Identification of amino acid residues responsible for differences in substrate specificity and inhibitor sensitivity between two human liver dihydrodiol dehydrogenase isoenzymes by site-directed mutagenesis

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Human liver dihydrodiol dehydrogenase isoenzymes (DD1 and DD2), in which only seven amino acid residues are substituted, differ remarkably in specificity for steroidal substrates and inhibitor sensitivity: DD1 shows 20α-hydroxysteroid dehydrogenase activity and sensitivity to 1,10-phenanthroline, whereas DD2 oxidizes 3α-hydroxysteroids and is highly inhibited by bile acids. In the present study we performed site-directed mutagenesis of the seven residues (Thr-38, Arg-47, Leu-54, Cys-87, Val-151, Arg-170 and Gln-172) of DD1 to the corresponding residues (Val, His, Val, Ser, Met, His and Leu respectively) of DD2. Of the seven mutations, only the replacement of Leu-54 with Val produced an enzyme that had almost the same properties as DD2. No significant changes were observed in the other mutant enzymes. An additional site-directed mutagenesis of Tyr-55 of DD1 to Phe yielded an inactive protein, suggesting the catalytically important role of this residue. Thus a residue at a position before the catalytic Tyr residue might play a key role in determining the orientation of the substrates and inhibitors.

INTRODUCTION

Dihydrodiol dehydrogenase (DD; *trans*-1,2-dihydrobenzene-1,2-diol:NADP$^+$ oxidoreductase, EC 1.3.1.20) catalyses the NADP$^+$-linked oxidation of *trans*-dihydrodiols of aromatic hydrocarbons to corresponding catechols. The enzymes of rat and human liver have also been shown to play multifunctional roles in the metabolism of steroids, prostaglandins and drug ketones, and in the transport of bile acids [1–5]. Human liver contains at least four DD (DD1–DD4), of which one minor form (DD3) is identified with aldehyde reductase [6]. Subsequently, our protein sequencing of the other enzyme forms, and cloning and expression of their cDNA species [7–9], showed that DD1, DD2 and DD4 are identical with a putative DD encoded by a cDNA cloned from the cDNA library of ethacrynic acid-resistant human carcinoma HT29 colon cells [10], human liver bile acid-binding protein [11] and human liver chlordecone reductase [12,13] respectively, which belong to the aldo–keto reductase family [14]. The three DDs are isoenzymes with a sequence identity of more than 85%. Especially, DD1 and DD2 show differences of only seven amino acid residues. However, the two DDs differ from each other in their specificities for steroidal substrates and inhibitor sensitivities [3,6]: DD1 shows 20α-hydroxysteroid dehydrogenase activity and is sensitive to 1,10-phenanthroline, whereas DD2 oxidizes some 3α-hydroxysteroids and is highly inhibited by bile acids.

In this study we performed site-directed mutagenesis of the seven residues of DD1 to the corresponding residues of DD2, and show that only one residue before the catalytic Tyr-55 plays a key role in determining the functional difference between DD1 and DD2.

EXPERIMENTAL

Materials

Restriction enzymes and chemicals used in this study were as specified elsewhere [5–9], except that *Pfu* polymerase and a Genepure kit for isolation of DNA were obtained from Stratagene and Nippongene respectively. The expression plasmid pKK223-3 containing the cDNA for DD1, pKKDD1, previously constructed [9] was used. Human liver DD2 was purified to homogeneity as previously described [6].

Site-directed mutagenesis

Mutagenesis was performed by the overlap-extension method [15], using *Pfu* polymerase and the following primer pairs, each of which was composed of forward and reverse oligonucleotides to alter a codon of DD1. The mutagenic 22- to 24-mer primers were synthesized to give the T38V (Thr-38 → Val), R47H (Arg-47 → His), L54V (Leu-54 → Val), C87S (Cys-87 → Ser), V151M (Val-151 → Met), R170H (Arg-170 → His) and Q172L (Gln-172 → Leu) mutant DDs; the respective amino acid codons for the positions of DD1 were replaced with those of DD2. An additional mutant, Y55F (Tyr-55 → Phe), was produced by using a 24-mer oligonucleotide with the amino acid codon TAC replacing the original Tyr-55 codon of TTT. The entire coding regions of the mutated cDNA species were amplified by PCR with two flanking primers that have the partial sequences of positions 22–41 and 4491–4510 respectively around the multiple cloning site of the pKK223-3 vector. Because the DD1 cDNA has two *Eco*RI sites at positions −6 from the initiation codon [9] and 851 in the coding region [7], a cDNA fragment of 860 bp was formed from the amplified cDNA by digestion with this restriction enzyme. The cDNA fragment encoding the N-terminal amino acid sequence (residues 1–284) of the enzyme was isolated by agarose-gel electrophoresis with the Genepure kit. This fragment was then ligated into a pKKDD1-L plasmid that had been prepared by digestion with *Eco*RI and encoded the partial C-terminal amino acid sequence (residues 285–323) of DD1. The mutations were verified by nucleotide sequence analyses of the mutated cDNA species [7].

Abbreviations used: DD, dihydrodiol dehydrogenase; rDD1, recombinant DD1.

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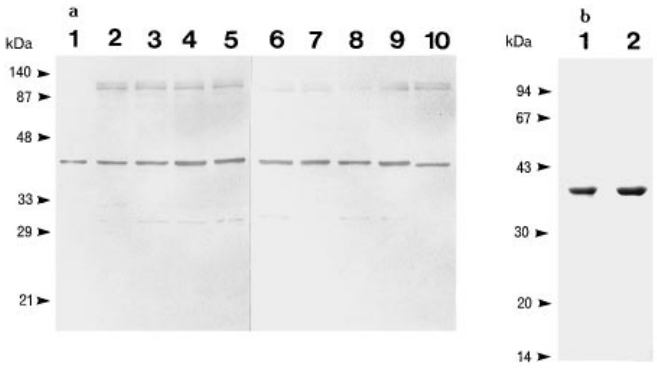

Figure 1 Western blot analysis of the wild-type and mutated DDs in *E. coli* cell extracts and SDS/PAGE of the purified rDD1 and L54V

(**a**) Western blot analysis of the purified rDD1 (0.1 μg, lane 1) and the extracts of recombinant DDs (each 10 μg of protein, other lanes). Lane 2, rDD1; lane 3, T38V; lane 4, R47H; lane 5, L54V; lane 6, Y55F, lane 7, C87S; lane 8, V151M; lane 9, R170H; lane 10, Q172L. (**b**) The proteins (each 2 μg) of the purified rDD1 (lane 1) and L54V (lane 2) were run on an SDS/12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

Expression and purification of recombinant enzyme

The wild-type and mutated cDNA species were expressed in *Escherichia coli* (JM109) cells, and the cell extracts (12000 ***g*** supernatant) were prepared as described [8,9]. The recombinant proteins in the extracts were detected by Western blot analysis [16] with an antibody against DD2 that reacts similarly with both DD1 and DD2 [4]. The wild-type DD1 (rDD1) and L54V were purified from the extracts of *E. coli* cells (each 2 litres of culture) essentially as described for the purification of human liver DD1 [6]. Protein concentration was determined by the method of Bradford [17] with BSA as the standard, and protein purity was examined by SDS/PAGE [18].

Enzyme assay

The dehydrogenase activity was assayed by measuring the NADPH fluorescence at 455 nm (excitation at 340 nm) [6]. The standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.25 mM $NADP^+$, 1.0 mM (*S*)-indan-1-ol and the enzyme, in a total volume of 2.0 ml. One unit of enzyme activity was defined as the amount that catalyses the production of 1 μmol of NADPH/min at 25 °C. To estimate the kinetic constants, the initial rate determination of each substrate was performed at five concentrations. The kinetic studies in the presence of inhibitors (at three concentrations for each of the inhibitors) were performed in an identical manner. The inhibition constants, K_{is} (slope effect) and K_{ii} (intercept effect), were determined as described ([19], pp. 57–60). The kinetic constants represent the means of duplicate or triplicate determinations.

RESULTS

The expression of all the 36 kDa recombinant enzymes mutated with the seven residues of DD1 was confirmed by Western blot analysis of the *E. coli* cell extracts (Figure 1a). Dehydrogenase activity for (*S*)-indan-1-ol was detected in the extracts of all the mutants (Table 1). Clear differences between DD1 and DD2 have been observed in their substrate specificities for steroidal substrates and in their inhibitor sensitivities [3,6]. Therefore 3α- and 20α-hydroxysteroid dehydrogenase activities and effects of representative inhibitors for the two isoenzymes on the (*S*)-indan-1-ol dehydrogenase activity were compared between the mutant enzymes, rDD1 and DD2. All the mutant enzymes except L54V showed 20α-hydroxysteroid dehydrogenase activity for 5β-pregnan-20α-ol-3-one and a high sensitivity to 1,10-phenanthroline, similar to those determined with rDD1. The 20α-hydroxysteroid dehydrogenase activity in the *E. coli* extract of L54V fell below the lower limit of the detection of the fluorimetric assay (10 pmol/min), and 0.1 mM 1,10-phenanthroline did not inhibit the (*S*)-indan-1-ol dehydrogenase activity of L54V. In addition, L54V exhibited 3α-hydroxysteroid dehydrogenase activity for androsterone and was highly inhibited by bile acids and ibuprofen, similarly to DD2. Furthermore, whereas the K_m values for (*S*)-indan-1-ol and 5β-pregnan-20α-ol-3-one of the other mutant enzymes were as low as those of rDD1, L54V showed a high K_m for (*S*)-indan-1-ol, similar to the value for DD2. These results obtained with the *E. coli* extracts of the mutant enzymes suggest that the single mutation of Leu-54 to Val, corresponding to the residue of DD2, converts DD1 to a DD2-like enzyme.

To test this possibility, the expressed L54V was purified from the cell extract, to obtain an almost homogeneous preparation (Figure 1b), with an overall yield of 14% (0.5 mg) and a

Table 1 Activities and inhibitor sensitivity of the recombinant DDs in *E. coli* cell extracts and the purified human liver DD2

The activities towards the hydroxysteroids are relative to that assayed with 1 mM (*S*)-indan-1-ol as the substrate, which is expressed as specific activity. The inhibition of (*S*)-indan-1-ol dehydrogenase activity was determined. Abbreviations: n.a., no activity was detected under the assay conditions; n.d., not determined.

Parameter	rDD1	T38V	R47H	L54V	C87S	V151M	R170H	Q172L	DD2	Y55F
(*S*)-Indan-1-ol dehydrogenase activity (m-units/mg)	1.7	3.4	2.4	1.0	1.9	2.1	1.6	1.8	–	n.a.
Relative activity (%) towards:										
25 μM 5β-Pregnan-20α-ol-3-one	19	12	18	n.a.	17	18	14	19	n.a.	n.a.
10 μM Androsterone	n.a.	n.a.	n.a.	5	n.a.	n.a.	n.a.	n.a.	5	n.a.
K_m (μM) towards:										
(*S*)-Indan-1-ol	45	63	56	500	47	60	52	40	520	n.d.
5β-Pregnan-20α-ol-3-one	5	3	4	n.d.	3	4	4	2	n.d.	n.d.
Inhibition (%) by:										
0.1 mM 1,10-Phenanthroline	56	78	76	0	58	55	62	53	0	n.d.
0.1 μM Lithocholic acid	6	0	0	70	0	0	0	0	57	n.d.
0.2 μM Ursodeoxycholic acid	6	0	0	85	0	0	0	0	76	n.d.
20 μM Ibuprofen	32	0	0	53	0	0	8	0	72	n.d.

Table 2 Comparison of substrate specificities and inhibitor sensitivities of the purified rDD, DD2 and L54V

The activities towards the substrates were determined at pH 7.4, except that 9α,11β-PGF_2 dehydrogenase activity was assayed at pH 10.0. The apparent kinetic constants of rDD1 and DD2 for the substrates are taken from [9]. The inhibition patterns of the three enzymes by 1,10-phenanthroline were of mixed type. Abbreviation: n.a., no activity was detected.

	rDD1		DD2		L54V	
Substrate	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
(*S*)-1,2,3,4-Tetrahydronaphth-1-ol	7	26	260	16	220	33
(*S*)-Indan-1-ol	45	25	520	9.5	520	36
Benzene dihydrodiol	870	8.7	5000	1.9	6600	7.2
9α,11β-Prostaglandin F_2	138	7.1	208	25	238	12
Androsterone	–	0	7.8	0.71	7.7	1.4
5β-Pregnan-3α,20α-diol	3	7.9	1	0.22	0.8	0.48
5β-Pregnan-20α-ol-3-one	2	4.8	–	0	–	n.a.
4-Pregnen-20α-ol-3-one	13	15	–	0	–	n.a.
Inhibitor	K_{is} (μM)	K_{ii} (μM)	K_{is} (μM)	K_{ii} (μM)	K_{is} (μM)	K_{ii} (μM)
1,10-Phenanthroline	20	68	270	820	280	900
Phenolphthalein	0.3	–	3.8	–	3.5	–
Flufenamic acid	0.8	–	0.1	–	0.1	–
Ibuprofen	24	–	1.1	–	1.1	–
Lithocholic acid	2.7	–	0.02	–	0.01	–
Ursodeoxycholic acid	8.8	–	0.03	–	0.03	–
Chenodeoxycholic acid	23	–	0.04	–	0.04	–

purification of 630-fold. The purified L54V oxidized xenobiotic alcohols, 3α-hydroxysteroids and 9α,11β-prostaglandin F_2 and showed an optimal pH of approx. 10, at which the oxidation rates for these substrates were 7–10-fold higher than those determined at pH 7.4. Even at the optimal pH L54V did not exhibit 20α-hydroxysteroid dehydrogenase activity for 5β-pregnan-20α-ol-3-one or 4-pregnen-20α-ol-3-one. Because the kinetic constants for the substrates have been reported at pH 7.4 for the native and recombinant DDs of human liver [3,6,9], the constants were also determined at this pH and compared with those of rDD1 and DD2. The K_m values of L54V for the substrates clearly differed from those of the purified rDD1, but were almost the same as those of DD2, although k_{cat} values of L54V were higher than those of DD2 (Table 2). As described previously with DD1 and DD2 from human liver [3,6], the lower K_m values for the steroids than those for the xenobiotic substrates indicate that the steroids are endogenous substrates for the two enzymes. When the inhibition patterns and constants for several inhibitors were compared between L54, rDD1 and DD2, all the inhibitors except 1,10-phenanthroline showed competitive inhibition with respect to (*S*)-indan-1-ol for the three enzymes, but the K_i values of L54V were almost identical with those of DD2.

A Tyr residue has been shown to be a catalytic residue in several members of the aldo–keto reductase family [20–28]. Because the Tyr residue is conserved at the position next to Leu-54 or Val-54 in the DD1 and DD2 sequences, an additional mutation of Tyr-55 of DD1 to Phe was performed. The production of the recombinant protein in the *E. coli* cell extract was confirmed by Western blot analysis (Figure 1a), but dehydrogenase activity for (*S*)-indan-1-ol or the hydroxysteroids was not detected (Table 1).

DISCUSSION

The aldo–keto reductase family includes more than 36 proteins [20,29] in which a number of amino acid residues are highly conserved. Of these, a Tyr residue (corresponding to Tyr-55 of DD1 and DD2) has been shown to be a catalytic residue by site-directed mutagenesis and X-ray crystallographic studies of several members of this family [21–28]. The role of the Tyr residue in DD1 was confirmed by the production of an enzymically inactive mutant protein of Y55F. Crystallographic studies of aldose reductase [23–25], rat liver 3α-hydroxysteroid dehydrogenase [26,27] and mouse FR-1 [28] have also shown or suggested 11–15 residues that are responsible for binding the substrates or competitive inhibitors. These residues do not correspond to the seven residues that differ between the primary sequences of DD1 and DD2, except that a residue at a position corresponding to Leu-54 of the DD1 sequence has been suggested as one of the hydrophobic residues in the active site pockets of human liver aldose reductase [25] and rat liver 3α-hydroxysteroid dehydrogenase [26,27]. So far there have been no reports of studies with site-directed mutagenesis to examine the roles of the residues corresponding to the seven residues of DD1.

The conversion of DD1 to DD2 by the single mutation of L54V, and not by the mutations of the other residues, clearly indicates an important role of the residue at position 54 (Leu in DD1 and Val in DD2) in determining the substrate specificity and the affinity for the competitive inhibitors of the two enzymes. In addition, the lack of apparent effects of the mutations of the other residues on the substrate specificity and inhibitor sensitivity of DD1 suggest that the residues at positions 38, 47, 87, 151, 170 and 172 are not involved in the binding of the substrates or inhibitors. When the secondary structures of DD1 are predicted based on the tertiary structure of rat liver 3α-hydroxysteroid dehydrogenase [26,27] (68% sequence identity with DD1 and DD2), at least the residues at positions 38, 151, 170 and 172 are equivalent to those on α-helices located on the exterior of the structure of the rat liver enzyme.

The almost identical specificities and kinetic constants for the substrates between L54V and DD2 suggest that the conformations of the active sites of the two enzymes are similar. Although a definitive conformational difference between the active sites of DD1 and L54V or DD2 must await a comparative

study on the high-resolution three-dimensional structures of the DD holoenzymes, it is likely that the replacement of Leu-54 with the less bulky Val residue at a position just before the catalytic Tyr residue causes a local spatial change, such as a space produced by the loss of a methyl group, around the Tyr residue in the active site. The newly produced space in L54V might allow the 3α-hydroxy group of the steroidal A-ring of androsterone to be correctly oriented towards the phenolic group of Tyr-55. In contrast, the conformational change might lead to unfavourable or loose interactions of the hydroxy groups of the other substrates with the catalytic Tyr residue, which would result in the elevation of K_m values for the non-steroidal substrates and the apparent loss of 20α-hydroxysteroid dehydrogenase activity.

Although the mechanism of inhibition by 1,10-phenanthroline is not clear at present, the other inhibitors probably bind to the active site of the L54V, rDD1 and DD2 because of their competitive inhibitions with respect to the alcohol substrate. The equality of the K_i values for these competitive inhibitors between L54V and DD2 supports the conformational similarities of the active sites of L54V and DD2. Of the competitive inhibitors, bile acids, flufenamic acid and ibuprofen have a carboxy group in their molecules, and the K_i values for them were decreased by the L54V mutation, whereas the value for phenolphthalein, without the carboxy group, increased. Thus the presence of a carboxy group in the inhibitor molecule is important for its tight binding to the active site of L54V or DD2. Recently, X-ray crystallographic studies of the ternary complex of aldose reductase with NADPH and its competitive inhibitors have shown that the carboxy group of the inhibitors binds to a positively charged anion-binding site formed by nicotinamide of NADPH and the active-site residues (Tyr-48 and His-110) [24,28,30]. Because the two residues are conserved in positions 55 and 117 in DD1 and DD2, the carboxy group of the DD inhibitors might interact with the active-site residues of the enzymes, similarly to the binding reported for the aldose reductase inhibitors. However, the inhibitors, especially bile acids, inhibited L54V and DD2 more than DD1. This again suggests the importance of the space around the catalytic Tyr residue of L54V and DD2, mentioned above, which would allow the large side chain carboxy group on the D-ring of bile acids to interact more tightly with the anion-binding site of L54V or DD2 than with that of DD1.

The aldo–keto reductase family includes various enzymes with distinct substrate specificities. As shown by three-dimensional structures of ternary complexes (with NADPH and inhibitors) of aldose reductase [23,24] and FR-1 [28], many residues are involved in substrate binding and the diversity of the residues probably determines the substrate specificity of the respective enzymes. The present study demonstrated that one residue, just before the catalytic Tyr residue, plays a key role in determining the difference in substrate specificities and inhibitor sensitivities of the two isoenzymes of human liver DD. Because the residue at this position shows a variety of exchanges in diverse members of this enzyme family [20,28,29], whether it plays such a significant role in other members remains to be determined. However, this first report on a great functional change by the small and conservative replacement of Leu with Val provides an impact on future structure–function relationship studies of the functionally similar members of the aldo–keto reductase family.

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