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I269S Mutation in Horse Liver Alcohol Dehydrogenase S Isoenzyme and its Reactivity for Steroids and Retinoids

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Ile-269 in horse liver alcohol dehydrogenase isoenzyme S(HLADH-S) was mutated to serine by phosphorothioate-based site-directed mutagenesis in order to study the role of the residue in coenzyme binding. The specific activity of the mutant(I269S) enzyme to ethanol was increased 49-fold. All turnover numbers of I269S enzyme toward 9 primary alcohols were increased. The mutant enzyme showed 3.6, 4.6, 11.6-fold higher catalytic efficiency for 5β-androstane-3,17-dione, 5β-cholanic acid-3-one and retinal than wild-type, respectively. The reaction mechanism of I269S enzyme was ordered bi bi as wild-type's. These results indicate that the hydrophobic interaction of Ile-269 residue with coenzyme plays an important role in dissociation of coenzyme from enzyme-coenzyme complex, which has been known as the rate limiting step of ADH reaction.

Key words : I269S, Horse liver alcohol dehydrogenase S isoenzyme, Site-directed mutagenesis, Coenzyme binding site, Steroids, Retinoids

INTRODUCTION

Alcohol dehydrogenase (ADH) is a well-characterized enzyme (Eklund and Brändén, 1987). The wide substrate specificities of mammalian ADHs suggest a general function in detoxification of alcohols. It has also been suggested that ADH is an enzyme with multiple functions involved in the transformation of many alcohols and aldehydes of physiological interest (Boleda *et al.*, 1993). Horse liver alcohol dehydrogenase (E and S isoenzyme, EC 1.1.1.1) is a NAD^+-dependent enzyme with a broad substrate specificity. The enzymatic mechanisms and structures of various enzyme complexes have been studied extensively (Brändén *et al.*, 1975; Eklund and Brändén, 1987). The tertiary structures for apoenzyme and holoenzyme of HLADH were determined by X-ray crystallography (Eklund *et al.*, 1982 ; Ramaswamy *et al.*, 1994). This structure has been used as a model for the enzyme-coenzyme-substrate ternary complex. The E and S isoenzymes of horse liver alcohol dehydrogenase appear to have very similar tertiary structures as they can form a heterodimer (Brändén *et al.*, 1975) but only the S isoenzyme is active on the steroid substrates. The complete amino acid sequence of the S isoenzyme was obtained from the cloned cDNA and used to construct a model of the S isoenzyme based on the known structure of the E isoenzyme (Park and Plapp, 1991). The activity of the S isoenzyme on steroids (Dworschack and Plapp, 1977; Ryzewski and Pietruszko, 1980) can be explained with this model. As compared to the E isoenzyme, the S isoenzyme has a deletion of Asp-115 and a substitution of Leu for Phe-110 in substrate binding pocket, which can allow Leu-116 to move and relieve steric hinderance with a steroid substrate (Park and Plapp, 1991).

The three-dimensional structure model suggests that Ile-269 residue is related to coenzyme binding and the side chain of Ile-269 residue forms hydrophobic interaction with adenine ring of coenzyme NAD^+. Also it has been suggested that the carbonyl oxygen of the residue participates in hydrogen-bonding to nicotinamide ribose-3'-hydroxyl group of NAD^+ (Eklund *et al.*, 1982; Ramaswamy *et al.*, 1994). In the present study, we mutated Ile-269 residue of S isoenzyme to serine and compared the kinetic properties, enzyme reaction mechanism and substrate specificities of the mutant enzyme to the wild-type's.

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MATERIALS AND METHODS

Materials

NAD^+, NADH, 2,2,2-trifluoroethanol, 5β-cholanic acid-3-one and all trans-retinoids were purchased from Sigma Chemical Co. Acetaldehyde, heptafluorobutanol and 5β-androstane-3,17-dione were obtained

from Fluka, Aldrich, and Steraloids INC., respectively. Primary alcohols and acetaldehyde were redistilled prior to use.

Strains

E. coli XL1-Blue containing phagemid pBPP/HLADH-S for template DNA preparation was obtained from Dr. Bryce V. Plapp, The University of Iowa (Park and Plapp, 1991). *E. coli* XL1-Blue strain was used for transformation and expression experiments.

Mutamer

The mutagenic oligonucleotide (the sites of mutation are bold-italic), TTT/GAA/GTC/$\overset{269}{\underset{GC}{\boldsymbol{AG}}}$T/GGT/CGG/CT, was synthesized and supplied by Korea Basic Science Center.

Media

TYP medium (1.6% tryptone, 1.6% yeast extract, 0.5 % NaCl, 0.25% K_2HPO_4) containing ampicillin (50 μg/ml) was employed for preparation of single-stranded template DNA in mutation. LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) was used for seed culture and preparation of double stranded template DNA in DNA sequencing. The LB medium containing 100 μg/ml of ampicillin was used for main culture.

Mutagenesis and Transformation

TYP broth (5 ml) was inoculated with 100 μl of seed culture of *E. coli* XL1-Blue containing pBPP/HLADH-S. The seed culture grew at 37°C for 15 hours in 1 ml of TYP medium containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) inoculated with single colony of the microorganism. When the 5 ml culture was grown at 37°C for about 30 min., helper phage R408 was added with 10 M.O.I. and further incubated for about 7 hours. The cells were removed from the culture by centrifugation (11,000 rpm, 15 min., 4°C). The supernatant was used to single-stranded template DNA preparation (Sambrook *et al.*, 1989). Mutation was carried out by using phosphorothioate based site-directed mutagenesis method (Nakamaye and Eckstein, 1986; Sayers and Eckstein, 1991). Transformation was carried out as described by Sambrook *et al.* (1989). Sequencing of double-stranded DNA of the plasmid miniprep. (Holmes and Quigley, 1981) was carried out by using dideoxy chain termination method (Sanger *et al.*, 1977).

Enzyme Isolation and Purification

Seed cultures in 50 ml of LB medium containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) were obtained by inoculating one loop of *E. coli* XL1-Blue containing pBPP/HLADH-S and incubating at 210 rpm and 37°C for 15 hours. 36 ml of these cultures was used to inoculate 4.5 liters of LB medium containing ampicillin (100 μg/ml) and cultivated at 30°C with agitation for 7-8 hours, when the bacterial culture was reached to 0.8 of A_{600}, IPTG (final concentration 0.2 mM) was added and further incubated for about 20 hours. The cells were collected by centrifugation on GS-3 rotor for 10 min at 6500 rpm and 4°C. The yield of cells was about 20 g. The cells were stored at -70°C until use. The alcohol dehydrogenase was purified by following the purification methods of Park and Plapp (1991) through lysis of cell by sonication, protamine sulfate treatment of the cell lysate, DEAE-Sepharose CL-6B column (2.5×10 cm), S-Sepharose Fast Flow column (2.5×10 cm), ultrafiltration with PM10 membrane, Sephadex G-50 column (1.0× 20 cm) and the second DEAE- Sepharose CL-6B column (2.5×10 cm) chromatography. The degree of purification was determined by using 12% SDS-PAGE (Blackshear, 1984; Laemmli, 1970) and scanning the gel with densitometer.

Enzyme Activity Assay

Enzyme activities were assayed in the reaction mixture containing 990 or 980 μl of the assay solution (Plapp, 1970) and 10 or 20 μl of enzyme sample at 25°C. The assay solution contained NAD^+ free acid (2 mM), $Na_4P_2O_7 \cdot 10H_2O$ (88 mM), semicarbazide · HCl (7.7 mM), glycine (19.8 mM) and ethanol (570 mM). The pH was 9.0. The absorbance change at 340 nm was measured and enzyme activity (U/ml) was calculated by equation of [(ΔA_{340}/min)×dilution factor/ (6.22× volume (ml) of the enzyme solution added to reaction mixture)]. The protein concentration was determined by Bio-Rad protein assay method (Bradford, 1976) using bovine serum albumin as a standard.

Kinetic Studies

The kinetic constants (K_m) of the purified HLADH-S enzyme to coenzyme (NAD^+, NADH) and substrate (ethanol, acetaldehyde) were determined. Initial velocity studies were carried out at 25°C in 33 mM sodium phosphate buffer containing 0.25 mM EDTA, pH 8.0. Activity was determined by measuring the change in absorbance at 340 nm. The initial velocity data were fitted to HYPER FORTRAN program (Cleland, 1979), and K_m and turnover numbers were obtained. Dead-end and product inhibition studies were carried out according to Park and Plapp (1991) and the data were fitted to an appropriate equation for COMP, NONCOMP or UNCOMP (Cleland, 1979).

Substrate Specificities

Specificities of the ADHs for the primary alcohols, various steroids, all trans-retinol, all trans-retinal, benzyl alcohol and cyclohexanol were studied. Kinetic constants were determined spectrophotometrically at 25°C in 83 mM potassium phosphate buffer (pH 7.3) containing 0.25 mM EDTA and 40 mM KCl. Assay solution for activity on steroids contained 0.3% (v/v) methanol. Activity on retinoids was measured spectrophotometrically at 400 nm according to the method of Parés and Julià (1990), with 0.1 M sodium phosphate buffer (pH 7.5) containing 40 mM NaCl and 0.02% Tween 80. The molar extinction coefficient of retinal at 400 nm was 29,500 $M^{-1}cm^{-1}$. The concentration of NAD^+ was fixed at 2 mM for primary alcohol or 4 mM for retinol and NADH at 0.2 mM for steroids or 0.77 mM for retinal.

RESULTS AND DISCUSSION

Confirmation of I269S mutation and expression of

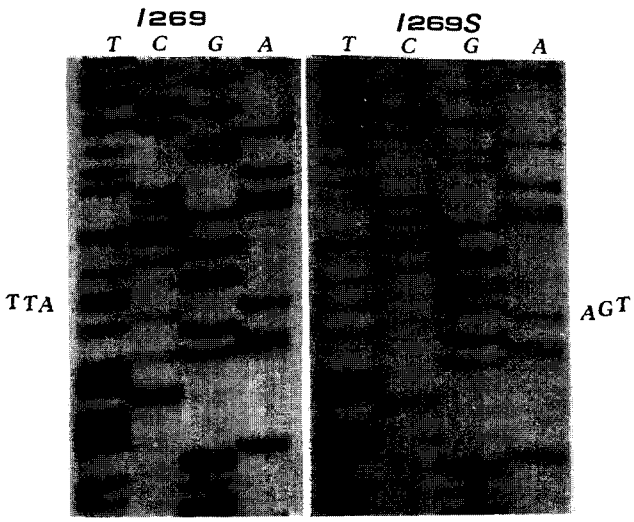

Fig. 1. Confirmation of site-directed mutagenesis in HLADH-S gene at the site 269 residue from isoleucine (ATT) to serine (AGT).

HLADH-S: Fig. 1 shows the mutation of the position of Ile-269 to serine. The expected change in codon sequence from ATT to AGT was confirmed. In the SDS-PAGE, the purified I269S HLADH-S protein was almost pure and its molecular weight was about 40 kD which was corresponding to subunit size of HLADH (Fig. 2), and the expression level of the protein was the same as that of wild-type (data not shown).

Enzyme reaction mechanism and kinetic constants: The specific activity of purified enzyme was 66.6 U/mg (Table I). The specific activity of the mutant enzyme was increased 49-fold compared to wild-type enzyme.

The kinetic constants were listed in Table II. Michaelis constants of mutant enzyme to NAD^+ (K_a) and

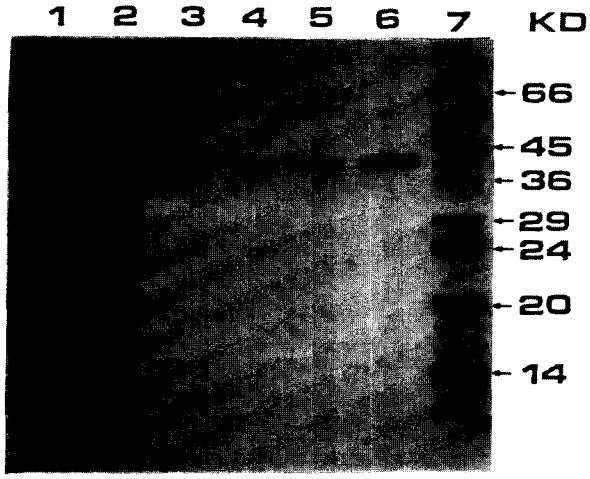

Fig. 2. 12% SDS-PAGE pattern of the mutant (I269S) HLADH-S during protein purification steps. SDS-PAGE was run at room temperature (Blackshear, 1984; Laemmli, 1970), stained with coomassie brilliant blue(0.125% coomassie brilliant blue in 10% acetic acid and 50% methanol), and destained with acetic acid : methanol : H_2O (75:100:825). Lane 1: Cell lysate, Lane 2: The first DEAE-Sepharose column chromatography, Lane 3: S-Sepharose column chromatography, Lane 4: Ultrafiltration, Lane 5: Sephadex G-50 column chromatography, Lane 6: The second DEAE-Sepharose column chromatography, Lane 7: Molecular weight markers.

Table I. Purification table of the wild-type (I269) and the mutant (I269S) HLADH-S proteins

Step of Purification	Compared Parameters					
		U/ml	mg/ml	U/mg	Yield (%)	Purification Fold
Cell Lysate	I269	0.56	14.3	0.039	100	1
	I269S	37.8	14.3	0.265	100	1
DEAE-Sepharose	I269	0.41	2.80	0.14	84.4	3.6
	I269S	24.5	1.83	13.4	81.0	5.1
S-Sepharose	I269	0.11	0.11	0.96	39.3	24.6
	I269S	9.5	0.18	52.5	41.9	19.8
Sephadex G-50	I269	0.31	0.32	0.96	28.6	24.6
	I269S	47	0.85	58.8	36.3	22.2
DEAE-Sepharose	I269	0.16	0.12	1.36	27.6	34.9
	I269S	29.3	0.44	66.6	33.2	25.1

Tabel II. Kinetic constants for the wild-type (I269) and the mutant (I269S) horse liver alcohol dehydrogenase. Kinetic constants were determined in initial velocity and product inhibition studies at 25°C in 33 mM sodium phosphate buffer (pH 8.0) containing 0.25 mM EDTA. Dead-end inhibition studies were carried out at 25°C in 83 mM potassium phosphate buffer (pH 7.3) containing 0.25 mM EDTA and 40 mM potassium chloride

Constants[a]	E[b]	I269[c]	I269S	I269S/I269	Constants[a]	E[b]	I269[c]	I269S	I269S/I269
K_a, μM	3.9	11	737	67	K_i,TFE, mM	0.0084	0.490	12.9	26
K_b, mM	0.35	4.8	304	63	K_i,HFB, mM	0.0053	0.0042	0.14	30
K_p, mM	0.40	24	468	20	K_pK_{iq}/K_q, mM	0.034	0.3	172	537
K_q, μM	5.8	7.3	78	11	V_1, S^{-1}	3.5	1.5	22	15
K_{ia}, μM	27	62	161	2.6	V_2, S^{-1}	47	73	297	4
K_{ib}, mM	27	74	638	9	V_1/K_b, $mM^{-1}S^{-1}$	10	0.31	0.07	0.2
K_{ip}, mM	0.52	[d]	11	[d]	V_2/K_p, $mM^{-1}S^{-1}$	120	3.0	0.63	0.2
K_{iq}, μM	0.50	1.5	29	19					

[a]K_a, K_b, K_p, and K_q are the Michaelis constants for NAD^+, ethanol, acetaldehyde, and NADH, respectively. K_i values are product inhibition constants. K_i,TFE and K_i,HFB are dissociation constant for substrate analogue trifluoroethanol and heptafluorobutanol, respectively. V_1 is the turnover number for ethanol oxidation and V_2 is the turnover number for acetaldehyde reduction. Kinetic constants were determined in initial velocity studies with an spectrophotometer Varian Cary 210. In initial velocity studies, the concentrations of substrate and coenzyme were varied. The concentration of ethanol was fixed while the concentrations of NAD^+ and NADH were varied for the inhibition studies.
[b]From Dworschack and Plapp (1977) and E indicates the 'E' isoenzyme of HLADH.
[c]From Park and Plapp (1992).
[d]Undefined.

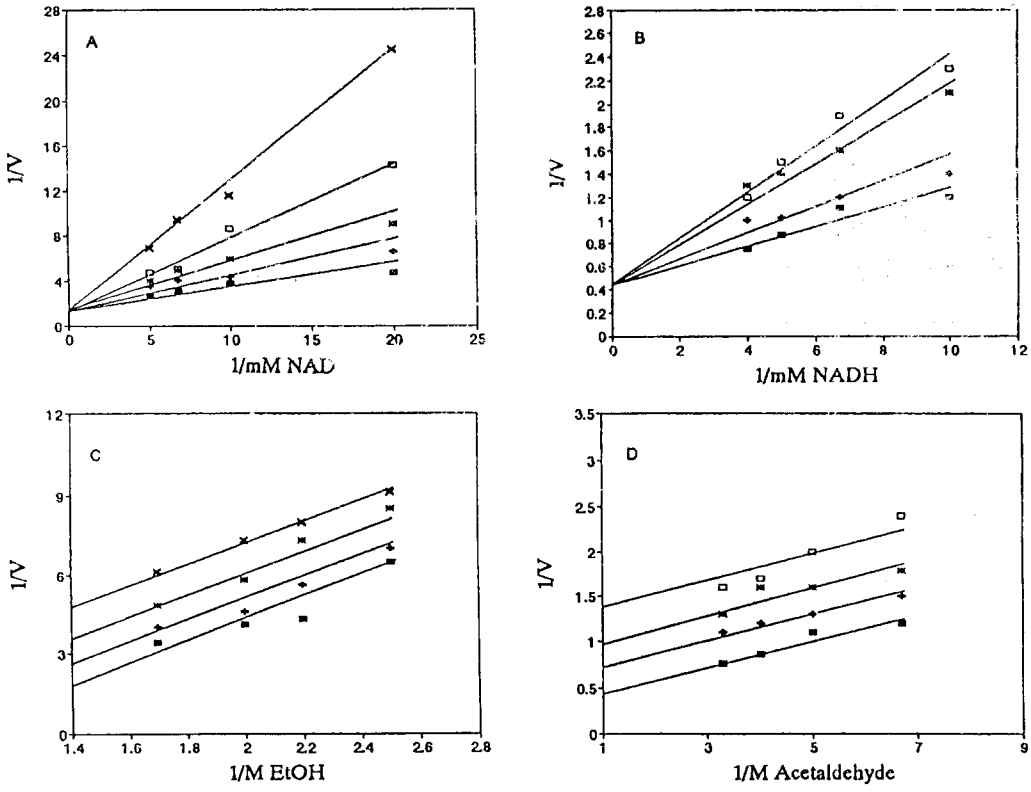

Fig. 3. Product inhibition patterns for the mutant (I269S) HLADH-S isoenzyme. The buffer was pH 8.0, 33 mM sodium phosphate containing 0.25 mM EDTA. Velocities were measured at 25°C. A: inhibition by NADH (■: 0, +: 0.02, *: 0.04, □: 0.08, ×: 0.2 mM) against NAD^+ (0.05, 0.1, 0.15, 0.2 mM) at 1 M ethanol, 0.052 nN enzyme. B: inhibition by NAD^+ (■: 0, +: 1, *: 2, □: 3 mM) against NADH (0.1, 0.15, 0.2, 0.25 mM) at 250 mM acetaldehyde, 0.026 nN enzyme. C: inhibition by acetaldehyde (■: 0, +: 5, *: 10, ×: 20 mM) against ethanol (400, 450, 500, 600 mM) at 4 mM NAD^+, 0.052 nN enzyme. D: inhibition by ethanol (■: 0, +: 1.25, *: 1.5, □: 1.75 mM) against acetaldehyde (100, 200, 300, 400 mM) at 0.4 mM NADH, 0.026 nN enzyme.

ethanol (K_b) were increased about 67- and 63-fold, respectively. K_p and K_q values of mutant enzyme were also 20 and 11 times larger than the wild-type's. The turnover numbers were increased 15-fold for ethanol

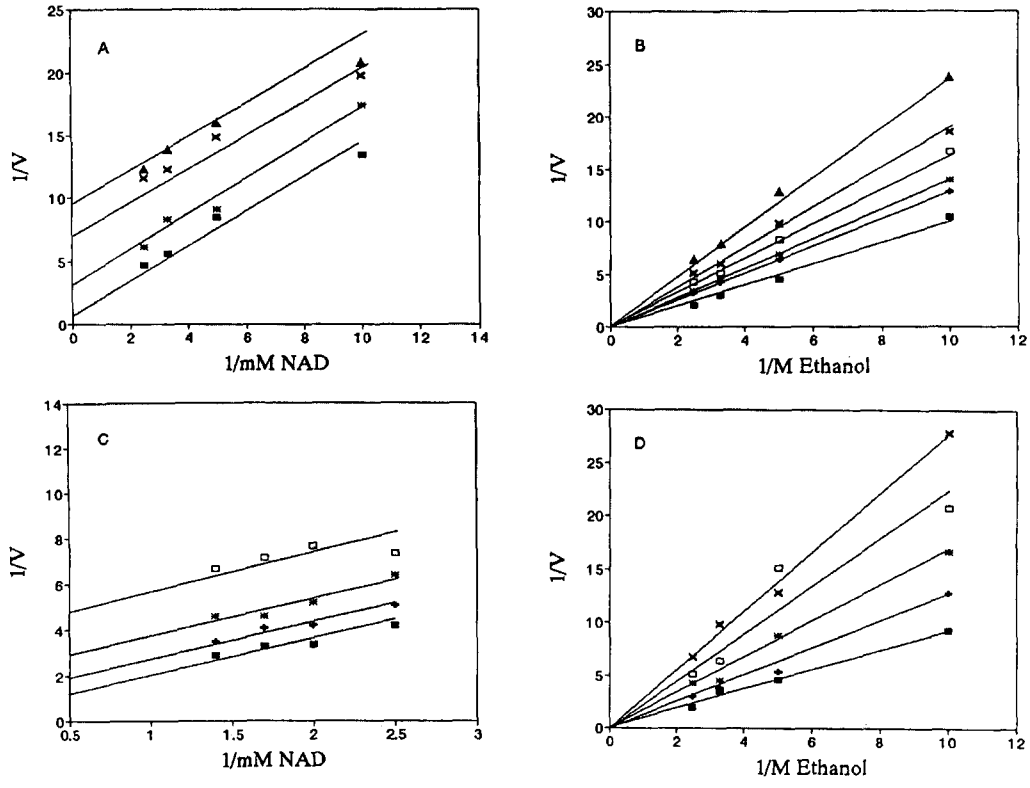

Fig. 4. Dead-end inhibition patterns for the mutant (I269S) HLADH-S isoenzyme. The buffer was pH 7.3, 83 mM potassium phosphate containing 0.25 mM EDTA and 40 mM potassium chloride. Velocities were measured at 25°C. A: inhibition by trifluoroethanol (■: 0, ∗: 10, ×: 35, ▲: 50 mM) against NAD^+ (0.1, 0.2, 0.3, 0.4 mM) at 500 mM ethanol, 0.27 nN enzyme. B: inhibition by trifluoroethanol (■: 0, +: 5, ∗: 7.5, □: 10, ×: 15, ▲: 20 mM) against ethanol (0.1, 0.2, 0.3, 0.4 mM) at 2 mM NAD^+, 0.27 nN enzyme. C: inhibition by heptafluorobutanol(■: 0, +: 80, ∗: 160, □: 320 μM) against NAD^+ (0.4, 0.5, 0.6, 0.7 mM) at 500 mM ethanol, 0.27 nN enzyme. D: inhibition by heptafluorobutanol (■: 0, +: 20, ∗: 40, □: 80, ×: 160 μM) against ethanol (0.05, 0.1, 0.2, 0.3, 0.4 mM) at 2 mM NAD^+, 0.27 nN enzyme.

oxidation and 4-fold for acetaldehyde reduction. The I269S substitution decreased the affinities for NAD^+ and NADH about 2.6- and 19-fold, respectively. These results suggest that the side chain of Ile-269 residue is involved in hydrophobic interaction with coenzyme. Consequently, the Ile-269 substitution with serine resulted in decreased affinity of the enzyme to coenzyme and dissociation rate of coenzyme from enzyme-coenzyme complex, which is rate-limiting step in ADH reaction, increased. These results agree well with the report (Eklund *et al.*, 1982) that the 269 residue lies in the coenzyme binding site and interacts with adenine ring of coenzyme.

Fig. 3 and 4 showed the product and dead-end inhibition patterns for I269S HLADH-S. Reduced and oxidized coenzyme were competitive against each other. Acetaldehyde and ethanol appeared to be an uncompetitive against each other. Trifluoroethanol (TFE), an analogue of ethanol, and heptafluorobutanol (HFB) were competitive against ethanol and uncompetitive against NAD^+. The substitution increased dissociation constants (K_i, TFE and K_i, HFB) 26-fold for trifluoroethanol and 30-fold for heptafluorobutanol (Table II). These results indicate that the substitution affects the substrate affinity too. Our results are different from the Fan and Plapp's results(1995) in which the I269S mutant enzyme of E isoenzyme did show little changes in dissociation constant of TFE, compared to wild-type enzyme. This suggests that there are some differences in cross interaction between coenzyme binding domain and substrate binding domain in E and S isoenzyme.

There have been conflicting reports about the kinetic mechanism of the S isoenzyme of horse liver ADH (Dworschack and Plapp, 1977; Ryzewski and Pietruszko, 1980). Although the inhibition pattern by acetaldehyde were different from wild-type (competitive or noncompetitive), the patterns of dead-end inhibition were consistant with an ordered binding of NAD^+ before ethanol. From these results, we could confirm that the reaction mechanism of mutant enzyme was not changed.

Substrate specificities: In Table III, the substrate specificities are listed. Mutant enzyme had 6~28-fold higher maximum velocities for the alcohols as compared to the corresponding values for the S isoen-

Table III. Substrate specificities of the wild-type (I269) and the mutant (I269S) horse liver alcohol dehydrogenase S isoenzyme

Substrate	V, S^{-1}			K_m, mM			V/K_m, $mM^{-1}S^{-1}$		
	E[a]	I269	I269S	E[a]	I269	I269S	E[a]	I269	I269S
Ethanol	5.8	2.2	14	0.54	58	505	11	0.038	0.027
1-Propanol	5.8	2.0	22	0.17	2.8	137	35	0.72	0.16
1-Butanol	4.9	1.7	28	0.13	0.26	15	39	6.5	1.8
1-Pentanol	5.3	1.3	22	0.14	0.10	1.3	37	12.8	17
1-Hexanol	3.7	0.78	21	0.056	0.07	0.59	65	12.0	36
1-Heptanol	-	0.79	7.85	-	0.021	0.17	-	38	46
1-Octanol	-	0.43	8.53	-	0.008	0.20	-	51	43
1-Nonanol	-	0.20	5.53	-	0.012	0.37	-	17	15
1-Decanol	-	0.15	3.85	-	0.012	0.92	-	33	4.18
Cyclohexanol	-	1.19	5.45	-	4.67	33.3	-	0.25	0.16
Benzylalcohol	-	1.85	6.77	-	0.146	4.78	-	13	1.42
Retinol	-	0.016	0.70	-	[b]	2.52	-	[b]	0.28
Retinal	-	0.0625	1.06	-	0.025	0.037	-	2.5	29
5β-Cholanic acid-3-one	-	0.92	1.70	-	0.097	0.04	-	9.4	43
5β-Androstane -3,17-dione	-	2.20	4.13	-	0.140	0.071	-	16	58

[a]: Data from Dworschack and Plapp (1977) and E indicates the 'E' isoenzyme of HLADH.
[b]: Undefined.

zyme. Michaelis constants were greatly increased in the mutant enzyme. Consequently catalytic efficiencies of the mutant enzyme were reduced in propanol, butanol and decanol, but increased in hexanol. While the wild-type enzyme showed the highest catalytic efficiency for octanol, heptanol was the best substrate for mutant enzyme. The mutant enzyme showed higher catalytic efficiency for 5β-cholanic acid-3-one, 5β-androstane-3,17-dione but the catalytic efficiencies for benzyl alcohol and cyclohexanol were decreased. The substitution resulted in low affinities for primary alcohols and aldehyde substrate, indirectly. According to X-ray crystallography data, the catalytic domain of the enzyme complex with NAD^+ or NADH and various substrates rotates toward coenzyme binding domain, and forms the closed form of enzyme-coenzyme complex (Eklund *et al.*, 1981 and 1984 ; Eklund and Brändén, 1987; Ramaswamy *et al.*, 1994). Also, Ramaswamy *et al.* (1994) indicated that Ile-269 also involved in hydrogen bonding to nicotinamide ribose-3'-hydroxyl group of NAD^+ by backbone carbonyl oxygen. It has been suggested that the initial coenzyme binding to enzyme induces proper positioning of the subsite of coenzyme toward the substrate binding site and the following substrate binding resulted in the enzyme reaction (Plapp *et al.*, 1986 ; Sekhar and Plapp, 1988 and 1990). In our mutation, the loss of the hydrophobic side chain of Ile-269 residue of S isoenzyme could affect conformational positioning of peptide backbone of the enzyme and resulted in the different activities for various substrates. Similar changes in affinities of coenzyme and substrate were reported in the case of D223G yeast enzyme (Fan, *et al.*, 1991). But the reduced affinities for coenzymes did not decrease the affinities for the steroids and retinoids substrates in I269S mutant enzyme (Table III). Although we could not compare the K_m value for retinol of wild-type with that of mutant directly because wild-type enzyme did not show the saturation phenomenon in the range of the concentration tested, the mutant enzyme showed highly increased V_{max} value for retinol than wild-type. The substitution of Ile-269 residue to serine made the enzyme more efficient for the steroid and retinoid by 3.6~4.6 and 11.6-fold, respectively.

From our results, we could confirm that the hydrophobic interaction of the nonpolar side chain of Ile-269 residue of S isoenzyme with the adenine base of the coenzyme NAD^+ play an important role in dissociation of coenzyme from enzyme-coenzyme complex and consequently increased the turnover numbers of mutant enzyme.

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