The role of cysteine in the alteration of bovine liver dihydrodiol dehydrogenase 3 activity

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Bovine liver NADP⁺-dependent dihydrodiol dehydrogenase (DD3) is extremely sensitive to SH reagents such as *N*-ethylmaleimide (NEM) and 5,5'-dithiobis(2-nitrobenzoic acid). NEM produced time- and concentration-dependent inactivation of DD3 in a pseudo-first-order reaction manner. This inactivation was prevented by NADP⁺, 3-acetylpyridine-adenine dinucleotide phosphate, 2',5'-ADP and 2'-AMP but not by substrates, NAD⁺, nicotinamide mononucleotide or 5'-ADP. DD3 was adsorbed by an affinity column of thiopropyl-Sepharose 6B, but enzyme incubated with both NEM and NADP⁺ was not. Moreover, one [¹⁴C]NEM molecule was incorporated into a cysteine of DD3 in the presence, and two cysteines of DD3 in the absence, of NADP⁺. These results suggested that two cysteine residues were modified per enzyme molecule by NEM, one was protected by NADP⁺ and the other had no significant function for the enzyme activity. Two radiolabelled peptides (P1 and P2) produced by the digestion with lysyl endopeptidase of [¹⁴C]NEM-modified DD3 could be separated by reverse-phase HPLC. P1, which was radiolabelled by [¹⁴C]NEM only in the absence of NADP⁺,

showed the following sequence; H₂N-Tyr-Lys-Pro-Val-Xaa-Asn-Gln-Val-Glu-NEM·Cys-His-Pro-Tyr-Phe-Asn-Gln-Ser-Lys-COOH (Xaa indicates a possible cysteine residue). This sequence was very similar to that of rat liver 3 α -hydroxysteroid/dihydrodiol dehydrogenase (3 α -HSD/DD) (residues 184 to 201) and was also highly conserved in the aldo-keto reductase superfamily. The sequence of P2, which had radioactivity in both the absence and presence of NADP⁺, also contained an NEM-modified cysteine and was similar in sequence to the regions located in loop A of rat 3 α -HSD/DD. The present study suggests that P1, which may have a cysteine residue corresponding to Cys-193 of rat 3 α -HSD/DD, functions in the alteration of DD3 activity depending on the modulation of NADP⁺-binding ability through a thiol/disulphide exchange reaction similar to that of rat 3 α -HSD/DD shown in our previous results; while P2, which may have a cysteine residue corresponding to Cys-145 of rat 3 α -HSD/DD, may be located near the surface of the enzyme molecule.

INTRODUCTION

Dihydrodiol dehydrogenase [DD; EC 1.3.1.20] catalyses the oxidation of non-K-region *trans*-dihydrodiols of polycyclic aromatic hydrocarbons such as benzo[a]pyrene, benz[a]anthracene, 5-methylchrysene and 7,12-dimethylbenz[a]anthracene. The enzyme can suppress the formation of the ultimate carcinogens, *anti*-diol epoxides, by oxidizing *trans*-dihydrodiol [1–3]. The evidence that the addition of the purified rat enzyme to the Ames test greatly reduced the mutagenicity of benzo[a]pyrene [4] and benz[a]anthracene [5] suggests the important role of DD in the metabolism of aromatic hydrocarbons *in vivo*. DDs were purified from some animal tissues, i.e. rat liver [1,6], rabbit liver [7], mouse liver [8] and guinea-pig testis [9]. Rat liver enzyme [rat 3 α -hydroxysteroid/dihydrodiol dehydrogenase (3 α -HSD/DD)] has been investigated in detail including its monomeric structure (*M_r* 37029), its 3 α -hydroxysteroid dehydrogenase (3 α -HSD) activity, the interconversion of androsterone/androstanedione [6], its role in bile acid biosynthesis [10], 9-, 11- and 15-hydroxyprostaglandin dehydrogenase activities [11] and carbonyl reductase activity [12]. The primary structure of this rat enzyme, deduced from its cDNA sequence [13], is similar to bovine lung prostaglandin F synthase (PGFS) [14], human liver chlordecone reductase [15] and human placenta and bovine lens aldose reductases [16,17], which are members of the aldo-keto reductase superfamily.

We have previously characterized three multiple forms of dihydrodiol dehydrogenase, namely, DD1 (*M_r* 35000), DD2 (*M_r*

36000) and DD3 (*M_r* 35500) purified from bovine liver [21]. All have monomeric structures and utilize NAD(P)H specifically as a coenzyme. DD1 has been identified as 3 α -HSD from the results of substrate specificity and immunological cross-reaction with rat 3 α -HSD/DD [22]. Since DD2 has a strong activity for D-glucuronic acid and D,L-glyceraldehyde, this should be classified into a typical high-*K_m* aldehyde reductase [23]. Among these three DDs, DD3 may be the major isoform in bovine liver with the highest dehydrogenase activity for *trans*-1,2-dihydrobenzene-1,2-diol (*trans*-benzenedihydrodiol), a model compound of aromatic hydrocarbon dihydrodiols, with the lowest *K_m* and the highest *k_{cat}* values [21]. DD3 shows a different substrate specificity for alcohols, aldehydes, steroids, ketones and quinones from the other typical aldo-keto reductases as reported previously [24]. However, the partial amino acid sequence of DD3 shows a great similarity to those of C-terminal sequences of rat 3 α -HSD/DD and bovine lung PGFS [25]. Additionally, we have demonstrated that the enzymic reaction of DD3 may proceed by an ordered Bi Bi mechanism, namely NADP⁺ binds first and NADPH leaves last [26], it being assumed that some conformational change of DD3 occurs when DD3 binds with NADP⁺. In contrast, NAD⁺ (*K_m* 138 μ M) is a less active cofactor for DD3 than NADP⁺ (*K_m* 2.7 μ M), suggesting that lack of a 2'-adenylate moiety leads to the lower affinity of nucleotide for the enzyme. Furthermore, we recently reported that DD3 has a novel dehydrogenase activity for some bile acids such as lithocholic acid, taurolithocholic acid and taurochenodeoxycholic

Abbreviations used: DD, dihydrodiol dehydrogenase; 3 α -HSD, 3 α -hydroxysteroid dehydrogenase; 3 α -HSD/DD, 3 α -hydroxysteroid/dihydrodiol dehydrogenase; PGFS, prostaglandin F synthase; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; NMN, nicotinamide mononucleotide; 3-APADP, 3-acetylpyridine-adenine dinucleotide phosphate; PTH, phenylthiohydantoin.

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acid which have no 12-hydroxy groups [26]. We also suggested that DD3 is a unique enzyme among various aldo-keto reductases.

Little is known about the cysteine residues which may have important roles in catalytic activity of aldo-keto reductases. Human placenta aldose reductase contains seven cysteine residues, three of which react readily with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); and the inactivation of aldose reductase by DTNB and *N*-ethylmaleimide (NEM) is prevented by saturating NADPH [27]. Aldose reductases are converted into more active forms by the oxidative modification [28–30], and K_m and k_{cat} values of bovine lens enzyme for D,L-glyceraldehyde, D-glucose and NADPH are increased by the carboxymethylation, but sensitivity to sorbinil is lost [31]. These results suggest that the enzymic activity, affinity for substrates and coenzyme, and inhibitor sensitivity are altered by the modification of cysteine residues. Furthermore, the results of site-directed mutagenesis and chemical modification of human placenta aldose reductase suggest that Cys-298 is present near the active site and constitutes a regulatory group which controls the catalytic activity and inhibitor sensitivity of the enzyme [32]. In the course of our studying DD, we have reported that 3 α -HSD/DD activity is modulated by SH-reagents and biological disulphides such as GSSG and cystine, and that NADP⁺ protected the enzyme from the inactivation, suggesting that cysteine residue(s) are important for activity [12]. Penning and co-workers [33,34] reported that Cys-217 of rat 3 α -HSD/DD functions in steroid and/or co-enzyme binding and that the other two cysteine residues are not essential for catalytic activity from studies of both chemical modification and site-directed mutagenesis.

In this paper, using NEM, we investigate the modification and the identification of important cysteine residue(s) for the alteration of DD3 activity depending on the modulation of NADP⁺-binding ability through a thiol/disulphide exchange reaction.

MATERIALS AND METHODS

Materials

NADP⁺, NAD⁺ and 5'-ADP were purchased from Oriental Yeast Co. NEM, dithiothreitol (DTT) and iodoacetic acid were obtained from Nacalai Tesque. 2'-AMP, 2',5'-ADP, β -nicotinamide mononucleotide (NMN), 3-acetylpyridine-adenine dinucleotide phosphate (3-APADP) and bovine pancreas insulin were the products of Sigma Chemical Co. DTNB, *Achromobacter lyticus* lysyl endopeptidase and mixtures of phenylthiohydantoin (PTH)-amino acids were from Wako Pure Chemical Industries. 5'-AMP and 1-acenaphthenol were supplied from Kohjin Co. and Aldrich Chemical Co., respectively. Nick-column and thiopropyl-Sepharose 6B was obtained from Pharmacia Biotechnology Inc. [*ethyl*-1-¹⁴C]NEM (1.4 GBq/mmol) was a product of Du Pont–New England Nuclear. *trans*-Benzenedihydrodiol was synthesized according to the method of Platt and Oesch [35]. All other chemicals were of the highest grade commercially available.

Enzyme activity measurement

The measurement of dehydrogenase activity was performed photometrically by monitoring the increase in the absorbance at 340 nm ($\epsilon = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$) with a double-beam Shimadzu UV-160A spectrophotometer. Assays were conducted at 25 °C in a 1-cm pathlength cuvette (1.0 ml final volume) in 100 mM glycine/NaOH buffer, pH 9.5, containing 67 μM NADP⁺ and 1.0 mM 1-acenaphthenol as coenzyme and substrate respectively. The enzyme assay was initiated by the addition of enzyme to the

assay mixture. One unit of enzyme activity was defined as 1 μmol of NADPH formation/min at 25 °C. Solutions of coenzyme and substrate were prepared daily.

Enzyme purification

Bovine liver DD was purified by the method previously reported [21] using 1-acenaphthenol as a substrate. Protein concentration was determined by the dye-binding method of Bradford [36]. Purified enzyme was identified as DD3 on the basis of chromatographic behaviour, substrate specificity, relative molecular mass and immunological cross-reaction with anti-DD3 antibody. Specific activity of the purified enzyme was estimated to be 1.1 $\mu\text{mol}/\text{min}$ per mg of protein.

Effect of SH-reagents on DD activity

Prior to the treatment with SH-reagents, DD3 was reduced by the incubation with 10 mM DTT at 25 °C for 30 min and then gel-filtered for the elimination of DTT by Nick-column equilibrated with 100 mM Tris/HCl buffer, pH 8.0, including 1 mM EDTA (buffer B), saturated with nitrogen gas. All of the SH-reagents and protectors were dissolved in buffer B. Reduced enzyme (1.5 μM) was then incubated with appropriate concentrations of SH-reagents in the absence or presence of protectors in buffer B at 25 °C. Aliquots of the reaction mixture were taken out for the measurement of enzymic activity at appropriate intervals.

Chromatography on thiopropyl-Sepharose 6B

DD3 reduced under the same conditions in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (buffer C) was chromatographed on thiopropyl-Sepharose 6B under the following conditions: (1) Reduced enzyme solution (100 μl) was applied to the column (0.4 cm $\times$ 2 cm) equilibrated with buffer C and the column was washed with 1 ml of buffer C followed by 1 ml of buffer C containing 100 mM 2-mercaptoethanol (2-ME). The adsorbed fraction was then eluted with buffer C with 1 M 2-ME. (2) The enzyme solution in 5 mM NADP⁺ was applied to the same column equilibrated with buffer C containing 5 mM NADP⁺, and then the elution was performed under the same conditions as (1) using buffer C which also contained 5 mM NADP⁺. (3) The enzyme was incubated with 0.1 mM NEM and 1 mM NADP⁺ at 25 °C for 30 min and then applied to the same column. Collected eluants of each fraction (100 $\mu\text{l}/\text{fraction}$) were assayed for enzymic activity.

Autoradiography

Reduced DD3 was incubated with 0.1 mM [¹⁴C]NEM in the absence or presence of 1 mM NADP⁺ at 25 °C for 15 min. The enzyme was also incubated with 0.1 mM DTNB for 15 min and then incubated with [¹⁴C]NEM. After treatment with NEM, the enzyme preparations were treated with 50 mM DTT to remove the excess SH-reagents. Each sample (0.2 μg) was analysed by SDS/12.5 %-PAGE.

Incorporation of [¹⁴C]NEM into DD3

Reduced DD3 (0.7–0.4 nmol/250 μl) was incubated with 0.1 mM [¹⁴C]NEM in the absence or presence of NADP⁺ (1 mM) in buffer B at 25 °C for 40 min. After the incubation, the enzyme solution was gel-filtered by Nick-column equilibrated with buffer B containing 1.5 M NaCl and then concentrated. The protein

concentration of each sample was determined at the end of the experiments and radioactivity was measured with an Aloka liquid scintillation system LSC-700.

Identification of cysteine residues modified with NEM

Reduced DD3 (5–6 nmol) was treated with 0.1 mM [^{14}C]NEM in the absence or presence of 1 mM NADP $^{+}$ in buffer B at 25 °C for 1 h. After gel filtration to remove excess [^{14}C]NEM, the enzyme was digested with lysyl endopeptidase (1/200, w/w) in buffer B containing 0.1% SDS for 18 h at 37 °C. The separation of digested peptides was performed on a Waters C18- μ Bondasphere column (3.9 mm $\times$ 150 mm) linked to a Gilson gradient HPLC system. Peptides were eluted with a linear gradient of 0–60% acetonitrile in the presence of 0.1% trifluoroacetic acid (with a flow rate of 1 ml/min over 120 min), monitoring the absorbance at 220 nm. Each peak was collected and then their ^{14}C radioactivity measured. The same results of the peptide mapping were obtained using unlabelled NEM under the same conditions. The peptide sequencing was carried out using a Shimadzu PSQ-1 automatic protein sequencer. PTH-NEM-modified cysteine was identified by the comparison with non- and NEM-modified bovine pancreas insulin as a standard. Pancreatic insulin (15 nmol) was dissolved in water containing both 10 mM DTT and 1% SDS, and then incubated at 25 °C for 2 h to reduce Cys-Cys bonds. Then, the separation of the α -chain and β -chain of insulin was performed with reverse-phase HPLC under the same conditions as described above. Each fraction was lyophilized and dissolved in buffer B. After incubation with or without 0.1 mM NEM for 1 h at 25 °C, each sample was subjected to re-chromatography with reverse-phase HPLC to remove excess NEM. Peak fractions were collected, lyophilized and the amino acid sequence of each was determined.

RESULTS

Inactivation by NEM

All modifications of enzyme by SH-reagents were performed in Tris/HCl buffer, pH 8.0. Modification by NEM, DTNB and iodoacetic acid clearly showed that DD3 is extremely sensitive to SH-reagents (Table 1). Only DTNB-induced inactivation of DD3 was reversible, i.e. DTNB-inactivated enzyme (remaining activity 10–20%) was effectively reactivated (> 90%) by incubation with 10 mM DTT for 30 min (results not shown). When DD3 was preincubated with DTNB, [^{14}C]NEM was not incorporated into the enzyme molecule (Figure 1, lane 2). These results suggested that both NEM and DTNB modified the same cysteine residue(s) in the enzyme molecule. NEM-induced inactivation of DD3, as a function of incubation time or concentration, as indicated in Figure 2. Estimated pseudo-first-order rate constants (k_{app}) were obtained from tangents of each line. Kinetic constants for the inactivation calculated by the method of Kitz and Wilson [38] were $K_1 = 1.3 \times 10^2 \mu\text{M}$, $k_{+2} = 1.2 \times 10^{-3} \text{ s}^{-1}$, where K_1 is the dissociation constant for the E·I

Figure 1 Incorporation of [^{14}C] NEM into DD3

The reduced enzyme, which is prepared as described in Table 1, was treated with 0.1 mM [^{14}C]NEM in the absence (lane 1) or presence (lane 3) of NADP $^{+}$ for 15 min at 25 °C in 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA. After pre-incubation with 0.1 mM DTNB for 15 min at 25 °C, the enzyme was further treated with [^{14}C]NEM under the same conditions (lane 2). Then, the enzyme preparations were treated with 50 mM DTT to remove the excess SH-reagents and each sample (0.2 μg) was subjected to SDS/12.5%-PAGE analysis.

Figure 2 Inactivation of DD3 by NEM

Reduced enzyme was incubated in 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA at 25 °C in the presence of increasing concentrations of NEM: ●, 0 μM ; ○, 25 μM ; ■, 50 μM ; □, 75 μM and ▲, 100 μM . Aliquots (10–20 μl) were taken from the reaction mixture and subjected to the standard (1.0 ml) assay for determination of the remaining activity. Rate constants for the inactivation reaction were calculated by drawing tangents to each progress line. Apparent pseudo-first-order rate constants were replotted against the concentration of NEM to yield the double-reciprocal plots for the inactivation of DD3 by NEM (insert of Figure).

complex and k_{+2} is the limiting rate constant for the formation of E·I complex (insert of Figure 2).

Protection from NEM-modified inactivation

To better understand the inactivation mechanism, the protective effects of coenzyme, substrates and their analogues on the NEM-inactivation of DD3 were further studied. The inactivation of DD3 by NEM was almost completely protected against by NADP $^{+}$ and 3-APADP, a coenzyme analogue (Table 2). On the other hand, substrates including *trans*-benzenedihydrodiol and 1-acenaphthenol did not prevent the inactivation of DD3 at all. As reported earlier, DD3 displayed an ordered Bi Bi mechanism in which pyridine nucleotide binds first and leaves last [26]. This suggests that substrates will only gain access to the active site if the E·NADP $^{+}$ complex exists. Considering the kinetic mechanism, the results from protective effects indicated that cysteine

Table 1 Inactivation of DD3 by GSSG and protection by NADP $^{+}$

Reagent	Concentration (mM)	Remaining activity (%)
No addition		98 $\pm$ 3.1
NEM	0.1	28 $\pm$ 2.0
DTNB	0.05	14 $\pm$ 0.9
Iodoacetic acid	5	44 $\pm$ 2.8

Table 2 Protection from *N*-ethylmaleimide-modified inactivation of DD3 by coenzymes and their analogues

Reduced enzyme was prepared as described in Table 1. The enzyme was incubated with 0.1 mM *N*-ethylmaleimide in the presence or absence of 0.5 mM protector for 15 min at 25 °C in 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and then the enzyme activity was measured. The values are means $\pm$ S.D. ($n = 3$). Abbreviations: 3-APADP, 3-acetylpyridine adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide.

Protector	Remaining activity (%)
No addition	28 $\pm$ 2.0
NADP ⁺	98 $\pm$ 2.6
NAD ⁺	40 $\pm$ 1.2
3-APADP	85 $\pm$ 2.7
NMN	38 $\pm$ 1.1
2',5'-ADP	76 $\pm$ 2.0
2'-AMP	71 $\pm$ 1.4
5'-ADP	35 $\pm$ 3.5
5'-AMP	24 $\pm$ 0.3

Figure 3 Elution profile of DD3 on thiopropyl-Sepharose 6B column chromatography

Reduced enzyme solution (100 μ l) was applied to the column (0.4 cm $\times$ 2 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, and washed with 1 ml of the buffer and 1 ml of 100 mM 2-ME. The adsorbed fraction was eluted with 1 M 2-ME (a). In the same experiments, the enzyme with 5 mM NADP⁺ was applied to the column equilibrated with the same buffer including 5 mM NADP⁺ (b) and pre-treated enzyme with both 0.1 mM NEM and 1 mM NADP⁺ at 25 °C for 30 min was applied to the column (c).

residue(s) might be located near the coenzyme-binding site of DD3. Additionally, both 2'-AMP and 2',5'-ADP showed a significant protective effect on the inactivation, but NAD⁺ (50-fold higher K_m value than that of NADP⁺), 5'-AMP, 5'-ADP and NMN did not have any protective abilities due to their lower affinities for the enzyme (Table 2), suggesting the 2'-adenylate moiety is essential for DD3 activity. Additionally, DD3 was also inactivated with 5 mM GSSG or 1 mM cystine to 58.3 or 50.9% remaining activity and protected in the presence of 1 mM NADP⁺ from inactivation (92.7 or 96.6% respectively).

Thiopropyl-Sepharose 6B column chromatography

Although the inactivation of DD3 through cysteine modification was protected against by NADP⁺, [¹⁴C]NEM was still incorporated into DD3 in the presence of NADP⁺ (Figure 1, lane 3). The incorporation of [¹⁴C]NEM into DD3 in the presence of NADP⁺ was clearly lower than that in the absence of NADP⁺ (Figure 1, lanes 1 and 3). The reduced enzyme (intact) was

Figure 4 Isolation of lysyl endopeptidase-digested peptides of DD3 labelled with [¹⁴C]NEM

Reduced enzyme was incubated with 0.1 mM [¹⁴C]NEM in 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA for 1 h at 25 °C in the absence (a and b) or presence (c and d) of NADP⁺. Excess [¹⁴C]NEM was removed by gel filtration equilibrated with 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and the enzyme was subjected to lysyl endopeptidase digestion (1/200, w/w) for 18 h at 37 °C. Digested peptides were subjected to peptides mapping on a C18- μ Bondasphere column in a linear gradient of 0–60% acetonitrile including 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min (0–10 min, 0% and 10–130 min, 0–60%) (a and c). Peptides were detected by their UV absorbance at 220 nm. Every peak was collected and then measured for ¹⁴C radioactivity (b and d).

completely adsorbed on a thiopropyl-Sepharose 6B column equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and was eluted by 1 M 2-ME (Figure 3a). When DD3 with 5 mM NADP⁺ was applied to the column equilibrated with buffer C containing 5 mM NADP⁺, DD3 was also adsorbed to the column (Figure 3b). The existence of NADP⁺ at a saturated concentration to form the E · NADP⁺ binary complex did not show any different elution profile on thiopropyl-Sepharose 6B column chromatography. However, the enzyme modified by NEM in the presence of NADP⁺, retaining its original activity, passed through that column completely (Figure 3c). These results suggested the presence of at least two distinct cysteine residues modified by NEM in the DD3 molecule: one located near the NADP⁺-binding domain and the other present on the surface of the DD3 molecule. The latter cysteine possibly interacts with the thiopropyl-Sepharose 6B column.

Figure 5 Identification of NEM-modified cysteine

(a) This shows the chromatogram of the tenth cycle resulting from the sequence analysis of P1 indicated in Figure 5. (b) and (c) show the chromatograms of the seventh cycle resulting from the sequence analysis of bovine pancreas insulin (β -chain) untreated and treated with NEM (see the Material and methods section) respectively. An arrow points to PTH-NEM-cysteine which appears as a duplex peak at the retention time of around 10 min.

Incorporation of NEM into DD3

To estimate the number of NEM molecules incorporated into DD3, radioactivity of DD3 treated with 0.1 mM [14 C]NEM for 40 min at 25 °C was determined. Based on both radioactivity incorporated into DD3 and estimated relative molecular mass (35 500), 2.23 ± 0.05 mol of NEM/mol of enzyme in the absence of NADP $^{+}$ and 1.26 ± 0.05 mol of NEM/mol of enzyme in the presence of NADP $^{+}$ were incorporated. These results indicated that one cysteine residue in DD3, among the two residues which could be modified by NEM, might be protected by NADP $^{+}$.

Identification of NEM-modified cysteine residues

In order to identify the modified cysteine residues in DD3, the peptide was sequenced. First of all, the separation of [14 C]NEM-labelled peptides of DD3 after digestion by lysyl endopeptidase was attempted with reverse-phase HPLC and the radioactivities in all peaks were determined. When NEM-inactivation of DD3 was performed in the absence of NADP $^{+}$, two radioactive peaks were obtained at the retention times of 81 and 95 min, namely P1 and P2 respectively (Figures 4a and 4b). However, only one radioactive peak was observed at the retention time of 95 min in the presence of NADP $^{+}$ (Figures 4c and 4d). The two radio-labelled peaks were isolated, lyophilized and then subjected to the peptide sequence analyses. α - and β -chains of bovine pancreas insulin were used as standards to detect NEM-modified cysteines. As shown in Figure 5, the peaks of NEM-modified cysteine were detected with duplex peaks at a retention time of 10 min. These duplex peaks might be identified as NEM-modified cysteine which formed a diastereomer during the modification by NEM. Sequences P1 and P2 were as follows: P1, H $_2$ N-Tyr-Lys-Pro-Val-Xaa-Asn-Gln-Val-Glu-NEM-Cys-His-Pro-Tyr-Phe-Asn-Gln-Ser-Lys-COOH; and P2, H $_2$ N-Ser-Leu-Asn-Asn-Leu-Gln-Leu-Asp-Tyr-Val-Asp-Leu-Tyr-Ile-Ile-His-Phe-Pro-Val-Ala-Leu-Lys-Pro-Gly-Glu-Thr-Leu-Phe-Pro-Thr-Asp-Glu-Asn-Gly-Lys-Pro-Ile-Phe-Asp-Ser-Val-Asp-Leu-NEM-Cys-. As described in the sequence data, NEM-Cys residues were detected in the P1 and P2 at the position of 10 and 44, respectively. Xaa in P1 means undetectable amino acid, possibly a cysteine.

Sequence similarities

These sequences were compared with those of rat 3 α -HSD/DD, bovine lung PGFS and human and bovine aldose reductases, which are members of the aldo-keto reductase superfamily. P1 showed great similarities with the partial sequences of rat 3 α -HSD/DD (residues 184 to 201) [13], bovine lung PGFS (183–200) [14], human placenta aldose reductase (178–195) [16] and bovine lens aldose reductase (177–194) [17] as shown in Figure 6. Peptide P1 was highly conserved among many aldo-keto reductases and similar to the corresponding regions having an important role in nucleotide binding. Moreover, the peptide sequence of P1 clearly showed that only one residue was modified by NEM, even though the other cysteine residue was in the vicinity. The cysteine residue modified by NEM in P1, which may be a corresponding residue to Cys-193 of rat 3 α -HSD/DD, was also conserved in all enzymes listed in Figure 6. Additionally, the other peptide, P2, also had homology with the regions 102–145 of rat 3 α -HSD/DD [13] and region 101–144 of bovine lung

DD3 (P1)		Tyr-Lys-Pro-Val- X -Asn-Gln-Val-Glu-Cys [*] -His-Pro-Tyr-Phe-Asn-Gln-Ser-Lys	
PGFS	183	Tyr-Lys-Pro-Val-Cys-Asn-Gln-Val-Glu-Cys-His-Pro-Tyr-Leu-Asn-Gln-Ser-Lys	200
3 α -HSD/DD	184	Tyr-Lys-Pro-Val-Cys-Asn-Gln-Val-Glu-Cys-His-Leu-Tyr-Leu-Asn-Gln-Ser-Lys	201
Human AR	178	Tyr-Lys-Pro-Ala-Val-Asn-Gln-Val-Glu-Cys-His-Pro-Tyr-Leu-Asn-Gln-Ser-Lys	195
Bovine AR	177	Tyr-Lys-Pro-Ala-Val-Asn-Gln-Val-Glu-Cys-His-Pro-Tyr-Leu-Asn-Gln-Ser-Lys	194

Figure 6 Comparison of sequences of P1 and aldo-keto reductases

The sequence of P1, which was obtained from the chromatography of Figure 5, was compared with the partial amino acid sequences of PGFS (bovine liver prostaglandin F synthase) [14], 3 α -HSD/DD (rat liver 3 α -hydroxysteroid/dihydrodiol dehydrogenase) [13], human AR (human placenta aldose reductase) [16] and bovine AR (bovine lens aldose reductase) [17]. X means undetectable amino acid. Bold-typed Cys with asterisk in P1 shows the cysteine modified by NEM. Exact matches are signified with a vertical line.

PGFS [14]. Modified cysteine in P2, which may be a residue corresponding to Cys-145 of rat 3α -HSD/DD, was also conserved in both rat 3α -HSD/DD and bovine lung PGFS.

DISCUSSION

Like other enzymes whose activity is modulated by biological disulphides through thiol/disulphide exchange reactions, i.e. phosphofructokinase [39], pyruvate kinase [40], glutathione S-transferase π [41] and rat 3α -HSD/DD [12], DD3 also possesses a cysteine residue which may be an important residue for its activity. On the other hand, the GSH concentration in rat hepatocytes decreases during metabolism of benzo[a]pyrene [42], suggesting that activity of DD3 may be modulated through the oxidation of a cysteine residue under oxidative stress produced by disulphides, radical formation or lipid peroxidation. Additionally, it has also been reported that the proteins which have an accessible thiol group(s) for activity are modified with disulphide to form mixed disulphides under oxidative stress, as reported previously [43–45]. These reports indicate that the mixed disulphides could be an important regulation system of protein functions. While some questions about the relationship between oxidative stress and DD activity *in vivo* remain, it has been shown the mixed disulphides between protein and low-molecular-mass disulphides increase under oxidative stress [46]. In our previous studies [21,25], we suggested that DD3 is effectively inactivated by biological disulphides coincident with the incorporation of disulphides and easily reactivated by thiol compounds the same as rat 3α -HSD/DD [12], suggesting that DD3 activity is also modulated through a thiol/disulphide exchange reaction.

In the present study, we report that bovine novel dihydrodiol dehydrogenase (DD3) is extremely sensitive to SH-reagents (DTNB, iodoacetic acid and NEM) and is protected very effectively by nucleotides which have 2'-phosphate groups, and not by those with no 2'-phosphate group, from NEM-inactivation, suggesting the 2'-adenylate moiety is essential for the nucleotide binding with DD3. This is similar to the results with rat 3α -HSD/DD in our previous report [12]. In addition, DD3 is also inactivated with biological disulphides in a similar manner to NEM (data not shown), as was rat 3α -HSD/DD [12]. When DD3 was modified with radiolabelled NEM, two radiolabelled peptides were obtained following lysyl endopeptidase digestion of DD3 (P1 and P2). Of these radiolabelled peptides, P1 may retain enzymic activity and coenzyme-binding, considering the recent reports of the structural analyses of aldo-keto reductases [47,48], namely that the peptide sequence of P1 is highly conserved in $\alpha 8/\beta 8$ barrel motifs, suggesting a novel motif for nucleotide binding in the aldo-keto reductase superfamily, e.g. in rat 3α -HSD/DD [13], bovine lung PGFS [14] and human placenta and bovine lens aldose reductases [16,17]. The region of rat 3α -HSD/DD (residues 184 to 201) corresponding with P1 is located in and near strand $\beta 6$ from crystallographic studies [49]. On the other hand, peptide P2 of DD3 may be located on the surface, without significant function for enzymic activity. Penning et al. have reported that when cysteines which contact C-3, C-11 and C-17 of the steroids' nucleus were affinity-modified with [14 C]bromoacetoxysteroid derivatives [50], Cys-170, Cys-217 and Cys-242 were identified [33]. Additionally, the results of site-directed mutagenesis and crystallographic studies suggest that only Cys-217 can be involved in either steroid and/or coenzyme binding and both Cys-170 and Cys-242 are located on helices $\alpha 5$ and H1 of an α/β barrel motif which is located on the exterior of the protein [34]. The study of site-directed mutagenesis also

shows that Cys-298 of aldose reductase may be an essential for substrate- and sorbinil-bindings [51–54]. However, the deduced amino acid sequence of DD3 cDNA clone (H. Nanjo, H. Adachi, M. Aketa, T. Mizoguchi, T. Nishihara and T. Terada, unpublished work) suggests that DD3 has five cysteine residues in its molecule and lacks four residues including Cys-217 of the nine cysteines of rat 3α -HSD/DD and five residues including Cys-298 of aldose reductase. This means the NEM-modifications of residues corresponding to these cysteines should be ruled out. Though the partial amino acid sequences of P1 and P2 showed great similarities with two regions of rat 3α -HSD/DD (residues 184–201 and 102–145, respectively), there may be some differences in the primary structure (about 70% similarity with rat 3α -HSD/DD). It is easy to understand that some difference in structure of DD3 from that of rat 3α -HSD/DD leads to the different behaviour of DD3 to SH-reagents from that of rat 3α -HSD/DD. Sequence analysis of P2, which has the alkylated cysteine, shows a similarity to the partial sequence of rat 3α -HSD/DD (residues 102–145) [13] or bovine lung PGFS (residues 101–144) [14], but relatively little to those of aldose reductases [51–54]. We speculated that the cysteine residue in P2 which has little effect on the enzymic activity may be located on the surface of the enzyme. This speculation can be strongly supported by the results of the column chromatography on thiopropyl-Sepharose 6B as shown in Figure 3. The results of the three-dimensional structure, modelled with the program Homology based on the deduced amino acid sequence of DD3 cDNA, suggest that the cysteine residues conserved in rat 3α -HSD/DD, i.e. Cys-145 or Cys-193 of DD3, may be located on the surface or positioned near the barrel (similar to Cys-217 of rat 3α -HSD/DD), respectively. This result suggests that Cys-193 of DD3 behaves in a similar manner to Cys-217 of rat 3α -HSD/DD in the catalytic process, i.e. through the modulation of NADP $^{+}$ binding due to its modification.

In summary, we would like to postulate that of the two cysteines modified with NEM which have been identified, one cysteine residue of DD3, being modified with NEM only in the absence of coenzyme, may be involved in the alteration of DD3 activity through a thiol/disulphide exchange reaction.

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