RESEARCH COMMUNICATION

Relationship of human liver dihydrodiol dehydrogenases to hepatic bile-acid-binding protein and an oxidoreductase of human colon cells

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We previously isolated three monomeric dihydrodiol dehydrogenases, DD1, DD2 and DD4, from human liver, and cloned a cDNA (C9) thought to encode DD2, which is identical with those for human bile-acid-binding protein and an oxidoreductase of human colon carcinoma HT29 cells. In the present study we have provided evidence that the C9 cDNA clone encodes DD1, not DD2. A recombinant enzyme expressed from the cDNA in a bacterial system was purified, and its catalytic properties, bile-

acid-binding ability and primary sequence were compared with those of the hepatic dihydrodiol dehydrogenases. The results show that DD1 encoded by C9 possesses prostaglandin F synthase activity but low affinity for lithocholic acid, whereas DD2, showing differences of six amino acid residues from the DD1 sequence, exhibited high-affinity binding for the bile acid. Refined relationship between dihydrodiol dehydrogenases and their related proteins of human tissues is proposed.

INTRODUCTION

Dihydrodiol dehydrogenase (DD, EC 1.3.1.20) catalyses the NADP⁺-linked oxidation of *trans*-dihydrodiols of aromatic hydrocarbons to the corresponding catechols, and is distributed in mammalian tissues. In human liver, DD exists in at least four multiple forms (DD1–DD4) with similar molecular masses around 36 kDa [1]. One minor form (DD3) is identified with aldehyde reductase, and the other three forms exhibit 3 α -hydroxysteroid dehydrogenase activity, but differ from one another in their specificity for the other substrates and inhibitor sensitivity [1–3].

Two cDNA clones, designated C9 and C14, that were presumed to encode DD2 and DD4 have been isolated from human liver [4]; evidence is reported in the present study (see below) that the C9 clone encodes DD1 rather than DD2. The C9 and C14 clones are virtually identical with those of human liver bile-acid-binding protein (HBAB) [5] and chlordecone reductase [6] respectively, which are members of the aldo-keto reductase family [7]. Recently, a cDNA for an oxidoreductase (H-37) that is overexpressed in ethacrynic acid-resistant human carcinoma HT29 colon cells has been shown to be also identical with that for HBAB [8] and C9. Although the identity of DD4 and chlordecone reductase has been demonstrated by expression of the recombinant enzyme from the cDNA for DD4 [9], a recombinant protein expressed from the cDNA for HBAB (corresponding to C9) [5] has lower affinity for bile acids than does HBAB purified from human liver [10]. In addition, the recombinant HBAB exhibits DD activity, but its activity is not inhibited by 50–100 nM bile acids, which give significant inhibition of the activity of human liver DD2 [2].

In order to understand more clearly the relationship among DDs, HBAB and H-37 in human tissues, amino acid sequencing data have been obtained from human liver DD1 and DD2. Furthermore, we have characterized human liver DD1 and DD2

and a recombinant DD (rDD1) expressed from the C9 clone, in terms of substrate specificity, inhibitor sensitivity and bile acid binding. The results obtained suggest that the cDNA, previously referred to as that for DD2 (accession no. D26124), HBAB (accession no. M86609) or H-37 (accession no. U05684), encodes DD1. Indeed, our data suggest that DD2 and HBAB are encoded by the cDNA isolated by Ciaccio and Tew [11] that was designated c81/RACE. The nature of the inducible H-37 protein that is overexpressed in ethacrynic acid-resistant HT29 cells requires clarification.

EXPERIMENTAL

Materials

Restriction enzymes and chemicals used in this study were specified elsewhere [1–4,9], except that bis-1-anilinoanthracene-8-sulphonate (ANS) was from Tokyo Kasei Kogyo (Tokyo, Japan). Human liver DDs (DD1 and DD2) [1] and recombinant DD4 [9] were purified to homogeneity as previously described. The C9 clone previously isolated [4] was used.

Plasmid construction, expression and enzyme purification

Since the insert of the C9 clone [4] lacks a 51-base coding region corresponding to the N-terminal 17 amino acids of the sequence deduced from HBAB cDNA [5], the following three oligonucleotides were synthesized to prepare a cDNA containing the entire coding region by PCR. DD2EN1 (CCGGAATTCCATGGATT-CGAAATATCAGTGTGTGAAGCTGAATGATGG) included an *Eco*RI site followed by the nucleotides 1–38 of the HBAB cDNA, DD2EL (GTGTGAAGCTGAATGATGGTCACTTC-ATGCCTGTCCTGGGATTTGGCAC) contained the underlined additional coding region of DD2EN1 to the nucleotides 39–68 of HBAB cDNA, and DD2EC (GGGATATCTTA-

Abbreviations used: DD, dihydrodiol dehydrogenase; HBAB, human liver bile-acid-binding protein; H-37, an oxidoreductase expressed in human HT29 colon cells; rDD1, recombinant DD; ANS, bis-1-anilinoanthracene-8-sulphonate; PG, prostaglandin. C9; this clone has previously been incorrectly referred to as DD2, but will henceforth be referred to in the present paper as C9.

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Table 1 Substrate specificity of rDD1, DD1 and DD2

The activities of 9 α ,11 β -prostaglandin (PG) F₂ dehydrogenase and PGD₂ reductase were assayed at pH 10.0 and 6.0, respectively. The apparent kinetic constants of DD1 and DD2 for the PGs are taken from [3], and the values of DD2 for the other substrates are from [2]. Abbreviation: tetralol, 1,2,3,4-tetrahydronaphth-1-ol.

Substrate	rDD1		DD1		DD2	
	K_m (μ M)	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat} (min ⁻¹)
Oxidation						
(S)-Tetralol	7	26	5	14	260	16
(S)-Indan-1-ol	50	25	38	10	520	9.5
Benzene dihydrodiol	870	8.7	580	3.2	5000	1.9
5 β -Pregnane-3 α ,20 α -diol	3	7.9	3	4.5	1	0.22
5 β -Pregnan-20 α -ol-3-one	2	4.8	3	2.9		0
4-Pregnen-20 α -ol-3-one	13	15	12	5.2		0
9 α ,11 β -PGF ₂	138	7.1	133	4.1	208	25
Reduction						
5 β -Pregnan-3 α -ol-20-one	1	1.9	2	1.6		0
5 β -Androstane-3,17-dione	2	2.6	1	1.1	0.3	0.6
PGD ₂	20	4.8	12	2.4	79	14

ATATTCATCAGAAAATGGATAAT) was complementary to the nucleotides 947–969 of HBAB cDNA and included an *EcoRV* site before the underlined stop codon.

To construct an expression plasmid for DD2 (pKKDD2), the coding region of the cDNA was amplified by PCR with two sets of primers; the first PCR was carried out using DD2ENL and DD2EC, and the PCR product was further amplified using DD2EN1 and DD2EC. The final PCR product was ligated into expression vector pKK223-3. The identity of the amplified clone was confirmed by DNA sequencing as described [4].

The pKKDD2 construct was transfected into the host strain *Escherichia coli* JM109, the *E. coli* cells were cultured in LB medium (1.8 litres), and the cell extract (12000 g supernatant) was prepared as described [9]. The rDD1 from the extract was purified essentially as described for the purification of human liver DD1 [1].

Enzyme assay

The dehydrogenase activity was assayed by measuring the NADPH fluorescence at 455 nm (excitation at 340 nm) [1]. The standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, 0.25 mM NADP⁺, 1.0 mM (S)-indan-1-ol and the enzyme, in a total volume of 2.0 ml. The buffer used in the assay of rDD1 in *E. coli* extracts and its purification steps was 0.1 M glycine/NaOH buffer, pH 10.0. The reductase activity was determined spectrophotometrically by measuring the NADPH oxidation in the reaction mixture (2.0 ml), which consisted of 0.1 M potassium phosphate buffer, pH 7.4, 0.1 mM NADPH, carbonyl substrate and the enzyme, unless otherwise noted. One unit of enzyme activity was defined as the amount that catalyses the production or oxidation of 1 μ mol of NADPH/min at 25 °C. The kinetic constants represent means of triplicate determinations. The activation energy for the (S)-indan-1-ol dehydrogenation catalysed by the enzyme was calculated by analysing the initial rates at 16–52 °C according to the Arrhenius plot [12].

Binding studies of lithocholic acid

The binding of lithocholic acid to the enzymes was directly assessed by determining the unbound fraction of the bile acid at

25 °C by ultrafiltration assay using spun microconcentrators (Microcon 10; Amicon). A volume of 0.4 ml of binding assay mixture (10 μ M enzyme and 10 μ M lithocholic acid or 20 μ M NADP⁺ in 0.1 M potassium phosphate, pH 7.4) was filtered by centrifugation at 13000 g for 20 min. As a control, the same volume of the mixture without the enzyme was filtered. The concentration of lithocholic acid in the filtrate was determined enzymically. The filtrate (0.2 ml) was added to a reaction mixture (1.0 ml) containing 0.1 M glycine/NaOH, pH 10.0, and 0.25 mM NADP⁺, and the reaction was started by addition of recombinant DD4 (8 μ g), which oxidizes the bile acid [9]. The fluorescence of NADPH was recorded until it was unchanged.

Binding of lithocholic acid to DD2 was also examined by the ANS-fluorescence inhibition technique [13], which has been used for determining the dissociation constants of bile acids for HBAB [10].

Other analytical methods

Protein concentration was determined, with BSA as the standard, by the method of Bradford [14]. SDS/PAGE [15], isoelectric focusing [16] and protein sequence determination, including the reductive pyridylethylation of protein, its digestion with lysyl-endopeptidase, isolation of the peptides, and sequencing by automated Edman degradation [4], were carried out as described in the references cited.

RESULTS AND DISCUSSION

The dehydrogenase activity for (S)-indan-1-ol (11 ± 1 m-units/mg) was detected in the extract of *E. coli* JM109 cells transfected with the pKKDD2 construct, but not in that with the vector only. The expressed rDD1 was purified 230-fold, with an overall yield of 30 % (2.1 mg), from the cell extract. The purified rDD1 showed a single protein on SDS/PAGE and gel-focusing analyses. The molecular mass of rDD1 was 36 kDa, which is similar to those of DD1 and DD2 [1], although its pI value of 8.4 was not consistent with those of DD1 (pI 9.1) and DD2 (pI 7.6). The activation energy of rDD1 for the (S)-indan-1-ol was 49 kJ/mol, which was similar to 50 kJ/mol for DD1 and slightly lower than 53 kJ/mol for DD2. It should be noted that the N-terminal sequence up to 20 residues, determined by direct

Table 2 Inhibitor sensitivity of rDD1, DD1 and DD2

Inhibitor	IC ₅₀ (μM)		
	rDD1	DD1	DD2
Hexestrol	8.3	9.5	2.8
1,10-Phenanthroline	65	55	2100
1,7-Phenanthroline	85	72	1500
Flufenamic acid	5.3	6.0	0.9
Indomethacin	130	140	75
Ibuprofen	1040	950	6.9
Lithocholic acid	22	25	0.07
Ursodeoxycholic acid	340	340	0.08
Chenodeoxycholic acid	540	570	0.13

sequencing of rDD1, was the same as those deduced from the pKKDD2 construct and the cDNA for HBAB [5].

Clear differences between DD1 and DD2 have been observed in their substrate specificity for steroidal substrates and inhibitor sensitivity [1]. The substrate specificity of rDD1 was almost the same as that of DD1, although k_{cat} values of rDD1 were higher than those of DD1 (Table 1). The inhibitor sensitivity of rDD1, DD1 and DD2 (Table 2) also supports the identity of rDD1 with DD1. Furthermore, the sequences (total of 252 residues) of 14 different peptides of DD1 completely matched the regions of the sequence deduced from C9 (i.e. the cDNA for HBAB), whereas there were differences of six amino acids between the deduced cDNA sequence and the partial sequence (total of 264 amino acids) of DD2 (Figure 1). The functional and structural data clearly indicate that the C9 clone, which has been thought to code for DD2, encodes DD1. Because of the high sequence identity between DD1 and DD2, we probably sequenced the identical regions of their sequences with DD2 peptides, so that the C9 clone was incorrectly identified [4].

As C9 (now the cDNA for DD1) is the same as the cDNA for HBAB [5], the specificity for xenobiotic substrates and low sensitivity to bile acids of rDD1 are similar to those of recombinant HBAB [5]. Although rDD1 exhibited 20 α -hydroxysteroid dehydrogenase activity, it showed very low 3 α -hydroxysteroid dehydrogenase activity (4 m-units/mg) for lithocholic acid, as reported for recombinant HBAB. However, this is not direct evidence that supports the identity of DD1 with HBAB at the protein level. To clarify this point, the bile-acid-

Table 3 Binding of lithocholic acid to the recombinant and native DDs determined by ultrafiltration assay

The mixture of 10 μM enzyme, 10 μM lithocholic acid and/or coenzyme was filtered through a Microcon 10 ultrafiltration membrane. The concentration of lithocholic acid in the filtrate is expressed as its ratio to that in the filtrate of the control mixture without enzyme. Abbreviation: rDD4, recombinant DD4.

Coenzyme	Fraction of free lithocholic acid			
	rDD1	DD1	DD2	rDD4
None	0.78	0.65	0.38	0.56
20 μM NADP ⁺	0.61	0.53	0.10	0.44

binding ability of recombinant and native DDs of human liver was compared by the ultrafiltration assay for binding of lithocholic acid in the absence or presence of NADP⁺ (Table 3). The binding of the bile acid to rDD1 and DD1 was low, whereas DD2, with high sensitivity to bile acids (Table 2), exhibited the highest ability to bind the bile acid. The dissociation constant of DD2 for lithocholic acid determined by the ANS displacement technique was 0.16 μM, which is similar to the value (0.03 μM) for native HBAB [10] rather than that (1.1 μM) of the recombinant HBAB [5]. Furthermore, the sequences of DD2 peptides are almost identical with those reported by protein sequencing of native HBAB [5], in which five amino acids have been detected as disagreement between the primary sequence of native HBAB and the deduced cDNA sequence. Four (His-47, Val-54, His-170, Leu-172) of the five amino acids detected in native HBAB were identified in the present sequencing of DD2, and are also involved in the sequence difference of six amino acids between DD1 and DD2. These results strongly suggest the identity of native HBAB with DD2, but not with DD1. In addition, the cDNA for DD1 is identical with that for H-37 of human carcinoma HT29 colon cells [8]. Although the properties reported for H-37 [8] cannot be used to discriminate its identity with DD1 or DD2, because of the high sequence identity and overlapping substrate specificity of the two DDs, the agreement of pI value (7.6) between native H-37 [8] and DD2 suggests the identity of the two enzymes. Thus DD2, HBAB and H-37 may be the same protein, but the cDNAs, previously isolated as those for these proteins, may encode DD1.

A complete cDNA sequence or amino acid sequence for DD2 has not been published, but four cDNAs for oxidoreductases similar to the human liver DDs have recently been cloned from

Figure 1 Alignment of the peptide sequences of human liver DD1 and DD2 with amino acid sequences deduced from HBAB cDNA and c81/RACE

The sequenced peptides derived from DD1 and DD2 are aligned above (DD1p) and below (DD2p), respectively, the sequence deduced from the cDNA for HBAB (cDNA) [5]. Matching amino acids between the peptide sequences and the sequence deduced from HBAB cDNA are denoted by hyphens. The regions that were determined by previous sequencing of DD2 peptides [4] are indicated by **bold** underlining. Since the amino acid sequence deduced from c81/RACE [11] differs from that of HBAB cDNA by seven amino acids in their N-terminal regions of positions from 38 to 172, only the seven amino acids are shown in the partial sequence of c81/RACE (1–220).

libraries of human liver [17] and human HT29 colon cells [11]. When the amino acid sequences deduced from these cDNAs were aligned with the partial sequence of DD2, perfectly matched alignment was obtained between the sequences of DD2 peptides and the sequence deduced from another cDNA (c81/RACE) cloned from the library of human HT29 cells [11] (Figure 1). This suggests that DD2 is identical with the putative c81/RACE protein: thus, the cDNA (c81/RACE) may encode a protein which has been designated as DD2, HBAB or H-37 by different researchers.

A gene with *cis*-acting elements in its 5'-upstream region has been cloned with the cDNA for HBAB (i.e. that for DD1) as a probe, and its mRNA has been identified in human hepatoma and lung carcinoma cells [18]. In addition, H-37 (probably corresponding to DD2) in the ethacrynic acid-resistant carcinoma HT29 colon cells is induced, through synthesis *de novo* from its mRNA, by Michael acceptor xenobiotics [8]. These studies have suggested a role in xenobiotic metabolism for the enzymes, because their endogenous substrates have not been identified. The substrate specificity of DD1 and DD2 (Table 1) suggests PGs and steroids as the endogenous substrates. Especially, the K_m values for PGD₂ of the two DDs are comparable with those of bovine PGF synthase isoenzymes [19,20]. In addition, DD1 and DD2 exhibited PGH₂ 9,11-endoperoxide reductase activity of 7.4 and 21 nmol of PGF_{2α} formed/min per mg, respectively, at pH 6.5 and 37 °C (A. Hara and K. Watanabe, unpublished work). Considering the regulation of the DDs and expression of their mRNA in the hepatoma and carcinoma cells, DD1 and DD2 may play an important role in the metabolism of PGs, which are regulators of cell growth.

Finally, the sequence data indicate that DD1 and DD2 are isoenzymes with high sequence identity, but they showed the above functional differences. This suggests that key residue(s), which determine the substrate specificity, inhibitor sensitivity and bile acid binding of the two isoenzymes, are involved in the six or seven residues that differed between the sequences. Further studies by site-directed mutagenesis of these residues will provide a better understanding of the structure-function relationship of

the two DDs and their functionally similar members of the aldo-keto reductase family.

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