ELSEVIER Chemico-Biological Interactions 130–132 (2001) 833–845

Chemico-Biological Interactions

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Site-directed mutagenesis studies of bovine liver cytosolic dihydrodiol dehydrogenase: the role of Asp-50, Tyr-55, Lys-84, His-117, Cys-145 and Cys-193 in enzymatic activity☆

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Abstract

A previous report on the cloning, bacterial expression and purification of bovine liver cytosolic dihydrodiol dehydrogenase (DD3) cDNA (1,330 bp in full length) using pKK223-3 expression vector characterize the properties of the recombinant DD3 in the aspects of substrate specificity and inhibitor sensitivity (Terada et al., Adv. Exp. Biol. Res. 414 (1997) 543–53). The nucleotide sequence of this DD3 cDNA completely matches that of bovine liver-type prostaglandin F synthase (PGFS) (Suzuki et al., J. Biol. Chem. 274 (1999) 241–8). In the present study, a large amount of recombinant DD3 (rDD3) was expressed in *Escherichia coli* BL21 (DE3) with a pET28a expression vector. The recombinant DD3 (rDD3) was easily and quickly purified to an apparent homogeneity with one step column chromatography of Ni^{2+}-affinity resin. The rDD3 showed essentially the same substrate specificity and inhibitor sensitivity as purified liver DD3 (DD3). To analyze the role of amino acid residues of DD3 in its enzymatic activity, site-directed mutagenesis of DD3 with PCR method was performed. The results of the analyses of these mutants in the aspects of substrate specificity and cofactor-binding suggested a variety of functions in the enzymatic

Abbreviations: AKR, aldo–keto reductase; DD, dihydrodiol dehydrogenase; DTNB, dithio bis(2-nitro benzoic acid); HSD, hydroxysteroid dehydrogenase; NEM, *N*-ethylmaleimide; PGFS, prostaglandin F synthase.

☆ The nucleotide sequence data reported in this paper is available in the DDBJ/EMBL/GenBank nucleotide sequence databases, accession number D49542.

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PII: S0009-2797(00)00239-8

activity: as an active site Tyr-55 may act as a general acid and Asp-50, Lys-84 and His-117 may play an important role in the control of protonation of Tyr-55 as a general acid in the dehydrogenase activity under higher pH conditions, though these residues may not be involved in reductase activity under lower pH conditions. Though the mutated DD3s (Cys to Ser) did not show significant differences in their substrate specificities, these mutants showed different sensitivities to SH-reagents. Present results indicate that Cys-193 may play an important role in the modulation of enzymatic activity under redox conditions generated with GSH + GSSG among five cysteines in DD3.

Keywords: Dihydrodiol dehydrogenase; Site-directed mutagenesis; Active site motif and modulation of enzymatic activity

1. Introduction

Dihydrodiol dehydrogenases (DDs) [EC 1.2.1.20] have been reported as playing important roles in many animal tissues in the metabolism of *trans*-dihydrodiols of polycyclic aromatic hydrocarbons [1–8]. The great advances in the structure analyses of these enzymes revealed that DDs belong to the aldo–keto reductase superfamily (AKR family) which includes 3α-hydroxysteroid dehydrogenase (3α-HSD) [3–5,7,8], 17β-hydroxysteroid dehydrogenase [9], 20α-hydroxysteroid dehydrogenase [6], aldose reductase (AR) [10] and aldehyde reductase (ALR) [11]. There are also indications that these enzymes may metabolize not only endogenous compounds, but xenobiotic compounds as well [1–12].

Recently, we reported that three bovine liver cytosolic DDs (DD1, DD2, and DD3) showed unique properties in their substrate specificities, inhibitor sensitivities and immunochemical cross reactions [13]. Both DD1 and DD2 have been identified with 3α-HSD and ALR, respectively. Though DD1 and DD3 showed a strong cross reaction in immunochemical analyses toward anti-DD1 and anti-DD3 antibodies, they differed in their substrate specificity and inhibitor sensitivity, suggesting that DD3 is a novel enzyme among these bovine liver DDs [13–15]. For example, unlike DD1 and DD2, DD3 showed highly specific activity toward the *trans*-dihydrodiols of aromatic hydrocarbons. Additionally, DD3 is competitively inhibited by androsterone which is a typical substrate of DD1 [13]. Furthermore, we reported that cysteine may play an important role in the modulation of enzymatic activity under a redox buffer [15].

In a recent report on the cloning of DD3 cDNA and the properties of bacterial recombinant DD3 [16], the nucleotide sequence of DD3 cDNA was found to completely match that of bovine liver-type prostaglandin F synthase [17]. Considering the substrate specificity of DD-activity, together with the results of bovine liver-type prostaglandin F synthase, DD3 may play an important role in the metabolism of cholic acid and xenobiotics as well as prostaglandis.

In the present study, we investigated the effects of amino acid substitutions on the enzymatic activity of DD3.

2. Methods and materials

2.1. Materials

A Geneclean II kit, restriction endonucleases, pBluescript KS II (+) and Gene Taq polymerase were obtained from Funakoshi, TOYOBO, NEB, and Nippon Gene, respectively. The pET28a expression vector was the product of Novagene, and the prestained protein marker was the product of NEB. All other reagents were of the highest grade commercially available.

2.2. Standard assay systems of DD3 activity

The dehydrogenase activity of DD3 was determined by monitoring the increase in the absorption at 340 nm at 25°C. A reaction mixture contained 100 mM glycine/NaOH, pH 9.5, 0.2 mM $NADP^+$, and 0.5 mM 1-acenaphthenol, with an appropriate amount of enzyme as described in our previous papers [13–15]. The reductase activity was determined using a reaction mixture of 100 mM Na–phosphate buffer, pH 6.5, 65 μM NADPH and 0.25 mM camphorquinone.

2.3. $NADP^+$-binding assay of DD3

The binding studies of $NADP^+$ to DD3 were performed according to a method recently reported [18–20]. Quenching of an emission at 336 nm following an excitation at 282 nm was detected with Hitachi Fluorescence Spectrophotometer. The apparent K_d-values were calculated from these results according to Wilkinson's method [21].

2.4. Construction of the expression plasmid

A cDNA encoding DD3 in pBluescript KS II (+) (pBS-dd3) was used as a template. Amplification of the cDNA carrying the internal *Eco*RI cleavage-site (dd3′) was performed using F01 and R01 (forward and reverse) primers. Underlines in the primer sequences indicate the restriction sites of *Eco*RI. The PCR was carried out by preheating at 94°C for 5 min, denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. After 30 cycles of amplification, the resulting PCR products were treated with 5 U of *Eco*RI for 2 h at 37°C. The *Eco*RI fragment of the PCR products (dd3′) was ligated into the expression vector pET28a. The resulting expression plasmid (pET-dd3′) was transformed into *Escherichia coli* BL21 (DE3).

F01	5′-TTC GAA TTC ATG GAT CCC AAA GGC-3′
R01	5′-GCG GAA TTC AAA TGG GTA CTC AGG GTG-3′

2.5. The expression of the recombinant DD3 protein (rDD3)

Transformed bacterial cells were cultured overnight in 25 ml of L-Broth culture medium, including 25 μg/ml of kanamycin at 37°C with vigorous shaking, and then further cultured in 250 ml of L-Broth under the same conditions. After culturing for 3 h, 0.1 mM IPTG was added to the medium and followed by 3.5 h incubation. The bacterial cells were harvested and washed with 20 mM Na–phosphate buffer, pH 7.5 (buffer A). The cells were sonicated with Model W-220 of Heat System Ultrasonic Inc. at 4°C.

2.6. Site-directed mutagenesis

Site-directed mutagenesis studies were performed with PCR using mutation primers and either F01 or R01 (see Table 1). The reaction mixture of PCR contained a 135 ng template of DD3 cDNA, 0.4 μM dNTP, each 1 μM reverse and mutation primers, and 1 U Gene Taq polymerase. The PCR was carried out by preheating at 94°C for 2.5 min, denaturing at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. After 25 cycles of amplification, the PCR products were subjected to a second PCR, amplified with either F01 or R01. After digestion with *Eco*RI and after confirming their nucleotide sequences with Shimadzu DSQ-1000L auto DNA sequencer, these PCR products were then inserted into the pET28a expression vector. The expression plasmid was also transformed into *E. coli* BL21 (DE3) cells and the expressed protein was then purified by the same method as wild type (WT). The primers for the mutation studies are described in Table 1 with mutated nucleotides expressed as small letters.

Table 1
Primers for site-directed mutagenesis[a]

Primer	Sequence
Common forward primer	5′-TGCAACCAGGTGGAATaTCACC-3′
Common reverse primer	5′-TGCAACCAGGTGGAATGTCACC-3′
Reverse primer	5′-CACCCTGAGTAcCCATTTGAATTCCGC-3′
Forward primer	5′-GATCCGAAtCcATGGATCCCA-3′
R01 primer (D50N)	5′-GGTTCCGCCATATTaAtAGTGCTC-3′
R02 primer (L54N)	5′-CATATTGACAGTGCTCATaatTACCAA-3′
R03 primer (Y55F)	5′-ATATTGACAGTGCTCATTTGTtCCAA-3′
R04 primer (K84N)	5′-TTTTCTACACTTCAAAtCTTTGGTCC-3′
R05 primer (Y55H)	5′-ATATTGACAGTGCTCATTTGcACCAA-3′
R06 primer (H117Q)	5′-ATGTCGATCTTTATATTATTCAaTTTCC-3′
R07 primer (H117Y)	5′-ATGTCGATCTTTATATTATTtATTTTCC-3′
F01 primer (C145S)	5′-GGATCTCTcTCGCACATGGG-3′
F02 primer (C154S)	5′-CTGGAGAAGTcTAAGGACGCA-3′
F03 primer (C188S)	5′-GCCCGTCTcCAACCAGGTGG-3′
F04 primer (C193S)	5′-GGAATcTCACCCTTATTTCAATC
F05 primer (C206S)	5′-GTTGGATTTCTcCAAGTCACATG-3′

[a] The primers for site-directed mutagenesis are listed. Small letter in the sequences are the mutations of nucleotide.

2.7. Inactivation studies

The inactivation studies of rDD3 were initiated with the addition of oxidants, 0.5 mM DTNB, 5 mM H_2O_2 or 50 μM NQ (naphthoquinone). Pyridine nucleotides (1 mM) were added into the reaction mixture, the mixture was incubated for 30 min, and appropriate samples ($\sim$5 μg) were then subjected to DD3 activity-determinations for 1-indanol. The reactions were performed using 100 μg/ml of rDD3 in 10 mM Na–phosphate buffer under anaerobic conditions with N_2 gas-saturated 10 mM Na–phosphate buffer, pH 7.5.

3. Results and discussion

3.1. Amino acid alignments of aldo–keto reductases

Amino acid alignments of aldo–keto reductases are summarized in Fig. 1. Open triangles indicate the amino acid residues which are targets for the site-directed mutagenesis in the present study. Among the amino acid residues, Asp-50, Tyr-55, Lys-84, His-117, Cys-188, Cys-193 and Cys-206 were completely conserved within these enzymes. Open squares are the AKR-motifs which are obtained from the motif analysis. The DYKH motif contained within Asp-50, Tyr-55, Lys-84 and His-117 (the DYKH tetrad) was conserved in the AKRs as an active site motif. Though there is a similarity in the amino acid sequences between these AKRs, these enzymes showed unique substrate specificities. It is likely that the differences between the amino acid sequences of these enzymes is what regulate substrate-binding or that the other factors affect the enzymatic activity.

3.2. Constructions of expression plasmids of mutants

Site-directed mutagenesis studies were investigated with PCR technique using primers which introduced the mutations listed in Table 1. Mutated DNA fragment was amplified with mutation primer and forward or reverse primer. Then the resulting DNA fragment was used as a primer with reverse or forward primer to amplify the mutated cDNA of DD3. The mutated cDNAs of DD3 were sequenced to confirm the mutations were introduced into the proper positions and no other mutations were not introduced during PCRs. The resulting cDNAs were also ligated with pET28a expression vector to construct the expression plasmids and then sequenced to confirm their proper open reading frames. Inductions and purifications were performed according to the same procedure described above. The purified mutants were analyzed as to their expressions and purifications with 12.5% SDS–PAGE. The expressions and purifications were essentially the same as WT.

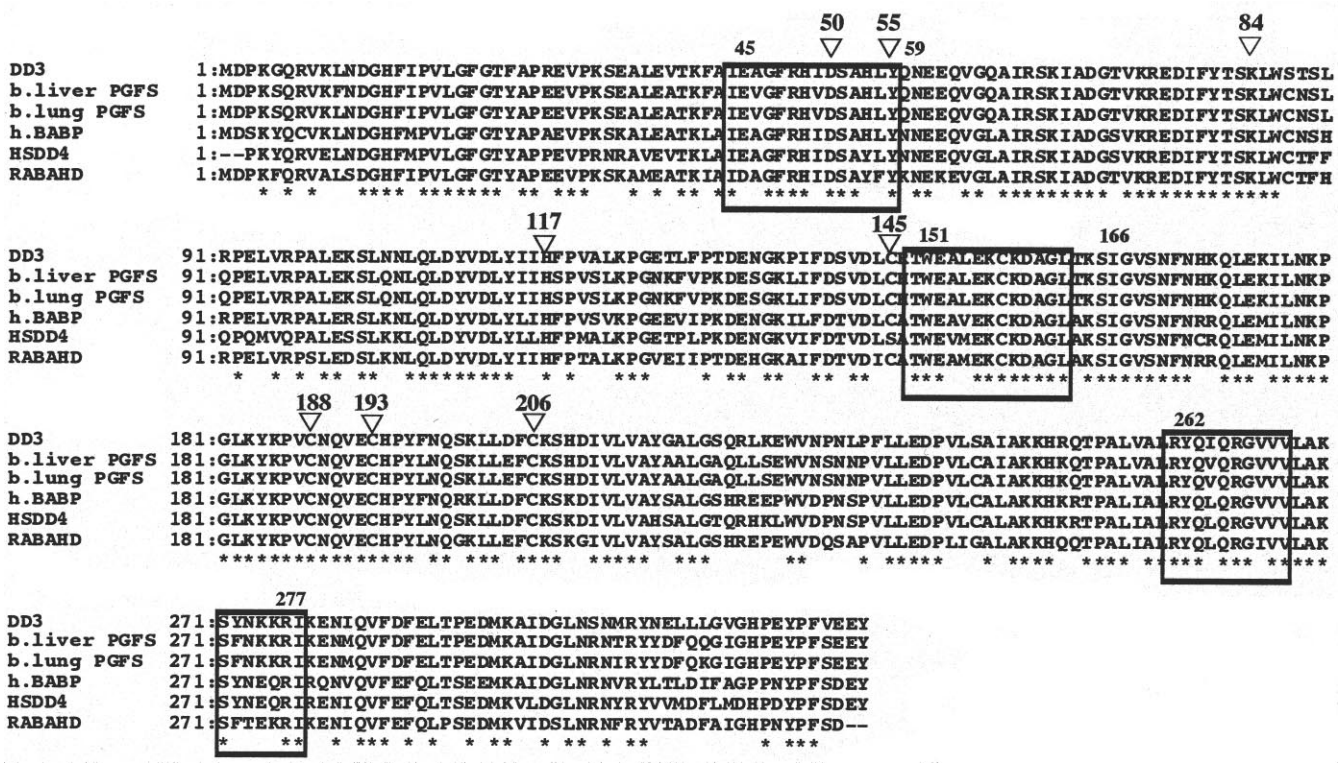

Fig. 1. Amino acid sequence alignments of enzymes belonging to the aldo-keto reductase superfamily. Deduced amino acid sequence of DD3 was compared with the enzymes. Triangles indicate amino acid residues which are targeted for site-directed mutagenesis.

3.3. CD-spectrum of the rDD3s

The CD-spectrum of WT and the mutated DD3 were determined by CD-spectrophotometer. The scanned spectrum did not show a significant pattern from 200 to 265 nm, suggesting the substitutions of amino acid residues caused insignificant structural changes in the mutated DD3. Comparing with WT clarifies that the inactivations of the mutants were not caused by the structural changes.

3.4. Substrate specificity of the rDD3s

Enzymatic activities of WT and the mutant rDD3s toward a variety of compounds are analyzed and summarized in Table 2. The values in the table represent the relative percentage of activity for the enzymes of WT toward *trans*-benzenedihydrodiol. The Y55F mutant showed an extremely low activity for these substrates, both in dehydrogenase and reductase activities, suggesting that Tyr-55 functions as the active center for the enzymatic activity of DD3. The Y55H mutant showed relatively higher activities for substrates than did Y55F, suggesting that His-55 is able to function as a proton donor/acceptor, though its efficacy is low (data not shown). Both D50N and K84N mutants showed low dehydrogenase activities, and the K84N mutant also lost its reductase activity. These results indicate that Lys-84 may function as an important amino acid residue in controlling the protonation of Tyr-55 under any pH conditions. Interestingly, reductase activity of the D50N mutant matched WT, suggesting that Asp-50 is involved in the protonation of Tyr-55 under higher pH conditions. The replacement of His-117 by Asn or Tyr also resulted in a great effect on the dehydrogenase activity. These results suggest that the DYKH tetrad is an essential motif for dehydrogenase activity and some of the residues are also involved in reductase activity through the control of Tyr-55 protonation in the native DD3. The results from the H117Q mutant showed greater activity than WT, suggesting that replaced Asn may have different effects on the Tyr-55 protonation under lower pH conditions. The most significant effects on the activation of reductase activity were obtained from the Y55H mutant (data not shown). At present, we suppose that the activation of Y55H is caused by the different pK_as of tyrosine and histidine.

3.5. Apparent K_d-values for $NADP^+$

The affinities of the rDD3s toward $NADP^+$ were determined according to the methods previously described based on the quenching of tryptophan in the enzyme molecule. The quenchings of tryptophan fluorescence (Ex. 282 nm and Em. 336 nm) generated from the $NADP^+$-binding were measured by the Hitachi Fluorescence Spectrophotometer. The relative quenchings were determined by comparing the exact fluorescence to the full fluorescence obtained from the rDD3s in the absence of $NADP^+$. Apparent K_d-values are summarized in Table 3. The mutants had values similar to WT. The replacements of amino acid residues in the present study may not be involved in the $NADP^+$-binding directly. Penning et al. reported from

Table 2
Substrate specificities of rDD3 and mutants[a]

Substrate (mM)	Relative activity (%)							
	Concentration	WT	D50N	L54N	Y55F	K84N	H117Q	H117Q & Y55F
[Dehydrogenase]								
trans-Benzenedihydrodiol	1.0	100	11	12	0.11	1.8	2.8	0.00
trans -naphthalenedihydrodiol	1.0	84	19	17	0.12	0.87	2.4	0.07
1-Acenaphthenol	0.5	170	31	35	0.34	2.6	11	0.30
1-Indanol	0.5	52	13	13	0.08	0.92	1.6	0.07
Taurochenodeoxycholic acid	1.0	48	0.96	1.3	0.03	0.67	1.3	0.05
[Reductase]								
(1*S*)-(+)-Camphorquinone	0.25	8.4	7.2	7.4	0.61	0.51	17	1.3
(1*R*)-(−)-Camphorquinone	0.25	6.3	6.4	7.4	0.33	0.28	16	0.96

[a] WT of rDD3 and mutants were subjected to the determinations of enzymatic activity towards substrates. The values in the Table represent the averages of three determinations.

Table 3
Apparent K_d-values of WT and mutants for $NADP^+$[a]

Mutant	K_d-values (nM)
	Averages ± S.D.
WT	67.6 ± 5.5
D50N	93.3 ± 3.9
Y55F	74.6 ± 3.0
Y55H	70.5 ± 2.5
K84N	93.7 ± 4.8
H117Q	78.3 ± 7.6
H117Y	97.0 ± 5.5
Y55F & H117Q	95.7 ± 2.4
C145S	84.0 ± 3.7
C193S	73.0 ± 2.1

[a] The affinities of WT and mutants for $NADP^+$ were determined by the method as described in the text. About 100 μg/ml of enzymes were subjected to binding to $NADP^+$ by measuring the quenching of fluorescence (Ex. 282 nm; Em. 336 nm) of the enzyme following incremental addition with S.D. of three determinations.

their crystallographic studies, that the following amino acid residues contributed to $NADP^+$-binding: Thr-24, Asp-50, Ser-166, Asn-167, Gln-190, Phe-216, Leu-219, Ser-221, Ser-271, Tyr-272, Arg-276 and Glu-279. These amino acid residues correspond to the same amino acids except for Phe-216 which corresponds to Tyr-216 of DD3. It seems that the mutated amino acid residues in the present study are not involved in $NADP^+$-binding.

3.6. pH-profiles of the rDD3s

The pH-profiles of the rDD3 dehydrogenase activities were investigated under various pH conditions (Fig. 2). The dehydrogenase activity of WT is greater under higher pH conditions. On the other hand, the activity of D50N reached its maximum at around pH 10, suggesting that the pH profile of D50N was shifted to lower pH conditions. Similar to D50N, the activity of K84N reached its maximum at around pH 10, but unlike D50N did not drop at the higher pH. Furthermore, the maximum enzymatic activity of H117Q shifted to a lower pH compared to the other mutants. By substituting the above amino acid residues, pH conditions surrounding Tyr-55 were altered and the protonation of Tyr-55 in the D50N, K84N and H117Q mutants were controlled in a different manner.

3.7. Apparent K_m-values of the rDD3s for substrates

Apparent K_m-values of WT, C145S and C193S were determined by the double reciprocal plot analysis. The enzymatic activities of the rDD3s were measured under varied concentrations of substrates. The data from these kinetics were

analyzed according to the method of Wilkinson and the apparent K_m-values are summarized in Table 4. The substitutions of cysteine residues by serine do not affect the enzymatic activities (data not shown) and affinities toward substrates. These results suggest that the cysteines are not directly involved in the enzymatic activity. The affinities of the rDD3s were also determined, the apparent K_m-values of Cys-mutants were essentially the same as those of WT.

3.8. Sensitivity of the rDD3s to SH-reagents

Sensitivity of WT, C145S and C193S mutants to SH-reagents were investigated (Table 5). The excess DTT (dithiothreitol) was eliminated from DTT-treated enzymes by gel filtration with Sephadex G-50. All the buffers used in this study were saturated with N_2 gas and all studies were performed under anaerobic conditions in a vacuum chamber saturated with N_2 gas. Aliquat samples were subjected to the determination of the remaining activity toward 1-indanol. The C145S mutant showed essentially the same sensitivity as WT to the SH-reagents, but the C193S mutant showed insignificant sensitivity to the SH-reagents. The other

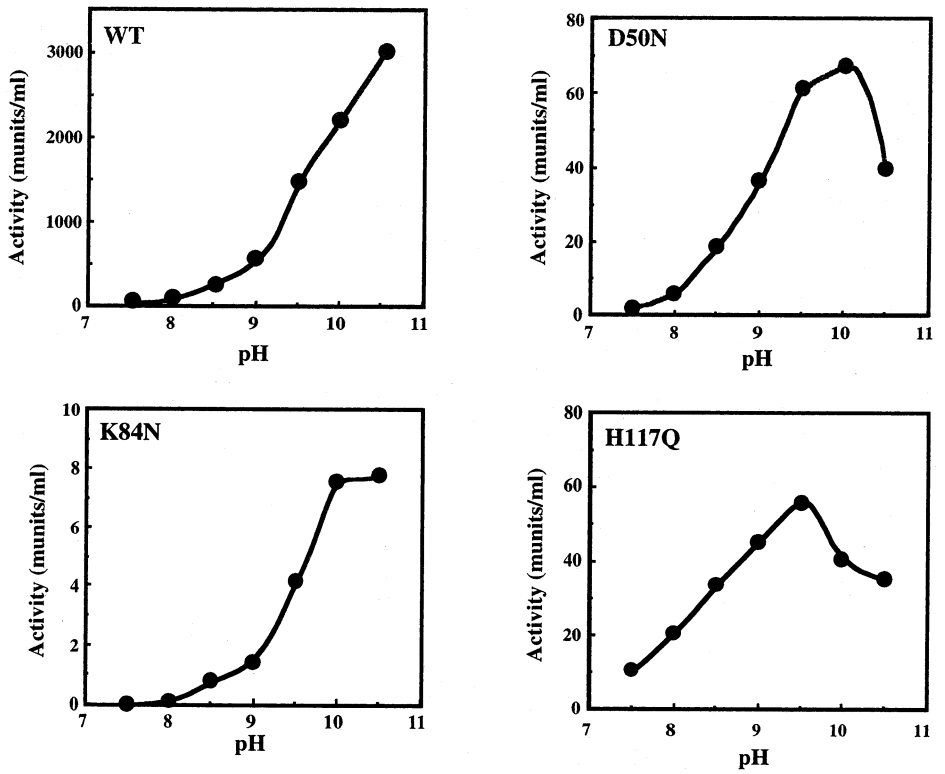

Fig. 2. pH-profiles of DD3 and its mutants. The enzymatic activities toward indanol were determined under various pH conditions.

Table 4
Kinetic parameters of rDD3 and mutants towards substrates and cofactors[a]

Compound	K_m (μM)		
	WT	C145S	C193S
[Substrate]			
1-Acenaphthenol	410	500	450
1-Indanol	290	370	340
Tauochenodeoxycholic acid	890	780	920
[Cofactor]			
$NADP^+$	2.3	2.1	2.6
NAD^+	440	400	380

[a] Apprent K_m-values were determined with various substrates and cofactors. About 5 μg of rDD3(~ 3.9 mU) was subjected to the enzymatic activity-determinations.

mutants (C154S, C188S and C216S) showed no differences in their substrate specificities or sensitivities to SH-reagents (data not shown). We have reported on the possible modulation of the enzymatic activity of rat 3αHSD under redox conditions made through a variety of ratios of GSH and GSSG. As indicated above, DD3 has five cysteine residues in its molecule. Among the nine cysteine residues of rat 3αHSD, these five cysteine residues of DD3 were found at positions corresponding to DD3, namely 145, 154, 188, 193 and 216. The results of computer simulation of the DD3 3D-structure suggest that Cys-145 may be located on the surface of the enzyme molecule of DD3. However, Cys-193 may be positioned in the barrel structure and may be located near or by the $NADP^+$-binding site. The replacement of Cys-193 by serine does not prevent $NADP^+$-binding, though some bulky compounds such as SH-reagents do prevent the binding of $NADP^+$ by obstructing the binding site. This hypothesis is strongly supported by our previous results where the corresponding Cys-145 was modified with [^{14}C]-*N*-ethylmaleimide

Table 5
Effects of SH-reagents on the enzymatic activities of rDD3 and mutatnts. DTT-treated rDD3 (100 μg/ml) were subjected to the SH-reagent-treatments[a]

Compound	mM	Remaining activity (%)		
		WT	C145S	C193S
No addition		95	98	99
GSSG	25	77	76	95
NEM	0.2	36	36	92
DTNB	0.1	39	38	94
$+NADP^+$		96	94	96
$+NAD^+$		43	44	90

[a] After 30 min incubation, samples (5 μg) were determined regarding their remaining activity (%). GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

in the absence/presence of $NADP^+$, but the Cys-193 was modified with NEM only in the absence of $NADP^+$ following its inactivation. No evidence was present that the other cysteines were modified with NEM. Furthermore, we have clarified with those previous results that the modification of DD3 by NEM was competitively prevented by DTNB. These results indicate that the remaining three cysteines (Cys-154, 188 and 216) may be located in the buried regions of the enzyme molecule and unable to interact with SH-reagents.

Further site-directed mutagenesis and crystallographic studies of DD3 will be necessary to clarify the role of amino acid residues of DD3 in its enzymatic activity.

Acknowledgements

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (08672509).

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