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MOUSE LIVER DIHYDRODIOL DEHYDROGENASES

IDENTITY OF THE PREDOMINANT AND A MINOR FORM WITH 17β-HYDROXYSTEROID DEHYDROGENASE AND ALDEHYDE REDUCTASE

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Abstract—A major and a minor form of dihydrodiol dehydrogenase were co-purified with 17β-hydroxysteroid dehydrogenase and aldehyde reductase, respectively, to apparent homogeneity from liver cytosol of male ddY mice. The activities of dihydrodiol dehydrogenase and testosterone dehydrogenase or aldehyde reductase of the two enzyme forms comigrated electrophoretically. The major form of the enzyme oxidized 17β-hydroxysteroids and nonsteroidal alicyclic alcohols and reduced 17-ketosteroids and various synthetic carbonyl compounds, showing higher affinity for steroids than for xenobiotics. The activity of this enzyme form toward benzene dihydrodiol and testosterone exhibited identical thermostability and susceptibility to inhibition by quercitrin, SH-reagents, nonsteroidal estrogens and anti-inflammatory agents. On the other hand, the minor form of the enzyme, which oxidized benzene dihydrodiol but not 17β-hydroxysteroids, also reduced various aldehydes well and was specifically inhibited by barbiturates and sorbinil. These results indicate that the major form of dihydrodiol dehydrogenase is identical to 17β-hydroxysteroid dehydrogenase and the minor enzyme form to aldehyde reductase.

Polycyclic aromatic hydrocarbons are widespread environmental contaminants and comprise one of the most potent classes of carcinogenic chemicals. Recent advances in research on polycyclic aromatic hydrocarbon carcinogenesis have led to identification of diol epoxide metabolites as the principal active forms of the hydrocarbons which are formed through the proximate metabolites, dihydrodiols [1, 2]. Rat liver soluble dihydrodiol dehydrogenase (EC 1.3.1.20) that converts benzene dihydrodiol (*trans*-1,2-dihydroxy-3,5-cyclohexadiene) into catechol [3] has been shown to decrease the mutagenicity of polycyclic aromatic hydrocarbons when tested in the Ames assay [4]. A recent study which indicates that the rat liver enzyme oxidizes dihydrodiols of polycyclic hydrocarbons as well as benzene dihydrodiol [5] has strongly suggested that dihydrodiol dehydrogenase plays an important role in detoxication of carcinogenic polycyclic aromatic hydrocarbons.

Although rat liver dihydrodiol dehydrogenase is identified as 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) [6, 7], four forms of the enzyme have been purified from mouse liver, three of which oxidize testosterone and oestradiol-17β but not 3α-hydroxysteroids, while the other form shows strict specificity for benzene dihydrodiol [8]. However, whether the former three forms of the mouse enzyme are identical to 17β-hydroxysteroid dehydrogenases has not been studied, and the nature of the latter enzyme form is unknown. The present studies were conducted to elucidate the physiological roles of the respective forms of mouse liver dihydrodiol dehydrogenase. We purified the multiple forms of the enzyme from mouse liver cytosol and examined the substrate specificity in the forward and backward reactions and inhibitor sensitivity.

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EXPERIMENTAL PROCEDURES

Materials. Male ddY mice (8- to 10-weeks-old) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Steroids, indomethacin, zomepirac, and standard proteins were obtained from the Sigma Chemical Co.; 1-acenaphthenol, 1-indanol, cyclohex-2-en-1-ol, 1,2,3,4-tetrahydro-1-naphthol and flufenamic acid were from the Aldrich Chemical Co.; and NADP and NADPH from the Oriental Yeast Co. (Tokyo, Japan). Sorbinil was a gift from Dr. Y. Ohta (Fujita-Gakuen Health University, Aichi, Japan). Benzene dihydrodiol was synthesized as described by Platt and Oesch [9]. HA-Ultrogel was purchased from LKB Produkter AB, and Sephadex G-100 and DEAE-Sephacel were from Pharmacia Fine Chemicals. Blue-Sepharose was prepared as described by Heyns and De Moor [10].

Enzyme assay. Dehydrogenase and reductase activities were assayed spectrophotometrically by measuring the absorbance at 340 nm for NADPH. The reaction mixture for the determination of the dehydrogenase activity contained, in a total volume of 2.0 ml, 100 mM glycine–NaOH buffer, pH 10.0, 0.25 mM NADP, 1.8 mM benzene dihydrodiol or 50 μM testosterone and enzyme, and that for the

reductase assay consisted of 80 mM potassium phosphate buffer, pH 6.0, 80 μM NADPH, 1.0 mM D-glucuronate or various concentrations of carbonyl compounds and enzyme. Steroids, menadione and inhibitors such as anti-inflammatory agents and synthetic estrogens were dissolved in methanol and added to the reaction mixture to give a final methanol concentration of 2.5%. The reactions were started by adding the enzyme. One unit of activity was defined as the oxidation or production of 1 μmol of NADPH/min at 25°. For the study of pH dependence of the dehydrogenase or reductase activity of the enzymes, 100 mM glycine–NaOH, 100 mM Tris–HCl, 80 mM potassium phosphate or 100 mM sodium citrate–phosphate buffers were used at various pH values.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [11] with bovine serum albumin as a standard.

Enzyme purification. Dihydrodiol dehydrogenases were purified from livers of mice according to the procedure as described [8], except that DEAE-Sephacel and Blue-Sepharose were employed instead of DEAE-cellulose and reactive red 120-agarose respectively. On the DEAE-Sephacel column (2.5 × 50 cm) chromatography, a high peak of enzyme activity (DD1) emerged in the effluent during washing of the column with 5 mM Tris–phosphate, pH 7.4, containing 5 mM 2-mercaptoethanol, and three peaks of activity (DD2–DD4) were eluted with a linear NaCl gradient (0–0.12 M). The four enzymes were obtained in a pure form as judged by polyacrylamide gel electrophoresis.

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed at 4° [12]. Sodium dodecyl sulfate (SDS)–polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli [13], using molecular weight standards. The gels were stained for protein with Coomassie brilliant blue R-250 and for enzyme activity toward benzene dihydrodiol, testosterone or D-glucuronate as described [14].

Molecular weight determination. The molecular weight of the enzyme denatured in 1% SDS and 1% 2-mercaptoethanol was estimated by SDS–slab gel electrophoresis, and that of the native enzyme was determined using a high performance liquid chromatograph with TSK gel G3000SW (Toyo Soda Manufacturing Co., 0.75 × 60 cm) in 10 mM potassium phosphate buffer, pH 7.0, containing 0.15 M KCl and 0.5 mM EDTA. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen A, and myoglobin at a flow rate of 0.5 ml/min.

RESULTS

When the dehydrogenase activities toward benzene dihydrodiol and testosterone were examined in the cytosol from male mouse liver, testosterone dehydrogenase activity was about 3-fold higher than benzene dihydrodiol dehydrogenase activity. Since Bolcsak and Nerland [8] have reported that three of four dihydrodiol dehydrogenase from mouse liver cytosol show 17β-hydroxysteroid dehydrogenase activity, and since hepatic aldehyde reductase from other animals [15, 16] also exhibits low benzene dihydrodiol dehydrogenase activity, we co-purified dihydrodiol dehydrogenase, testosterone dehydrogenase and aldehyde reductase from liver cytosol (Table 1). At the DEAE-Sephacel chromatography

Table 1. Co-purification of benzene dihydrodiol and testosterone dehydrogenase activities and aldehyde reductase activity from mouse liver cytosol

		Specific activity* on				
Step	Total protein (mg)	(A) Benzene dihydrodiol (units/mg)	(B) Testosterone (units/mg)	(C) D-Glucuronate (units/mg)	(B/A)	(C/A)
Cytosol	11,500	0.003	0.009	0.004	3.0	1.3
$(NH_4)_2SO_4$ fraction	7,330	0.004	0.010	0.005	2.5	1.3
DEAE-Sephacel						
DD1	1,510	0.010	0.035	0.0	3.5	0.0
DD2	123	0.017	0.0	0.23	0.0	13
DD3	179	0.017	0.001	0.0	0.06	0.0
DD4	200	0.019	0.002	0.0	0.11	0.0
Blue-Sepharose						
DD1	128	0.11	0.38	0.0	3.5	0.0
DD2	15.5	0.12	0.0	1.6	0.0	13
Sephadex G-100						
DD1	30.4	0.40	1.4	0.0	3.5	0.0
DD2	7.1	0.25	0.0	2.9	0.0	12
HA-Ultrogel						
DD1	7.6	0.99	3.5	0.0	3.5	0.0
DD2	4.5	0.27	0.0	3.1	0.0	12

* At each stage of purification, the dehydrogenase activities were determined at pH 10.0 with 1.8 mM benzene dihydrodiol or 50 μM testosterone, and the reductase activity was at pH 6.0 with 1.0 mM D-glucuronate.

step, the recoveries of dihydrodiol dehydrogenase activity in DD1–DD4 were 52, 7, 7 and 13%, respectively, while 80% of testosterone dehydrogenase activity was coeluted with DD1 and 1.2% of the activity with DD3 and DD4. The aldehyde reductase activity was recovered only in DD2 fractions which did not exhibit testosterone dehydrogenase activity. The dehydrogenase activities on benzene dihydrodiol and testosterone of DD1 and the dihydrodiol dehydrogenase and aldehyde reductase activities of DD2 were not separated in the subsequent purification steps, and the ratios of dihydrodiol dehydrogenase activity to testosterone dehydrogenase activity for DD1 or to aldehyde reductase activity for DD2 were essentially constant at subsequent purification steps. The two enzyme preparations showed single protein bands on polyacrylamide disc gel electrophoresis, in which the activity toward benzene dihydrodiol was stained at the same mobility as that of testosterone dehydrogenase activity for DD1 or as that of aldehyde reductase activity for DD2 (Fig. 1). DD1 and DD2 also exhibited single protein bands with molecular weights of 36,000 and 39,000, respectively, on SDS–gel electrophoresis (Fig. 2); similar values of 30,000 and 32,000 were obtained by gel permeation chromatography of the native DD1 and DD2.

The other two minor forms of dihydrodiol dehydrogenase (DD3 and DD4) were also purified to electrophoretic homogeneity (Fig. 2). The specific dihydrodiol dehydrogenase activities of the purified DD3 and DD4 were 2.90 and 3.30 units/mg protein with overall recoveries of 3 and 4% respectively. The

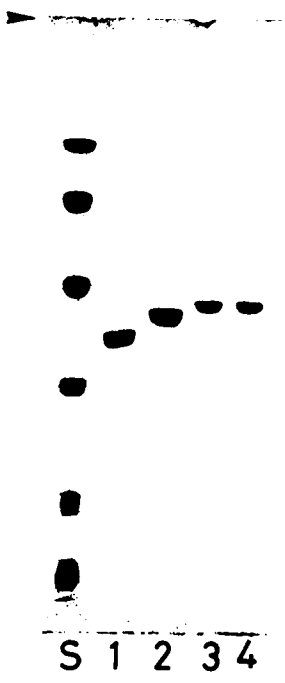

Fig. 2. SDS–polyacrylamide slab gel electrophoresis of four dihydrodiol dehydrogenases from mouse liver cytosol. About 10 μg of DD1 (1), DD2 (2), DD3 (3) and DD4 (4) was run on a 10% polyacrylamide gel. The following proteins, phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (45K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K) and α-lactoalbumin (14.4K), were used as standards (S). The arrowhead indicates the origin of the separating gel (cathode).

Fig. 1. Polyacrylamide disc gel electrophoresis of mouse liver dihydrodiol dehydrogenases. About 5μg of DD1 and DD2 was run on a 7.5% polyacrylamide gel. The gels were stained for protein (p) and activities of benzene dihydrodiol dehydrogenase (b), testosterone dehydrogenase (t) and D-glucuronate reductase (g). The direction of migration was from top (cathode) to bottom (anode), and the arrowheads indicate the origin of the separating gel.

two enzymes showed the same molecular weight of 41,000 on SDS–gel electrophoresis and 47,000 by gel permeation chromatography.

The substrate specificities of DD1 and DD2 in the forward and backward reactions were examined with various alcohols and carbonyl compounds (Table 2). DD1 oxidized alicyclic alcohols and 17β-hydroxysteroids but was not active toward primary alcohols such as ethanol, *n*-butanol and *n*-octanol and 3-hydroxysteroids such as androsterone, epiandrosterone and aetiocholanolone. The K_m values for 17β-hydroxysteroids were much lower than those for alicyclic alcohols and benzene dihydrodiol. In contrast, DD2 oxidized only benzene dihydrodiol among the alcohols tested. When the reverse reaction was examined with NADPH as a cofactor, DD1 reduced various types of carbonyl compounds as well as 17-ketosteroids at an optimal pH of around 5.8. The enzyme showed higher affinity for 17-ketosteroids than for xenobiotic carbonyl compounds except for 2-nitrobenzaldehyde and pyridine-4-aldehyde. DD2 reduced aldehydes, sugars and diacetyl but not 17-ketosteroids, aromatic aldehydes and menadione. Although the K_m values of DD2 for most of the aldehydes were slightly lower than that for benzene dihydrodiol, the V_{max} values for the aldehydes were much higher than that for benzene dihydrodiol. The maximum rate in the pyridine-4-aldehyde reduction by DD2 was observed at broad pH ranges from 5.8 to 6.5.

The substrate specificities of the other two minor forms of dihydrodiol dehydrogenase (DD3 and DD4) were similar to each other but different from those of DD1 and DD2; of the alcohols listed in Table 2, DD3 and DD4 oxidized only 50 μM testosterone, 50 μM 5α-dihydrotestosterone and 0.5 mM 1-acenaphthenol at rates of less than 10% of the benzene dihydrodiol dehydrogenase activity, and exhibited low reductase activities on 1 mM pyridine-4-aldehyde, 4-nitrobenzaldehyde and diacetyl which are less than 50% of the benzene dihydrodiol dehydrogenase activity. The K_m values for benzene dihydrodiol of DD3 and DD4 were 5.1 and 5.3 mM respectively.

To confirm the possibility that the activities toward benzene dihydrodiol and 17β-hydroxysteroids reside in the same protein of DD1, we compared the thermal stability and inhibitor sensitivity of the two activities. The activities toward benzene dihydrodiol and testosterone showed almost the same thermal stability; the times required for 50% inactivation of the original two activities at 42° and 44° were 11.2 and 3.6 min respectively. The two activities of DD1 were also inhibited similarly by the inhibitors listed in Table 3. SH-reagents, synthetic estrogens, quercitrin and nonsteroidal anti-inflammatory agents, rat liver 3α-hydroxysteroid dehydrogenase inhibitors [7], similarly inhibited the two activities of this enzyme, but Δ^4,3-ketosteroids, which are also inhibitors of rat liver enzyme [6], showed low inhibitions of the two activities of this mouse enzyme. The inhibition by hexestrol was competitive with respect to either testosterone ($K_i = 0.9$ μM) or benzene dihydrodiol ($K_i = 0.8$ μM). Furthermore, no additive formation of NADPH was observed when benzene dihydrodiol was mixed with testosterone at concentrations of their K_m values. Both dehydrogenase activities were inhibited by the addition of 2-nitrobenzaldehyde and aetiocholanolone which were good substrates in the reverse reaction. These results suggest that benzene dihydrodiol and testosterone may bind to the same site of the enzyme.

DD2 was different from DD1 in inhibitor sensitivity. The enzyme was inhibited by barbiturates and sorbinil, aldehyde reductase (EC 1.1.1.2) inhibitors [17, 18], but not by the anti-inflammatory agents and 3-ketosteroids.

DISCUSSION

The present studies have demonstrated that the major form (DD1) of the four dihydrodiol dehydro-

Table 2. Substrate specificities of mouse liver dihydrodiol dehydrogenases*

	DD1		DD2	
Substrate	K_m (μM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
1-Acenaphthenol	1120	5.90	—	ND†
Testosterone	19	3.80	—	ND
Tetrahydro-1-naphthol	230	3.78	—	ND
Cyclohex-2-en-1-ol	169	3.41	—	ND
1-Indanol	750	3.20	—	ND
5β-Dihydrotestosterone	7	3.17	—	ND
5α-Dihydrotestosterone	9	1.90	—	ND
Oestradiol-17β	14	1.42	—	ND
Benzene dihydrodiol	110	1.16	5.0	0.55
NADP‡	15	—	0.01	—
2-Nitrobenzaldehyde	2	2.61	0.26	1.45
Diacetyl	14	2.33	5.0	23.8
Menadione	23	1.93	—	ND
3-Nitrobenzaldehyde	58	1.43	1.3	6.62
Pyridine-4-aldehyde	4	1.36	0.71	62.3
Aetiocholanolone	2	1.29	—	ND
4-Nitroacetophenone	15	1.19	—	ND
4-Nitrobenzaldehyde	24	1.01	0.29	62.3
Androsterone	9	0.82	—	ND
Epiandrosterone	8	0.76	—	ND
n-Butyraldehyde	308	0.64	5.0	23.0
Pyridine-3-aldehyde	103	0.63	1.1	37.9
4-Benzoylpyridine	30	0.50	—	ND
Androst-4-ene-3,17-dione	4	0.44	—	ND
Oestrone-3-sulfate	9	0.36	—	ND
4-Carboxybenzaldehyde	—	ND	0.063	72.9
D-Glucuronate	—	ND	4.3	36.1
D-Xylose	—	ND	222	6.06
D-Ribose	—	ND	100	2.26
NADPH‡	1	—	0.006	—

* The dehydrogenase activity was assayed at pH 10.0 and the reductase activity at pH 6.0.

† ND, no activity was detected.

‡ The values for NADP were determined with 1.8 mM benzene dihydrodiol as a substrate, and those of DD1 and DD2 for NADPH with 50 μM aetiocholanolone and 14 mM D-glucuronate respectively.

Table 3. Effects of inhibitors on mouse liver dihydrodiol dehydrogenases

Inhibitor	Conc (mM)	Inhibition (%) of activity*		
		DD1		DD2
		Testosterone	Benzene dihydrodiol	Benzene dihydrodiol
Hexestrol	0.01	72	68	58
Dienstrol	0.01	62	69	58
$HgCl_2$	0.01	52	60	20
PCMPS†	0.01	49	42	13
Sorbinil	0.01	0	0	72
Aetiocholanolone	0.05	81	79	0
2-Nitrobenzaldehyde	0.05	78	78	25
Zomepirac	0.05	55	50	5
Progesterone	0.05	38	32	0
Predonisone	0.05	9	8	0
Corticosterone	0.05	9	6	0
Flufenamic acid	0.1	81	73	0
Quercitrin	0.1	72	61	68
Indomethacin	0.1	60	58	0
Phenobarbital	1.0	3	4	61
Barbital	1.0	0	2	50
Benzene dihydrodiol	1.8	32	—	—

* The dehydrogenase activities of the enzymes were assayed with 50 μM testosterone and/or 1.8 mM benzene dihydrodiol as a substrate.

† PCMPS, *p*-chloromercuriphenylsulfonic acid.

genases in mouse liver is 17β-hydroxysteroid dehydrogenase (EC 1.1.1.62) because of its higher affinity for 17β-hydroxysteroids and 17-ketosteroids and its co-purification with 17β-hydroxysteroid dehydrogenase. In addition, we found that the enzyme oxidized xenobiotic alicyclic alcohols and reduced various carbonyl compounds, which suggests that the enzyme is also identical to indanol dehydrogenase (EC 1.1.1.112) and carbonyl reductase (EC 1.1.1.184). Indanol dehydrogenase has been studied only in rabbit and bovine liver [19], but its physiological role is unknown. Carbonyl reductase has been detected in mouse liver [20], but it has not been purified and characterized. The broad substrate specificity of DD1 resembles those of 3α-hydroxysteroid dehydrogenase from rat liver [4, 7] and testosterone 17β-dehydrogenase (EC 1.1.1.64) from guinea pig liver [15, 21] which have been identified as dihydrodiol dehydrogenase and carbonyl reductase in these animal livers. We have reported recently that seven forms of dehydrogenase for alicyclic alcohols and benzene dihydrodiol from rabbit liver cytosol exhibit high affinity for 3α- or 17β-hydroxysteroids [22]. These data collectively indicate that cytosolic 3α- and 17β-hydroxysteroid dehydrogenases, at least in liver of rodents and rabbit, regardless of species difference in steroid specificity, can act as carbonyl reductase and dihydrodiol dehydrogenase for xenobiotics such as carbonyl compounds and dihydrodiols derived from aromatic hydrocarbons.

The high aldehyde reductase activity of the purified DD2, the co-purification of dehydrogenase activity towards benzene dihydrodiol and aldehyde reductase towards D-glucuronate, and the electrophoretical co-migration of the two activities indicate that they are identical. Tulsiani and Touster [23] have purified two aldehyde reductases, designated as aldehyde reductase I and II, from mouse liver. Judging from the substrate specificity, molecular weight, optimal pH and phenobarbital inhibition, DD2 corresponds to aldehyde reductase II reported by these authors. The ability to oxidize benzene dihydrodiol also has been observed with aldehyde reductases from guinea pig [15] and hamster liver [16]. Although the dihydrodiol dehydrogenase activity of aldehyde reductase is lower than the reductase activity in this and other studies [15, 16], the enzyme may be responsible for the dihydrodiol oxidation in tissues in which dihydrodiol dehydrogenases similar to hepatic hydroxysteroid dehydrogenases are not present.

Bolcsak and Nerland [8] previously reported that two of four dihydrodiol dehydrogenases (DD3 and DD4) from Swiss–Webster mouse liver are dimeric proteins associated with high 17β-hydroxysteroid dehydrogenase activity. We also isolated the two enzymes from ddY mouse liver, but the properties of the enzymes are not consistent with those of the enzymes from Swiss–Webster mouse liver. The present enzymes seem to be monomers based on the results of the molecular weight determination and showed higher K_m values for benzene dihydrodiol and lower testosterone dehydrogenase activity than the enzymes from Swiss–Webster mouse liver. The present enzymes reduced the limited aldehydes and diacetyl at low rates. Although these discrepancies between the present and previous studies on the two minor enzymes may be due to a different strain of mouse, the natures and physiological functions of the enzymes remain to be explored.

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