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Substitution of Gly-224 Residue to Ile in Yeast Alcohol Dehydrogenase and Enzyme Reaction Mechamism

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Gly-224 residue of yeast alcohol dehydrogenase was mutated by site-directed mutagenesis to isoleucine, which is the corresponding amino acid residue of horse liver alcohol dehydrogenase. The mutated gene on M13 vector was subcloned in YEp13 and used to transform *Saccharomyces cerevisiae* 302-21#2 strain, and the expressed protein was purified. The turnover numbers of mutant enzyme for ethanol and acetaldehyde were decreased compared to wild-type enzyme. The results of product inhibition studies indicated that the reaction mechanism was changed to Iso Theorell-Chance from Ordered Bi Bi. We supposed that Gly-224 was related to the enzyme reaction mechanism.

Key words: YADH I, Gly-224, Site-directed mutagenesis, Isoleucine, Iso Theorell-Chance, Ordered Bi Bi

INTRODUCTION

The structure and mechanism of alcohol dehydrogenase (EC 1.1.1.1, ADH) have been extensively studied (Eklund and Branden, 1987). Various alcohol dehydrogenases from different sources have been discovered, isolated and reported. Horse liver alcohol dehydrogenases (HLADH) and yeast alcohol dehydrogenase (YADH) have been major objects of studies(Branden *et al.*, 1975; Klinman, 1981). The tertiary structures for apoenzyme and holoenzyme of HLADH were determined by X-ray crystallography (Eklund *et al.*, 1982). The tertiary structure of YADH has not been identified but its gene was cloned and the amino acid sequence is known (Bennetzen *et al.*, 1982). Between HLADH and YADH, there are many differences in amino acid sequence, but the amino acid residues of sites related to reaction such as substrate and coenzyme binding site are somewhat conserved (Jornvall *et al.*, 1978). On the basis of the tertiary structure of HLADH, an active site conformation of YADH was proposed in which Gly-224 (according to HLADH amino acid sequence) is located at coenzyme binding site (Plapp *et al.*, 1987). In this paper we have mutated Gly-224 residue to isoleucine, which is the corresponding amino acid residue of HLADH, and compared the kinetic properties of the mutant enzyme and native enzyme. Through initial velocity and product inhibition studies the enzyme reaction mechanism was investigated.

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MATERIALS AND METHODS

Microorganisms and vectors

M13mp19-YADH I virus for template DNA preparation was obtained from Dr. Bryce V. Plapp, The University of Iowa (Ganzhorn and Plapp, 1988). *E. coli* CSH50, CJ236 and XL1-Blue strains as host cell for mutation, YEp13 plasmid as a shuttle subcloning vector and *Saccharomyces cerevisiae* 301-21#2 (Y02; MATa, *adh 1-11, leu2, trp1*) which could not produce active alcohol dehydrogenase as host cell for YEp13-ADH were prepared by Dr. E. T. Young, The University of Washington. For native enzyme production, *Saccharomyces cerevisiae* Y24 strain was used.

Media

The culture medium for *E. coli* containing M13mp 19-YADH I DNA was YT medium, *E. coli* strains containing YEp13-YADH I was grown in LB medium containing ampicillin (50 μg/ml). 2YT medium was used for preparation of single-stranded template DNA of DNA sequencing and Sc-Leu selective medium and YPD medium containing antimycin A (1 μg/ml) for selection of the yeast cells transformed with YEp13-YADH I. For YADH I purification, seed culture medium was Sc-Leu medium and main culture medium was YPD medium.

Reagents and apparatuses

The mutagenic oligonucleotide (the sites of mutation are underlined), GGT/ATT/GAC/ATT/GGT/GAA/GG (Ile-224) was synthesized and supplied by Korea Basic Science Center. Klenow fragment and T4 DNA ligase were obtained from KOSCO. Restriction enzymes *Sph*I and *Eco*RI were obtained from Pharmacia. The sequenase kit was from USB. The column materials for ADH purification, DEAE-Sepharose CL-6B and Octyl-Sepharose CL-4B were purchased from Pharmacia, standard protein marker for SDS-PAGE, NAD^+Li salt (99%) and NADH (98%) as coenzyme from Sigma Chemical Co. 95% Ethanol and 99% acetaldehyde for substrate from Ducksan Pharmaceutical Co. and Fluka, respectively, were redistilled prior to use.

A Varian Cary 210C spectrophotometer was used for enzyme activity assay by measuring the change in absorbance at 340 nm.

Mutagenesis and transformation

Mutation was carried out by using two primer method (Zoller and Smith, 1987). Single-stranded M13 template DNA containing uracil was prepared by infection of *E. coli* CJ236 with M13mp19-ADH I virus (Kunkel *et al.*, 1987). The mutated DNA sequence was confirmed by dideoxy DNA sequencing method (Sanger *et al.*, 1977). After confirming the mutation, double-stranded M13mp19-ADH I DNA was isolated from phage-infected *E. coli* XL1-Blue (Stratagene plasmid mini boiling preparation protocol) and digested with SphI restriction enzyme. The 1.6 kb ADH I DNA fragment was cut out from 1% low-melting agarose gel after electrophoresis, and ligated to YEp13 plasmid DNA which was prepared by *Sph*I digestion and bacterial alkaline phosphatase treatment. The ligation mixture was added to competent *E. coli* XL1-Blue cells and spreaded on the 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 1.6 kb ADH I insert in YEp13 plasmid was confirmed by EcoRI and SphI digestion. The plasmid containing the insert was used for the transformation of yeast strain *Saccharomyces cerevisiae* 302-21 # 2 (Y02) by the lithium acetate method (Ito *et al.*, 1983). Transformants were selected on Sc-Leu minimal medium plate in order to identify the acquisition of the LEU2 marker gene. The transformants on Sc-Leu plate were tested for ADH I gene expression on YPD agar plate containing antimycin A (1 μg/ml).

Enzyme isolation and purification

Seed cultures in 50 ml Sc-Leu minimal liquid medium were obtained by inoculating one-loop of the yeast transformant cells and incubation for 48 hours at 175 rpm and 30℃ ($A_{600} \simeq 5.0$). These cultures were used to inoculate 6 liters of YPD medium and cultivated with agitation at 30℃ for 48 hours ($A_{600} \simeq 10.0$). The cells were collected by centrifugation on GSA rotor at 6500 rpm and 4℃ for 5 min. The yield of cells was 10~15 g/liter (wet weight). The cells were stored at −70℃ until use. The alcohol dehydrogenase was purified by following the purification methods of Ganzhorn *et al.* (1988) through lysis of cell by glass bead beating, 10~18% polyethylene glycol 4000 fractionation, DEAE-Sepharose CL-6B column (2.0×20 cm) and Octyl-Sepharose CL-4B column (3.0×10 cm) chromatography. The degree of purification was determined with 12% SDS-PAGE (Laemmli, 1970).

ADH enzyme activity assay

Enzyme activities were assayed in the reaction mixture containing 995 or 990 μl of the assay solution and 5 or 10 μl of enzyme sample. 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, 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 equation of [(ΔA_{340}/min)×dilution factor/(6.22×volume(ml) of the enzyme solution added to reaction mixture)].

Kinetic studies

The kinetic constants of the purified ADH I enzyme to coenzyme (NAD^+, NADH) and substrate (ethanol, acetaldehyde) were determined. Initial velocity studies were carried out at 30℃ in 83 mM potassium phosphate buffer containing 40 mM KCl, pH 7.3. 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 varing the concentration of the other substrate in the presence of a product. A physiological buffer of 83 mM potassium phosphate and 40 mM KCl at pH 7.3 and 30℃ was used (Cornell, 1983). A Fortran program was used to estimate initial velocities by a linear or parabolic fit of the data. Initial velocities were fitted to the appropriate equations with the programs COMP, NONCOMP or UNCOMP (Cleland, 1979).

RESULTS AND DISCUSSION

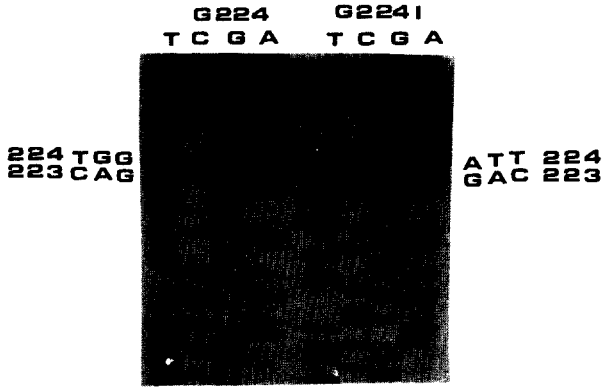

Fig. 1. Confirmation of site-directed mutagenesis in YADH I gene at the site 224 residue from glycine (GGT) to isoleucine (ATT) by Sanger dideoxy DNA sequencing method.

Fig. 1 shows the mutation of the position of Gly-224 to isoleucine. The expected change in codon sequence from GGT to AAT was confirmed. Insertion of 1.6 kb ADH I gene to YEp13 plasmid was confirmed by the presence of 1.6 kb ADH I gene fragment when treated with *Sph*I and of 6.0 kb fragment band with *Eco*RI. *Saccharomyces cerevisiae* 302-21 # 2 (Y02) transformed with YEp13-YADH I could grow in Sc-Leu plate and was resistant to antimycin A due to expression of the ADH I gene.

The purified G224 and G224I YADH proteins were nearly pure and molecular weights were about 36 kDa (corresponding to subunit molecular weight of YADH I: 36250 dalton). The fractions with the highest activity of Octyl-Sepharose column fractions were used for kinetic studies and specific activities were calculated from the YADH I concentration (mg/ml) and enzyme activity (U/mg). The protein concentration was determined from the absorbance at 280 nm, by using a factor 1.26 A/cm per 1 mg/ml (Hayes and Velick, 1954). The specific activities of purified proteins were 289 U/mg for G224I-mutant enzyme and 828 U/mg for wild-type enzyme (Table I).

The reactivity 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). The turnover numbers were decreased 10-fold for ethanol and 4-fold for acetaldehyde. Michaelis constants for NAD^+, ethanol and NADH increased by 5.5-fold, 3-fold and 3-fold, respectively, but were nearly unchanged for acetaldehyde. Fig. 2 shows the inhibition pattern by NADH as product inhibitor against NAD^+ for G224 and G224I YADH. The pattern of G224 wild-type enzyme showed competitive inhibition pattern (Fig. 2A), which is consistent with the Ordered BiBi mechanism, but G224I mutant enzyme showed noncompetitive product inhibition pattern (Fig. 2B). We carried out further product inhibition studies in order to decide the

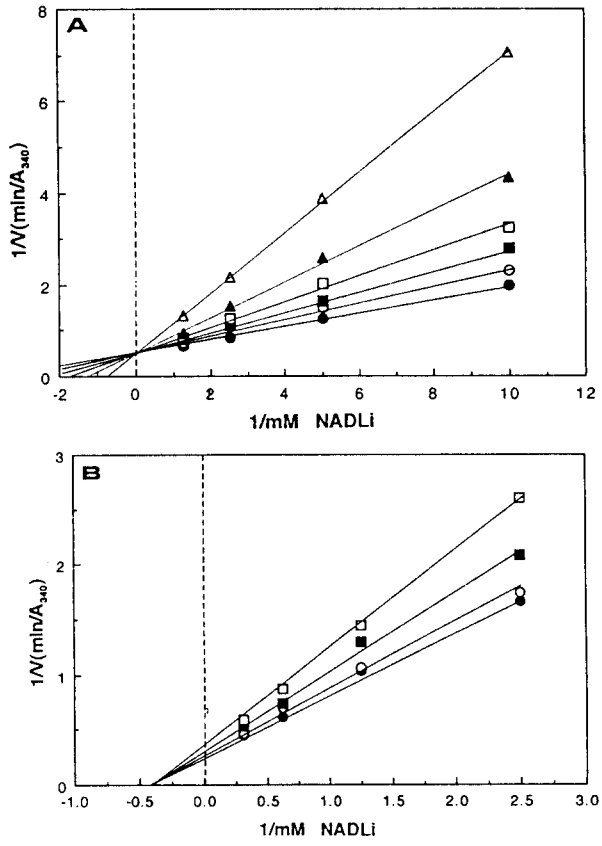

Fig. 2. Product inhibition patterns for G224 and G224I yeast alcohol dehydrogenase. A, inhibition by NADH (●, 0 mM: ○, 0.0125 mM: ■, 0.025 mM: □, 0.05 mM: ▲, 0.10 mM: △, 0.20 mM) at 500 mM ethanol, 2.42 nN G224 wild-type enzyme. B, inhibition by NADH (●, 0 mM: ○, 0.05 mM: ■, 0.10 mM: □, 0.20 mM) at 500 mM ethanol, 6.47 nN G224I mutant enzyme. 1/V is the reciprocal initial velocity in $(\Delta A/min)^{-1}$.

Table I. Purification table of wild type and G224I YADHI proteins

	Total Unit(U)		Total Protein(mg)		Specific Activity(U/mg)	
	G224	G224I	G224	G224I	G224	G224I
Protamine sulfate	86925	17268	1373	752	63	23
PEG precipitate	44289	8751	375	308	118	28
DEAE-Sepharose	42297	3233	104	27	407	120
Octyl-Sepharose	24829	3183	30	11	828	289

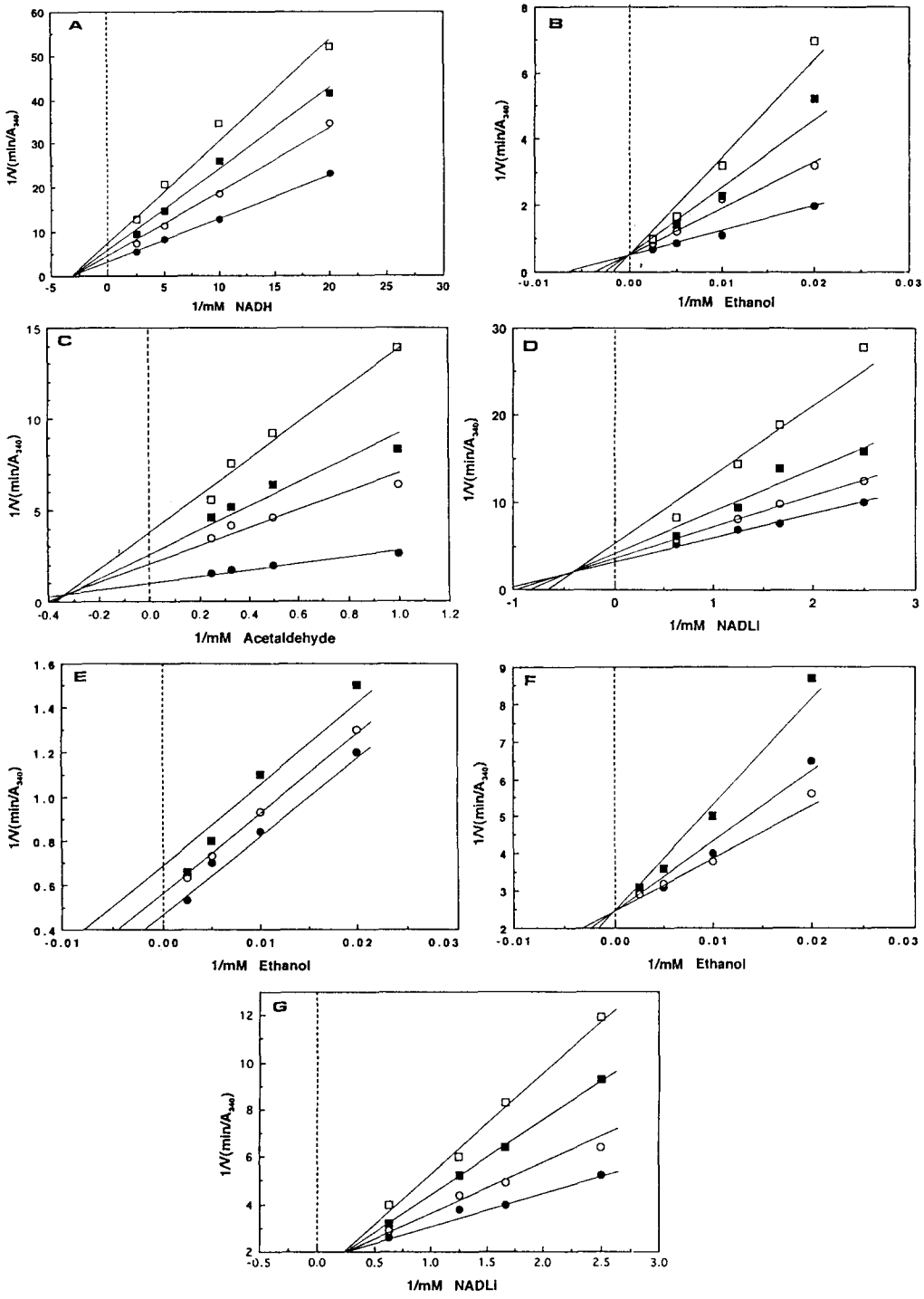

Fig. 3. Product inhibition patterns for G224I yeast alcohol dehydrogenase. The buffer was 83 mM potassium phosphate, 40 mM KCl, pH 7.3. Velocities were measured at 30°C. A, inhibition by NAD^+ (●, 0 mM; ○, 2 mM; ■, 4 mM; □, 8 mM) against NADH (0.05, 0.1, 0.2 and 0.4 mM) at 25 mM acetaldehyde, 11.2 nN enzyme. B, inhibition by acetaldehyde (●, 0 mM; ○, 0.125 mM; ■, 0.3 mM; □, 0.5 mM) against ethanol (50, 100, 200 and 400 mM) at 10 mM NADLi, 56 nN enzyme. C, inhibition by ethanol (●, 0 mM; ○, 125 mM; ■, 250 mM; □, 500 mM) against acetaldehyde (1, 2, 3 and 4 mM) at 0.4 mM NADH, 11.2 nN enzyme. D, inhibition by NADH (●, 0 mM; ○, 0.05 mM; ■, 0.1 mM; □, 0.2 mM) against NAD^+ (0.4, 0.6, 0.8 and 1.6 mM) at 25 mM ethanol, 56 nN enzyme. E, inhibition by NADH (●, 0 mM; ○, 0.1 mM; ■, 0.2 mM) against ethanol (50, 100, 200 and 400 mM) at 5 mM NADLi, 112 nN enzyme. F, inhibition by acetaldehyde (●, 0 mM; ○, 0.125 mM; ■, 0.3 mM) against ethanol (50, 100, 150 and 200 mM) at 0.9 mM NADLi, 56 nN enzyme. G, inhibition by acetaldehyde (●, 0 mM; ○, 0.06 mM; ■, 0.12 mM; □, 0.24 mM) against NADLi (0.4, 0.6, 0.8 and 1.6 mM) at 25 mM ethanol, 112 nN enzyme.

Table II. Kinetic constants of G224 wild-type(G224), G224I mutant yeast alcohol dehydrogenase I

		G224[a]	G224I	EHLADH[b]
Km(mM)	NAD^+	0.16~0.17	0.92	0.0039
	Ethanol	17~21	53	0.35
	Acetaldehyde	0.74~1.1	0.96[c]	0.40
	NADH	0.0994~0.11	0.34[c]	0.0058
V_1 (sec^{-1})		340~360	39	
V_2 (sec^{-1})		1700~1800	471	
Activity (sec^{-1})		400	201	

Initial velocity studies were performed at pH 7.3 and 30℃ in a physiological buffer of 83 mM potassium phosphate and 40 mM KCl (Cornell, 1983). Km is the Michaelis constant. V_1 and V_2 are the turnover numbers of forward and reverse reaction, respectively.
[a]Data from Ganzhorn *et al.* (1987) and Gould and Plapp (1990).[b]Data from Park and Plapp (1991). [c]The kinetic constants for acetaldehyde reduction were determined from assays at 366 nm using a 10 mm path-length cuvette. The highest NADH concentration was 0.4 mM.

reaction mechanism of G224I mutant enzyme. The results are shown in Fig. 3 and summarized in Table III. Under the condition of saturated (>5×Km) or unsaturated NAD^+ concentration, the inhibition pattern by acetaldehyde was competitive (Fig. 3B, F) and at unsaturated NADH, the inhibition pattern by ethanol was noncompetitive (Fig. 3C). But the inhibition pattern by acetaldehyde at unsaturated concentration of ethanol was noncompetitive (Fig. 3G).

Comparing the amino acid residues of the coenzyme-binding site of the active site between HLADH and YADH I, Asp, Lys and Phe around the adenine ribose of the active site are the same but 224 amino acid residue is different (HLADH : Ile, YADH : Gly). It has been known that the affinity of HLADH to coenzyme and substrate are much higher than those of YADH as shown in Table II. In our Ile-224 mutant enzyme, the expected increments of affinity to coenzyme were not seen (Table II). But we could observe from the results of product inhibition studies (Table III) that substitution Gly-224 to Ile changed the reaction mechanism to Iso Theorell-Chance (Fig. 4) (Laidler and Bunting, 1973). In this mechanism, the formation of the ternary complex of enzyme, coenzyme and substrate is insignificant kinetically and release of the first product is accompanied by the conformational change of enzyme. We assumed that the substitution of Gly-224 residue to isoleucine resulted in instability of the ternary complex of YADH I. The computer modeling on the basis of X-ray crystallography of YADH I is need-

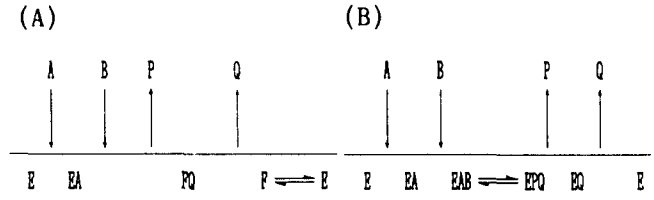

Fig. 4. Scheme for Iso Theorell-Chance (A) and Ordered Bi Bi (B) mechanism of yeast alcohol dehydrogenase.
E: YADH I, F: isomerized YADH I, B and P: ethanol and acetaldehyde, A and Q: NAD^+ and NADH.

Table III. The summary of product inhibition studies of G224I yeast enzyme

SUBSTRATE (mM) FIXED	VARIED	INHIBITOR (mM)	Km (mM)	Kis (mM)	Kii (mM)	V (min^{-1})	Inhibition pattern G224I	G224[a]	Fig. 2 or 3
EtOH	NAD^+	NADH							
500	0.4-3.2	0-0.2	2.00	0.35	1.20	3.6	NC	C	2A, 2B
CH_3CHO	NADH	NAD^+							
25	0.05-0.4	0-8	0.37	4.3	8.1	0.35	NC	C	3A
NAD^+	EtOH	CH_3CHO							
10	50-400	0-0.5	140	0.19		1.9	C	NC	3B
NADH	CH_3CHO	EtOH							
0.4	1-4	0-500	1.1	66	170	0.79	NC	NC	3C
EtOH	NAD^+	NADH							
25	0.4-1.6	0-0.2	0.70	0.075	0.49	0.28	NC	C	3D
NAD^+	EtOH	NADH							
5	50-400	0-0.2	99		0.62	2.3	UC	NC	3E
NAD^+	EtOH	CH_3CHO							
0.9	50-200	0-0.3	120	0.98		0.58	C	NC	3F
EtOH	NAD^+	CH_3CHO							
25	0.4-1.6	0-0.24	0.84	0.13	0.92	0.58	NC	NC	3G

[a]Product inhibition patterns of wild-type YADH I with Ordered Bi Bi mechanism (Gould and Plapp, 1990; Ganzhorn *et al.*, 1987).

ed to explain the effect of the substitution in detail.

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REFERENCES CITED

Bennetzen, J. and Hall, B. D., The primary structure of the *Saccharomyces cerevisiae* and gene for alcohol dehydrogenase I. *J. Biol. Chem.,* 257, 3018-3025 (1982).

Branden, C. I., Jornvall, H., Eklund, H. and Furugren, B., Alcohol dehydrogenase: In *Enzymes* (3rd Ed.), 11, 1975, pp. 103-190.

Cleland, W. W., Statistical analysis of enzyme kinetic data. *Methods Enzymol.,* 63, 103-138 (1979).

Cornell, N. W., Properties of alcohol dehydrogenase and ethanol oxidation in vivo and in hepatocytes. *Pharmacol. Biochem. Behav.,* 18, Suppl. 1, 215-221 (1983).

Eklund, H., and Brändén, C. I., *Active Sites of Enzymes,* Jurnak. F. A. and McPherson, A. (Eds), Wiley, New York, 1987, pp. 73-142.

Eklund, H., Plapp, B. V., Samama, J. P. and Brändén, C. I., Binding of substrate in a ternary complex of horse liver alcohol dehydrogenase. *J. Biol. Chem.,* 257, 14349-14358 (1982).

Ganzhorn, A. J., Green, D. W., Hershey, A. D., Gould, R. M. and Plapp, B. V., Kinetic characterization of yeast alcohol dehydrogenase. *J. Biol. Chem.,* 262, 3754-3761 (1987).

Ganzhorn, A. J. and Plapp, B. V., Carboxyl groups near the active site zinc contribute to catalysis in yeast alcohol dehydrogenase, J. Biol. Chem., 263, 5446-5454 (1988).

Gould, R. M., Plapp, B. V., Substitution of arginine for histidine-47 in the coenzyme binding site of yeast alcohol dehydrogenase I. *Biochemistry,* 29, 5463-54 68 (1990).

Hayes, J. E., Jr., and Velick, S. F., Yeast alcohol dehydrogenase; Molecular weight, coenzyme binding and reaction equilibria, J. Biol. Chem., 207, 225-244 (1954).

Ito, H., Fukuda, Y., Murata, K. and Kimura, A., Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.,* 153, 163-168 (1983).

Jornvall, H., Eklund, H. and Branden, C. I., Subunit conformation of yeast alcohol dehydrogenase. *J. Biol. Chem.,* 253, 8414-8419 (1978).

Klinman, J. P., Probes of mechanism and transition state structure in alcohol dehydrogenase reaction. *Crit. Rev. Biochem.,* 10, 37-78 (1981).

Kunkel, T. A., Roberts, J. D. and Zakour, R. A., Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol.,* 154, 367-382 (1987).

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-685 (1970).

Laidler, R. J. and Bunting, P. S., *The Chemical Kinetics of Enzyme Action* (2nd Ed.), Clarendon Press, Oxford, 1973.

Park, D. H. and Plapp, B. V., Isozymes of horse liver alcohol dehydrogenase active on ethanol and steroids; cDNA cloning, expression and comparison of active sites. *J. Biol. Chem.,* 266, 13296-13302 (1991).

Plapp, B. V., Ganzhorn, A. J., Gould, R. G., Green, D. W. and Hershey, A. D., Aldehyde dehydrogenase, aldo-keto reductase and alcohol dehydrogenase, In Weiner, H. and Flynn, T. G., (Eds.), *Enzymology and Molecular Biology of Carbonyl Metabolism 3, Progress in Clinical and Biological Research,* Alan R. Liss, New York, 1987, Vol. 232, pp. 227-236.

Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: a Laboratory Mannual, CSH, New York, 1989, p4.31.

Sanger, F., Nicklen, S. and Coulson, A. R., DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.,* 74, 5463-5467 (1977).

Zoller, M. J. and Smith, M., Oligonucleotide-directed mutagenesis; A simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol.,* 154, 329-350 (1987).