Roles of the C-terminal domains of human dihydrodiol dehydrogenase isoforms in the binding of substrates and modulators: probing with chimaeric enzymes

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Human liver dihydrodiol dehydrogenase (DD; EC 1.3.1.20) exists in isoforms (DD1, DD2 and DD4) composed of 323 amino acids. DD1 and DD2 share 98 % amino acid sequence identity, but show lower identities (approx. 83 %) with DD4, in which a marked difference is seen in the C-terminal ten amino acids. DD4 exhibits unique catalytic properties, such as the ability to oxidize both (*R*)- and (*S*)-alicyclic alcohols equally, high dehydrogenase activity for bile acids, potent inhibition by steroidal anti-inflammatory drugs and activation by sulphobromophthalein and clofibric acid derivatives. In this study, we have prepared chimaeric enzymes, in which we exchanged the C-terminal 39 residues between the two enzymes. Compared with DD1, CDD1-4 (DD1 with the C-terminal sequence of DD4) had increased k_{cat}/K_m values for 3α-hydroxy-5β-androstanes and bile acids of 3–9-fold and decreased values for the other substrates by 5–100-fold. It also became highly sensitive to DD4 inhibitors such as phenolphthalein and hexoestrol. Another chimaeric enzyme, CDD4-1 (DD4 with the C-terminal sequence of DD1), showed the same (*S*)-stereospecificity for the alicyclic alcohols as DD1, had decreased k_{cat}/K_m values for bile acids with 7β- or 12α-hydroxy groups by more than 120-fold and was resistant to inhibition by betamethasone. In addition, the activation effects of sulphobromophthalein and bezafibrate decreased or disappeared for CDD4-1. The recombinant DD4 with the $His^{314} \rightarrow Pro$ (the corresponding residue of DD1) mutation showed intermediate changes in the properties between those of wild-type DD4 and CDD4-1. The results indicate that the binding of substrates, inhibitors and activators to the enzymes is controlled by residues in their C-terminal domains; multiple residues co-ordinately act as determinants for substrate specificity and inhibitor sensitivity.

INTRODUCTION

Dihydrodiol dehydrogenase (DD; EC 1.3.1.20) catalyses the $NADP^+$-linked oxidation of *trans*-dihydrodiols of aromatic hydrocarbons to the corresponding catechols. The enzyme exists in multiple forms in mammalian tissues [1–5], and major forms of the enzyme purified from several mammalian livers have been demonstrated, or suggested, to be identical to 3α-, 17β- and/or 3(20)α-hydroxysteroid dehydrogenases (HSDs), some of which exhibit additional oxidoreductase activity for prostaglandins [2,4]. Thus DD plays a role not only in the metabolism of xenobiotic hydrocarbons, but also in the metabolism of endogenous steroids and prostaglandins. The enzymes in several mammalian tissues have been shown to be members of the aldo-keto reductase (AKR) superfamily [6,7], including aldose reductase and 3α-HSD, which are therapeutic targets for the development of drugs for diabetic complications [8] and prostatic diseases [9].

We previously purified four forms (DD1–DD4) of human liver DD with molecular masses of approx. 36 kDa [5]. The minor form (DD3) is aldehyde reductase and the other forms show distinct specificity for 3α- and/or 20α-hydroxysteroids and prostaglandins [5,10–12]. DD1 shows 3(20)α-HSD activity, DD2 oxidizes some 3α-hydroxysteroids, and DD4 exhibits broad and high 3α-HSD activity for various steroids, including bile acids. They also differ in inhibitor sensitivity; 1,10-phenanthroline, bile acids and steroidal anti-inflammatory drugs are selective potent inhibitors of DD1, DD2 and DD4 respectively. In addition, DD4 oxidizes the (*S*)- and (*R*)-isomers of xenobiotic alicyclic alcohols equally, in contrast to the (*S*)-stereospecificity of DD1 and DD2 [10], and is uniquely activated by sulphobromophthalein (BSP) [13] and clofibric acid derivatives [14]. The sequences of the three isoforms are identical to those deduced from cDNAs for human oxidoreductases [12, 15–18]. DD1, DD2 and DD4 are composed of 323 amino acids with 83–98 % sequence identities, belong to the AKR superfamily, and have recently been named AKR1C1, 1C2 and 1C4 respectively [7]. Previous site-directed mutagenesis studies of DD1 [19] and DD4 [20] have suggested that Tyr^{55} is the catalytic residue and that two basic residues (Lys^{270} and Arg^{276}) are involved in NADP(H) binding. A residue, positioned closer to the N-terminus than the catalytic tyrosine residue, has been shown to be responsible for the difference in substrate specificity and inhibitor sensitivity between DD1 and DD2 (which show only seven amino acid differences) [19]. This residue is a leucine for DD1 and a valine for DD2. However, leucine at this position is conserved in DD4 and other members of the AKR family, which suggests that other amino acids are involved in the differences in substrate specificity, inhibitor sensitivity and the effects of the activating drugs between DD4 and DD1 (or DD2).

Recent crystallographic studies of the AKR-family proteins have proposed a putative substrate-binding site, which includes the following three components: (1) an oxyanion-binding site delineated by the nicotinamide ring of the coenzyme and the side

Abbreviations used: AKR, aldo-keto reductase; BSP, sulphobromophthalein; CDD1-4, DD1 with the C-terminal sequence of DD4; CDD4-1, DD4 with the C-terminal sequence of DD1; DD, dihydrodiol dehydrogenase; GST, gluthathione S-transferase; HSD, hydroxysteroid dehydrogenase; H314P, mutant DD4 enzyme.

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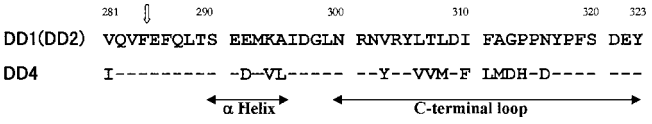

Figure 1 Alignment of amino acid sequences in the C-terminal regions of DD1 (or DD2) and DD4

This figure shows partial sequences (position 281–323) of DD1 and DD4, in which matching amino acids are denoted by hyphens in the DD4 sequence. The partial sequence of DD2 is identical to that of DD1. Secondary structures predicted from rat liver 3α-HSD [24] are shown under the DD4 sequence. The chimaeric enzymes were prepared by exchanging the C-terminal sequences from the position indicated by an open arrow.

chains of Tyr^{55} and His^{117} at the enzyme active site, (2) residues at the edge of the active site, and (3) residues from three loops which are called the A-loop, B-loop and C-terminal loop [21,22]. The A-loop contributes to one side of the apolar substrate-binding cleft, with the opposite side being formed by the B-loop and the C-terminal loop. The residues in the active site, along its edge (Ala^{52}, Leu^{54}, Trp^{86} and Phe^{118}) and in the B-loop (Trp^{227}) are conserved in both DD1 and DD4, but those in the A-loop (positions 120, 128 and 129) and in the C-terminal loop (Figure 1) are different between the two human liver enzymes. Although the C-terminal domain of the AKR-family proteins is characterized by pronounced sequence divergence, more than three residues in this region have been shown to interact with competitive inhibitors in the ternary complexes of several AKRs [22–25]. Studies of aldose reductase [26] and aldehyde reductase [27,28] by deletion and site-directed mutagenesis of their C-terminal residues have suggested that, of the above components of the active site, the C-terminal loop is critical for their substrate specificity and inhibitor sensitivity. The present study was carried out to clarify the roles of the C-terminal loops in the differential catalytic properties of DD1 and DD4, using chimaeric proteins in which the C-terminal 39 residues were exchanged. In addition, a DD4 mutant enzyme, H314P, was prepared to determine the possible contribution of the unique structure of two successive proline residues in the DD1 sequence to the observed effects.

MATERIALS AND METHODS

Materials

Pfu DNA polymerase and a Genepure kit for isolation of DNA were purchased from Stratagene and Nippongene respectively. Restriction endonucleases were obtained from Takara Shuzo Co. Ltd. (Shiga, Japan), and plasmids, *Escherichia coli* host strain JM109, Sephadex G-100 and Q-Sepharose were from Pharmacia. Steroids and anti-inflammatory drugs were from Sigma. Matrex Red A was obtained from Amicon and sodium bezafibrate was from Kissei Pharmaceutical Co. Ltd. (Nagano, Japan). Fenofibric acid was synthesized from fenofibrate as described previously [14]. The expression plasmid pKK 223-3 containing the cDNA for DD1 (pKKDD1) or DD4 (pKKDD4), constructed previously [11,12], was used.

Construction of expression plasmids

The expression plasmids for CDD1-4 (DD1 with the C-terminal sequence of DD4; pKKDD1/4) and CDD4-1(DD4 with the C-terminal sequence of DD1; pKKDD4/1) were prepared first. The cDNAs for DD1 and DD4 have two *Eco*RI sites at the identical positions of -6 from the initiation codon and 851 in the coding regions. The digestion of pKKDD1 and pKKDD4 with *Eco*RI yielded long cDNA fragments (860 bp) and the remaining short cDNA fragments (nucleotides 852–972) in the pKK 223-3 plasmids. They were isolated, and then the long cDNA fragments for DD1 and DD4 were ligated into the pKK 223-3 plasmids containing the short cDNA fragments for DD4 and DD1 respectively, as described previously [19,20]. When the constructs were transfected into *E. coli* cells and the chimaeric enzymes were expressed, the expression efficiency of CDD4-1 was as high as that described for DD4 [11], but the efficiency of CDD1-4 was as low as that of DD1 [12]. Therefore, we prepared other constructs, DD1/pGEX and CDD1-4/pGEX, using expression vector pGEX-2T to obtain larger amounts of the recombinant DD1 and CDD1-4 as fusion proteins with glutathione S-transferase (GST). The coding regions of the cDNAs for DD1 and CDD1-4 were amplified by PCR using pKKDD1 and pKKDD1/4 respectively as the templates and *Pfu* DNA polymerase. The 5′-sense and 3′-antisense primers were pgexDD1NF and DD2EC respectively for amplification of DD1 cDNA, and the respective primers were pgexDD1NF and C1 for amplification of CDD1-4 cDNA. The common primer, pgexDD1NF, had the sequence 5′-CGTGGATCCCCGGGAATGGATTCGAAATATCAGTGTGTG-3′ that corresponded to nucleotides 1–24 of DD1 cDNA and included the underlined *Sma*I restriction site, and the 3′-antisense primers include an *Eco*RV restriction site [11,12]. PCR products were digested with *Sma*I and *Eco*RV, and ligated into the pGEX-2T plasmids that had been digested with *Sma*I followed by dephosphorylation with calf-intestine alkaline phosphatase.

To construct the expression plasmid for H314P, PCR was performed with pKKDD4 as a template and the primers EH and H314pr. EH, the 5′-sense primer, had the sequence at positions 22–41 around the multiple cloning site of the pKK233-3 vector [20]. H314pr had the complementary sequence 5′-CGGAAGCTTTTAATATTCATCAGAAAATGGATAATCAGGGGGGTCCATAAGAAA-3′ corresponding to nucleotides 928–972 and which included a mutated codon (underlined) and a *Hin*dIII restriction site beneath the stop codon. Since DD4 cDNA has a *Hin*dIII restriction site at position 250, the PCR product was digested with *Hin*dIII, and the long DNA fragment (730 bp) was isolated by use of a Genepure kit. The fragment was ligated into the pKK 223-3 plasmid containing the short cDNA fragment (nucleotides 1–250 of DD4 cDNA) that had been isolated after *Hin*dIII digestion of pKKDD4.

The entire coding regions of the cDNAs in the expression plasmids were sequenced as described previously [15], in order to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

Expression and purification of the recombinant enzymes

Expression of the recombinant enzymes in *E. coli* and the subsequent protein purification were carried out according to the methods described for recombinant DD4 [11], except that DD1 and CDD1-4, expressed as fusion proteins with GST, were purified as follows. The expression of the GST-fused DDs was confirmed by Western blot analysis using anti-DD4 IgG [11] and by assaying the activity of DD and GST. The proteins of the crude-cell extracts were fractionated with ammonium sulphate [40 % and 80 % saturation], and dissolved in buffer A (20 mM Tris/HCl, pH 8.0, containing 5 mM 2-mercaptoethanol, 0.5 mM EDTA and 0.15 M KCl). The sample was filtered through a Sephadex G-100 column (2×90 cm) equilibrated with buffer A. The enzyme fractions were concentrated by ultrafiltration using an Amicon YM-10 membrane, dialysed against buffer A without KCl, and then applied to a Q-Sepharose column (1.5×20 cm) equilibrated with the same buffer. The

GST-fused DDs were eluted with a linear 0–0.15 M NaCl gradient in the same buffer. The enzyme fractions were concentrated by ultrafiltration and then digested with thrombin (at 25 °C) as described previously [18]. After almost complete digestion was confirmed by SDS-polyacrylamide gel electrophoresis on a 12.5 % slab gel [29], the reaction mixture was dialysed against buffer B (40 mM Tris/HCl, pH 8.5, containing 5 mM 2-mercaptoethanol, 0.5 mM EDTA and 20 % glycerol). The dialysed solution was applied to a Matrex Red A column (1.2 × 10 cm) equilibrated with buffer B. DD activity was eluted with buffer B containing 0.1 M NaCl and 0.5 mM $NADP^+$. The enzyme fractions were concentrated by ultrafiltration, and stored at −20 °C. The above procedures, except the thrombin treatment, were carried out at 4 °C.

Enzyme assay

Dehydrogenase activity of DD was assayed fluorimetrically by recording the production of NADPH as described previously [5]. The standard reaction mixture consisted of 0.1 M potassium phosphate, pH 7.4, 0.25 mM $NADP^+$, 1 mM (*S*)-indan-1-ol and enzyme, in a total volume of 2.0 ml. The enzyme activity during the purification was assayed with 0.1 M glycine/NaOH buffer, pH 10.0, instead of the phosphate buffer in the standard reaction mixture, and the specific activities of the purified enzymes were expressed as the values determined with this glycine/NaOH buffer. One unit of the enzyme activity was defined as the amount catalysing the formation of 1 μmol NADPH/min at 25 °C. Kinetic constants for the substrates were determined by Lineweaver–Burk analyses with at least five different substrate concentrations at a saturating $NADP^+$ concentration of 0.25 mM. IC_{50} (apparent concentration required for 50 % inhibition) values for inhibitors were estimated in the standard reaction mixtures containing five different inhibitor concentrations. The kinetic study in the presence of inhibitor was carried out in a similar manner, and the inhibition constants, K_{is} (slope effect) and K_{ii} (intercept effect), were determined as described [30]. These values represent the means of triplicate determinations.

Other methods

Protein concentration was determined by the Bradford method using bovine-serum albumin as a standard [31]. The products from the oxidation of 5β-pregnan-20α-ol-3-one were analysed by thin-layer chromatography as described in [10]. The molecular lengths of clofibric-acid derivatives were calculated using the program CS Chem3D Pro (CambridgeSoft Co., Cambridge, MA, U.S.A.).

RESULTS AND DISCUSSION

Purification of wild-type and chimaeric DDs

The wild-type (DD1 and DD4) and chimaeric (CDD1-4 and CDD4-1) enzymes were purified from the extracts of *E. coli* cells to homogeneity based on SDS/PAGE (Figure 2). The overall yields of DD1, CDD1-4, DD4 and CDD4-1 were 13, 10, 40 and 52 % respectively, and the respective purification factors were 11-, 8-, 51- and 60-fold. In this study, DD1 and CDD1-4 were expressed as the GST-fused proteins to improve the low expression efficiency of recombinant DD1 using a pKK 223-3 expression vector [12]. The low yields of DD1 and CDD1-4 were mainly caused by low recoveries (approx. 30 %) of the homogeneous and active enzymes at the purification step of thrombin digestion of the fused enzymes followed by Matrex Red A chromatography. However, large amounts of the fused proteins of the two enzymes were expressed, as indicated by their low purification factors, and 6–8 mg of the purified enzyme preparations were always obtained from 1 litre of cultured cells. The specific activities of DD1 (2.7 units/mg), DD4 (2.5 units/mg) and CDD4-1 (3.0 units/mg) are similar to reported values for recombinant DD1 and DD4 [11,12]. CDD1-4 showed a low specific activity of 0.63 unit/mg, which is partly due to the high K_m value for (*S*)-indan-1-ol which was used as the substrate in the enzyme assay (Table 1).

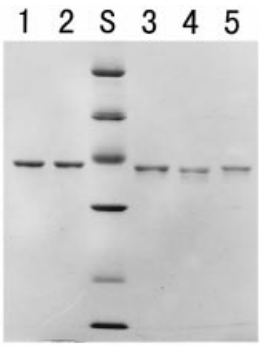

Figure 2 SDS/PAGE of the purified wild-type, chimaeric and mutated enzymes

The recombinant enzymes (1 μg) were electrophoresed and stained for protein with Coomassie Brilliant Blue R-250. Lane 1, DD1; lane 2, CDD1-4; lane 3, DD4; lane 4, CDD4-1; and lane 5, H314P. Molecular weight marker proteins in lane S are 67, 43, 30, 20.1 and 14.44 kDa from the top.

Alterations to substrate specificity caused by exchanging the C-terminal domains of DDs

The present recombinant DD1 had an additional four amino acids (Gly-Ser-Pro-Gly) at the N-terminus of the native DD1 sequence, but its substrate specificity and kinetic constants for alicyclic alcohols and 20α-hydroxysteroids (Table 1) were almost the same as those reported for hepatic and recombinant DD1 without the additional amino acids [12]. Since the specificity of DD1 for 3α-hydroxysteroids has not been studied because of the low activity for the steroids, we determined the kinetic constants for various 3α-hydroxysteroids using the present DD1 preparation. The enzyme was active towards the steroids, except for bile acids with 7β- and/or 12α-hydroxy groups (ursodeoxycholic acid, deoxycholic acid and cholic acid), but the K_m and k_{cat} values for most 3α-hydroxysteroids were much higher and lower respectively than those for 20α-hydroxypregnanes. This suggests that the substrate-binding cleft of the enzyme accepts the access of the steroid molecule from the C-20 hydroxy group near the D ring in preference to the C-3 hydroxy group on the A ring.

The effect of replacing the C-terminal 39 residues of DD1 with those of DD4 on the substrate specificity was assessed by comparing the kinetic constants, for various types of substrate, for DD1 and CDD1-4. The replacement produced a 10–37-fold increase in the K_m values for indan-1-ols and 1,2,3,4-tetrahydronaphth-1-ols, and a 10–100-fold decrease in the k_{cat}/K_m values for the dehydrogenation of the alicyclic alcohols. For 3α-HSD activity, CDD1-4 had significantly decreased reactivity towards 5α-androstanes, but had increased k_{cat}/K_m values for 5β-androstanes, lithocholic acid and chenodeoxycholic acid by 3–9-fold. This change was mainly caused by a more than 4-fold increase in the k_{cat} values, in contrast to the kinetic change for the xenobiotic alicyclic alcohols. In addition, the chimaeric enzyme exhibited a low but distinct 3α-HSD activity for ursode-

Table 1 Comparison of kinetic constants of the wild-type (WT) and chimaeric enzymes

The kinetic constants are means; S.E.M.s are $< 20\%$, except where indicated ($* < 30\%$); na, no activity was detected. Values in parentheses were calculated from the activities using 50 μM steroids. Tetralol, 1,2,3,4-tetrahydronaphth-1-ol.

	DD1			CDD1-4			Ratio CDD1-4/WT			DD4			CDD4-1			Ratio CDD4-1/WT		
Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m	k_{cat}	k_{cat}/K_m	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m	k_{cat}	k_{cat}/K_m
Alicyclic alcohols																		
(*S*)-Indan-1-ol	36	26	720	700	13	18	16	0.5	0.03	146	6.1	42	90	13	140	0.6	2.1	3.3
(*R*)-Indan-1-ol	340	6.2	18	3200	7.0	2.2	9.4	1.1	0.1	250	6.5	26	5000	16	3.2	20	2.5	0.1
(*S*)-Tetralol	7.0	25	3500	260	9.4	36	37	0.4	0.01	110	12	110	16	15	940	0.2	1.3	8.5
(*R*)-Tetralol	180	1.1	6.1	3600	1.4	0.4	20	1.3	0.06	160	11	69	1600	7.4	4.6	10	0.7	0.07
Steroids with hydroxy group on the A ring																		
Androsterone	15*	0.21*	14*		(0.04)			0.2		0.5*	2.6*	5200*	69	6.0	87	130	2.3	0.02
5β-Androstan-3α-ol-17-one	14	0.72	51	30	6.1	200	2.1	8.4	4.0	0.9	2.9	3200*	14	5.0	350	16	1.7	0.1
5α-Androstane-3α,17β-diol	18	0.25*	14*		(0.05)			0.2		0.8	5.2	6200*	30	3.4	110	37	0.7	0.02
5β-Androstane-3α,17β-diol	39	0.45	12*	45	2.9	64	1.2	4.4	4.6	1.2	2.8	2300	25	5.4	220	21	1.9	0.1
Steroids with hydroxy group on the D ring																		
5β-Pregnan-20α-ol-3-one	1.0	5.6	5600	0.3*	0.19*	630*	0.3	0.03	0.1	0.3*	0.5*	1700*	0.5*	0.58*	1200*	1.7	1.2	0.7
5β-Pregnane-3α,20α-diol	1.5	9.6	6400	0.7*	0.80*	1140*	0.5	0.08	0.2	0.2*	1.2	6000*	1.3	2.3	1770	6.5	1.9	0.3
Bile acids																		
Lithocholic acid	0.4*	0.18*	500*	1.3	1.9	1460	3.2	11	3.0	1.0	1.9	1900	2.6	2.6	1000*	2.6	1.4	0.5
Chenodeoxycholic acid	3.8*	0.11*	29*	6.5	1.7	260	1.7	15	9.0	1.1	1.9	1700	5.4	1.2	220	5.4	0.6	0.1
Ursodeoxycholic acid		na		3.1	0.08*	26*				18	4.8	270	30*	0.03*	1.0*	1.7	0.006	0.004
Deoxycholic acid		na			na					9.7	5.1	530	21*	0.07*	3.3*	2.2	0.01	0.006
Cholic acid		na		110	0.14*	1.3*				59	12	200	280	0.47	1.7	4.7	0.04	0.008

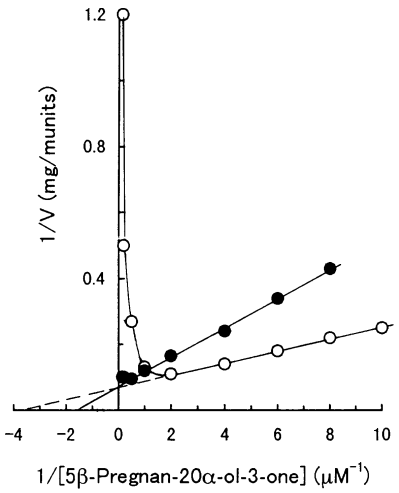

Figure 3 Double-reciprocal plots of velocity versus 5β-pregnan-20α-ol-3-one concentration for DD4 (○) and CDD4-1 (●)

oxycholic acid and cholic acid, which was not detected for DD1. The 20α-HSD activity of CDD1-4 was lowered due to large decreases in the k_{cat} values in addition to the changes in the K_m values. Thus replacing the C-terminal sequence of DD1 with that of DD4 affects the steroid specificity of DD1 mainly by altering the catalytic centre activities for steroidal substrates; CDD1-4 binds the hydroxy group on the A ring of 5β-steroid molecules more efficiently than DD1. These results suggest that the C-terminal domain of DD1 is one of the components in its substrate-binding site.

To confirm the role of the C-terminal domain in determining the substrate specificity of human liver DDs, the reverse chimaeric enzyme, CDD4-1, was prepared and its kinetic constants for the substrates were compared with those of DD4 (Table 1). One of characteristics for DD4 is the ability to oxidize the (*S*)- and (*R*)-isomers of indan-1-ol and 1,2,3,4-tetrahydronaphth-1-ol equally. CDD4-1 became an (*S*)-stereospecific enzyme like DD1, due to the elevation of the k_{cat}/K_m values for the (*S*)-isomers and lowering of the values for the (*R*)-isomers. The ratios of k_{cat}/K_m values for the (*S*)-isomers to the (*R*)-isomers were 44 and 204 respectively for CDD4-1, which were comparable to the respective values of 40 and 570 for DD1 and much higher than the values of approx. 1.6 for DD4. The replacement of the C-terminal domain increased the K_m values for 3α-hydroxysteroids and bile acids to different extents depending on the substrates. Although the changes in the k_{cat} values for most substrates were minor, the values for bile acids with 7β- and/or 12α-hydroxy groups were significantly decreased, which caused a more than 120-fold decrease in the k_{cat}/K_m values. The kinetic alterations caused by exchanging the C-terminal domains of DD1 and DD4 were reflected in the k_{cat} or K_m values, depending on the structural differences of the substrates, which strongly suggests that the C-terminal domains of the enzymes play an important role in determining the substrate specificity.

DD4 has been reported not to exhibit 20α-HSD activity [5,10,11]. However, DD4 showed low activity for 5β-pregnan-20α-ol-3-one at very low concentrations, but at higher concentrations potent substrate inhibition was observed (Figure 3). The oxidized product was identified as 5β-pregnane-3,20-dione by thin-layer chromatography. Thus DD4 is a bifunctional 3(20)α-HSD, the same as DD1. In addition to the low k_{cat} value for this substrate, the high substrate inhibition would lead this activity to be undetectable in the previous studies. The kinetic constants for CDD4-1 for this substrate were not changed significantly compared with those of DD4, but CDD4-1 did not show such high substrate inhibition. Since DD4 also showed high affinity for 3α-hydroxysteroids, 5β-pregnan-20α-ol-3-one may bind to the active site of the enzyme from its A-ring side at higher concentrations, and thereby would act as a competitive inhibitor for the oxidation of the C-20 hydroxy group of this steroid. In the case of CDD4-1, such an alternative binding of this steroid to the active site of the enzyme may not occur because the K_m value for 5β-pregnan-20α-ol-3-one was much lower compared with those for the 3α-hydroxysteroids.

Inhibitor sensitivity of chimaeric enzymes

The inhibitor sensitivities of CDD1-4 and CDD4-1 were compared with those of the respective wild-type enzymes (Table 2). In addition to phenolphthalein and flufenamic acid reported previously [19], hexoestrol, medroxyprogesterone acetate and indomethacin were competitive inhibitors of DD1 with respect to (*S*)-indan-1-ol. The conversion of DD1 into CDD1-4 produced different effects on the sensitivity to the competitive inhibitors. Sensitivity to hexoestrol, phenolphthalein and medroxyprogesterone acetate significantly increased (more than a 16-fold decrease in their IC_{50} and K_i values), whereas sensitivity to indomethacin was only slightly affected and sensitivity to flufenamic acid conversely fell. Similarly, the inhibitory potencies of betamethasone and 1,10-phenanthroline were increased and decreased respectively. An almost inverse alteration in the sensitivity to inhibitors was observed by replacing the C-terminal sequence of DD4 with that of DD1. Compared with DD4, CDD4-1 decreased the inhibitory potencies of hexoestrol, phenolphthalein, medroxyprogesterone acetate and betamethasone, and became more sensitive to flufenamic acid and 1,10-phenanthroline. These results suggest that different residues in the C-terminal domains of DD1 and DD4 are involved in the binding of these structurally different inhibitor molecules. For the competitive inhibitors, flufenamic acid may bind to the enzymes in a different manner from the cases of hexoestrol, phenolphthalein and medroxyprogesterone acetate. Jez et al. [21] have suggested that members of the AKR family retain a common (α/β)8-barrel three-dimensional fold similar to that of rat liver 3α-HSD [22] and porcine and human aldose reductases [25,32], in which inhibitors with a carboxy group are thought to bind the oxyanion-binding site at the enzyme active site. The carboxy group of flufenamic acid may interact with the oxyanion-binding site of human DD, which would cause the different effects of the replacement of the C-terminal domains on the inhibitory potencies of the competitive inhibitors without the carboxy group.

The outstanding change induced by the conversion of DD4 into CDD4-1 was the large decrease in inhibitory potency of the steroidal inhibitors, medroxyprogesterone acetate and betamethasone. This, together with the similar effects on the catalytic efficiency for bile acids with 7β- and/or 12α-hydroxy groups, suggests that functional groups on the B and C rings of the planar steroid molecules contribute to their tight binding through residues in the C-terminal domain of DD4. In addition, the inhibition patterns of medroxyprogesterone acetate, indomethacin and phenolphthalein were changed by the replacement. The C-terminal loop and A-loop of aldose reductase have been reported to flex upon inhibitor binding [23,25] and the conformational changes of the enzyme induced by the two structurally distinct inhibitors have been shown to be different [25]. The different patterns of inhibition produced by medroxyprogesterone acetate or indomethacin with DD4 and DD1 or the

Table 2 Comparison of inhibitor sensitivity among the wild-type (WT) and chimaeric enzymes

The inhibition constants are means; S.E.M.s are $< 20\%$, except where indicated ($^* < 35\%$); nd, the inhibition constant was not determined. Competitive inhibitors give only K_{is}, and mixed-type inhibitors yield both K_{is} and K_{ii}. The values in parentheses are the percentage inhibition with 50 μM medroxyprogesterone acetate and 0.2 mM betamethasone.

	DD1			CDD1-4			Ratio CDD1-4 /WT		DD4			CDD4-1			Ratio CDD4-1 /WT	
Inhibitor	IC_{50} (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50}	K_{is}	IC_{50} (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50}	K_{is}
Hexoestrol	8.3	1.6	–	0.2	0.05*	–	0.02	0.03	0.6	0.2	–	31	4.4	–	52	22
Phenolphthalein	4.6	0.3	–	0.02	0.003*	–	0.01	0.01	0.08	0.02*	–	0.8*	0.3*	0.8	10	15
Medroxyprogesterone acetate	0.7	0.07	–	0.02	0.004*	–	0.03	0.06	0.03	0.04*	0.03*	(11%)	9.2	–	–	220
Indomethacin	130	15	–	72	10	–	0.6	0.7	51	18	37	200	48	–	3.9	2.7
Flufenamic acid	5.3	0.8	–	9.5	3.1	–	1.8	3.9	110	20	–	9.4	0.9	–	0.09	0.05
1,10-Phenanthroline	65	20	68	1200	nd	nd	18	–	1500	nd	nd	700	nd	nd	0.47	–
Betamethasone	(10%)	nd	nd	210	184	290	–	–	2.4	7.5	3.6	(12%)	nd	nd	–	–

chimaeric enzymes might result from the differences in their conformational changes upon inhibitor binding. The competitive inhibition of DD1 by the two inhibitors was not altered upon replacement of the C-terminal sequence with that of DD4, but the inhibition patterns of DD4 became competitive upon replacement of the C-terminal sequence with that of DD1. Therefore, we speculate that, upon inhibitor binding, the C-terminal loop and A-loop of DD4 would move in concert to create a unique conformational change (leading to the mixed type of inhibition) that differs from those of DD1 and the chimaeric enzymes. Likewise, in the case for noncompetitive inhibition of CDD4-1 by phenolphthalein, the conformational change, which is created by the movement of the C-terminal loop of DD1 and A-loop of DD4 upon inhibitor binding, would be different from those of the other enzymes.

Effects of activators of DD4 on the chimaeric enzymes

BSP and clofibric acid derivatives are activators of DD4, and mutagenesis studies have suggested that their sulphonyl or carboxy groups interact with Lys^{270} and/or Arg^{276} of the enzyme [14,20]. In addition, comparative studies with their various derivatives have led to the proposal that the hydrophobic parts of the activator molecules interact with other residues of the enzyme [13,14]. DD1 and CDD1-4 were not activated, but rather inhibited by BSP, clofibric acid, fenofibric acid and bezafibrate. The conversion of DD4 into CDD4-1 affected the stimulatory effects of the compounds differently (Figure 4). While the effects of clofibric acid on DD4 and CDD4-1 were almost identical, those of BSP and fenofibric acid on CDD4-1 were significantly decreased compared with those on DD4, and bezafibrate did not activate CDD4-1. The data suggest that the C-terminal domain is also involved in the binding of the activators, except clofibric acid, to the enzyme. The molecular lengths from the carbon atom of the carboxy group to the chlorine atom of the opposite side chain were calculated to be 7, 10 and 14.8 Å for clofibric acid, fenofibric acid and bezafibrate respectively. The chlorine or neighbouring aromatic ring of fenofibric acid and bezafibrate may interact with residues in the C-terminal domain of DD4, while the smallest molecule, clofibric acid, may not have such a region and therefore its stimulatory effect was not affected by the replacement of the C-terminal sequence. As bezafibrate showed an inhibitory effect on CDD4-1, its binding to the enzyme may be different from its binding to DD4, and its long side chain might interfere with the access of the substrate to the active site of this chimaeric enzyme. Although the part of BSP which interacts with the C-terminal residues cannot be speculated about because of the presence of two sulphonyl groups in the molecule, similar interactions as described for the clofibric acid derivatives would occur. The interaction of the activators with the C-terminal domain is not crucial to the activation, but it may be participating in the proper orientation of the large activator molecules, since the dissociation constants of bezafibrate and fenofibric acid are much lower than that of clofibric acid [14].

Properties of H314P

There are 13 amino acid differences between the C-terminal sequences of DD1 and DD4 that were exchanged in the chimaeric enzymes. The two enzymes show approx. 68% amino acid sequence identities with rat liver 3α-HSD, the three-dimensional structure of which has been solved [22]. When the secondary structures of DD1 and DD4 were predicted based on the structure of the rat enzyme, ten of the C-terminal 13 amino acids different between the human two enzymes were positioned in the C-terminal loops (Figure 1). The observed alterations in catalytic

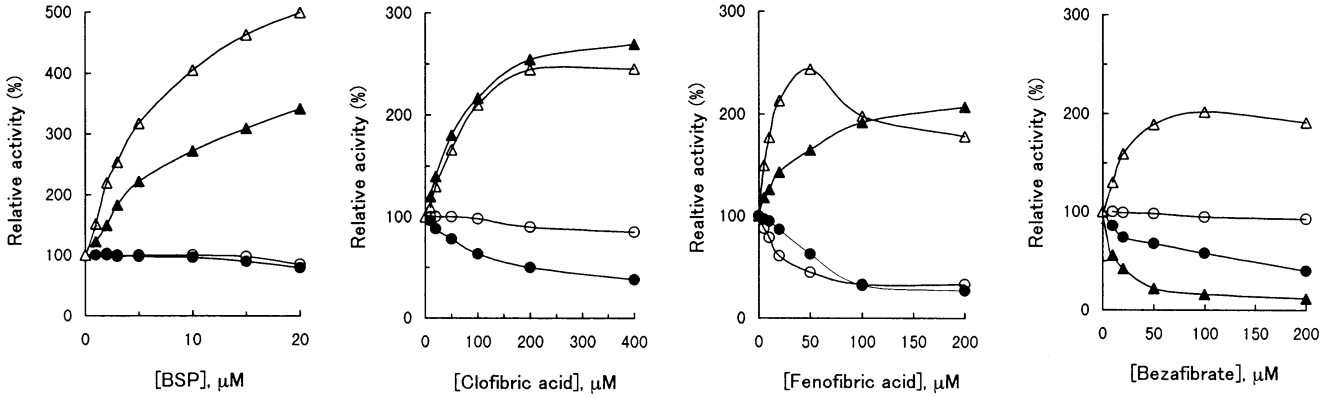

Figure 4 Effects of BSP and clofibric acid derivatives on the dehydrogenase activities of the wild-type and chimaeric enzymes

The activities of DD1 (○), CDD1-4 (●), DD4 (△) and CDD4-1 (▲) were assayed with 2 mM (*S*)-indan-1-ol as the substrate in the absence or presence of BSP, clofibric acid, fenofibric acid, or bezafibrate.

Table 3 (A) substrate specificity and (B) inhibitor sensitivity of H314P

The values are means; S.E.M.s are $< 20\%$, except where indicated (* $< 28\%$). WT, wild type.

(A)

	H314P			Ratio H314P/WT		
Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} · mM^{-1})	K_m	k_{cat}	k_{cat}/K_m
Androsterone	9.4	6.0	640	19	2.3	0.1
5β-Androstane-3α,17β-diol	3.8	7.5	2000	3.2	2.7	0.9
Lithocholic acid	0.8	1.8	2200	0.8	0.9	1.2
Deoxycholic acid	17	1.4	82	1.7	0.3	0.2
Cholic acid	39	2.9	74	0.7	0.2	0.4
5β-Pregnan-20α-ol-3-one	0.4*	0.2*	500	0.7	0.2	0.4
5β-Pregnane-3α,20α-diol	1.3	6.9	5300	6.5	5.7	0.9

(B)

Inhibitor	IC_{50} (μM)	Ratio H314P/WT
Hexestrol	3.1	5.5
Phenolphthalein	2.0	25
Medroxyprogesterone acetate	1.4	45
Indomethacin	54	1.1
Flufenamic acid	54	0.5
Betamethasone	180	75

properties by formation of the chimaeric enzymes may result from the replacement of the ten amino acids. In the crystal structure of rat liver 3α-HSD complexed with NADPH and testosterone, residues at positions 306 and 310 (numbering is the same between the rat enzyme and human DDs) have been suggested to be located close to the competitive inhibitor, testosterone [22]. Although no site-directed mutagenesis study of these residues has been done for 3α-HSD or DDs, the mutation of residues at position 298 (corresponding to position 306 of human DDs) of both aldehyde reductase [27] and aldose reductase [33] moderately affects the kinetic constants for several substrates. Arg^{311} of aldehyde reductase has been reported to determine the preference of the enzyme for carboxy-containing substrates and the specificity of several inhibitors [28], but the corresponding amino acid in the sequences of aldose reductase, rat 3α-HSD or human DDs is unknown because of the uniquely long C-terminal sequence of aldehyde reductase. In addition, the mutation of Cys^{303} (corresponding to position 311 of human DDs) of aldose reductase has been shown not to affect the kinetic constants for the substrates [26,33]. However, a recombinant mutant DD4 in which Leu^{311} was replaced with valine showed almost half the k_{cat}/K_m values for the various substrates compared with DD4 (T. Kume, H. Iwasa and A. Hara, unpublished work). Thus the C-terminal residues that participate in the binding of substrates and inhibitors may be different depending on the respective members of the AKR family, in addition to the structural differences of the substrates and inhibitors as described above for human DDs. In the alignment of the C-terminal sequences of DD1 and DD4, DD1 has successive proline residues at positions 314 and 315, that could influence substrate specificity and/or in-

hibitor sensitivity by forming a rigid-turn structure. The two residues also correspond to the residues involved in the binding of zopolrestat, a competitive inhibitor of aldose reductase, to a fibroblast growth factor-induced reductase, a member of the AKR family [23]. To test this possibility, His^{314} of DD4 was mutated into proline, which produced the Pro-Pro sequence at positions corresponding to the sequence of DD1. The recombinant enzyme, H314P, was purified and its substrate specificity, for representative steroids, and inhibitor sensitivity compared with those of the wild-type DD4 (Table 3). The mutation produced moderate changes in the kinetic constants for the substrates and the IC_{50} values for the inhibitors, which are intermediate between the values for the wild-type DD4 and for CDD4-1. The residue at position 314, or the Pro-Pro sequence, may be involved in the binding of the substrates and inhibitors, but was not a critical residue or sequence for determining the substrate specificity and inhibitor sensitivity. This suggests that multiple residues in the C-terminal loop of human DD may co-ordinately determine the orientation of the substrates and inhibitors in the active site.

The present work demonstrates the important role of the C-terminal loop in controlling the substrate specificity, inhibitor sensitivity and activation of human DDs. In addition, we suggest that critical residues, which participate in binding to specific parts of the substrates or modulators with diverse structures, are present in the C-terminal ten amino acids, which differ between DD1 (or DD2) and DD4. However, the replacement of the C-terminal loop did not produce complete conversion of DD1 into DD4 (or DD4 into DD1), which suggests that amino acids in the other regions of the substrate-binding site are also important for determining the substrate specificity and modulating inhibitor binding. Of the residues in the proposed substrate-binding site of the AKR-family proteins [21], only the residues in the A-loop and C-terminal loop are different between DD1 and DD4. Ongoing site-directed mutagenesis studies using chimaeric enzymes show that, in addition to the residues in the A-loop, some residues responsible for the orientation of the coenzyme and for creating the substrate-binding cavity serve to further modulate substrate specificity and inhibitor binding (A. Hara and T. Ohta, unpublished work). Since the human DDs exhibit 3α- and 20α-HSD activities, such structure/function studies may facilitate rational drug design targeting for 3α- and 20α-HSDs, the inhibitors of which have been thought to have roles in the management of prostatic disease and in the maintenance of pregnancy respectively [9].

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