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Journal of Steroid Biochemistry & Molecular Biology 88 (2004) 393–398
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

3α/β,20β-Hydroxysteroid dehydrogenase (porcine testicular carbonyl reductase) also has a cysteine residue that is involved in binding of cofactor NADPH

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Received 28 August 2003; accepted 29 December 2003

Abstract

Besides residue of the catalytic triad that is conserved in the short-chain dehydrogenase/reductase (SDR) superfamily, a Cys side chain reportedly plays functional roles in NADP-dependent 15-hydroxyprostaglandin dehydrogenase and human carbonyl reductase (CR). The three-dimensional structure of porcine 3α/β,20β-hydroxysteroid dehydrogenase, also known as porcine testicular carbonyl reductase, demonstrates the proximity of the Cys 226 side chain to the bound NADP. However, no clear explanation with respect to the basis of the catalytic function of the Cys residue is yet available. By chemical modification, point mutation, and kinetic analysis, we determine that two Cys residues, Cys 149 and Cys 226, are involved in the enzyme activity. Furthermore, we found that pretreatment with NADP markedly protects the enzyme from inactivation by 4-(hydroxyl mercury) benzoic acid (4-HMB), thereby confirming that Cys 226 is involved in binding of the cofactor. On the basis of the tertiary structure of 3α/β,20β-HSD, the possible roles of Cys residues, especially that of Cys 226, in enzyme action and in the binding of cofactor NADPH are discussed.

Keywords: Short-chain dehydrogenase/reductase; 3α/β,20β-Hydroxysteroid dehydrogenase; Chemical modification; Carbonyl reductase; Cysteine residue

1. Introduction

Porcine 3α/β,20β-hydroxysteroid dehydrogenase (3α/β,20β-HSD), also known as porcine testicular carbonyl reductase, metabolizes androgens and progestins. The enzyme was first isolated from neonatal pig testes, suggesting that 3α/β,20β-HSD may regulate steroid hormone concentrations in the testes during development [1–3]. Later studies revealed that porcine 3α/β,20β-HSD is expressed in many other tissues including the kidney, liver, brain, lung, thymus, heart, spleen, and adrenal gland, and belongs to the short-chain dehydrogenase/reductase (SDR) superfamily [4,5]. The wide distribution of 3α/β,20β-HSD suggested a broader role for the enzyme in steroid hormone metabolism. Interestingly, porcine 3α/β,20β-HSD shares a high degree of amino acid homology with human, rat, mouse, and rabbit monomeric carbonyl reductases (CRs) [6–9]. However, porcine 3α/β,20β-HSD contains 12 amino acids at its carboxyl-terminus that are absent in other mammalian monomeric carbonyl reductases. Nearly all members of the SDR superfamily possess a triad of amino acids—Tyr, Lys, and Ser—that has been identified as catalytically important [10]. The same catalytic triad is also present in 3α/β,20β-HSD and the Tyr and Lys side chains are involved in the catalytic reaction [11]. On the other hand, a Cys residue, not conserved in the SDR superfamily but present in both mammalian NAD-dependent 15-hydroxyprostaglandin dehydrogenase and human carbonyl reductase (also known as 15-hydroxyprostaglandin dehydrogenase/9-ketoprostaglandin reductase) reportedly influences the enzyme activity and reacts with organomercurials [12,13]. The sequence homology between 3α/β,20β-HSD and human carbonyl reductase is estimated to be about 85% [4]. The tertiary structure of 3α/β,20β-HSD has been determined recently, revealing structural features of a mammalian carbonyl reductase, the first monomeric member of the SDR superfamily [14]. The structure showed the presence of the Cys 226 side chain in close proximity to the nicotinamide ring of the bound NADP molecule.

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doi:10.1016/j.jsbmb.2003.12.013

In this paper, we report the results of modification of the reactive Cys residues in porcine 3α/β,20β-HSD by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and 4-(hydroxyl mercury) benzoic acid (4-HMB), and discuss the possible roles of Cys 226 in the action of the enzyme and in the binding of cofactor NADPH.

2. Materials and methods

2.1. Chemicals

4-Nitrobenzaldehyde was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Menadione, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 4-(hydroxyl mercury) benzoic acid, sodium salt (4-HMB) and isopropyl-β-D-(−)thiogalactopyranoside (IPTG) were obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). NADPH, $NADP^+$, NAD^+, and various nucleotides were obtained from Sigma–Aldrich Japan (Tokyo, Japan). DE-52 was purchased from Whatman International Ltd. (Maidstone, UK). All other reagents were of the highest commercially available grade.

2.2. Expression and isolation of 3α/β,20β-HSD mutants

Site-directed mutagenesis was performed using a Mutant-Express K_m kit and the protocol (Takara Biomedicals, Tokyo, Japan) according to the oligonucleotide-directed dual amber method [3]. The following oligonucleotides were used: C25A: GAACTGCCGGGCCAGGTCCCG; C73A: CAGGAAGTCGGCCAGGGCACG; C121A: AAGCTCTGTGGCCACATTTCG; C149A: TTCAGGGCTGGCCTCGTTAAG; C225A: CCCTGGGCAGGCGGCATTCAG; C226A: CCACCCTGGGGCGCAG GCATT. All mutant 3α/β,20β-HSD were sequenced to confirm the presence of correct codon substitutions for C25A, C73A, C121A, C149A, C225A, and C226A. The vector pKK223-3 was used for the construction of different mutants and their expression in *E. coli* JM105 cells. *E. coli* cells transformed with the different vectors were seeded into LB media containing 50 μg/ml of ampicillin. IPTG was added to the media for 18 h at 37 °C to induce expression of 3α/β,20β-HSD. The *E. coli* cells were collected by centrifugation and suspended in KPB buffer (3 mM potassium, pH 7.4) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM pepstatin, 0.1 mM EDTA, and 0.1 mM DTT. The *E. coli* cells were disrupted by sonification and the homogenate was centrifuged for 60 min at 105,000 × *g*. The supernatant was dialyzed with KPB containing 0.1 mM EDTA and DTT. The enzyme was purified with DE-52 column chromatography according to the method of Nakajin et al. [15]. Wild-type 3α/β,20β-HSD and its mutants were identified in column fractions by an enzyme assay or a Western blot that is specific for 3α/β,20β-HSD [5]. The purified enzyme was concentrated with an ultrafiltration apparatus using a PM-10 diaflo-membrane (Amicon Inc., Beverly, MA, USA) and stored at −30 °C until use. The protein content of each sample was determined by BCA assay (Pierce Chemical Co., Rockford, IL, USA). Purity was checked using sodium dodecyl sulfate gel electrophoresis (SDS–PAGE) according to the method of Laemmli [16]. The molecular weight markers (molecular weight in parentheses) used for SDS–PAGE were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.3. Enzyme assay

Aldo-keto reductase activity was determined spectrophotometrically by measuring the oxidation rate of NADPH with an enzyme rate calculator apparatus (Hitachi 200-0045) attached to a spectrophotometer (Hitachi 200-20) at 37 °C. For the determination of kinetic constants for substrates, menadione, or 4-nitrobenzaldehyde (4-NBA), the assay mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 60 μM NADPH, substrate at various concentrations, and enzyme in a total volume of 1.2 ml. For the determination of kinetics for the cofactor, enzyme assays were carried out with menadione at fixed concentrations (250 μM), and the NADPH concentration was changed from 2.5 to 50 μM. The reaction was initiated by addition of NADPH to the assay mixture, and enzyme activity was calculated as nmol/min mg of enzyme from duplicate assays. Blanks without substrate or enzyme were routinely included. The substrates were dissolved in ethanol. The final concentration of the solvent in the assay mixture did not exceed 1% (v/v). The kinetic parameters were calculated from the Lineweaver–Burk plots.

2.4. Chemical modification of Cys residues

DTNB and 4-HMB were used as the thiol-modifying reagents and were dissolved in ethanol. The final concentration of ethanol in the reaction mixture did not exceed 1% (v/v). Chemical modification of Cys residues by DTNB or 4-HMB was performed by incubation of the enzyme in 100 mM potassium phosphate buffer (pH 7.4) in appropriate concentrations of DTNB or 4-HMB and at an appropriate incubation time at 37 °C (see figure legend for details).

3. Results and discussion

3.1. The location of Cys residues on the tertiary structure of 3α/β,20β-HSD

The 3α/β,20β-HSD tertiary structure has a basic SDR fold including a seven-stranded parallel β-sheet (βA to βG) flanked by three parallel helices on each side (αB, αC, αG and αD, αE, αF), except for the 41-residue insertion (four helices, αF′-1 to αF′-4) before αF and the addition of a C-terminal strand βH [14]. All six Cys residues of the enzyme are displayed on the tertiary structure shown in Fig. 1a.

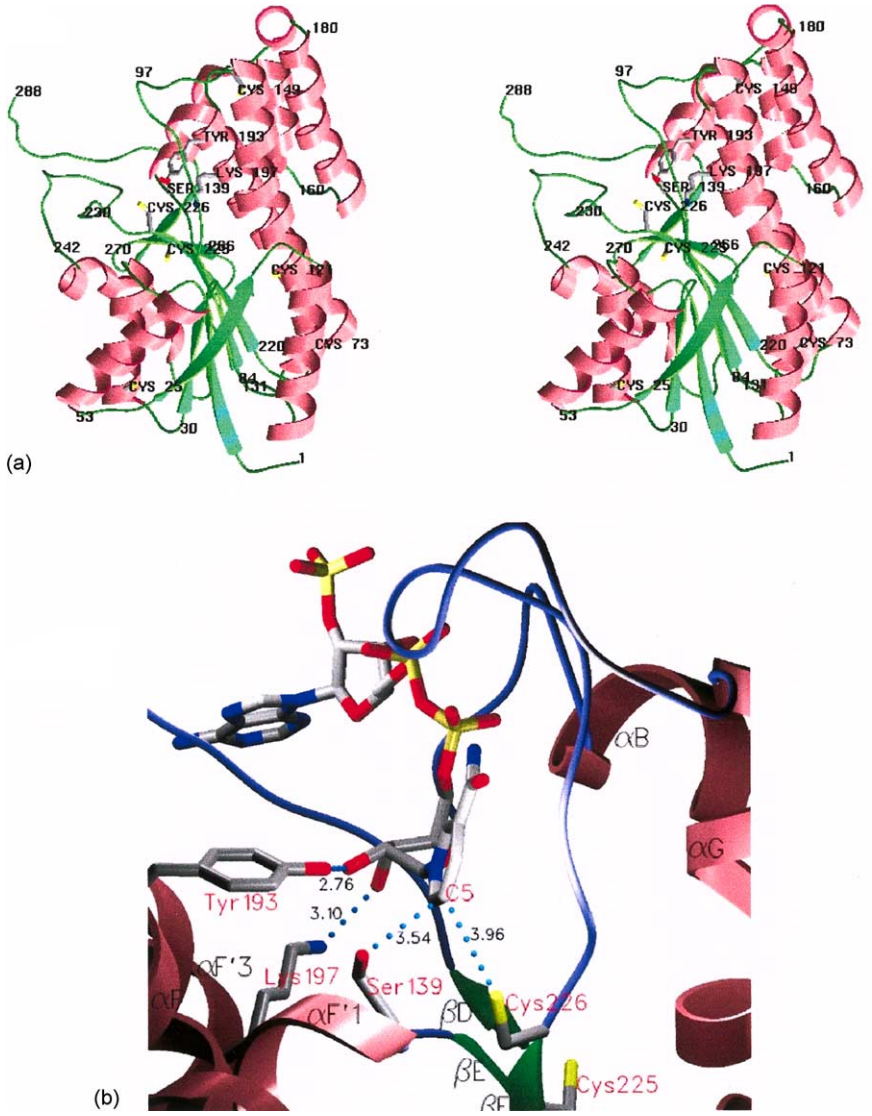

Fig. 1. A stereo view of the location of the Cys residues on the tertiary structure of 3α/β,20β-HSD (a) and a close-up view of the surrounding neighborhood of the active site (b). The α-helices are drawn in rose pink and the β-strands in green in the stereo ribbon diagram of 3α/β,20β-HSD structure derived from a previous report [14]. The atoms are color-coded as follows: carbon, gray–white; nitrogen, blue; oxygen, red; and sulfur and phosphorus, yellow. The direct contacts with the nucleotide derived from nicotinic acid are shown schematically in b, and distances are given in Å.

Residues Cys 25, Cys 73, and Cys 121 belong to helices αB, αD, and αE, respectively. Cys 149 is on the hinge part of αF′-1 between the αF′-2 strand, whereas Cys 225 and Cys 226 are on the C-terminus of βF. Residues of the catalytic triad, Ser 139, Tyr193, and Lys 197, involved in cofactor binding and catalysis, are also shown in Fig. 1a, as well as in Fig. 1b, which depicts a close-up view of the surrounding neighborhood of the nicotinamide ring of bound NADP. Fig. 1b also shows the interactions of important side chains, including Cys 225 and 226, with the nicotinamide-ribose moiety of the coenzyme.

3.2. Inactivation of 3α/β,20β-HSD by reaction with DTNB

The time-dependent inactivation of 3α/β,20β-HSD by DTNB is shown in Fig. 2. A plot of the log of residual activity against time of DTNB inactivation gives a straight line. According to the method of Levy et al. [17], a plot of log(the reciprocal of the half-time for inactivation) against log(DTNB) was used to calculate the order of the inactivation reaction. The points fit a straight line with a slope equal to 1.96, indicating that an average at least 2 mol of DTNB bind to 1 mol of 3α/β,20β-HSD when inactivation occurs.

3.3. Catalytic efficiency of the Cys to Ala mutants

To identify the cysteine residues that are involved in enzyme activity, the substituted mutants from Cys 25, 73, 121, 145, 225 and 226 to Ala were constructed and assayed the enzyme activity. Table 1 summarizes the results of enzyme assay of wild-type and all mutant enzymes with the two

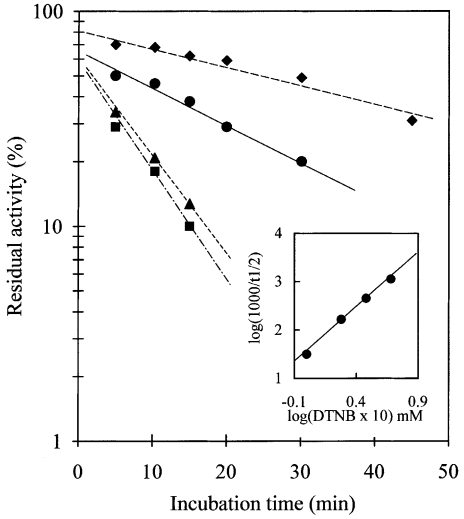

Fig. 2. Time-dependent inactivation of 3α/β,20β-HSD by DTNB. Modification of Cys residues was carried out using 12.5 μM wild-type 3α/β,20β-HSD enzyme with 0.1 mM (◆), 0.2 mM (●), 0.3 mM (▲) and 0.5 mM (■) DTNB in 100 mM Tris–glycine buffer (pH 8.3) at 37 °C. After 5–45 min, aliquots were removed from the incubation mixture and immediately assayed for enzyme activity (250 μM 4-NBA) and residual activity was determined at the indicated times. Inset is the plot used to calculate the order of the inactivation reaction.

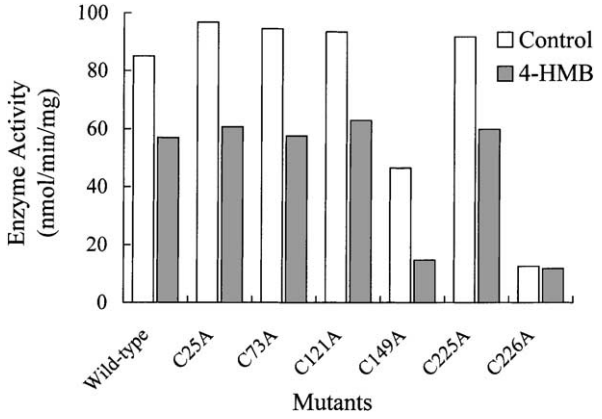

Fig. 3. Inactivation by modification of Cys residue on wild-type 3α/β, 20β-HSD and Cys to Ala mutants with 4-HMB. Wild-type 3α/β,20β-hydroxysteroid dehydrogenase and Cys to Ala mutants (3.0 μM each) were modified by 4-HMB (3.0 μM) for 3 min at 37 °C in 100 mM potassium phosphate buffer (pH 7.4). After incubation, aliquots were removed from the incubation mixture and immediately assayed for enzyme activity (250 μM menadione). Enzyme activity is expressed as the mean of two independent experiments.

different substrates, menadione and 4-NBA. The values of catalytic efficiency indicated with V_{max}/K_m of the four mutants, C25A, C73A, C121A, and C225A, are comparable with those of wild-type enzyme for the two substrates. In contrast, the catalytic efficiency of C149A for menadione and 4-NBA decreased to 19 and 42%, respectively. The catalytic efficiency of C226A for menadione and 4-NBA decreased markedly to 0.6 and 1.1%, respectively, on account of a higher K_m and lower V_{max}. The K_m value of C226A mutant for NADPH also increased 22-fold, indicating a decline in the affinity for the cofactor NADPH. These results indicate that Cys 149 and 226 are involved in the enzyme activity, and Cys 226 is strongly involved in enzyme activity.

3.4. Inactivation of the substituted mutants from Cys to Ala by reaction with 4-HMB

All substituted mutants from Cys 25, 73, 121, 149, 225, and 226 to Ala and the wild-type enzyme were incubated with 4-HMB. As shown in Fig. 3, the specific enzyme activities of the substituted mutants, C25A, C73A, C121A, and C225A, were similar to that of the wild-type enzyme, whereas the enzyme activities of the substituted mutants C149A and C226A decreased to 50 and 18%, respectively. By incubation with 4-HMB, the enzyme activities of C25A, C73A, C121A, and C225A decreased to 61–67% and C149A to 32% when compared with no 4-HMB treatment (control). However, the enzyme activity of C226A did not decrease further, that is, Cys 226 was almost insensitive to inactivation by 4HMB. These results also indicate that Cys 226 is the 4-HMB reacting residue and is involved in enzyme activity.

Table 1
Kinetic parameters of wild-type 3α/β,20β-hydroxysteroid dehydrogenase and Cys to Ala substitution mutant

Mutants	4-Nitrobenzaldehyde			Menadione			NADPH (K_m (μM))
	K_m (μM)	V_{max} (nmol/min mg)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min mg)	V_{max}/K_m	
Wild-type	12	94	7.8	13	100	7.7	19
C25A	11	142	12.5	12	133	11.1	15
C73A	14	133	9.5	9	125	13.9	16
C121A	10	104	10.4	9	95	10.6	13
C149A	10	33	3.3	33	50	1.5	21
C225A	9	67	7.4	12	80	6.7	15
C226A	24	2	0.083	1580	8	0.005	413

Enzyme activity was determined spectrophotometrically at 340 nm and 37 °C. To estimate the kinetic constant for substrate, the assay mixture consisted of enzyme sample and substrate, 20–100 μM 4-nitrobenzaldehyde and menadione, in 100 mM potassium phosphate buffer (pH 7.4). The enzyme reaction was initiated by the addition of 60 μM NADPH. To estimate the kinetic constant for cofactor, the assay mixture consisted of enzyme sample and substrate, 250 μM menadione in 100 mM potassium phosphate buffer (pH 7.4), and the reaction was initiated by the addition of 10–50 μM NADPH.

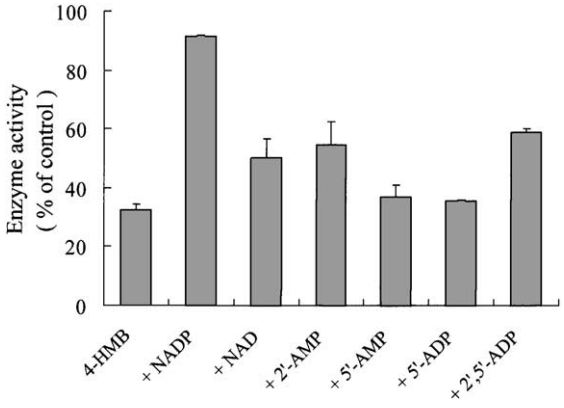

Fig. 4. Protection effect of cofactor and its analogues or substrate against 4-HMB treatment for wild-type 3α/β,20β-HSD. Wild-type enzyme (3.0 μM) was modified with 4-HMB (6.0 μM) in 100 mM potassium phosphate buffer (pH 7.4) in the absence or presence of $NADP^+$, its analogues (3.0 μM each) for 3 min at 37 °C. After incubation, aliquots were removed from the incubation mixture and immediately assayed for enzyme activity (250 μM menadione). Enzyme activity is expressed as % of control with ±S.E. from three independent experiments. Control means the vehicle instead of 4-HMB.

3.5. Protection effect of cofactor and its analogs against inactivation by 4-HMB

Protection of the enzyme against inactivation by 4-HMB was examined by pretreatment of cofactor and its analogue (Fig. 4). The enzyme activity decreased to 33% by incubation with 4-HMB. Pretreatment of $NADP^+$ markedly protected the inactivation by 4-HMB (86.5%). An analog of NADP, NAD^+, and the nucleotides 2′-AMP and 2′,5′-ADP also protected against the inactivation by 4-HMB (50.3, 54.5, and 58.6%, respectively). However, the nucleotides 5′-AMP and 5′-ADP afforded little protection against the inactivation by 4-HMB. These results suggest that the reactive Cys 226 is closely involved in the binding of the cofactor NADPH.

It has been reported that apart from a triad of Tyr, Lys, and Ser residues, a Cys residue, which is not conserved in the SDR superfamily but is present in NAD-dependent 15-hydroxyprostaglandin dehydrogenase and human CR (15-hydroxyprostaglandin dehydrogenase/9-ketoprostaglandin reductase) is involved in enzyme activity [12,13]. However, no clear explanation with regard to its functional role has yet been provided. By site-directed mutagenesis and inactivation of mutants by 4-HMB, Wermuth and coworkers reported that human CR is highly susceptible to inactivation by organomercurials, suggesting the presence of reactive Cys residues in, or close to, the active site, and that Cys 227 (Cys 226 in our numbering) was the most reactive residue. However, at that time, the location of reactive Cys and the functional role of a reactive Cys residue were speculative for lack of a tertiary structure. Recently, the tertiary structure of 3α/β,20β-HSD, which is highly homologous to human CR, has been elucidated and the precise location of the Cys residue has been determined.

In this study, we have investigated whether reactive Cys residues involved in enzyme activity are present in 3α/β,20β-HSD. By performing chemical modification with DTNB, kinetic analysis of the Cys-to-Ala mutant enzymes and inactivation of the Cys-to-Ala mutants by 4-HMB, we elucidated that two of the six Cys residues are involved in the enzyme activity. DTMB and 4-HMB are specific chemical modification reagents for Cys residues. There is a difference in reaction mechanism. We used the both reagents to find out more information for functional Cys residues.

One of the two, Cys 149, is located at the hinge part of αF′-1 between the αF′-2 strand. Its side chain sticks into the interior of the all-helix sub-domain such that the sulfur atom has several hydrophobic contacts with residues 188, 187, 146, and 145 [14]. Substituting Cys by Ala could thus interfere with the packing of this sub-domain against helices E and F by destabilizing helix F′-1. As the N-terminus of the helix F′-1 is close to the active site in proximity to Ser 139 and Tyr193, the enzyme activity may thus be affected. The other Cys residue is Cys 226, which is at the C-terminus of βF, and close to the active site. Its side chain protrudes into the active site that contains the triad of a Tyr, a Lys, and a Ser. In contrast, the side chain of Cys 225 faces the opposite side of the strand, away from the active site. As shown in Fig. 1b, the Cys 226 sulfur atom is virtually at a van der Waals contact distance from C5 of the nicotinamide ring, as is the hydroxyl oxygen of Ser 139. This architecture probably helps to stabilize the nicotinamide ring in a proper orientation so that the hydride at C4 can be transferred. We also found that pretreatment of $NADP^+$ markedly protected the inactivation by 4-HMB. This result also suggests that Cys 226 has an important role in binding of the cofactor.

A few other SDRs, such as 17β-HSD type 1, has a Cys residue in a similar position. Furthermore, this Cys residue, Cys 185, was labeled with a mercury compound and the enzyme derivative was used to determine the tertiary structure of 17β-HSD type 1 [18]. By superimposition of the three-dimensional structures, we find that Cys 185 of 17β-HSD type 1 corresponds to Cys 226 of 3α/β,20β-HSD. However, Cys 185 is not close enough to the cofactor to have contact with the nicotinamide ring, and thus, may have little influence in the cofactor binding. Therefore, the role of Cys 226 described in this paper could be unique to NAD-dependent 15-hydroxyprostaglandin dehydrogenase, human carbonyl reductase, or 3α/β,20β-HSD.

Acknowledgements

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan. DG is supported in part by National Institutes of Health, USA, grant GM62794.

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