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HUMAN ALCOHOL DEHYDROGENASE: DEPENDENCE OF SECONDARY ALCOHOL OXIDATION ON THE AMINO ACIDS AT POSITIONS 93 AND 94

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Summary: The human liver $\alpha\alpha$ and $\beta_1\beta_1$ isoenzymes are straight-chain alcohol dehydrogenases with different efficiencies toward secondary alcohols. Two of the 24 amino acid substitutions in $\alpha\alpha$ (A for F93 and I for T94) were made by site-directed mutagenesis of $\beta_1\beta_1$ and the substrate specificity of β93A94I was examined. The V_{max}/K_M values of β93A94I for secondary alcohols (especially R enantiomers) are similar to that of $\alpha\alpha$ and as much as 4000-fold greater than $\beta_1\beta_1$, but the dependences of V_{max}/K_M on primary alcohol chain length are similar to $\beta_1\beta_1$, but not $\alpha\alpha$. Thus, the substitutions of A for F93 and I for T94 in $\beta_1\beta_1$ account for the increased efficiency towards secondary alcohols and stereoselectivity for enantiomeric alcohols, but not for the effects of chain length on the V_{max}/K_M for primary alcohols seen with $\alpha\alpha$.

There are multiple molecular forms of human alcohol dehydrogenase (EC 1.1.1.1). Each of these isoenzymes are dimers of about 80,000 molecular weight and contain between 373-377 amino acids per subunit (1). The isoenzymes that have been well studied are the products of five separate genes (ADH_1-ADH_5) that encode the α, β, γ, π, and χ subunits, respectively (2). Polymorphism has been demonstrated at ADH_2 (β_1, β_2, β_3) and ADH_3 (γ_1, γ_2) (2). The α, β, and γ subunits share at least 93% sequence identity and have been shown to form heterodimers (eg. $\alpha\beta_1$, $\beta_2\gamma_1$) (3). In contrast, the π and χ subunits share only 50-60% sequence identity with each other or with the α, β, or γ subunits and form only homodimers (eg. $\pi\pi$ or $\chi\chi$) (1,3). The isoenzymes exhibit large differences in both affinity and maximal oxidation rate for different alcohol substrates (3).

Only two alcohol dehydrogenases have been studied by X-ray crystallography; horse liver EE and human liver $\beta_1\beta_1$ (4-6). Molecular modelling studies of the horse EE alcohol dehydrogenase have identified potentially important amino acids lining the substrate binding site that determine substrate specificity for the group of human isoenzymes (7,8). The molecular modelling studies have been used to predict the internal topographies of the substrate binding pockets of the human alcohol dehydrogenase isoenzymes and how amino

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Abbreviations: DEAE, diethylaminoethyl; DTT, dithiothreitol; HEPES, N-[2-hydroxyethylpiperazine-N'-(2-ethanesulfonic acid)]; EDTA, ethylenediaminetetraacetic acid.

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acid substitutions appearing in the different human isoenzymes may affect substrate specificity. These studies suggest that the substitutions within the alcohol binding pocket and their potential effects can be separated into three regions, depending on their location from the catalytic zinc atom that coordinates the alcoholate anion (7,8). This approach has been used to explain the differences in substrate specificity among the various human alcohol dehydrogenase isoenzyme forms; for example, the unique ability of the human $\alpha\alpha$ isoenzyme to oxidize secondary alcohols with high efficiency is thought to arise from substitutions of Ala for Phe 93 and/or Thr for Ser 48 (7,9).

The recently determined structure of the human $\beta_1\beta_1$ alcohol dehydrogenase isoenzyme (6) and the ability to make specific mutations in $\beta_1\beta_1$, and express the mutant enzymes (10), provide an opportunity to assess the relative contributions of selected amino acid substitutions toward substrate specificity among the human alcohol dehydrogenase isoenzymes. We have used site-directed mutagenesis to substitute Phe 93 by Ala and Thr 94 by Ile in the human $\beta_1\beta_1$ enzyme to determine the contributions of these residues toward the substrate specificity of the recombinant enzyme for primary and secondary alcohols.

Materials and Methods: The mutagenesis and expression of the human $\beta_1\beta_1$ cDNA was performed as described by Hurley *et al.* (10) with the following modifications. After the lysis and initial batch chromatographic step over DEAE-cellulose[1], the enzyme was exchanged into 7 mM Hepes, pH 8.0, 2 mM DTT, 1 mM EDTA, and 1 mM benzamidine (C. Stone, unpublished work) by repeated concentration and redilution using a MINITAN tangential flow concentrator (Millipore). The buffer-exchanged enzyme was chromatographed over S-Sepharose (Pharmacia) that had been equilibrated with exchange buffer. After washing the column with two column volumes of exchange buffer, the enzyme was eluted with 110 mM NaCl in exchange buffer. The eluted enzyme was dialyzed into 20 mM sodium phosphate, pH 7.5, 0.5 mM DTT and applied to a Affi-Gel Blue column (Biorad) equilibrated in dialysis buffer. The bound enzyme was washed extensively to remove traces of coenzyme. The enzyme was then eluted with 0.85 M NaCl in column buffer. Alcohol dehydrogenase activity was followed at 340 nm in a standard assay containing 2.4 mM NAD^+ and 33 mM ethanol in 100 mM glycine, pH 10.0 using an extinction coefficient of 6.22 $cm^{-1}mM^{-1}$ for NADH. Protein concentrations were determined using the dye-binding procedure of Bradford (11) with bovine serum albumin as the standard.

The substrate specificity of the purified enzyme was determined by varying the concentration of alcohol at a fixed concentration of NAD^+ (2.4 mM) in 100 mM sodium phosphate, pH 7.5. All alcohols were the highest grade available and were used without further purification (Aldrich or Schweizerhall). The K_M value for NAD^+ was obtained by varying the concentration of NAD^+ (grade 1, Boehringer Mannheim) at a fixed concentration of ethanol (33 mM). The kinetic constants were determined from the initial velocity data by Cleland's kinetic programs (12). All other reagents were from Sigma and were of the highest grade commercially available.

Results: Between 5 and 10 mg of recombinant enzyme was obtained per liter of *E. coli* culture after final purification. The enzyme eluted from the S-Sepharose column was judged pure by SDS-polyacrylamide electrophoresis. However, it contained bound NADH as indicated by the absorbance at 322 nm. Therefore, the enzyme was chromatographed over an Affi-Gel Blue column in order to remove most of the bound coenzyme (13). The

specific activity of the purified β93A94I enzyme was 0.23 U/mg at pH 10.0, similar to that of the recombinant human $\beta_1\beta_1$ enzyme (10).

The substitution of Ala for Phe 93 and Ile for Thr 94 resulted in 3 to 8-fold increases in the K_M values for primary alcohols and between 9 and 5000-fold decreases in the K_M for secondary alcohols compared to $\beta_1\beta_1$ (Table 1). Ethanol and S-3-methyl-2-butanol exhibited the highest K_M values for β93A94I, about 0.3 mM, while R-2-butanol, R-2-pentanol, and cyclohexanol had the lowest K_M values, about 10 μM. In contrast, the K_M value for NAD^+ (Table 1) and the maximal velocities for substrate oxidation (obtained by multiplying V_{max}/K_M in Table 2 by K_M in Table 1) of the mutant enzyme were similar to values reported for $\beta_1\beta_1$ (9,10). On average, the maximal velocities for the oxidation of the five primary alcohols were approximately 20% lower than those obtained for the oxidation of the eight secondary alcohols, but within each class of substrates the maximal velocity did not vary by more than 10%. When the V_{max}/K_M values for substrates having the same number of carbon atoms are compared (Tables 2 and 3), the large decrease in K_M and the near constant V_{max} of β93A94I results in a higher efficiency for secondary alcohols than for primary alcohols (except for S-3-methyl-2-butanol). Similar to that observed for $\alpha\alpha$, β93A94I has a clear preference for the R-configuration over the S-configuration of enantiomeric secondary alcohols, the opposite trend is observed for $\beta_1\beta_1$ (Tables 2 and 3).

For primary alcohols, $\alpha\alpha$, $\beta_1\beta_1$, and β93A94I exhibited linear relationships between the log of the octanol:water partition coefficient [log(P)] and the log of V_{max}/K_M for the substrate (Figure 1). The human $\alpha\alpha$ enzyme shows a strong correlation between the

Table 1

K_M Values for Substrates
(μM)

Alcohol	$\beta_1\beta_1$[a]	β93A94I[b]	$\alpha\alpha$[a]
Ethanol	50	268±60	6,100
1-propanol	19	127±50	600
1-butanol	12	92±30	32
1-pentanol	19	66±25	14
1-hexanol	22	65±20	--
R-2-butanol	2,200	9.2±3	150
S-2-butanol	360	29±7	290
R-2-pentanol	6,100	10±4	57
S-2-pentanol	200	24±4	59
R-3-methyl-2-butanol	76,000	14±1	140
S-3-methyl-2-butanol	24,000	276±5	400
2-propanol	--	49±5	--
Cyclohexanol	3,900	10±5	12
NAD^+	15	23±3	12

[a]The kinetic constants for $\beta_1\beta_1$, and $\alpha\alpha$ are taken from Stone *et al.* (9).
[b]All values are the mean of three determinations ± SD.

Table 2

V_{max}/K_M for Alcohol Substrates
($min^{-1}\mu M^{-1} x\ 10^3$)

Alcohol	Log(P)[a]	$\beta_1\beta_1$[b]	β93A94I[b]	$\alpha\alpha$[b]
Residue 93		Phe	Ala	Ala
Residue 94		Thr	Ile	Ile
Ethanol	-0.32	80	12	2.7
1-propanol	0.34	160	29	36
1-butanol	0.88	240	39	640
1-pentanol	1.4	190	48	1100
1-hexanol	2.0	200	49	--
R-2-butanol	0.61	2.9	490	140
S-2-butanol	0.61	18	140	38
R-2-pentanol	1.3	1.0	450	290
S-2-pentanol	1.3	34	180	360
R-3-methyl-2-butanol	1.3	0.08	340	180
S-3-methyl-2-butanol	1.3	0.28	16	11
2-propanol	0.05	--	86	--
Cyclohexanol	1.2	0.76	420	2100

[a]The values for log(P) of the alcohols are taken from (16).
[b]The V_{max}/K_M values for $\alpha\alpha$, and $\beta_1\beta_1$ are taken from (9) and are based on a subunit molecular weight of 40,000 daltons.

log(P) and log(V_{max}/K_M) of the substrate, with a slope of 1.6. However, the log(V_{max}/K_M) values of substrates with $\beta_1\beta_1$ and β93A94I are relatively insensitive to log(P) and these enzymes exhibit slopes of 0.16 and 0.25, respectively.

Discussion: The combination of X-ray crystallography and site-directed mutagenesis have enabled investigators to probe the structure-function relationships of many enzyme systems. Thus, as more information is gained with regard to the role of particular amino

Table 3

Ratio of V_{max}/K_M of Secondary Versus Primary Alcohols

Substrate Ratio	$\beta_1\beta_1$	β93A94I	$\alpha\alpha$
R-2-Butanol/1-Butanol	0.012	12	0.21
S-2-Butanol/1-Butanol	0.076	3.6	0.060
R-2-Pentanol/1-Pentanol	0.0052	9.3	0.27
S-2-Pentanol/1-Pentanol	0.18	3.6	0.33
Cyclohexanol/1-Hexanol	0.0037	8.5	--

Ratios were calculated from V_{max}/K_M values in Table 2.

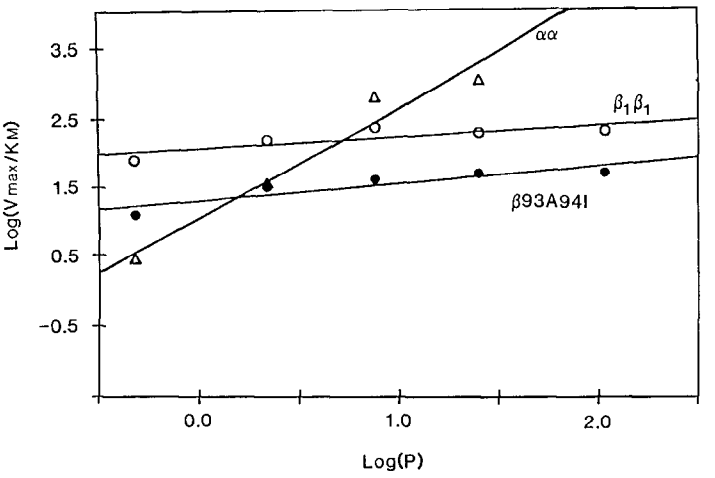

Figure 1. **Correlations between the $Log(V_{max}/K_M)$ for an alcohol substrate versus its log(P).** The values for the log of the octanol:water coefficient [log(P)] were taken from (16). Linear regressions of the data from the individual enzymes yielded slopes of 1.6 (r=0.98), 0.16 (r=0.79), and 0.25 (r=0.89) for $\alpha\alpha$ (△), $\beta_1\beta_1$ (○), and β93A94I (●), respectively.

acids in substrate recognition and catalysis, it becomes possible to use mutagenesis to create novel activities by altering the enzyme and its interactions with substrates. These approaches have been used to transpose the substrate specificities of related enzymes, such as malate and lactate dehydrogenase (14), and to engineer novel substrate specificities into subtilisin (15). Alcohol dehydrogenase is a good enzyme for substrate engineering, in that it exhibits broad substrate specificity for alcohols. Differences in substrate efficiencies are thought to result from changes in active site amino acid side chain volume and/or flexibility (7,8). In this experimental study, we have used site-directed mutagenesis of the human $\beta_1\beta_1$ cDNA to substitute Ala for Phe 93 and Ile for Thr 94 and created a catalytically active mutant enzyme (β93A94I), which has an active site size very different from $\beta_1\beta_1$. The substitutions are two of the six amino acid exchanges that occur within the substrate binding site of $\alpha\alpha$ versus $\beta_1\beta_1$, and results from our laboratory have demonstrated that $\alpha\alpha$ is unique among the human alcohol dehydrogenase isoenzymes in its ability to oxidize secondary alcohols with efficiencies approaching those of primary alcohols (9). However, there are 22 other amino acid substitutions between $\alpha\alpha$ and $\beta_1\beta_1$, including five within the substrate binding pocket at positions 57, 94, 116, 318, and 319, which could also affect primary and/or secondary alcohol specificity.

Like horse EE alcohol dehydrogenase and the human $\gamma_1\gamma_1$ enzyme (9), $\alpha\alpha$ exhibits a strong correlation (slope = 1.6 in Figure 1) between the log(P) of the substrate and $log(V_{max}/K_M)$, while the $log(V_{max}/K_M)$ is much less dependent on log(P) for $\beta_1\beta_1$ (slope = 0.2 in Figure 1). These differences in the correlation between log(P) and $log(V_{max}/K_M)$ are not the result of substitutions at position 93 and 94, since β93A94I behaves like $\beta_1\beta_1$ (93F94T) and not like $\alpha\alpha$ (93A94I). Thus other amino acid substitutions in $\alpha\alpha$ versus $\beta_1\beta_1$ must affect the dependence of $log(V_{max}/K_M)$ on log(P).

From a comparison of the sequences and substrate specificities of β93A94I and $\alpha\alpha$, it is likely that substitutions at positions 57, 116, 318, and/or 319 cause the increase in efficiency of the enzyme toward the longer chain primary alcohols, such as 1-butanol and 1-pentanol (Table 2 and Figure 1). Our modelling experiments indicate that the Met for Leu substitution at position 57 and the Ile for Val substitution at position 318 should be the primary contributors to this effect. The Ile for Thr substitution at position 94 probably has little effect on substrate specificity, and its major effect is to increase the hydrophobicity of the alcohol binding pocket near the catalytic zinc atom.

The β93A94I enzyme exhibits three major catalytic differences versus $\beta_1\beta_1$. First, the efficiencies toward primary alcohols are reduced by about 80%. The loss of a favorable van der Waals contact between the substrate and the side chain of Phe 93 could account for the decreased efficiency of β93A94I versus $\beta_1\beta_1$ for primary alcohols. The relatively high efficiency of $\alpha\alpha$ (Ala 93, Ile 94) with 1-butanol and 1-pentanol may result from compensating substitutions of Met for Leu 57 and Ile for Val 318 in the outer region of the alcohol binding site.

Second, the efficiencies toward all secondary alcohols are increased by 5- to 4200-fold. In fact, the efficiency of β93A94I toward all secondary alcohols, with the exception of S-3-methyl-2-butanol, is greater than that observed toward any primary alcohol (Table 2). When the ratios of V_{max}/K_M for a particular secondary alcohol versus its straight-chain homologue are compared, it is obvious that β93A94I prefers secondary over primary alcohols as substrates by 4- to 12-fold, whereas $\beta_1\beta_1$ clearly prefers primary alcohols (Table 3). The large increase in efficiency of β93A94I toward secondary alcohols versus $\beta_1\beta_1$ can be attributed to the loss of the steric constraint of the large Phe side chain at position 93. The fact that the efficiency of β93A94I toward secondary alcohols approaches or surpasses that of $\beta_1\beta_1$ toward primary alcohols suggests that the extra bulk of the secondary alcohols appear to have regained the favorable van der Waals contacts within the substrate binding pocket that is lost for primary alcohols with β93A94I.

Third, the reversal in efficiency for stereoisomers is undoubtedly due to a preference for orienting the larger part of the substrate (eg., the isopropyl group of 3-methyl-2-butanol) toward Ala 93 instead of Thr 48 in β93A94I. This is most clearly seen with the stereoisomers of 3-methyl-2-butanol, where β93A94I prefers the R-isomer over the S-isomer by 22-fold (Table 2). In $\beta_1\beta_1$, neither orientation is particularly favorable, as evidenced by the low V_{max}/K_M values, but the steric restrictions caused by Phe 93 appear to outweigh those caused by Thr 48. This is confirmed in the human $\gamma_1\gamma_1$ isoenzyme (Ser 48, Phe 93), where the substitution of Ser for Thr 48 does not effect the stereoselectivity for 3-methyl-2-butanol (9).

In conclusion, site-directed mutagenesis of $\beta_1\beta_1$ to form a mutant with Ala for Phe 93 and Ile for Thr 94 creates an enzyme with catalytic efficiencies toward secondary alcohols that exceed efficiencies toward primary alcohols and a stereoselectivity for enantiomeric alcohols similar to that exhibited by the human $\alpha\alpha$ isoenzyme. However, the relative dependency of primary alcohol oxidation on chain length (hydrophobicity) for $\alpha\alpha$, $\gamma_1\gamma_1$

and horse EE alcohol dehydrogenase versus $\beta_1\beta_1$ must result from substitutions further away from the catalytic zinc atom than Phe 93 and Thr 94.

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