Purification and Properties of Multiple Forms of Dihydrodiol Dehydrogenase from Monkey Liver

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Two major and two minor forms of dihydrodiol dehydrogenase with similar molecular weights of around 36000 were purified from monkey liver cytosol. All the forms oxidized *trans*-dihydrodiols of benzene and naphthalene and reduced aromatic aldehydes, but showed differences in charge, specificity for other substrates and inhibitor sensitivity. One major (pI 8.7) and one minor (pI 7.9) form of the enzyme exhibited high activity for alicyclic alcohols and sensitivity to *o*-phenanthroline. The other major form (pI 6.2) oxidized 3α-hydroxysteroids and was inhibited by dexamethasone and indomethacin, whereas the other minor form (pI 5.8) showed high reductase activity for aldehydes including D-glucuronate and sensitivity to barbital and sorbinil, and cross-reacted with human aldehyde reductase. The results indicate that the multiple forms of monkey liver dihydrodiol dehydrogenase are indanol dehydrogenases, 3α-hydroxysteroid dehydrogenase and aldehyde reductase.

Keywords dihydrodiol dehydrogenase; indanol dehydrogenase; aldehyde reductase; 3α-hydroxysteroid dehydrogenase; enzyme multiplicity; monkey liver

A soluble nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent dihydrodiol dehydrogenase (EC 1.3.1.20), that converts *trans*-1,2-dihydroxy-3,5-cyclohexadiene (benzene dihydrodiol) into catechol, has been suggested to play an important role in detoxification of carcinogenic polycyclic aromatic hydrocarbons.[1] Recent studies on dihydrodiol dehydrogenase have shown that the enzyme exists in multiple forms in rodent liver,[2] and the multiple forms have been identified as genetically distinct monomeric enzymes such as 3α-hydroxysteroid dehydrogenase,[3] 17β-hydroxysteroid dehydrogenases and/or aldehyde reductase.[4] On the other hand, a dimeric dihydrodiol dehydrogenase, distinct from the monomeric enzymes from rodent liver, has been isolated from monkey kidney.[5]

We have recently isolated a monomeric indanol dehydrogenase with a molecular weight of 36000 from monkey liver, and found that the enzyme exhibits low dihydrodiol dehydrogenase activity.[6] During the purification of indanol dehydrogenase to elucidate its relationship to dihydrodiol dehydrogenase, we found the existence of four dihydrodiol dehydrogenases in this tissue. This paper describes the purification and properties of the multiple forms of the enzyme from monkey liver.

Experimental

Materials Livers of Japanese monkey were obtained from the Primate Research Institute, Kyoto University. Chemicals used in this study were specified elsewhere,[4,6] except that Matrex Red A was obtained from Amicon Co.

Enzyme Purification The purification of dihydrodiol dehydrogenase was performed at 4 °C. Monkey liver (80 g) was homogenized in 300 ml of 10mM Tris–HCl, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid (EDTA), 0.14 M KCl and 1 mg/ml trypsin inhibitor, and the homogenate was centrifuged for 1 h at 105000 × *g*. The supernate was fractionated by addition of solid ammonium sulfate. The 35—70% ammonium sulfate precipitate was collected by centrifugation for 15 min at 12000 × *g*, dissolved in 30 ml of 10 mM Tris–HCl, pH 8.0, containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A), and then passed through a 5 × 95 cm Sephadex G-100 column in buffer A plus 0.15 M KCl. The enzyme-active fractions were concentrated to 30 ml by ultrafiltration using an Amicon YM-10 membrane, dialyzed against buffer A, and then applied to a 2.6 × 30 cm Q-Sepharose column equilibrated with the buffer. The column was washed with buffer A and the buffer plus 20% glycerol, and the adsorbed proteins were eluted with a linear 0—0.1 M NaCl gradient in buffer A plus 20% glycerol. This resulted in resolution of four enzyme-active peaks (DD1—DD4). The four enzymes were separately purified by chromatography on Matrex Red A and HA-Ultrogel, in which the buffers used for the purification of DD4 were supplemented with 20% glycerol to stabilize this enzyme. The four enzymes were adsorbed on 1.6 × 10 cm Matrex Red A columns equilibrated with buffer A, and were eluted with linear 0—1.0 M NaCl gradients. The four enzyme fractions were separately concentrated by ultrafiltration, dialyzed against buffer A without EDTA, and then applied to 1.2 × 5 cm HA-Ultrogel columns equilibrated with the buffer. While DD3 was eluted during washing of the column with the buffer, the other enzymes were eluted with 0—0.08 M potassium phosphate gradients in the buffer.

Analytical Methods Dehydrogenase and reductase activities were assayed spectrophotometrically or fluorometrically by measuring the production and oxidation of NADPH as described.[6] The standard assay mixture for dihydrodiol dehydrogenase contained 100 mM glycine–NaOH, pH 10.0, 0.25 mM $NADP^+$, 1.8 mM benzene dihydrodiol and enzyme in a total volume of 2.0 ml. One unit of activity was defined as the oxidation or production of 1 μmol of NAD(P)H/min at 25 °C.

Protein concentration was determined by the methods of Lowry *et al.*[7] with bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[8] and isoelectric gel focusing[9] were carried out as described. The determination of relative molecular mass (M_r) of the enzyme was performed as previously described.[6]

Immunodiffusion and immunoprecipitation using the antibody against human liver aldehyde reductase[10] were performed by the method of Sawada *et al.*[11]

Results and Discussion

The results of purification of the four dihydrodiol dehydrogenases from monkey liver are summarized in Table I. The enzyme activity of the ammonium sulfate fraction was eluted as a single peak around M_r of 35000 on Sephadex G-100 filtration, but was resolved into two major (DD1 and DD4) and two minor peaks (DD2 and DD3) in the Q-Sepharose step (Fig. 1). When indanol dehydrogenase activity in these peaks was assayed, 70% of the enzyme activity was coeluted with DD1, and 18 and 9% of the activity were recovered in DD2 and DD4, respectively, but only 3% of the activity was detected in DD3. The dihydrodiol dehydrogenase and indanol dehydrogenase activities of DD1, DD2 and DD4 were not separated in the subsequent purification steps. DD1, DD3 and DD4 showed single protein bands with similar M_rs of 36000, 39000 and 38000, respectively, on SDS-PAGE, but DD2 contained some faint bands around 28000 besides a main protein band with M_r of 36000. DD1—DD4 had distinct pI values of 8.7, 7.9, 5.8, and 6.2, respectively. Of the enzymes with indanol dehydrogenase activity, the M_r and pI values

of DD1 are identical to those reported for monkey liver indanol dehydrogenase,[6] which indicates that this enzyme is indanol dehydrogenase.

Since DD2, DD3 and DD4 were different from indanol dehydrogenase in M_r and/or pI values, their substrate specificity (Table II) and inhibitor sensitivity (Table III) were compared to those of DD1. DD2 showed substrate specificity similar to that of indanol dehydrogenase (DD1)[6]: it oxidized alicyclic alcohols more effectively than *trans*-dihydrodiols of benzene and naphthalene, showed a preference for (*S*)-(+)-1-indanol over its (*R*)-(−)-form, and reduced aromatic aldehydes with NADPH as a cofactor at a pH optimum of 5.8. In addition, the inhibitor sensitivity of DD2 was almost identical to that of indanol dehydrogenase, which is inhibited by *o*-phenanthroline.[6] Since indanol dehydrogenase has been reported to exist in two forms in monkey liver,[6] DD2 may correspond to the minor form of the enzyme which has not been characterized yet.

DD3 showed high specificity for the *trans*-dihydrodiols, and exhibited high NADPH-dependent reductase activity towards aldehydes including D-glucuronate at a pH optimum of 6.0. The substrate specificity is similar to that of aldehyde reductases from rodent[4] and monkey liver.[10] The enzyme was specifically inhibited by aldehyde reductase inhibitors such as barbital and sorbinil. The antiserum against human liver aldehyde reductase, which has been reported to cross-react with monkey liver aldehyde reductase,[10] formed a precipitin line only against DD3 of the four dihydrodiol dehydrogenases on the immunodiffusion test, and the dihydrodiol dehydrogenase activity was com-

Table I. Purification of Dihydrodiol Dehydrogenases from Monkey Liver

Step	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Recovery (%)
Crude supernate	9890	31.1	0.003	100
Ammonium sulfate fraction	4830	29.7	0.006	95
Sephadex G-100	440	28.0	0.064	90
Q-Sepharose				
DD1	24.5	4.84	0.198	16
DD2	12.4	2.19	0.177	7
DD3	10.8	0.59	0.055	2
DD4	119	6.09	0.051	20
Matrex Red A				
DD1	21.7	4.52	0.208	15
DD2	5.0	1.21	0.243	4
DD3	4.6	0.24	0.052	0.7
DD4	15.0	3.37	0.225	11
HA-Ultrogel				
DD1	10.7	3.32	0.310	11
DD2	2.4	0.55	0.229	2
DD3	2.3	0.15	0.066	0.5
DD4	2.5	0.62	0.248	2

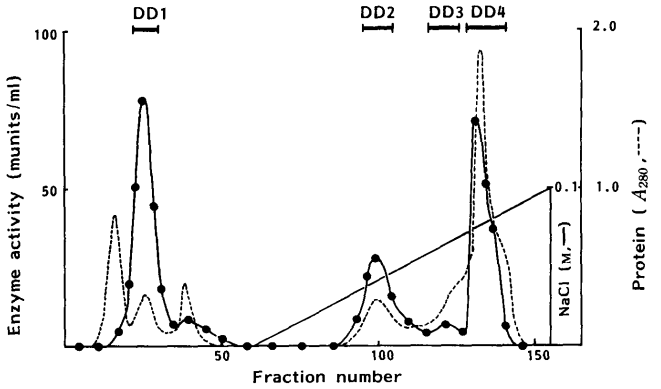

Fig. 1. Resolution of Multiple Forms of Monkey Liver Dihydrodiol Dehydrogenase on Column Chromatography with Q-Sepharose

The enzyme activity was resolved into four peaks during washing with buffer A plus 20% glycerol and eluting with a linear 0—0.1 M NaCl gradient (——) in the buffer at a flow rate of 60 ml/h. The fractions (10 ml) were analyzed for protein (------) and for the enzyme activity (●).

Table II. Substrate Specificity of Monkey Liver Dihydrodiol Dehydrogenases

Substrate	Concentration (mM)	DD1	DD2		DD3		DD4	
		V (%)	*V* (%)	K_m (mM)	*V* (%)	K_m (mM)	*V* (%)	K_m (mM)
Dehydrogenation[a]								
Benzene dihydrodiol	1.8	100	100	5.9	100	>20	100	1.4
Naphthalene dihydrodiol[c]	1.8	89	129	nd	157	17	136	4.6
1-Acenaphthenol	0.5	1310	621	0.21	0	nd	251	1.4
(*S*)-(+)-1-Indanol	1.0	681	713	nd	0	nd	122	nd
(*R*)-(−)-1-Indanol	1.0	36	12	nd	0	nd	176	nd
5α-Androstane-3α,17β-diol	0.005	1	0	nd	0	nd	375	0.001
Androsterone	0.005	0	0	nd	0	nd	187	0.006
5β-Androstane-3α,17β-diol	0.005	1	0	nd	0	nd	147	0.001
$NADP^+$	0.25	100	100	0.010	100	0.01	100	0.032
NAD^+	0.8	3	4	nd	1	nd	18	2.2
Reduction[b]								
3-Nitrobenzaldehyde	1.0	157	169	0.012	1020	2.6	62	nd
4-Nitrobenzaldehyde	1.0	80	120	0.12	9950	0.18	63	0.29
D-Glucuronate	10.0	0	0	nd	9280	5.4	0	nd
5β-Androstan-17α-ol-3-one	0.05	11	0	nd	0	nd	90	0.001
NADPH		—	—	0.006	—	0.001	—	0.006

The activities (*V*) with indicated substrate concentrations are relative to the benzene dihydrodiol dehydrogenase activity of the respective enzymes. *a*) The activities and apparent K_m values for the substrate were determined with 0.25 mM $NADP^+$ as a cofactor and those for $NAD(P)^+$ with 1.8 mM benzene dihydrodiol as a substrate at pH 10.0. *b*) The values for the carbonyl substrates were determined with 0.08 mM NADPH as a cofactor and those for NADPH with 1.0 mM 4-nitrobenzaldehyde as a substrate at pH 6.0. *c*) *trans*-1,2-Dihydroxy-1,2-dihydronaphthalene.

Table III. Effects of Inhibitors on Monkey Liver Dihydrodiol Dehydrogenases

Inhibitor	Concentration (mM)	Inhibition (%)			
		DD1	DD2	DD3	DD4
Hexestrol	0.01	91	84	0	82
Sorbinil	0.01	1	0	95	0
Dexamethasone	0.01	0	0	0	50
Indomethacin	0.1	50	39	0	88
o-Phenanthroline	1.0	66	79	5	1
Barbital	1.0	9	0	88	0

The inhibitor was added to the assay mixture before the reaction was started by adding the enzyme solution.

pletely immunoprecipitated by the antiserum. The results indicate that DD3 is identical to aldehyde reductase.

DD4 oxidized 3α-hydroxysteroids as well as the substrates for DD1 and DD2 in the presence of either $NADP^+$ or NAD^+. When benzene dihydrodiol and 5β-androstane-3α, 17β-diol were mixed at concentrations equivalent to their K_m values, the rate of dehydrogenation was 62% of the sum of the rates obtained with individual substrates, which suggests that a single enzyme protein in DD4 catalyzes the two reactions. The lower K_m values for steroids than those for the xenobiotic substrates indicate that DD4 acts physiologically as 3α-hydroxysteroid dehydrogenase. In addition, the enzyme was potently inhibited by dexamethasone and indomethacin. The properties of DD4 are similar to those of rat liver 3α-hydroxysteroid dehydrogenase.[3)]

In conclusion, monkey liver contained at least three distinct enzyme species associated with dihydrodiol dehydrogenase activity: one enzyme was identified as indanol dehydrogenase, which is clearly different from dihydrodiol dehydrogenases in livers of other species, whereas the other enzymes were aldehyde reductase and 3α-hydroxysteroid dehydrogenase.

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