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Articles

Purification and Steady-State Kinetic Characterization of Human Liver $\beta_3\beta_3$ Alcohol Dehydrogenase†

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Received December 30, 1988; Revised Manuscript Received May 3, 1989

ABSTRACT: Human liver alcohol dehydrogenase catalyzes the NAD^+-dependent oxidation of alcohols. Isoenzymes are produced in liver by five different genes, two of which are polymorphic. We have studied the three $\beta\beta$ isoenzymes produced at *ADH2* because they exhibit very different kinetic properties and they appear with different frequencies in different racial populations. The $\beta_3\beta_3$ isoenzyme which appears in 25% of black Americans was purified to homogeneity, and conditions were found to stabilize this labile isoenzyme. The comparison of substrate specificity among $\beta\beta$ isoenzymes for primary straight-chain alcohols indicates that there is a positive correlation between V_{max}/K_M and the log octanol/water partition coefficient for alcohols with $\beta_2\beta_2$ and $\beta_3\beta_3$ but not with $\beta_1\beta_1$. Methyl substitutions at C1 or C2 of these alcohols reduce the catalytic efficiency with all three isoenzymes. The K_M and K_i values of $\beta_3\beta_3$ for NAD^+ and NADH are substantially higher than values for $\beta_1\beta_1$ or $\beta_2\beta_2$. The V_{max} of $\beta_3\beta_3$ for ethanol oxidation is 90 times that of $\beta_1\beta_1$. Sequencing of the β_3 subunit and gene indicates that the polymorphism results from a single amino acid exchange of Cys-369 in β_3 for Arg-369 in β_1 and β_2 [Burnell et al. (1987) *Biochem. Biophys. Res. Commun. 146*, 1227-1233]. In horse alcohol dehydrogenase and $\beta_1\beta_1$, the guanidino group of Arg-369 is thought to stabilize the NAD(H)-enzyme complex by bonding to one of the pyrophosphate oxygens. Thus, the substitution of Cys-369 in $\beta_3\beta_3$ explains its weak binding of coenzymes and high activity.

The majority of ingested ethanol is metabolized in the liver through acetaldehyde, to acetate. The rate-limiting step in this process is the oxidation of ethanol to acetaldehyde which is catalyzed by the NAD^+-dependent alcohol dehydrogenase (ADH, EC 1.1.1.1). In human liver, this enzyme exists in multiple molecular forms which are the products of five genes. The subunits encoded by *ADH1* (α), *ADH2* (β), and *ADH3* (γ) form homodimeric and heterodimeric class I ADH isoenzymes (Vallee & Bazzone, 1983; Smith, 1986; Bosron & Li, 1987). Polymorphism has been observed at two of these loci, resulting in the production of three different β subunits, β_1, β_2, and β_3 (Smith et al., 1971; Bosron et al., 1983a), and two different γ subunits, γ_1 and γ_2 (Smith et al., 1972). It has been proposed that differences in the catalytic properties of these polymorphic liver ADH isoenzymes may account for part of the observed genetic variability of ethanol metabolic rate in humans (Bosron & Li, 1981, 1987).

We have focused our recent efforts on the catalytic and structural characterization of the $\beta\beta$ isoenzymes because they exhibit widely different kinetic properties and appear with different frequencies in different racial groups. β_1 is found

†This work was supported by National Institute on Alcohol Abuse and Alcoholism Research Grant AA02342 and Training Grant AA07462.
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0006-2960/89/0428-6810$01.50/0

in all racial populations and is the predominant β ADH subunit observed in white Americans and Europeans (Bosron & Li, 1981). β_2 appears in about 85% of Japanese and Chinese, but less than 15% of white Americans and Europeans (Yin et al., 1984a). β_3 is observed only in about 25% of black Americans (Bosron et al., 1983a).

$\beta_3\beta_3$ had been purified from liver in low yield, but its activity was not stable (Bosron et al., 1979, 1980). The initial kinetic studies had indicated that it exhibited a higher V_{max} for ethanol oxidation and higher K_M values for NAD^+ and ethanol than those of $\beta_1\beta_1$. Here, we report a new procedure for the isolation of $\beta_3\beta_3$ from human liver which allows recovery of the pure, stable enzyme in yields necessary for structural and catalytic characterization. We have identified the amino acid substitution in the C-terminal region of β_3 vs β_1 (Cys for Arg-369; Burnell et al., 1987). We report here the steady-state kinetic constants of $\beta_3\beta_3$ for ethanol oxidation and substrate specificity and comparison of $\beta_3\beta_3$ kinetic properties with those of $\beta_1\beta_1$ and $\beta_2\beta_2$.

Materials and Methods

Source of Livers and Determination of ADH Phenotype. Autopsy liver specimens were obtained by the Marion County Coroner, Indianapolis, IN, from individuals who had succumbed to sudden traumatic death. Livers were stored at −70 °C. Phenotypes at *ADH2* and *ADH3* were determined by starch gel electrophoresis and by comparing the ethanol oxidizing activities of liver homogenate–supernatants at pH 7.0, 8.5, and 10 (Bosron et al., 1983a). $\beta_3\beta_3$ was purified from liver samples which exhibited the ADH2 3-3 ADH3 1-1 phenotype.

Assay of ADH Activity. Alcohol dehydrogenase activity was assayed by monitoring the formation or utilization of NADH in a Gilford Response spectrophotometer at 340 nm. Enzyme activity units are expressed as micromoles of NADH formed or utilized per minute based on the absorptivity of NADH at 340 nm of 6.22 $A \cdot mM^{-1} \cdot cm^{-1}$. Initial rates were estimated by linear regression of the change in absorbancy with time, and standard errors were less than 15% of the linear rate. Enzyme assays were performed at 25 °C with 33 mM ethanol and 2.4 mM NAD^+ in 0.1 M NaP_i–NaOH, pH 7.0. Protein concentrations were determined by the procedure of Lowry et al. (1951), using bovine serum albumin as standard. Specific activities of $\beta_3\beta_3$ are expressed as units of ethanol oxidizing activity at pH 7.0 per milligram of protein. All kinetic experiments were performed at 25 °C in 0.1 M NaP_i–NaOH, pH 7.5. Kinetic data were obtained in duplicate or triplicate, and kinetic constants were calculated by using the computer programs described by Cleland (1979). For individual experiments, the coefficients of variation of K_M and V_{max} were less than 10%, and that for K_i was less than 15%.

Purification of $\beta_3\beta_3$ ADH. Approximately 100 g of frozen liver tissue was minced and suspended in an equal volume (w/v) of 10 mM Tris-HCl, pH 8.0. The tissue was homogenized by using a Brinkmann Polytron and centrifuged at 91000*g* for 30 min. The homogenate–supernatant was passed through a 300-mL bed of DEAE-cellulose [(diethylaminoethyl)cellulose; DE52, Whatman] equilibrated with 10 mM Tris-HCl, pH 8.0. The ADH which eluted in the void volume was collected.

Tris-HCl, pH 8.2, was added to the DEAE pool to a final concentration of 0.1 M, and it was applied to a 2.5 × 12.5 cm agarose–hexane–AMP (AGAMP-Type II, Pharmacia) affinity chromatography column which had been equilibrated with 0.1 M Tris-HCl, pH 8.2. It was washed with buffer until the absorbancy of the effluent at 280 nm stabilized. ADH was eluted with 0.5 mM NADH and 0.5 M NaCl in buffer. ADH was precipitated by using 0.6 g/mL $(NH_4)_2SO_4$, centrifuged at 12000*g* for 30 min, and stored at −70 °C.

Table I: Purification of Human $\beta_3\beta_3$ ADH[a]

	total units	mg of protein
(1) homogenate–supernatant	500	6000
(2) DEAE-cellulose	600	1500
(3) agarose–AMP	500	500
(4) CM-cellulose, pH 8.6	50	20
(5) CM-cellulose, pH 7.7	20	7

[a] Average values for the recovery of ethanol oxidizing activity determined at pH 7.0 and protein for seven preparations are shown.

$\beta_3\beta_3$ was separated from other class I ADH isoenzymes by cation-exchange chromatography on CM-cellulose [(carboxymethyl)cellulose; CM-52, Whatman]. The precipitated protein was resuspended in 10 mM Tris-HCl, pH 8.2, at room temperature and desalted by gel filtration on Bio-Gel P-6DG (Bio-Rad) in 10 mM Tris-HCl, pH 8.2, at room temperature. NAD^+ was added to a final concentration of 1 mM, and ADH was applied to a 1.5 × 25 cm CM-cellulose column equilibrated with 10 mM Tris-HCl, 1 mM NAD^+, and 1 mM DTT, pH 8.6, at 4 °C. The column was washed with buffer until the absorbancy at 280 nm stabilized, and ADH was eluted with a 400-mL 0–35 mM NaCl linear gradient in buffer. The fractions containing $\beta_3\beta_3$ were pooled and concentrated, and 10% v/v of 1 M Tris-HCl, pH 8.2, was added. NAD^+ and DTT were added each to final concentrations of 2 mM, and an equal volume of glycerol was added to stabilize enzyme activity during storage at −20 °C.

$\beta_3\beta_3$ was rechromatographed on CM-cellulose in 5 mM Tris–H_3PO_4, pH 7.7, at 4 °C. The $\beta_3\beta_3$ pool in 50% glycerol was equilibrated with column buffer by gel filtration on Bio-Gel P-6DG at room temperature. NAD^+ was added to 1 mM concentration, and the pool was applied directly to an 1.5 × 25 cm CM-cellulose column equilibrated with 10 mM Tris–H_3PO_4 and 1 mM NAD^+, pH 7.7, at 4 °C. The column was washed with buffer until the absorbancy at 280 nm stabilized, and $\beta_3\beta_3$ was eluted with a 300-mL linear gradient of 15–35 mM NaCl in column buffer. $\beta_3\beta_3$ was stored at −20 °C in NAD^+, DTT, and glycerol as described above.

Electrophoresis and Isoelectric Focusing. SDS–polyacrylamide gel electrophoresis was performed using a separating gel of 10% polyacrylamide with a stacking gel of 4% polyacrylamide as described for the Hoefer Mighty Small gel system. High-voltage starch gel electrophoresis was performed in 13% starch gels at pH 8.0 as described by Bosron et al. (1979). Agarose isoelectric focusing was performed according to the procedure of Yin et al. (1984b), except that the pH 9–11 Ampholine (Pharmacia LKB) concentration was increased from 3.2% to 3.8%.

Protein was visualized on SDS–polyacrylamide gels using the basic silver strain (Wray et al., 1981). Both starch gels and agarose isoelectric focusing gels were stained for ethanol oxidizing activity with thiazolyl blue dye (Bosron et al., 1979) using 120 mM ethanol and 75 mM 1-butanol as substrates. Agarose isoelectric focusing gels were stained for protein with Coomassie Blue.

Results

Purification of $\beta_3\beta_3$ ADH. The ADH isoenzymes were isolated by affinity and ion-exchange chromatography from autopsy liver samples which contained isoenzymes with β_3 but not β_2 or β_1 subunits (Table I). All class I (α, β, and γ) isoenzymes in the homogenate–supernatant did not bind to DEAE-cellulose but bound to the AGAMP affinity column. They were identified at each step by starch gel electrophoresis

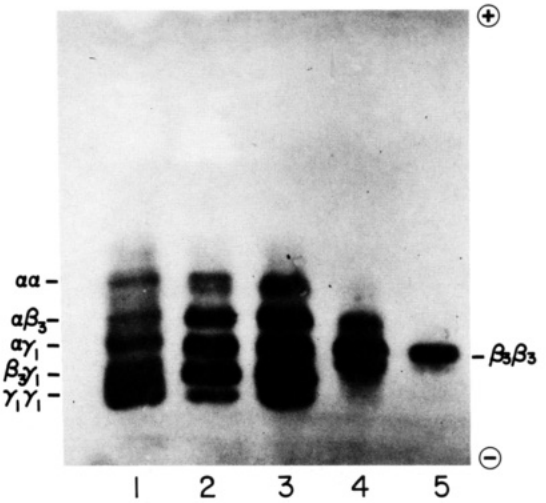

FIGURE 1: Agarose isoelectric focusing of ADH isoenzymes during purification of $\beta_3\beta_3$. Lane 1 contains the ADH2 3-3 ADH3 1-1 liver homogenate–supernatant, lane 2 the void pool from DEAE-cellulose chromatography, lane 3 the AGAMP pool, lane 4 the pH 8.6 CM-cellulose peak IV, and lane 5 the $\beta_3\beta_3$ pool from the pH 7.7 CM-cellulose column. The gel was stained for alcohol oxidizing activity as described under Materials and Methods.

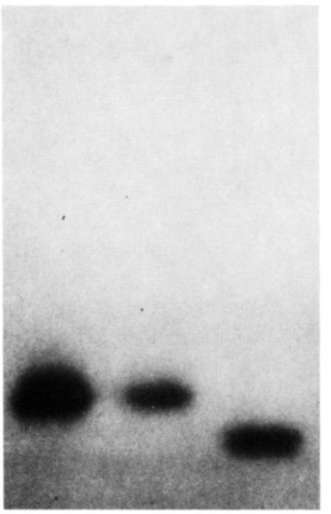

FIGURE 3: Agarose isoelectric focusing gel of purified $\beta\beta$ isoenzymes. Purified $\beta_2\beta_2$, $\beta_3\beta_3$, and $\beta_1\beta_1$ were applied to an agarose gel and subjected to isoelectric focusing as described under Materials and Methods. The gel was stained for alcohol oxidizing activity.

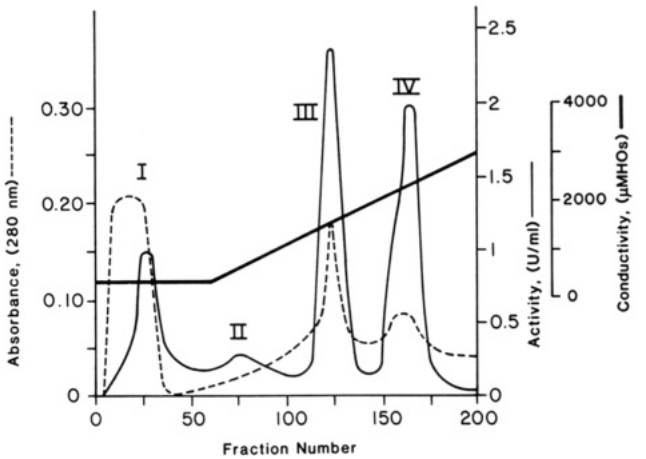

FIGURE 2: Elution profile from β_3 ADH isoenzyme separation by ion-exchange chromatography on CM-cellulose at pH 8.6. ADH purified by AGAMP chromatography was applied to a CM-cellulose column equilibrated at pH 8.6 as described under Materials and Methods. Enzyme activity at pH 7.0 (solid line) and protein (dashed line) were identified in fractions eluted with a 0–35 mM NaCl gradient. Peak IV contains the $\beta_3\beta_3$ isoenzyme.

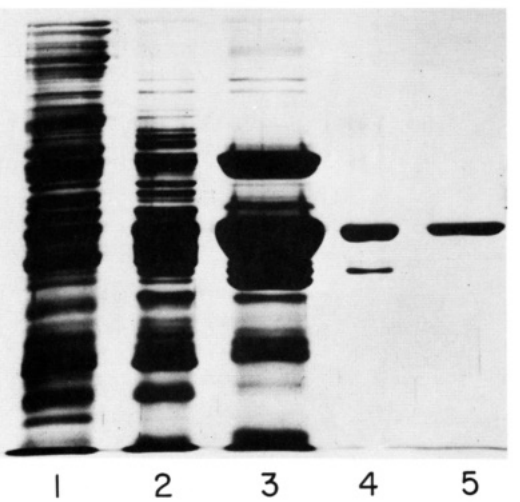

FIGURE 4: SDS–polyacrylamide gel electrophoresis of ADH isoenzymes during purification. The five samples described in Figure 1 were applied to the gel, electrophoresed, and stained for protein as described under Materials and Methods.

and agarose isoelectric focusing (Figure 1). The $\beta_3\beta_3$ was purified from the other isoenzyme forms by cation-exchange chromatography on CM-cellulose at pH 8.6 (Figure 2). $\alpha\alpha$, $\alpha\gamma_1$, and $\gamma_1\gamma_1$ eluted from the column in the unbound fraction (peak I). Three isoenzyme peaks were eluted by the linear salt gradient. Peak II contained $\alpha\beta_3$ and some $\beta_3\gamma_1$, and peak III contained $\beta_3\gamma_1$. Peak IV contained only $\beta_3\beta_3$, and it eluted last from the column at a concentration of about 20 mM NaCl (2300 $\mu\Omega^{-1}$). On starch gel electrophoresis, peak IV exhibited a single band of alcohol oxidizing activity which was the most cathodic band observed in the original liver homogenate–supernatant. However, three bands were identified by agarose isoelectric focusing of the $\beta_3\beta_3$ peak, a major band flanked by two minor bands (Figure 1, lane 4). These minor bands were observed both with the activity and with protein stain.

$\beta_3\beta_3$ was purified to homogeneity by chromatography on CM-cellulose at pH 7.7. It eluted at approximately 30 mM NaCl. As shown in Table I, the yield of $\beta_3\beta_3$ was about 4% of the homogenate–supernatant activity at pH 7.0. Purified $\beta_3\beta_3$ exhibited a single band on agarose isoelectric focusing. $\beta_3\beta_3$ migrated anodically to $\beta_1\beta_1$; its mobility was similar to $\beta_2\beta_2$ (Figure 3). On SDS–polyacrylamide gel electrophoresis, purified $\beta_3\beta_3$ showed a single band of approximately 40 000 daltons (Figure 4). CNBr peptides of the β_3 subunit prepared by this procedure have been sequenced from residues 41–55 and 367–374 (Burnell et al., 1987). The α, β, and γ subunits differ at five different positions in these regions; hence, purified $\beta_3\beta_3$ is not contaminated with isoenzymes containing α or γ subunits. The specific activity of $\beta_3\beta_3$ was 2.9 units/mg (Table I).

Kinetics of Ethanol Oxidation by $\beta_3\beta_3$. The steady-state kinetic constants of $\beta_3\beta_3$ for ethanol, acetaldehyde, and coenzymes were determined at pH 7.5 (Table II). The enzyme obeyed simple Michaelis–Menten kinetics when ethanol concentration was varied between 2 and 200 mM. Above 500 mM, substrate inhibition by ethanol was observed. The K_M and K_i values for NAD^+, the K_M for ethanol, and the V_{max} for ethanol oxidation were determined by simultaneously varying both NAD^+ and ethanol concentrations (Table II). The in-

Table II: Steady-State Kinetic Constants of Human Liver $\beta\beta$ ADH Isoenzymes[a]

constant	$\beta_1\beta_1$	$\beta_2\beta_2$	$\beta_3\beta_3$
K_M(EtOH) (mM)	0.022	0.84	36 ± 3 (11)
K_M(NAD^+) (μM)	7.4	180	710 ± 130 (6)
K_i(NAD^+) (μM)	90	340	2300 ± 300 (3)
K_M(AcH) (mM)	0.085	0.24	3.4 ± 1.5 (4)
K_M(NADH) (μM)	6.4	105	260 ± 87 (3)
$V_{max,fwd}$ (units/mg)	0.085	8.6	7.9 ± 1.4 (6)
$V_{max,rev}$ (units/mg)	6.0	98	64 ± 18 (3)
pH optimum	10.5	8.5	7.0

[a] Kinetic constants for all isoenzymes were determined in 0.1 M NaP_i–NaOH, pH 7.5, at 25 °C. Values for $\beta_3\beta_3$ are mean ± standard deviation for the indicated number of trials. K_M(AcH) (acetaldehyde) was determined over range of 1–20 mM AcH at 0.4 mM NADH. K_M(NADH) and $V_{max,rev}$ of $\beta_3\beta_3$ were determined at 20 mM AcH. K_M and $V_{max,fwd}$ of $\beta_1\beta_1$ and $\beta_2\beta_2$ for ethanol oxidation are derived from Tables III and IV. Other values for $\beta_1\beta_1$ were taken from Bosron et al. (1983b). Other values for $\beta_2\beta_2$ were taken from Yin et al. (1984b).

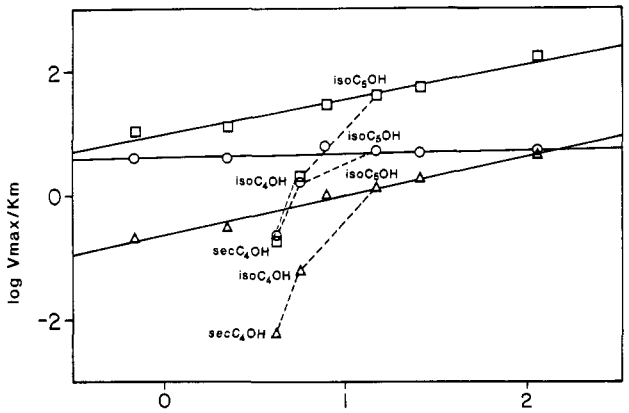

FIGURE 5: Hydrophobicity relationships for alcohol oxidation by $\beta\beta$ isoenzymes. Activity of purified $\beta_1\beta_1$ (○), $\beta_2\beta_2$ (□), and $\beta_3\beta_3$ (△) with alcohols was determined in 0.1 M NaP_i at pH 7.5. The log P values for parimary alcohols are the following from left to right: ethanol = −0.16, propanol = 0.34, butanol = 0.88, pentanol = 1.40, hexanol = 2.03. The log P values for secondary and branched-chain alcohols are *sec*-butyl alcohol = 0.61, isobutyl alcohol = 0.73, isopentyl alcohol = 1.16 (Leo et al., 1971; Hansch et al., 1972).

tersecting pattern is consistent with an ordered Bi-reactant mechanism. NADH was a competitive inhibitor of NAD^+ with a K_i of 56 μM. 4-Methylpyrazole was a competitive inhibitor of ethanol with a K_i of 2 μM. When acetaldehyde concentration was varied between 1 and 100 mM ([NADH] = 0.4 mM), plots of $1/v$ vs 1/[acetaldehyde] exhibited a downward curvature, and above 100 mM acetaldehyde, substrate inhibition was observed. For these reasons, the K_M value of $\beta_3\beta_3$ for acetaldehyde was determined by varying the acetaldehyde concentration from 1 to 20 mM (Table II). An equilibrium constant was calculated from the Haldane relationship, $K_{eq} = \{(V_{max,fwd})[K_M(AcH)][K_i(NADH)](10^{-7.5})\}/\{(V_{max,rev})[K_M(EtOH)][K_i(NAD^+)]\}$ (Cleland, 1963), to be 9.0×10^{-12}. This agrees well with values determined for horse and yeast alcohol dehydrogenases which are 8.6×10^{-12} and 11.5×10^{-12}, respectively (Theorell & Bonnischen, 1951; Racker, 1950).

Substrate Specificity of $\beta_3\beta_3$. The K_M and V_{max} values of $\beta_3\beta_3$, $\beta_2\beta_2$, and $\beta_1\beta_1$ for the oxidation of primary straight-chain aliphatic alcohols at pH 7.5 were determined. The V_{max} and V_{max}/K_M values are shown in Tables III and IV, respectively. For the primary straight-chain alcohols from two to six carbons, the plot of log (V_{max}/K_M) vs the log of the octanol/water partition coefficient (log P; Hansch et al., 1972) is linear (Figure 5). Simple regression analysis of the plots of log (V_{max}/K_M) for each isoenzyme vs log P of each substrate

Table III: V_{max} Values (units/mg) of Human Liver $\beta\beta$ ADH Isoenzymes for Oxidation of Aliphatic Alcohols at pH 7.5[a]

substrate	$\beta_3\beta_3$	$\beta_2\beta_2$	$\beta_1\beta_1$
ethanol	7.9	8.6	0.085
n-propyl alcohol	5.0	7.1	0.074
n-butyl alcohol	4.0	9.8	0.071
n-pentyl alcohol	4.6	6.8	0.092
n-hexyl alcohol	4.6	6.8	0.11
sec-butyl alcohol	0.4	3.4	0.080
isobutyl alcohol	2.7	6.6	0.097
isopentyl alcohol	3.4	7.8	0.073
methanol	NA	3.6	0.054
cyclohexanol	NA	ND	0.067

[a] V_{max} values of $\beta_3\beta_3$ were determined by multiplying V_{max}^{app} at 4.8 mM NAD^+ by 1.15 to correct for subsaturating [NAD^+]. V_{max} values of $\beta_2\beta_2$ and $\beta_1\beta_1$ were determined by varying each [substrate] at 2.4 mM NAD^+. NA indicates not active, and ND indicates not determined.

Table IV: V_{max}/K_M Values [(units/mg)/mM] of Human Liver $\beta\beta$ ADH Isoenzymes for Oxidation of Aliphatic Alcohols at pH 7.5[a]

substrate	$\beta_3\beta_3$	$\beta_2\beta_2$	$\beta_1\beta_1$
ethanol	0.22	10	3.9
n-propyl alcohol	0.29	12	3.9
n-butyl alcohol	0.98	29	5.9
n-pentyl alcohol	1.8	52	4.8
n-hexyl alcohol	4.6	170	5.1
sec-butyl alcohol	0.006	0.19	0.24
isobutyl alcohol	0.061	1.8	1.6
isopentyl alcohol	1.3	39	5.2
methanol	NA	0.0012	0.0049
cyclohexanol	NA	ND	0.0172

[a] Values are calculated from V_{max} values in Table III; K_M values are not shown. NA indicates not active, and ND indicates not determined.

results in the following equations, regression coefficients (r), and standard errors (s):

$$\log (V_{max}/K_M) = 0.64 (\log P) - 0.64 \quad \text{for } \beta_3\beta_3 \ (1)$$
$$r = 0.99 \ (n = 5); \ s = 0.081$$
$$\log (V_{max}/K_M) = 0.57 (\log P) + 0.99 \quad \text{for } \beta_2\beta_2 \ (2)$$
$$r = 0.98 \ (n = 5); \ s = 0.107$$
$$\log (V_{max}/K_M) = 0.062 (\log P) + 0.62 \quad \text{for } \beta_1\beta_1 \ (3)$$
$$r = 0.66 \ (n = 5); \ s = 0.060$$

For $\beta_3\beta_3$ and $\beta_2\beta_2$, the linear regression is significant at $p < 0.01$, but not for $\beta_1\beta_1$, $p > 0.05$.

The oxidation of *sec*-butyl alcohol (1-methyl-1-propanol) and isobutyl alcohol (2-methyl-1-propanol) by all three $\beta\beta$ isoenzymes resulted in log (V_{max}/K_M) values (Table IV) which were much less than those predicted by eq 1–3 (Figure 5). However, the V_{max}/K_M values for the oxidation of isopentyl alcohol (3-methyl-1-butanol) fit eq 1–3 for the three isoenzymes (Figure 5). As shown in Table III, the V_{max} values for the oxidation of straight and branched-chain primary alcohols and secondary alcohols by $\beta_1\beta_1$ are relatively constant, varying from 0.071 to 0.11 unit/mg. However, the V_{max} values of $\beta_2\beta_2$ and $\beta_3\beta_3$ for some alcohols are quite different. For example, $\beta_2\beta_2$ and $\beta_3\beta_3$ oxidize *sec*-butyl alcohol with V_{max} values which are only 35% and 10%, respectively, of values for 1-butanol. $\beta_3\beta_3$ oxidizes isobutyl alcohol and isopentyl alcohol with V_{max} values that are 68% and 74% of that for the straight-chain homologues.

The kinetics of propionaldehyde and butyraldehyde reduction with 0.4 mM NADH were also examined. As found with the primary alcohols, the K_M values for aldehydes decreased with increasing chain length. The K_M values for propionaldehyde and butyraldehyde were 3.2 and 0.65 mM, respec-

tively. In contrast to the kinetics of acetaldehyde, both longer chain aldehydes exhibited simple Michaelis–Menten kinetics in a 50-fold concentration range around K_M. The curvature observed with acetaldehyde could result from binding to other sites in the enzyme.

Discussion

The amino acid sequences of the three polymorphic human β ADH subunits differ only by single amino acid substitutions at position 47 or 369 (Jörnvall et al., 1987; Burnell et al., 1987). In the β_1 subunit, Arg residues are found at these two positions. A His residue is substituted for Arg-47 in the β_2 subunit, and a Cys residue is found at position 369 in β_3. X-ray crystallography of the horse EE isoenzyme indicates that the basic Arg residues at positions 47 and 369 interact with the negatively charged oxygens of the nicotinamide phosphate of NAD(H) to help stabilize the enzyme–coenzyme complex (Eklund et al., 1984). The horse and human enzymes have greater than 85% sequence identity (Jörnvall et al., 1987).

Jörnvall et al. (1984) suggested that the substitution of the bulky and less basic His residue at position 47 in the β_2 subunit can account for the 24- and 4-fold increases in the K_M and K_i values for NAD^+, the 100-fold increase in the V_{max}, and the decrease in the pH optimum for ethanol oxidation from 10.5 to 8.5 of $\beta_2\beta_2$ vs $\beta_1\beta_1$ (Yin et al., 1984b). The substitution of Cys at position 369 of the β_3 subunit also appears to alter coenzyme binding. For example, the K_i value of $\beta_3\beta_3$ for NAD^+ is 26 times greater than that of $\beta_1\beta_1$ (Table I). In the ordered Bi-Bi reaction mechanism of ADH, the K_i for NAD^+ is equivalent to a dissociation constant for the enzyme–coenzyme complex (Dalziel, 1963; Plapp, 1973). The V_{max} of $\beta_3\beta_3$ is 93 times greater than that of $\beta_1\beta_1$, and the pH optimum for ethanol oxidation is decreased from 10.5 to 7.0 (Bosron et al., 1980). The decrease in $\beta_3\beta_3$ activity above about pH 8.5 is consistent with an electrostatic repulsion of NADH by the thiolate anion formed by the deprotonation of Cys-369. This could accelerate the dissociation of NADH from the enzyme, the step which is partially rate limiting for horse ADH and $\beta_1\beta_1$ (Dworschack & Plapp, 1977; Bosron et al., 1983b). The dissociation rate constant of β_3 for NADH calculated from the kinetic relationship, rate = $(V_{max,rev})[K_i(NADH)]/[K_M(NADH)]$ (Cleland, 1963), was 14 units/mg or 550 min^{-1} per 40 000 molecular weight subunit of $\beta_3\beta_3$. The turnover number of $\beta_3\beta_3$, 320 min^{-1}, calculated from $V_{max,fwd}$, 7.9 units/mg, is only 40% slower; hence, NADH dissociation appears to be partially rate limiting for alcohol oxidation.

The K_M value of $\beta_3\beta_3$ for ethanol is 1600 times greater than that of $\beta_1\beta_1$ (Table II), and the K_M values of $\beta_3\beta_3$ for other substrates also are much greater than those of $\beta_1\beta_1$ (data can be calculated from Tables III and IV). It is not clear why the Cys for Arg-369 substitution should affect the K_M for alcohol so much. In addition to alcohol and aldehyde rate constants, the expression for this K_M contains constants for NADH dissociation and hydride transfer in the ternary complex. Changes in the NADH and hydride transfer constants have been shown to account for most of the difference in K_M for ethanol between the horse EE and SS isoenzymes (Dworschack & Plapp, 1977). The amino acid substitution in the coenzyme binding site of $\beta_3\beta_3$ could have similar effects on the K_M for alcohol.

The reduced affinity of $\beta_3\beta_3$ for coenzymes also affects the interaction of $\beta_3\beta_3$ with immobilized ligands used in ADH purification. $\beta_1\beta_1$, $\beta_2\beta_2$, and other class I ADH isoenzymes bind effectively to the CapGapp–Sepharose affinity column (Lange & Vallee, 1976; Bosron et al., 1983b; Yin et al., 1984b). These isoenzymes bind to this affinity resin by forming a ternary complex of ADH, NAD^+, and immobilized pyrazole. $\beta_3\beta_3$ does not bind to this affinity column (Bosron et al., 1980), even though it is inhibited by micromolar concentrations of 4-methylpyrazole. It is assumed that becase $\beta_3\beta_3$ forms a weak enzyme–NAD^+ complex (Table II), it cannot form a strong enzyme–NAD^+–immobilized CapGapp ternary complex. The fact that $\beta_3\beta_3$ binds to the AMP ligand of the AGAMP column suggests that the AMP binding site of β_3 may not be substantially altered by the Cys-369 substitution. The substitution of Cys for Arg-369 may also contribute to the instability of β_3 vs β_1 or β_2 and the necessity to include thiol reagents (DTT) in storage buffers.

The substrate binding pocket of the horse ADH E subunit is lined with apolar amino acid side chains (Eklund et al., 1974). Thus, substrates and inhibitors with long apolar aliphatic chains bind best in the substrate binding pocket (Hansch et al., 1972; Eklund & Samama, 1982). In the β subunits, either all of these apolar residues are conserved or the substituted amino acids also are apolar in nature (e.g., Tyr for Phe-110 and Val for Ile-318; Jörnvall et al., 1987). Thus, binding of the alcohol substrates in the $\beta\beta$ active sites should show the same relationship between the length of the aliphatic side chains and substrate binding. The K_M values of $\beta_3\beta_3$ and $\beta_2\beta_2$ decrease 36- and 21-fold, respectively, as the alcohol side chain increases in length from C_2 to C_6, while that of $\beta_1\beta_1$ is relatively independent of the straight-chain alcohol substrate (data can be calculated from Tables III and IV). For the V_{max}/K_m vs log P expressions of $\beta_3\beta_3$ and $\beta_2\beta_2$ (eq 1 and 2), the r values near unity also indicate that the efficiency of alcohol oxidation by these isoenzymes is directly related to the polarity of straight-chain alcohols. However, for $\beta_1\beta_1$ (eq 3), the r value (0.66, $p > 0.05$) and the slope term (0.062) are low.

The efficiency of oxidation of branched-chain alcohols by the human $\beta\beta$ ADH homodimers deviates from the linear relationships shown in eq 1–3. The V_{max}/K_M values for the oxidation of *sec*-butyl alcohol and isobutyl alcohol by $\beta_3\beta_3$, $\beta_2\beta_2$, or $\beta_1\beta_1$ are much lower than those of the corresponding straight-chain primary alcohols (Figure 5). However, the V_{max}/K_M value for the oxidation of isopentyl alcohol by each isoenzyme fits the relationships obtained for the oxidation of the straight-chain primary alcohols (eq 1–3). Thus, it appears that a methyl group substituted at C1 or C2 greatly reduces the efficiency of the oxidation (relative to that predicted by using eq 1–3), whereas substitution at the C3 position does not. X-ray crystallography of horse EE ADH–NAD^+–*p*-bromobenzyl alcohol complex has shown that the substrate binding site is narrow near the active-site zinc residue between the side chains of Ser-48 and Phe-93 (Eklund et al., 1982). This narrow area appears to enclose C1 and C2 of the bound substrate. In the β ADH subunits, Phe-93 is conserved, but the Ser-48 has been replaced by a more bulky Thr residue (Jörnvall et al., 1987).

These studies suggest that the structure of the homologous horse ADH can be used as a reference to interpret coenzyme and alcohol specificity of the human $\beta\beta$ isoenzymes. Specifically, the substitution of Cys for Arg-369 in the coenzyme binding site of human liver $\beta_3\beta_3$ isoenzyme results in large increases in coenzyme and alcohol K_M values, and maximal ethanol oxidizing activity vs $\beta_1\beta_1$. On the basis of these kinetic relationships, we propose that the pharmacokinetics of ethanol elimination in individuals with β_3 isoenzymes should differ from those of individuals with β_1 isoenzymes. For example, the rate of ethanol oxidation by $\beta_3\beta_3$ assayed at 100 mg % alcohol (22 mM) and near-physiological concentrations of

NAD^+ (0.5 mM), NADH (5 μM), and acetaldehyde (50 μM) at pH 7.4 and 37 °C is 4 times that of $\beta_1\beta_1$ (Burnell et al., 1987). The pharmacokinetics of blood ethanol elimination in caucasians has been found to be linear with time, starting from about 50 mg % or 11 mM alcohol concentration (Wagner et al., 1976). This is because the isoenzymes with α, β_1, γ_1, and γ_2 are nearly saturated at these concentrations. However, $\beta_3\beta_3$ is less than half-saturated at 11 mM ethanol; thus, the initial pharmacokinetics should be nonlinear. The recent development of methods to amplify the *ADH2* (β) gene from human white cell DNA and determine the *ADH2* genotype using allele-specific oligonucleotides (Gennari et al., 1988; Xu et al., 1988) will allow us to study the relationship between the presence of β_1, β_2, or β_3 isoenzymes and the pharmacokinetics of alcohol elimination.

Acknowledgments

We thank Colleen Harden and Leslie Magnes for expert technical assistance.

Registry No. ADH, 9031-72-5; NAD, 53-84-9; NADH, 58-68-4; AcH, 75-07-0; L-Arg, 74-79-3; L-Cys, 52-90-4; EtOH, 64-17-5; *sec*C_4OH, 78-92-2; isoC_4OH, 78-83-1; isoC_5OH, 123-51-3; *n*-propyl alcohol, 71-23-8; *n*-butyl alcohol, 71-36-3; *n*-pentyl alcohol, 71-41-0; *n*-hexyl alcohol, 111-27-3; methanol, 67-56-1; cyclohexanol, 108-93-0.

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