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The Kinetic Characteristics of K228G Mutant Horse Liver Alcohol Dehydrogenase

Sun Hyoung Cho, Ji Won Ryu and Kang Man Lee

College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

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The kinetic constants and the reaction mechanism of the K228G mutant horse liver alcohol dehydrogenase isoenzyme E (HLADH-E) were compared to the wild-type enzyme. All the Km and Ki constants of the mutant enzyme for NAD^+, ethanol, acetaldehyde and NADH were larger than those of the wild-type enzyme. The dissociation constants for the NADH and NAD^+ (K_{iq} and K_{ia}) were greatly increased by 130- and 460-fold, respectively. The product inhibition patterns suggested that the reaction mechanism of the mutant enzyme was changed to Random Bi Bi. These results could attribute to the increase in the dissociation rate of coenzyme with the substitution at Lys-228 residue.

Key words : K228G, Horse liver alcohol dehydrogenase, Kinetic constants, Random Bi Bi

INTRODUCTION

Alcohol dehydrogenase from horse liver (EC 1.1.1.1) is a well-characterized enzyme (Eklund and Brändén, 1987) and have been major objects of studies (Brändén *et al.*, 1975; Klinman, 1981). The tertiary structures for apoenzyme and holoenzyme of horse liver alcohol dehydrogenase were determined by X-ray crystallography (Eklund *et al.*, 1976 and 1981; Ramaswamy *et al.*, 1994). The three-dimensional structure of horse liver alcohol dehydrogenase suggests that Lys-228 is involved in coenzyme binding (Eklund *et al.*, 1984; Ramaswamy *et al.*, 1994). The amino group of Lys-228 forms hydrogen bond with 3'-hydroxyl group of adenine ribose of NAD^+. We compared the kinetic properties of the K228G mutant enzyme with those of wild-type enzyme. Through initial velocity and product inhibition studies, the enzyme reaction mechanism was investigated.

MATERIALS AND METHODS

Microorganisms

E. coli XL1-Blue containing mutant K228G phagemid pBPP/HLADH-E was originated from the previous mutagenesis result (Cho *et al.*, 1995) and used for double-stranded DNA preparation and mutant enzyme preparation. *E. coli* XL1-Blue strain was used for transformation in subcloning experiment.

Correspondence to: Kang Man Lee, College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

Enzyme isolation and purification

K228G mutant and wild-type HLADH were purified from the *E. coli* XL1-Blue cell containing K228G mutant and wild-type pBPP/HLADH-E, respectively, as described in the previous paper (Cho *et al.*, 1995) and Park and Plapp (1991). The degree of purification was determined by 12% SDS-PAGE and scanning the gel with densitometer. The purified enzymes were used for kinetic studies and specific activities were calculated from the HLADH-E protein concentration (mg/ml) and enzyme activity (U/ml). The protein concentration was determined by Bio-Rad protein assay method.

ADH enzyme activity assay

Enzyme activities were assayed in the reaction mixture containing 990 or 980 μl of the assay solution and 10 or 20 μl of enzyme sample at 30°C. The assay solution was made of 1.35 mg of NAD^+Li and 1 ml of the reaction solution containing $Na_4P_2O_7 \cdot 10H_2O$ 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.

Kinetic studies

The kinetic constants 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 HYPER FORTRAN program (Cleland, 1979) for personal computer (modified by Dr. B. V. Plapp) and Km and turnover numbers were determined.

Product inhibition studies

Product inhibition studies were performed by holding the concentration of one substrate while varying the concentration of the other substrate in the presence of a product. 33 mM Sodium phosphate buffer containing 0.25 mM EDTA at pH 8.0 and 25°C was used (Park and Plapp, 1991). Initial velocities were fitted to an appropriate computer program for competitive inhibition, noncompetitive inhibition or uncompetitive inhibition (Cleland, 1979).

Subcloning of K228G mutant DNA fragment into wild-type HLADH-E gene

Double-stranded pBPP/HLADH-E DNA was isolated from *E. coli* XL1-Blue containing pBPP/HLADH-E (Stratagene plasmid mini boiling preparation protocol) and digested with restriction enzymes *Nde*I and *Bst*EII. The 294 bp DNA fragment of K228G horse liver alcohol dehydrogenase gene (corresponding to the amino acid sequence from 189 to 286) and about 4.2 kb wild-type pBPP/HLADH-E without the 294 bp fragment were separated and cut out from 1.5% low-melting agarose gel after electrophoresis, and ligated to one another. The ligation mixture was added to competent *E. coli* XL1-Blue cells and spreaded on LB-ampicillin plate, according to Sambrook *et al.* (1989). The transformants were cultured in LB-ampicillin liquid medium and plasmids were isolated by plasmid mini boiling preparation method. The presence of 294 bp K228G HLADH-E insert was confirmed by *Nde*I and *Bst*EII digestion. Then, the enzyme was purified from this transformant and kinetic studies were carried out.

RESULTS AND DISCUSSION

The purified K228 and K228G HLADH-E proteins were nearly pure (94% and 92%, respectively) and molecular weights were about 40 kDa corresponding to subunit molecular weight of HLADH (Cho *et al.*, 1995). The specific activities of purified proteins were 2.5 U/mg for wild-type enzyme and 9.3 U/mg for K228G mutant enzyme as stated in the previous report.

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 I). The turnover numbers were increased 2.6-fold for ethanol oxidation and decreased 5-fold for acetaldehyde reduction. The K228G substitution also decreased the affinities for NAD(H); K_{ia} and K_{iq}, which were dissociation constants for NAD^+ and NADH, increased about 460- and 130-fold, respectively; Michaelis constants for NAD (K_a) and ethanol (K_b) increased by about 345- and 49-fold, respectively.

Table II shows the inhibition patterns for K228 HLADH-E. The inhibition patterns by NAD^+ against NADH and by NADH against NAD^+ were competitive. The inhibition patterns by acetaldehyde against ethanol and by ethanol against acetaldehyde were noncompetitive. The inhibition pattern by acetaldehyde against NAD^+ was uncompetitive. From these results we could refer that the wild-type enzyme had product inhibition patterns consistent with the Ordered Bi Bi mechanism (Wratten and Cleland, 1963; Plapp, 1970; Park and Plapp, 1991).

Table III and Fig. 1 shows the inhibition patterns for K228G mutant enzyme. The inhibition patterns were the same as wild-type in the case of inhibition by acetaldehyde against ethanol and ethanol against acetaldehyde as noncompetitive inhibition. But the inhibition patterns of NAD^+ against NADH (Fig. 1C, D), NADH against NAD^+ (Fig. 1A, B), and acetaldehyde against NAD^+ (Fig. 1E) were noncompetitive, which were different from the competitive or uncompetitive patterns of the wild-type enzyme (Table II). If secondary plots of inhibitor concentrations versus intercept values of Fig. 1 were drawn, they showed a nonlinear pattern. From the inhibition and secondary plot patterns of the mutant enzyme, the substitution of Lys-228 to glycine changed the reaction mechanism to Random

Table I. Kinetic constants for the wild-type (K228) and the mutant (K228G) HLADH-E

K (μM)	K228G	K228	Ratio of K228G/ K228	E[c]
K_a, μM	2240.0±302	6.5±1.20	345	3.0
K_b, μM	104±31.0	0.7±0.07	148	0.35
K_p, μM	1.8±0.53	0.3±0.06	6.0	0.40
K_q, μM	158.0±50.0	3.2±0.50	49.0	5.8
K_{ia}, μM	30800±9330	67.0±7.00	460	27
K_{ib}, μM	191±42.5	32.6±18.8	5.8	27
K_{ip}, μM	2.5±0.56	0.8±0.43	3.1	0.52
K_{iq}, μM	132±53.3	1.0±0.10	132	0.50
TN_f, s^{-1}	5.8	2.2	2.6	2.4
TN_r, s^{-1}	7.3	37.7	0.2	

Kinetic constants were determined by initial velocity and product inhibition studies. Michaelis constants (K)- K_a, K_b, K_p, and K_q; slope inhibition constants (K_{is})- K_{ia} and K_{iq}; intercept inhibition constants (K_{ii})- K_{ib} and K_{ip}. The letters $_{a, b, p}$ and $_q$ represent NAD^+, ethanol, acetaldehyde and NADH, respectively. TN_f and TN_r are the turnover numbers of the forward reaction and the reverse reaction, respectively. The fixed substrates were saturated.

[c]Alcohol dehydrogenase from horse liver (Boehringer Mannheim). Data from Dworschack and Plapp (1977).

Bi Bi (Rudolph, 1979). It has been suggested that horse liver alcohol dehydrogenase changes conformation from open form to closed one upon coenzyme binding (Eklund *et al.*, 1981). The K228G substitution could make the transition from the open to the closed conformation less favored. Therefore, NAD^+ and ethanol may bind randomly to the open form with lower affinity (Table I).

As a similar result, Fan *et al.* (1991) reported that substitution of Asp-223 to Gly in yeast alcohol dehydrogenase changed the reaction mechanism from preferred ordered to a more random one for the reaction

Table II. Product inhibition studies of wild-type HLADH-E. Product inhibition studies were performed at 25°C in 33 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA

Substrate		Inhibitor	K_m	K_{is}[a]	K_{ii}^D	V	Pattern[c]
Fixed (mM)	Varied (mM)	mM	mM	mM	mM	min^{-1}	
C_2H_5OH (8)	NAD^+ (0.1~0.5)	NADH (0~0.005)	0.2±0.03	0.001±0.0001		0.4±0.02	C
CH_3CHO (6)	NADH (0.005~0.04)	NAD^+ (0~0.160)	0.02±0.002	0.07±0.007		0.2±0.01	C
NAD^+ (1.6)	C_2H_5OH (1~4)	CH_3CHO (0~0.6)	0.7±0.17	0.08±0.02	0.8±0.4	0.2±0.01	NC
NADH (0.16)	CH_3CHO (1~6)	C_2H_5OH (0~3)	0.4±0.06	0.8±0.19	31.6±18.9	0.15±0.004	NC
C_2H_5OH (0.4)	NAD^+ (0.2~1.0)	NADH (0~0.004)	1.5±0.32	0.002±0.0003		0.3±0.04	C
CH_3CHO (0.4)	NADH (0.005~0.04)	NAD^+ (0~0.16)	0.003±0.0005	0.02±0.003		0.1±0.04	C
NAD^+ (0.008)	C_2H_5OH (5~40)	CH_3CHO (0~0.03)	0.6±0.25	0.003±0.001	0.06±0.01	0.04±0.001	NC
NADH (0.005)	CH_3CHO (1~6)	C_2H_5OH (0~2.0)	0.5±0.11	0.4±0.11	5.5±2.3	0.07±0.002	NC
C_2H_5OH (8)	NAD^+ (0.05~0.25)	CH_3CHO (0~0.6)	0.04±0.009		0.2±0.02	0.14±0.009	UC

[a]Slope inhibition constant.
[b]Intercept inhibition constant.
[c]C, competitive inhibition; NC, noncompetitive inhibition; UC, noncompetitive inhibition.

Table III. Product inhibition studies of K228G mutant HLADH-E. Product inhibition studies were performed at 25°C in 33 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA

Substrate		Inhibitor	K_m	K_{is}[a]	K_{ii}[b]	V	Pattern[c]
Fixed (mM)	Varied (mM)	mM	mM	mM	mM	min^{-1}	
C_2H_5OH (600)	NAD^+ (1~6)	NADH (0~0.08)	3.8±0.67	0.1±0.05	0.2±0.07	0.2±0.02	NC
CH_3CHO (12)	NADH (0.07~0.30)	NAD^+ (0~32)	0.5±0.11	30.8±9.33	46.0±40.0	0.3±0.05	NC
NAD^+ (14)	C_2H_5OH (75~500)	CH_3CHO (0~4)	236.8±29.74	1.7±0.37	2.5±0.56	0.2±0.01	NC
NADH (1)	CH_3CHO (2.4~20)	C_2H_5OH (0~20)	6.3±0.63	58.8±9.33	190.5±42.5	0.3±0.01	NC
C_2H_5OH (100)	NAD^+ (1~6)	NADH (0~0.08)	6.1±1.50	0.1±0.07	0.1±0.05	0.3±0.04	NC
CH_3CHO (2)	NADH (0.07~0.30)	NAD^+ (0~32)	0.2±0.05	11.5±4.47	35.8±31.9	0.2±0.03	NC
NAD^+ (2)	C_2H_5OH (75~500)	CH_3CHO (0~4)	106.0±14.89	1.3±0.28	8.2±3.2	0.1±0.01	NC
NADH (0.15)	CH_3CHO (2.4~20)	C_2H_5OH (0~20)	2.8±0.38	11.7±3.09	72.6±28.0	0.2±0.01	NC
C_2H_5OH (600)	NAD^+ (1~6)	CH_3CHO (0~12)	7.2±1.68	15.5±6.78	11.7±6.45	0.4±0.06	NC

[a]Slope inhibition constant.
[b]Interpt inhibition constant.
[c]NC, noncompetitive inhibition.

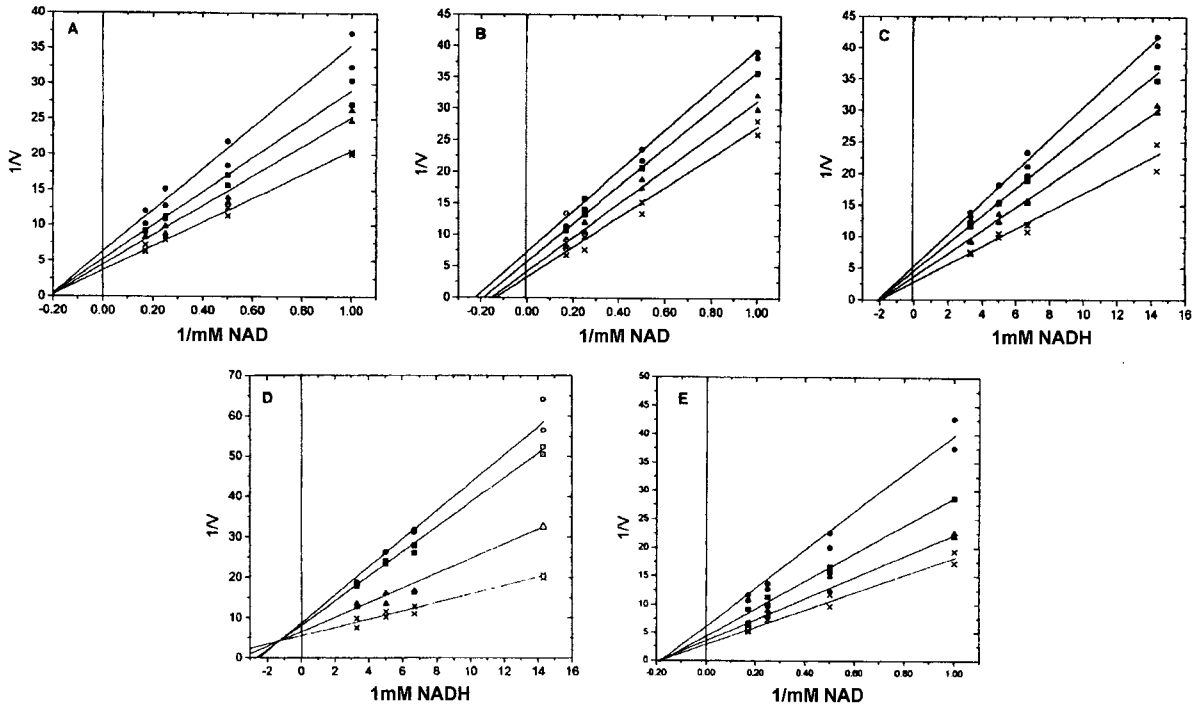

Fig. 1. Product inhibition patterns for the K228G mutant HLADH-E. The buffer was pH 8.0, 33 mM sodium phosphate containing 0.25 mM EDTA. Velocities were measured at 25°C and have units of ΔA_{340}/min for A, B and E and ΔA_{366}/min for C and D. A: inhibition by NADH (×: 0, ▲: 0.04, ■: 0.06, ●: 0.08 mM) against NAD^+ (1, 2, 4, 6 mM) at 600 mM ethanol, 27.25 nM enzyme. B: inhibition by NADH (×: 0, ▲: 0.04, ■: 0.06, ●: 0.08 mM) against NAD^+ (1, 2, 4, 6 mM) at 100 mM ethanol, 27.25 nM enzyme, C: inhibition by NAD^+ (×: 0, ▲: 8, ■: 16, ●: 32 mM) against NADH (0.07, 0.15, 0.20, 0.30 mM) at 12 mM acetaldehyde, 27.25 nM enzyme, D: inhibition by NAD^+ (×: 0, ▲: 8, ■: 16, ●: 32 mM) against NADH (0.07, 0.15, 0.20, 0.30 mM) at 2 mM acetaldehyde, 27.25 nM enzyme, E: inhibition by acetaldehyde (×: 0, ▲: 2, ■: 8, ●: 12 mM) against NAD^+ (1, 2, 4, 6 mM) at 600 mM ethanol, 27.25 nM enzyme.

Table IV. Product inhibition studies of subcloned pBPP/HLADH-E/K228G. Product inhibition studies were performed at 25°C in 33 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA

Substrate		Inhibitor	K_m	K_{is}[a]	K_{ii}[b]	V	Pattern[c]
Fixed (mM)	Varied (mM)	mM	mM	mM	mM	min^{-1}	
C_2H_5OH (600)	NAD^+ (1~6)	NADH (0~0.18)	3.4±0.5	0.15±0.04	0.13±0.03	0.5 ±0.03	NC
C_2H_5OH (600)	NAD^+ (1~6)	CH_3CHO (0~60)	2.9±0.3	15.9±3.40	27±7	0.23±0.008	NC

[a]Slope inhibition constant.
[b]Intercept inhibition constant.
[c]NC, noncompetitive inhibition.

with NAD^+. Lee & Ryu (1993) also reported that substitution of Gly-224 to Ile made the formation of the ternary complex of enzyme, coenzyme and substrate insignificant kinetically and release of the first product accompanied by the conformational change of enzyme, and changed the reaction mechanism to Iso Theorell-Chance. The K228G substitution also appeared to affect the enzyme dynamics. The previous paper using K228G mutant (Cho *et al.*, 1995) indicated that K228 residue was involved in the activation phenomenon during acetimidylation of the wild-type enzyme (Morris and McKinley-McKee, 1972; Plapp, 1970; Dworschack *et al.*, 1975). Therefore, lysine residue at 228 plays an improtant role in coenzyme association and dissociation to control enzyme activity and reaction mechanism.

To confirm no other mutations on the gene except K228G, the subcloning of the K228G fragment into wild-type pBPP/HLADH-E was carried out. No mutation in other sites of ADH gene could be detected except the K228G. Also the purified enzyme showed the same product inhibition as K228G mutant enzyme (Table IV). These results confirmed the changes in kinetic constants and reaction mechanism was due to K228G mutation in the protein.

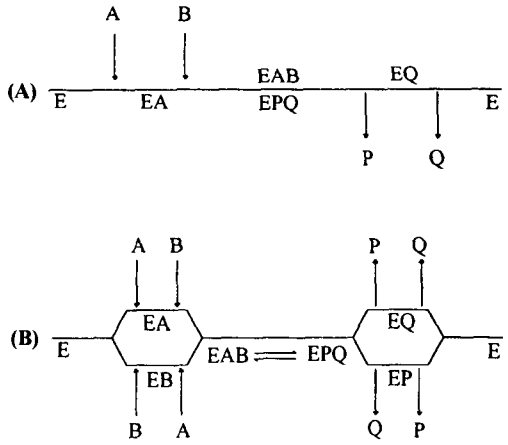

Fig. 2. Scheme for Ordered Bi Bi (A) and Random Bi Bi (B) mechanism of HLADH-E. The abbreviations used are: E, HLADH-E; B and P, ethanol and acetaldehyde; A and Q, NAD^+ and NADH.

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