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The Role of Lys-228 Residue in Horse Liver Alcohol Dehydrogenase Activity

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Lys-228 in horse liver alcohol dehydrogenase isoenzyme E (HLADH-E) was mutated to glycineby site-directed mutagenesis. The specific activity of the mutant enzyme was increased about 4-fold and Michaelis constants for $NAD^+(K_a)$ and NADH (K_q) increased by about 350- and 50-fold, respectively. The wild-type enzyme and K228G mutant enzyme were treated with ethylacetimidate. Acetimidylation of the wild-type enzyme increased the activity about 10-fold, but the mutant enzyme was little affected. These results confirm that Lys-228 residue plays an important role in the activity of the enzyme through forming the hydrogen bond with adenosine ribose of NAD^+.

Key words : Lys-228, Horse liver alcohol dehydrogenase, Site-directed mutagenesis, Glycine, NAD^+

INTRODUCTION

Horse liver alcohol dehydrogenase (E isoenzyme, EC 1.1.1.1) is an NAD-dependent enzyme with a broad substrate specificity. The enzymatic mechanism and structures of various enzyme complexes have been extensively studied (Bradèn *et al.*, 1975; Eklund & Bradèn, 1987). The enzyme has a molecular weight of 80,000 and is a dimer of two identical subunits, each of which has a coenzyme-binding and a catalytic domain. The coenzyme-binding domains have structures similar to those found in several other NAD^+-dependent dehydrogenases and they form a 12-stranded β-pleated sheet structure that makes up the central core of the dimer (Eklund *et al.*, 1976). The three-dimensional structure of a complex with NAD and p-bromobenzyl alcohol was determined at 2.9-Å resolution (Eklund *et al.*, 1982a). This structure has been used as a model for the enzyme-coenzyme-substrate ternary complex. The three-dimensional structure of HLADH suggests that Lys-228 is related to coenzyme binding (Eklund *et al.*, 1981). The amino group of Lys-228 forms a hydrogen bond with the 3'-hydroxyl group of adenosine ribose. Lys-228 residue was identified as the residue that is responsible for the enhancement of enzyme activity upon acetimidylation (Plapp, 1970; Dworschack *et al.*, 1975). In the present study we have mutated Lys-228 residue to glycine, which cannot form a hydrogen bond with the 3'-hydroxyl group of adenosine ribose and cannot be acetimidylated, and we could confirm the effect of Lys-228 residue on enzyme activity by kinetic studies and acetimidylation of lysine residues of the wild-type enzyme and the mutant.

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MATERIALS AND METHODS

Strains

E. coli XL1-Blue containing phagemid pBPP/HLADH-E 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 20-mer mutagenic oligonucleotide (the sites of mutation are bold-italic), AC/AAA/GAC/$\begin{pmatrix} G\,G\,G \\ A\,A\,C \\ C \end{pmatrix}$GGG/TTT/GCA/AAG, 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-strand-

ed template DNA in mutation and DNA sequencing. LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) for seed culture and ampicillin (100 μg/ml) for main culture was used.

Mutagenesis and transformation

The seed culture was grown at 37°C for 15 hours in 1 ml TYP medium containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) inoculated with single colony of the microorganism. TYP broth (5 ml) was inoculated with 100 μl of seed culture of *E. coli* XL1-Blue containing pBPP/HLADH-E. The culture was grown at 37°C for about 7 hours and used for preparation of single-stranded template DNA (Sambrook et al., 1989). Mutation was carried out by using the phosphorothioate-based site-directed mutagenesis method (Rickwood and Hames, 1991). Transformation was carried out as described by Sambrook et al. (1989). The mutated DNA sequence was confirmed by the 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-E and incubating at 210 rpm and 37°C for 15 hours. 36 ml of these cultures were 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 to an A_{600} of 0.8 at which time IPTG was added (final concentration 0.2 mM). Incubation was continued for about 20 hours. The cells were collected by centrifigation on GSA 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 precipitation, DEAE-Sepharose CL-6B column (2.5× 10 cm), S-Sepharose Fast Flow (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 12% SDS-PAGE (Blackshear, 1984;Laemmli, 1970) and scanning the gel with a densitometer.

Enzyme activity assay

Enzyme activities were assayed in the reaction mixture containing 990 or 980 μl of the assay solution and 10 or 20μl of enzyme sample at 30°C. The assay solution was made of 1.35 mg of NAD^+ free acid and 1 ml of the reaction solution containing $Na_4P_2O_7 \cdot H_2O$ (19.62 g), semicarbazide · HCl (0.429 g), glycine (0.746 g) and 95 % ethanol (17.1 ml) in 500 ml H_2O(pH 9.0). The absorbance change at 340 nm was measured and enzyme activity (U/ml) was calculated by the following equation : $[(\Delta A_{340}/\text{min}) \times \text{dilution factor}/(6.22 \times \text{volume (ml) of the enzyme solution added to reaction mixture})]$.

Kinetic studies

The kinetic constants (Km) of the purified HLADH-E 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 the HYPER FORTRAN program (Cleland, 1979), and Km was obtained.

Effects of acetimidylation

Enzyme (K228 : 4.70 nN, K228G : 2.54 nN) in 400 μl of 0.5 M triethanolamine. HCl buffer (pH8.0) was treated with 1/20 volume of 2.1 M ethyl acetimidate at 25°C. The reagent was added 4 times at 1 hr intervals. The reaction proceeded for 4 hours at 25°C (Plapp, 1970) and the enzyme activity was measured every 20 minutes during the modification reaction.

RESULTS AND DISCUSSION

Fig. 1 shows the mutation of the position of Lys-228 to glycine. The expected change in codon sequence from AAG to GGG had confirmed. We identified indirectly that no mutation except Gly-228 had occured by subcloning the mutated DNA fragment cor-

Fig. 1. Confirmation of site-directed mutagenesis in HLADH-E gene at the site 228 residue from lysine (AAG) to glycine (GGG)

responding to the 189-286 amino acid sequence into the rest of wild type ADH gene and comparing the activity with K228G mutant and K228 wild type ADH. When the SDS-PAGE gel was scanned with a densitometer, the purities of K228 and K228G HLADH-E proteins were 94.4% and 91.6%, respectively and molecular weights were about 40 kD (corresponding to a subunit molecular weight of HLADH) (Fig. 2). But the expression level of K228G enzyme was lower than K228 wild type (Fig. 2 and Table I). The active fractions of the second DEAE-Sepharose CL-6B column fractions were used for kinetic studies and acetimidylation experiments. The specific activities were calculated from the HLADH-E protein concentration (mg/ml) and enzyme activity (U/ml). The protein concentration was determined by the Bio-Rad protein assay method. The specific activities of purified proteins were 2.5 U/mg for wild-type enzyme and 9.3 U/mg for K228G mutant enzyme (Table I). The activities of the purified enzymes for NAD^+ and NADH as coenzyme and for ethanol and acetaldehyde as substrate were assayed, and the kinetic parameters were determined (Table II). Michaelis constants for $NAD^+(K_a)$ and ethanol (K_b) increased by about 350- and 150-fold, respectively. K_p and K_q values for mutant enzyme were 6 and 50 times larger than the wild-type's. These results accord with the previous proposals that Lys-228 residue is near the coenzyme ribose binding site and plays an important role in coenzyme binding and releasing. We propose that the primary effect of the mutation on the coenzyme binding (K_a and K_q) results in a lower affinity to substrates (K_b and K_p) also. Similar changes in affinities of coenzyme and substrate were reported in the case of D223G yeast enzyme (Fan, et al., 1991). It was known that modification of the amino groups of the enzyme resulted in higher activities and increased Km values, and the reason was assumed to be an increased dissociation rate of enzyme-coenzyme complex (Plapp, 1970). The K228G mutant enzyme cannot form a hydrogen bond with the 3′-hydroxyl group of adenosine ribose of NAD^+ and NADH (Eklund et al., 1982a,b), in which the coenzyme may bind to the K228G mutant enzyme with lower affinity. The release of the coenzyme from the enzyme-coenzyme complex, the rate-limiting step in the enzyme reaction, becomes faster and the activity of the K228 mutant appears to be larger than that of the wild-type enzyme. The in-

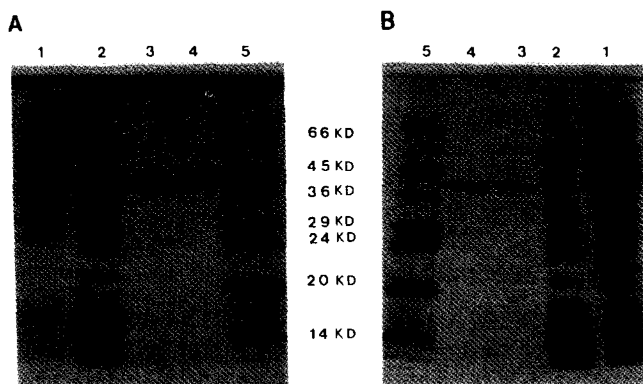

Fig. 2. 12% SDS-PAGE pattern of K228 (A) and K228G (B) HLADH-E during protein purification steps. SDS-PAGE was run at room temperature (Blackshear, 1984; Laemmli, 1970), stained with coomassie brilliant blue (0.125% coomassie brilliant blue in 10% acetic acid and 50% methanol), and destained with acetic acid:methanol:H_2O (75:100:825). Lane 1: Protamine sulfate precipitation, Lane 2: The first DEAE-Sepharose column chromatography, Lane 3: S-Sepharose column chromatography, Lane 4: The second DEAE-Sepharose column chromatography, Lane 5: Molecular marker. The specific activities of the final enzyme preparations were 9.3 U/mg for K228G mutant enzyme and 2.5 U/mg for wild-type enzyme. The purities of proteins were 91.6% for K228G mutant enzyme and 94.4% for wild-type enzyme.

Table I. Purification table of HLADH-E protein from *E. coli* XL1-Blue containing pBPP/HLADH-E.

K228	Activity[a] units	Protein[b] mg	S.A[c] units/mg	Yield %	Purification -fold
			K228		
PS[d]	147.0	1073.4	0.1	100.0	1
DEAE	145.2	265.1	0.6	98.8	6
S	121.8	66.4	1.8	82.9	18
DEAE	70.8	28.2	2.5	48.2	25
			K228G		
PS[d]	69.0	1056.0	0.1	100.0	1
DEAE	43.0	223.6	0.2	62.3	2
S	38.0	10.4	3.7	55.1	37
DEAE	30.3	3.2	9.3	43.9	93

The results are based on the purification of HLADH-E from about 20g of *E. Coli* wet cells obtained from a 4.5-liter culture broth. [a]One unit of ADH catalyzes formation of 1 μmol of NADH in 1 min at 30°c in assay mixture containing 550 mM ethanol and 1.76 mM NAD^+ at pH 9.0 (Plapp, 1970). [b]Protein concentrations were determined by Bradford dye-binding procedure using bovine serum albumin as a standard. [c]Specific activity, [d]protamine sulfate

Table II. Kinetic constants for the wild-type and the mutant HLADH-E. Kinetic constants were determined by initial velocity. The letters a, b, p and q represent NAD^+, ethanol, acetaldehyde and NADH, respectively.

	K228G	K228	ratio of K228G/K228
Ka, µM	2200±300	6.5±1.2	350
Kb, mM	100±30	0.7±0.07	150
Kp, mM	1.8±0.53	0.3±0.06	6
Kq, µM	160±50	3.2±0.5	50

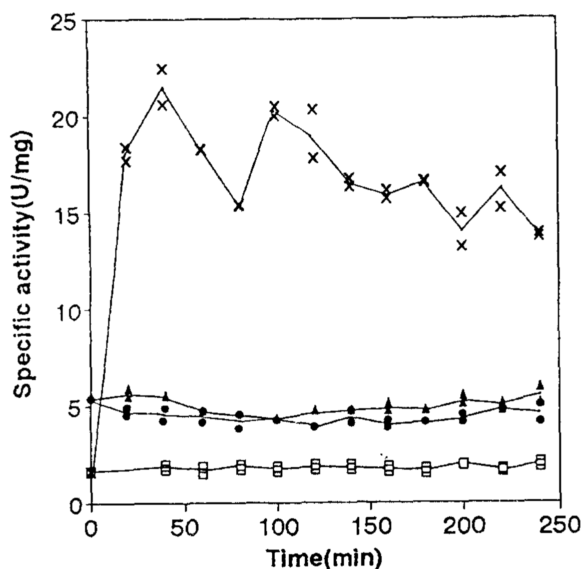

Fig. 3. The effect of acetimidylation of HLADH-E by ethyl acetimidate on enzymatic activity. Enzyme in 0.5 M triethanolamine. HCl buffer (pH 8.0) was treated with 1/20 volume of 2.1 M ethyl acetimidate at 25°C. The reagent was added 4 times at 1 hr intervals. The reaction proceeded for 4 hours at 25°C. K228(□—□), Acetimidylated K228(×—×), K228G(▲—▲) and acetimidylated K228G(●—●)

volvement of Lys-228 in the enzyme activity was tested through acetimidylation. The activity of acetimidylated wild-type enzyme was increased about 10-fold. However, the activity of acetimidylated mutant enzyme was little affected (Fig. 3). The results of the present work directly confirm the important role of Lys-228 residue in coenzyme binding of horse liver alcohol dehydrogenase, which has been suggested by previous crystallographic data (Eklund et al., 1982a,b) and chemical modification studies (Plapp, 1970; Dworschack et al., 1975)

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