Residues Specific for Class III Alcohol Dehydrogenase. Site-Directed Mutagenesis of the Human Enzyme†

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ABSTRACT: Human class III alcohol dehydrogenase (with both glutathione-dependent formaldehyde dehydrogenase and alcohol dehydrogenase activities) was expressed, and studied by site-directed mutagenesis corresponding to three amino acid residues that are affecting the substrate-binding pocket of class I (with alcohol dehydrogenase activity only). A Thr48Ala exchange results in an enzyme essentially without alcohol dehydrogenase activity but with some glutathione-dependent formaldehyde dehydrogenase activity retained. This indicates that coordination to the enzyme of *S*-hydroxymethylglutathione is mediated by interactions additional to, or different from, those utilized for primary and secondary alcohols. An Asp57Leu mutation causes considerable loss of the formaldehyde dehydrogenase activity, showing that a negative charge at position 57 is a prerequisite for this class III-type of activity, in the same manner as a positive charge at position 115 has been previously demonstrated to be crucial. Therefore, Asp57 and Arg115 appear to contribute equally to the interactions with *S*-hydroxymethylglutathione, compatible with defining the class III-type of specificity and possibly explaining the dependence on glutathione. A Tyr93Phe mutant exhibits decreased k_{cat} values for substrates in general and correlates with inhibition of alcohol dehydrogenase activity by 4-methylpyrazole, a potent inhibitor of the class I enzymes. In a double mutant, Asp57Leu/Tyr93Phe, the effects of the two mutations are potentiating one another, yielding a fall in k_{cat}/K_m for hydroxymethylglutathione by a factor of 1250, i.e., a still further loss of class III-type activity. At the same time, the alcohol dehydrogenase activity of Asp57Leu/Tyr93Phe has gained a characteristic class I property, complete inhibition by 4-methylpyrazole at concentrations only partially reducing the activity of the wild-type class III enzyme.

The relationships of presently characterized classes of mammalian alcohol dehydrogenase (ADH)[1] have been defined (Jörnvall et al., 1993; Zheng et al., 1993). They are dimeric enzymes, and, as judged from species variability, class III (with χ subunits) is the one most conserved. It also exists in invertebrate organisms and is considered to represent an ancestral form (Danielsson & Jörnvall, 1992; Kaiser et al., 1993; Parés et al., 1994; Danielsson et al., 1994). Studies of the distribution of class III ADH have revealed fairly equal levels in all tissues examined (Giri et al., 1989; Estonius et al., 1993), and expression of the enzyme has been suggested to be constitutive (Smith, 1986; Hur & Edenberg, 1992). This ADH class is identical to glutathione (GSH)-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989), the role of which in cytoprotective scavenging of formaldehyde (Uotila & Koivusalo, 1989) suggests a protective function for the whole enzyme system (Jörnvall et al., 1993).

The kinetic properties of class III ADH differ greatly from those of the other ADH classes. It is the only ADH capable of oxidizing *S*-hydroxymethylglutathione (HMGSH), a GSH adduct formed spontaneously in the presence of formaldehyde (Koivusalo et al., 1989; Holmquist & Vallee, 1991), yielding the GSH-dependent formaldehyde dehydrogenase activity (FDH activity) characteristic for class III. Thus far, Arg115 is one amino acid residue that has been shown to entail specific enzymatic properties characteristic of class III ADHs. It has been proposed to interact with a carboxyl group of HMGSH, thereby providing the class III-specific catalytic efficiency toward this substrate (Engeland et al., 1993; Holmquist et al., 1993), and further to interact with fatty acid carboxylates in activating the class I-type of ADH activity of the enzyme (Moulis et al., 1991). Apart from the HMGSH specificity, the low affinity of class III for small substrates like ethanol, which cannot saturate that enzyme, is another substrate difference between class III and I enzymes (Vallee & Bazzone, 1983).

Beyond these major class III-specific substrate characteristics, the patterns of pyrazole inhibition constitute a class difference. 4-Methylpyrazole, a potent inhibitor of class I ADH enzymes, inhibits class III to only a minor extent. The ethanol and pyrazole affinities of the enzyme are related and have been attributed to available space in the substrate-binding pocket (Eklund et al., 1990) and to the presence of charged residues in the class III pocket. This is exemplified by Tyr93 in class III versus Phe93 in typical class I enzymes, and by Asp57 in class III as contrasted with Leu or other hydrophobic residues in mammalian class I ADHs. Therefore, several residues, not only Arg115, might be essential in bringing about class-specific properties toward substrates and inhibitors.

In an attempt to study the relative importance of residues involved in differentiation of classes, and possibly to transform class III ADH enzymatic properties toward those of class I, we have examined the functions of Asp57 and

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[1] Abbreviations: ADH, alcohol dehydrogenase; GSH, glutathione; HMGSH, *S*-hydroxymethylglutathione; FDH, formaldehyde dehydrogenase; 12-HDA, 12-hydroxydodecanoic acid.

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Table 1: Oligonucleotides Used for Site-Directed Mutagenesis, with the Mismatching Bases Underlined

mutation	oligonucleotide
T48A	5′-GGCATCGGCGTGGCAAA-3′
D57L	5′-ACCCTACAGGAAGAGCTCCACTC-3′
Y93F	5′-TGTGGGATGAAAAGTGGGA-3′

Tyr93 in the oxidation of neutral and charged alcohols and of HMGSH, by altering the polarity and size of these substrate pocket residues. In addition, we have replaced Thr48 with Ala, a mutation deleterious to class I ADH activity (Höög et al., 1992), and then find differences in the oxidation of HMGSH specific for class III and of aliphatic alcohols typical of class I.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis. The entire coding part of a class III ADH cDNA (Sharma et al., 1989) was isolated by cleavage with *Eco*RI and *Ssp*I. The liberated 1360 bp fragment was ligated into *Eco*RI/*Sma*I digested plasmid pKK 223-3 (Pharmacia Biotech), which contains a strong *tac* promoter and a *rrn*B ribosomal terminator. This resulted in an expression vector for class III ADH, similar to those previously used for class I (Höög et al., 1992), with 15 bp between the ribosome binding site and the initiation codon.

A 390 bp *Eco*RI/*Kpn*I fragment derived from the 5′ coding region of the class III expression vector was ligated into M13mp18, and site-directed mutagenesis was performed according to the single priming method of Taylor et al. (1985), by which the mutations T48A, D57L, Y93F, and D57LY93F were obtained. For this purpose, three oligonucleotides were used (Table 1). All mutations were confirmed by dideoxy chain termination sequence analysis (Sanger et al., 1977) before and after religation into the expression plasmid.

Expression, Purification, and Detection of Proteins. Five recombinant class III ADH enzymes were expressed in 3-L LB cultures of *Escherichia coli* strain TG1, *lacI*^{q}, after induction with 0.15 mM isopropyl β-D-thiogalactopyranoside: rχwt (wild-type), rχT48A (Thr48 replaced with Ala), rχD57L (Asp57 replaced with Leu), rχY93F (Tyr93 replaced with Phe), and rχD57LY93F (Asp57 and Tyr93 replaced with Leu and Phe, respectively). Cells were harvested, and disrupted in 10 mM Tris-HCl, pH 8.0, by intermittent sonication prior to centrifugation for 40 min at 48000*g*. The supernatant, approximately 30 mL, was applied to a 150-mL DEAE column (DE-52, Whatman) equilibrated with 10 mM Tris-HCl, pH 8.0, and the recombinant protein was recovered by isocratic elution. The pooled fractions containing the recombinant enzyme, approximately 50 mL, were applied to a 25-mL column of AMP-Sepharose (Pharmacia Biotech), and the UV absorption of the effluent was monitored at 280 nm. The column was washed with equilibration buffer, 100 mM Tris-HCl, pH 8.0, until the UV absorption had resumed the prechromatographic level. The bound protein was eluted with 1 mM NAD^+ added to the same buffer and then dialyzed extensively against 0.1 mM dithiothreitol/10 mM Tris-HCl, pH 8.0. Final purification and concentration was achieved by FPLC on a 1-mL MonoQ anion-exchange column (Pharmacia Biotech), using a 20 mL linear gradient of 0−0.5 M NaCl in 10 mM Tris-HCl, pH 8.0, with a flow of 1 mL/min. Throughout the purifications, FDH activity was assayed in 0.1 M sodium pyrophosphate, pH 8.0, containing 1 mM GSH/1 mM formaldehyde and 2.4 mM NAD^+. Protein amounts were determined colorimetrically (Bradford, 1976) with bovine serum albumin as standard, and protein purity was confirmed by SDS−polyacrylamide gel electrophoresis. Protein sequence analysis was accomplished by Edman degradation in a MilliGen ProSequencer 6600 solid-phase instrument equipped with on-line HPLC.

Steady-State Kinetics. Formaldehyde was diluted from 20% solutions (Ladd Research Industries Inc.), and alcohols were of analysis grade. Pentanol, octanol, 12-hydroxydodecanoic acid (12-HDA), and cyclohexanol were dissolved in methanol yielding a final concentration of 3.3% methanol (v/v) in the assay mixtures, a concentration which does not affect class III ADH activity (Engeland et al., 1993). Enzymatic activities were determined spectrophotometrically using a Pharmacia Biochrom 4060 instrument to monitor NADH formation. A molar absorptivity of 6.22×10^3 M^{-1} cm^{-1} for NADH at 340 nm was used, and the enzyme activity reducing 1 μmol of NAD^+/min was defined as one unit. All kinetic determinations were performed at 24 °C, and 1−5 μg of enzyme was used per milliliter of assay mixture. Oxidation rates of ethanol, pentanol, octanol, cyclohexanol, and 12-HDA were determined with 2.4 mM NAD^+ in 0.1 M glycine−NaOH, pH 10. FDH activity was determined with 1 mM GSH/2.4 mM NAD^+ (rχwt and rχY93F) or 10 mM GSH/10 mM NAD^+ (rχT48A, rχD57L, and rχD57LY93F) in 0.1 M sodium pyrophosphate, pH 8.0. The K_m for NAD^+ at pH 8.0 was determined with 1 mM GSH/1 mM formaldehyde (rχwt and rχY93F) or 10 mM GSH/10 mM formaldehyde (rχT48A, rχD57L, and rχD57LY93F) in 0.1 M sodium pyrophosphate, and at pH 10.0 with 1.6 mM octanol in 0.1 M glycine−NaOH.

Inhibition of enzyme activity was measured with 1, 10, and 50 mM 4-methylpyrazole and the following substrates: ethanol (500 mM), pentanol (50 mM), and octanol (3.2 mM) at pH 10.0. The K_i for 4-methylpyrazole of rχD57LY93F was determined with 10 and 25 mM inhibitor at pH 10.0, with pentanol as substrate. A weighted nonlinear regression analysis program (Lutz et al., 1986) was used to calculate kinetic constants, which were obtained from three protein batches separately purified. Standard errors were less than 15% of the values determined. In addition, all recombinant proteins were screened for their pH optima in 0.1 M sodium acetate (pH 4.0−6.0), 33 mM sodium phosphate (pH 6.0−7.5), 33 mM sodium pyrophosphate (pH 7.5−9.0), and 0.1 M glycine−NaOH (pH 9.0−12.0), using 1.6 mM octanol, and 1 mM GSH/1 mM formaldehyde as substrates.

RESULTS

Purification, Yield, Specific Activities, and pH Optima of Recombinant Class III ADHs. The recombinant enzymes were purified in three successive chromatographic steps (Table 2), yielding homogeneous proteins as judged by SDS−polyacrylamide gel electrophoresis. The N-terminal amino acid sequence of rχwt was determined by Edman degradation and was found to be identical to that of human liver class III ADH (Kaiser et al., 1988) with the exception of lack of the acetyl group which is blocking the α-amino group of the liver enzyme (Egestad et al., 1990). The yield

Table 2: Purification Protocol of the Wild-Type Recombinant Class III ADH[a]

	protein (mg)	specific activity (units/mg)	purification (-fold)	yield (%)
crude extract	2640	0.1	1	100
DE-52	94	0.9	9	32
AMP-Sepharose	14	3.4	34	18
MonoQ	7.6	4.0	40	12

[a] The values apply to rχwt, obtained from a 3-L overnight culture. The yield after the DE-52 chromatography appears lower than it is because of the presence in the crude extract of *E. coli* class III ADH, which is eliminated in the DEAE step.

Table 3: Specific Activities and Apparent pH Optima of Recombinant Class III ADHs[a]

	1 mM GSH/1 mM formaldehyde		1.6 mM octanol
	specific activity[b] (units/mg)	pH optimum[c]	pH optimum[c]
rχwt	4.0	9.5	11.0
rχT48A	0.1	7.0	TA[d]
rχD57L	1.0	9.5	11.0
rχY93F	0.7	9.5	11.7
rχD57LY93F	0.2	9.5	11.7

[a] At pH 8.0, the formaldehyde dehydrogenase activity of rχT48A was about 60% of that detected at pH 7.0. For other recombinants, the ratio of HMGSH activities at pH 9.5 to those at pH 7.0 ranged from 2 to 4. Similarly, the ratios of octanol activities at their pH optima to those at pH 7.0 were in the range of 20−40. [b] Assayed in 2.4 mM NAD^+/0.1 M sodium pyrophosphate, pH 8.0. [c] Screened with 2.4 mM NAD^+ in 0.1 M sodium acetate (pH 4.0−6.0), 33 mM sodium phosphate (pH 6.0−7.5), 33 mM sodium pyrophosphate (pH 7.5−9.0), and 0.1 M glycine (pH 9.0−12.0). [d] Trace activity (TA) was detected at pH 8.0.

of the different mutant proteins was in the same range as for rχwt, approximately 2.5 mg/L culture (Table 2). In contrast, their specific activities toward HMGSH varied considerably. Values of 4, 0.1, 1, 0.7, and 0.2 units/mg were obtained for rχwt, rχT48A rχD57L, rχY93F, and rχD57LY93F, respectively (Table 3). The pH optima for oxidation of octanol were 11.0 for rχwt and rχD57L, and 11.7 for rχY93F and rχD57LY93F (Table 3). The formaldehyde dehydrogenase activity of all recombinants had an apparent maximum at pH 9.5, except for rχT48A, which catalyzed this reaction most efficiently at pH 7.0 (Table 3).

Characterization of ADH and FDH Activities. The effects of the point mutations on the oxidation rates were examined with both neutral and charged alcohols, and with the thiohemiacetal HMGSH (Table 4). For all substrates examined, the kinetic constants of the recombinant wild-type enzyme were essentially the same as those reported for the native human liver enzyme (Table 4). Methanol in concentrations up to 2.5 M was found not to be a substrate for any of the five recombinant enzymes. For all but rχT48A, plots of v versus [S] were linear up to 2 M ethanol, and therefore only the k_{cat}/K_m values were calculated (Table 4). The linearity also applied to cyclohexanol, with v versus [S] linear to 100 mM, yielding k_{cat}/K_m values of 0.7−0.8 mM^{-1} min^{-1}.

In concentrations which completely inhibit the activity of class I ADH enzymes, 4-methylpyrazole was virtually ineffective toward class III. Higher concentrations of the inhibitor (50 mM) diminished the catalytic efficiency (Table 5), and the oxidation of ethanol and pentanol by rχD57LY93F was abolished, yielding a K_i for 4-methylpyrazole of 10 mM. rχY93F was also inhibited by 4-methylpyrazole in oxidation of ethanol, pentanol, and octanol (Table 5).

rχT48A did not oxidize primary alcohols and cyclohexanol. Even at high concentrations of the mutant, 200 μg/mL of assay mixture, no activity was detected at pH 10.0 or 8.0 with substrate concentrations up to 3.5 M ethanol or 100 mM pentanol. Using 10 mM 12-HDA or 100 mM cyclohexanol at pH 10.0, and 3.2 mM octanol at pH 8.0, trace activities in the range of 10^{-3} unit/mg could be detected. The enzyme had FDH activity, but with a k_{cat}/K_m decreased by a factor of 7100, as a result of both a lowered k_{cat} and a 1100-fold increase in K_m.

The HMGSH-catalyzing efficiency of rχD57L was reduced by a factor of 550, mainly due to a 150-fold increase in K_m. The constants for 12-HDA remained unaltered, but a decrease in K_m for octanol and pentanol was noticed. In addition, the K_m values for NAD^+ at both pH 8.0 and 10.0 were increased.

The k_{cat}/K_m for HMGSH was one-fifth with rχY93F, attributable entirely to a decrease in k_{cat}. The K_m values for NAD^+ were unaffected, but a general decrease in k_{cat} for pentanol, octanol, and 12-HDA was detected. The K_m values for pentanol and octanol were identical to those of rχwt, but a decrease in K_m for 12-HDA lowered the catalytic efficiency by a factor of 3.6.

rχD57LY93F exhibited a marked decrease in k_{cat}/K_m for HMGSH, a consequence of a 125-fold increase in K_m and a 10-fold decrease in k_{cat}. The effects of the mutation on the constants for pentanol, octanol, and 12-HDA were similar, overall lowering both k_{cat} and K_m, resulting in a reduced net catalytic efficiency for these substrates (Table 4).

DISCUSSION

Ser or Thr are found at position 48 in all ADHs and are known to interact with both the substrate and the coenzyme via hydrogen bonding (Eklund et al., 1990). In class I, removal of the side-chain hydroxyl of this residue results in an inactive enzyme (Höög et al., 1992). This loss of classical ADH activity applies also to class III, where rχT48A exhibited essentially no activity toward the primary and secondary alcohols tested. However, the class III-type FDH activity was retained, although with a substantial decrease in k_{cat}/K_m (Table 4). In rχT48A, the loss of ADH but not FDH activity indicates interactions of HMGSH with the enzyme that are additional to, or different from, those affecting aliphatic alcohols. This is compatible with the conclusion that one of the two carboxyl groups of HMGSH interacts with Arg115 (Engeland et al., 1993) and that the α-amino group of γ-Glu could interact with Asp57 (below). Since the primary and secondary alcohols tested showed no activity, the side-chain hydroxyl of residue 48 appears to be a prerequisite for exact positioning of the hydroxyl group of those substrates that, in contrast to HMGSH, have no other polar interactions with the protein.

Exchange of Asp57 to Leu did not affect the oxidation of ethanol but increased the catalytic efficiency (k_{cat}/K_m) toward both pentanol and octanol, due to a lowered K_m for both substrates (Table 4). However, the major effect of the mutation was the reduction of the HMGSH-oxidizing efficiency, with the k_{cat}/K_m value reduced by a factor of 550. This decrease in catalytic efficiency of rχD57L toward HMGSH is in the same order of magnitude as the decrease

Table 4: Kinetic Constants for the Recombinant Wild-Type and Mutated Class III ADHs[a]

enzyme	ethanol	cyclohexanol	pentanol			octanol			12-HDA			HMGSH			NAD^+ (pH 8.0)	NAD^+ (pH 10.0)
	k_{cat}/K_m (mM^{-1} min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	K_m (mM)
wt[b]	0.045		24	174	7	1.2	220	180	0.06	170	2800	0.004	200	50000	0.009	0.07
rχwt	0.045	0.08	20	150	7.5	0.5	160	320	0.06	160	2700	0.004	200	50000	0.01	0.07
rχT48A	NA[d]	<0.01			NA[d]			<0.05[e]			<0.015	4.4	30	7	0.62	ND[f]
rχD57L	0.045	0.07	11	130	12	0.3	160	530	0.06	160	2700	0.6	55	90	0.52	0.30
rχY93F	0.045	0.07	20	35	1.7	0.5	35	70	0.04	30	750	0.004	40	10000	0.01	0.07
rχD57LY93F	0.045	0.08	10	30	3	0.1	30	300	0.02	25	1250	0.5	20	40	0.15	0.15
rχR115A[c]	0.015	ND[f]			ND[f]	1.2	150	125	0.68	122	180	0.28	97	350	ND[f]	ND[f]
rχR115D[c]	0.015	ND[f]			ND[f]	2.2	240	110	1.30	97	75	0.91	36	40	ND[f]	ND[f]

[a] k_{cat} values were calculated per subunit with the molecular mass of 40 kDa. [b] Values given for human liver class III ADH are from Wagner et al. (1984) (ethanol), Moulis et al. (1991) (pentanol), Eklund et al. (1990) (octanol, 12-HDA, and NAD^+ at pH 10.0), Holmquist and Vallee (1991) (HMGSH), and Uotila and Koivusalo (1989) (NAD^+ at pH 8.0). [c] Values given for this class III mutant are from Engeland et al. (1993). [d] No activity. [e] Detected at pH 8.0. [f] Not determined.

previously found for an R115D or an R115A exchange (Engeland et al., 1993). Significantly, this establishes that Asp57 is crucial to the FDH activity, like Arg115 which has previously been considered a specificity-determining residue. The diminished activity of R115D/A was attributed to the loss of charge interaction between HMGSH and Arg115. Analogously, the attenuated FDH activity of rχD57L could be explained by the lack of a side-chain carboxyl at position 57, resulting in deterred interactions of HMGSH with the enzyme. Notably, HMGSH has amino and carboxyl groups conceivably binding to the charged residues of Asp57 and Arg115, respectively, in class III. However, in class I the side-chain directions of these residues deviate from those required for proper interactions of HMGSH (Figure 1B). This substrate cannot yet be modeled into a class III enzyme because no corresponding three-dimensional structure is still available, although crystallographic analyses are in progress (Hurley et al., 1993; El-Ahmad et al., 1994). For class III HMGSH binding, local adjustments of the fold or side-chain orientations, especially of residue 115, would be required. In the latter case, distances appear to suggest that it is the α-carboxyl of γ-Glu and not the one of Gly in HMGSH that binds to Arg115 (Figure 1).

An enhanced inhibition of ethanol activity at high 4-methylpyrazole concentrations was manifest in rχY93F (Table 5). Presumably, the level of inhibition correlates with the hydrophobicity of the active site pocket (Eklund et al., 1990), where an increase in hydrophobicity caused by the Y93F exchange facilitates binding of 4-methylpyrazole. Another finding is that essentially without affecting the K_m values, the Y93F substitution results in similarly decrased k_{cat} for all substrates (Table 4), which may indicate that this residue replacement could affect a rate-limiting step common to the turnover of both HMGSH and aliphatic alcohols.

In resemblance with rχY93F, the sensitivity of aliphatic alcohol oxidation to inhibition by 4-methylpyrazole is pronounced with rχD57LY93F (Table 5). At high inhibitor concentrations, the ethanol and pentanol activities of rχD57LY93F are abolished. This enhanced inhibition could be explained partly by the Y93F substitution (Table 5), but evidently the effects of both Y93F and D57L exchanges potentiate one another in extinction of short and medium chain alcohol turnover (Table 5). In addition, the combined effect of the mutations result in an overall lowered catalytic efficiency (Table 4). The k_{cat} values are approximately one-fifth of those determined for rχwt and thus roughly the same as with the Y93F substitution, indicating that it is the latter that causes the reduction in k_{cat} (Table 4). The decrease in k_{cat} for HMGSH is of the same order of magnitude as for other alcohols tested, but a 100-fold increase in K_m for HMGSH is noticed. In this case, the D57L exchange of the double mutant appears to cause the increase in K_m for HMGSH, because the same effect is manifest with the Asp57Leu mutant (Table 4). Since the latter mutation also reduces k_{cat} for HMGSH but not for other substrates, this could indicate either alternative modes of substrate binding or different rate-limiting steps, with respect to the oxidation of aliphatic alcohols on the one hand and of HMGSH on the other.

All information obtained through the active site mutations now carried out yields a consistent pattern showing that a few positions are important for class differentiations. Exchanges at these positions, from the class III wild-type to

Table 5: Transition of Class III ADH Properties toward Those of Class I[a]

protein	residue at position 57	93	115	% ethanol activity at 50 mM 4-MP	% pentanol activity at 50 mM 4-MP	% octanol activity at 50 mM 4-MP	HMGSH activity (% of rχwt)
wild-type class III, rχwt	Asp	Tyr	Arg	20	20	20	100
mutated class III, rχD57L	**Leu**	Tyr	Arg	15	20	25	0.2
mutated class III, rχY93F	Asp	**Phe**	Arg	10	4	4	20
mutated class III, rχD57LY93F	**Leu**	**Phe**	Arg	0	0	5	0.1
mutated class III, rχR115A[b]	Asp	Tyr	**Ala**	ND[c]	ND[c]	ND[c]	0.7
mutated class III, rχR115D[b]	Asp	Tyr	**Asp**	ND[c]	ND[c]	ND[c]	0.1
wild-type class I	Leu	Phe	Asp	0	0	0	0

[a] This table shows the degrees of 4-methylpyrazole (4-MP) inhibition of alcohol dehydrogenase activity and the levels of HMGSH activity of all recombinant class III ADHs. Boldface indicates residues exchanged, and as shown by the percent values for the mutant forms, they have gained a class I-type of 4-methylpyrazole inhibition and have concomitantly lost considerable class III-specific HMGSH activity, only retaining a fraction. HMGSH activity is expressed as percent of k_{cat}/K_m values (Table 4) with that of rχwt defined as 100%. HMGSH activity of the wild-type class I refers to the human ββ isozyme (Holmquist & Vallee, 1991). Values for ethanol, pentanol, and octanol activities refer to percent of rates obtained in the absence of inhibitor. The activity of class I at 50 mM 4-methylpyrazole is given as 0, all class I K_i values for the inhibitor being in the micromolar range. The recombinant enzymes were assayed in 0.1 M glycine/NaOH pH 10.0, 2.4 mM NAD^+, with 0.5 M ethanol, 50 mM pentanol, and 3.2 mM octanol, respectively, all in the presence of 1, 10, and 50 mM 4-methylpyrazole. The 1 and 10 mM values are not shown because no significant differences among the recombinants were detected at these concentrations. Assays were started with the addition of substrate after incubations for 3 min. rχT48A showed no activity with ethanol, pentanol, or octanol at pH 10.0 and is not included. [b] Values are from Engeland et al. (1993). [c] ND, not determined.

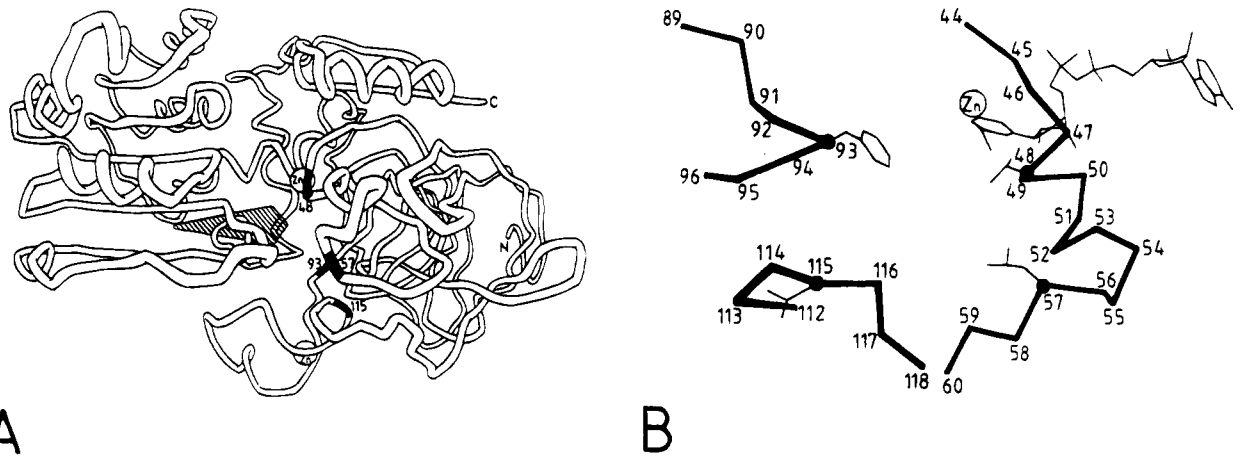

FIGURE 1: Active site relationships. (A) Schematic view of the subunit of class I mammalian liver ADH. The main-chain positions of substrate pocket residues exchanged are drawn in black. (B) View of the substrate pocket of class I ADH as seen from the direction of the arrow in panel A. Residues outlined by side-chains (Thr48, Leu57, Phe93, and Asp115, with main-chain attachment positions indicated by solid spheres) are from analysis of the class I enzyme (Hurley et al., 1991), while class III is not available from crystallographic data. Therefore, glutathione should not be understood to interact with the side chains shown, but rather with the class III alternatives in their conformation. Although deviations locally are possible, classes I and III are similarly folded (Eklund et al., 1990), and the two charged residues exchanged are separated (positions 57 and 115, bottom part of panel B) at the substrate pocket, allowing for the interactions with HMGSH. Zn denotes the catalytic zinc atom, and the coenzyme (top, right) is positioned as bound to the enzyme. Redrawn from a plot obtained using a program supplied by *Protein Science* (Richardson & Richardson, 1992), with positions 44−60, 89−96, and 112−118 of the main chain outlined from the Protein Data Bank coordinates (Bernstein et al., 1977; Abola et al., 1987) of the human class I enzyme (Hurley et al., 1991).

the class I wild-type residues, transform the enzyme properties. The residues are Arg115 (Engeland et al., 1993), Asp57, and Tyr93 of class III, corresponding to Asp115, Leu57, and Phe93 of class I, respectively. Their relationships at the substrate-binding pocket are summarized schematically in Figure 1, and the class-distinguishing effects of these exchanges are shown in Table 5. The ADH enzyme classes differ fundamentally; class III is active with HMGSH but class I is not; class III is hardly affected by 4-methylpyrazole and cannot be saturated with ethanol, while class I is inhibited readily and oxidizes ethanol efficiently. It is clear that the HMGSH activity and 4-methylpyrazole sensitivity are altered in a direction toward those of the other class by substitution of class III-specific substrate pocket residues with those typical of class I enzymes. In effect, therefore, we have converted class III toward class I, a transition defining the residues at positions 57 and 93, in addition to the one at 115 (Engeland et al., 1993), as essential to class characteristics.

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