzymes and bile acid 3-oxoreductases suggest that human liver contains several 3HSD isoenzymes

which are distinct in their specificity for steroids, in contrast with the presence of one 3HSD species with broad substrate specificity for C<sub>19</sub>-, C<sub>21</sub>-, C<sub>24</sub>- and C<sub>27</sub>-steroids in the liver cytosol of rats [3–6,28], mice [11], hamsters [13] and monkeys [15].

Both non-steroidal and steroidal anti-inflammatory drugs have been shown to be potent inhibitors of 3HSDs from animal livers [8,11,12,14]. DD4, although having a substrate specificity similar to those of the animal liver enzymes, was inhibited by the anti-inflammatory steroids, but not potently by the non-steroidal anti-inflammatory drugs. In addition, the non-competitive inhibition of DD4 by indomethacin and anti-inflammatory steroids contrast with the competitive inhibitions of the animal liver 3HSDs by the drugs [8,11]. Conversely, DD2 was competitively inhibited by the drugs, and the inhibitory potencies of the non-steroidal drugs were higher than those of the steroidal drugs. The inhibition patterns of the anti-inflammatory drugs suggest not only that the inhibitors bind near the substrate-binding site of DD2, but also that they bind to a certain site on two different kinetic forms of DD4: the free enzyme form (or the enzyme-cofactor complex) and the enzyme-substrate complex (or the ternary complex composed of the enzyme, substrate and cofactor). Further kinetic studies of the reaction mechanism are necessary in order to elucidate the different mechanisms by which these drugs inhibit the two enzymes.

The most striking characteristic of DD2 is the high sensitivity to bile acids. Since glycochenodeoxycholic acid was a less potent inhibitor than the corresponding free acid and the enzyme was not inhibited by 5 $\beta$ -cholestan-3 $\alpha$ -ol and cholesterol, the saturated side chain with a carboxy group at C-24 on bile acid molecules may be important for the binding to the enzyme. The importance of the presence of a carboxy group on the inhibitor molecule is also suggested by the non-steroidal anti-inflammatory drugs being of higher inhibitory potency than the anti-inflammatory steroids. The bile acids with 3 $\alpha$ -hydroxy groups were competitive inhibitors, but not substrates, for DD2. Although the bile acid may bind near the active site of the enzyme, its binding through the carboxy group could in itself prevent its proper alignment as a substrate in the active site or could cause a conformational change in the enzyme that distorts the substrate-binding site.

The high inhibitory potency of bile acids suggests that the activity of DD2 is regulated by intrahepatic bile acid concentration, although the physiological relevance of the inhibition remains unknown. Stolz *et al.* [29] have identified rat liver 3HSD as one of bile-acid-binding proteins, and Takikawa *et al.* [30] have recently reported that human liver bile-acid-binding protein, with an  $M_r$  similar to that of the rat liver protein, possesses dihydrodiol dehydrogenase activity, but not 3HSD activity, towards bile acids. Although we have not determined directly the ability of the two human liver dehydrogenases to bind bile acids, the extremely low  $K_i$  values of DD2 for several bile acids and its lack of 3HSD activity towards bile acids suggest identity between this enzyme and the human liver bile-acid-binding protein. In addition, we cannot exclude the possibility that DD4 exhibits a bile-acid-binding nature because of its similarity to rat liver 3HSD in substrate specificity. A further comparison between the

two human liver dehydrogenases in respect of their primary structures and possible role as bile-acid binders should be the subject of future investigation.

This work was supported by the Tokyo Biochemical Research Foundation and by the Kato Memorial Bioscience Foundation.

## REFERENCES

- Gower, D. B. (1984) in *Biochemistry of Steroid Hormones*, 2nd edn. (Makin, H. L. J., ed.), pp. 230–292, Blackwell Scientific Publications, Oxford
- Penning, T. M., Smithgall, T. E., Askonas, L. J. & Sharp, R. B. (1986) *Steroids* **47**, 221–247
- Koide, S. S. (1969) *Methods Enzymol.* **15**, 651–656
- Ikeda, M., Hayakawa, S., Ezaki, M. & Ohmori, S. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 511–520
- Ikeda, M., Hattori, H., Ikeda, N., Hayakawa, S. & Ohmori, S. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 377–391
- Usui, E. & Okuda, K. (1986) *Biochim. Biophys. Acta* **877**, 158–166
- Vogel, K., Bentley, P., Platt, K.-L. & Oesch, F. (1980) *J. Biol. Chem.* **255**, 9621–9625
- Wörner, W. & Oesch, F. (1984) *FEBS Lett.* **170**, 263–267
- Penning, T. M., Mukharji, I., Barrows, S. & Talalay, P. (1984) *Biochem. J.* **222**, 601–611
- Ikeda, M., Hattori, H. & Ohmori, S. (1984) *Biochem. Pharmacol.* **33**, 3957–3961
- Hara, A., Inoue, Y., Nakagawa, M., Naganeo, F. & Sawada, H. (1988) *J. Biochem. (Tokyo)* **103**, 1027–1037
- Sawada, H., Hara, A., Nakagawa, M., Tsukada, F., Ohmura, M. & Matsuura, K. (1989) *Int. J. Biochem.* **21**, 367–375
- Ohmura, M., Hara, A., Nakagawa, M. & Sawada, H. (1990) *Biochem. J.* **266**, 583–589
- Nakagawa, M., Harada, T., Hara, A., Nakayama, T. & Sawada, H. (1989) *Chem. Pharm. Bull.* **37**, 2852–2854
- Hara, A., Nakagawa, M., Taniguchi, H. & Sawada, H. (1989) *J. Biochem. (Tokyo)* **106**, 900–903
- Kudo, K., Amuro, Y., Hada, T. & Higashimoto, K. (1990) *Biochim. Biophys. Acta* **1046**, 12–18
- Hara, A., Taniguchi, H., Nakayama, T. & Sawada, H. (1990) *J. Biochem. (Tokyo)* **108**, 250–254
- Takikawa, H., Stolz, A., Kuroki, S. & Kaplowitz, N. (1990) *Biochim. Biophys. Acta* **1043**, 153–156
- Platt, K. & Oesch, F. (1977) *Synthesis* **7**, 449–450
- Platt, K. & Oesch, F. (1983) *J. Org. Chem.* **48**, 265–268
- Nakayama, T., Hara, A., Yashiro, K. & Sawada, H. (1985) *Biochem. Pharmacol.* **34**, 107–117
- Dixon, M., Webb, E. C., Thorne, C. J. R. & Tipton, K. F. (1979) *Enzymes* 3rd Ed., pp. 164–182, Longman Group, London
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Hara, A., Deyashiki, Y., Nakagawa, M., Nakayama, T. & Sawada, H. (1982) *J. Biochem. (Tokyo)* **92**, 1753–1762
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Hara, A., Kariya, K., Nakamura, M., Nakayama, T. & Sawada, H. (1986) *Arch. Biochem. Biophys.* **249**, 225–236
- Penning, T. M. & Sharp, R. B. (1990) *Carcinogenesis* **11**, 1203–1208
- Smithgall, T. E. & Penning, T. M. (1988) *Biochem. J.* **254**, 715–721
- Stolz, A., Takikawa, H., Sugiyama, Y., Kuhlenkamp, J. & Kaplowitz, N. (1987) *J. Clin. Invest.* **79**, 427–434
- Takikawa, H., Stolz, A., Sugiyama, Y., Yoshida, H., Yamada, M. & Kaplowitz, N. (1990) *J. Biol. Chem.* **265**, 2132–2136