Three-dimensional structures of the three human class I alcohol dehydrogenases

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Abstract

In contrast with other animal species, humans possess three distinct genes for class I alcohol dehydrogenase and show polymorphic variation in the *ADH1B* and *ADH1C* genes. The three class I alcohol dehydrogenase isoenzymes share ~93% sequence identity but differ in their substrate specificity and their developmental expression. We report here the first three-dimensional structures for the *ADH1A* and *ADH1C*2* gene products at 2.5 and 2.0 Å, respectively, and the structure of the *ADH1B*1* gene product in a binary complex with cofactor at 2.2 Å. Not surprisingly, the overall structure of each isoenzyme is highly similar to the others. However, the substitution of Gly for Arg at position 47 in the ADH1A isoenzyme promotes a greater extent of domain closure in the ADH1A isoenzyme, whereas substitution at position 271 may account for the lower turnover rate for the ADH1C*2 isoenzyme relative to its polymorphic variant, ADH1C*1. The substrate-binding pockets of each isoenzyme possess a unique topology that dictates each isoenzyme's distinct but overlapping substrate preferences. ADH1*B1 has the most restrictive substrate-binding site near the catalytic zinc atom, whereas both ADH1A and ADH1C*2 possess amino acid substitutions that correlate with their better efficiency for the oxidation of secondary alcohols. These structures describe the nature of their individual substrate-binding pockets and will improve our understanding of how the metabolism of beverage ethanol affects the normal metabolic processes performed by these isoenzymes.

Keywords: Alcohol dehydrogenase; isoenzymes; X-ray crystallography; substrate binding

The diversity of alcohol dehydrogenase isoenzymes found in humans is unique in the animal kingdom. Seven separate genes have been identified, characterized, and mapped to a gene cluster located on Chromosome 4 (Edenberg 2000). The different alcohol dehydrogenase isoenzymes have been classified into distinct classes based on enzymatic and DNA/protein sequence characteristics (Duester et al. 1999; Edenberg 2000). This article uses the newly suggested gene nomenclature for alcohol dehydrogenase (ADH), which is based on this classification system (Duester et al. 1999).

Thus, genes classified as *ADH1* are those that code for proteins closely resembling the class I isoenzymes, *ADH2* the class II isoenzymes, and so on for the six known classes in mammalian systems.

The human system has additional complexity due to a gene triplication and polymorphism that occurs among the class I isoenzymes, giving rise to the following class I genes in the human population: *ADH1A*, *ADH1B*1*, *ADH1B*2*, *ADH1B*3*, *ADH1C*1*, and *ADH1C*2*. The protein products of these genes, previously named α , β_1 , β_2 , β_3 , γ_1 , and γ_2 , can form random associations to yield both homo- and heterodimeric forms (Edenberg and Bosron 1997). In contrast, the products of the *ADH2*, *ADH3*, *ADH4*, and *ADH5* genes appear to only undergo homodimeric associations. Several animal species, other than humans, have been found to express two class I isoenzymes, specifically monkeys, ba-

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boons, horses, and lizards, but to date only humans show this triplication and extensive polymorphism (Duester et al. 1999).

The expression patterns of the different ADH isoenzymes have been well characterized. The ADH1 and ADH2 isoenzymes are expressed predominantly in the liver, although Northern analysis has shown minor levels in other tissues (Estonius et al. 1996). The class I isoenzymes are believed to participate in the general detoxification of various biogenic and dietary alcohols and aldehydes and are the major contributors to beverage ethanol metabolism (Edenberg and Bosron 1997). The metabolism of various physiologic substrates by these liver specific enzymes has been studied in vitro, but little has been shown conclusively in vivo. The most compelling information on the roles of the different ADH isoenzymes in mammals has come from recent gene knockout studies in mice (Deltour et al. 1999). These studies showed that mice lacking a functional *ADH1* gene showed a significantly decreased clearance of an intoxicating dose of ethanol and an increase in the time required to regain the loss of righting reflex after such a dose (Deltour et al. 1999). Mice deficient in ADH3 activity were shown to be more susceptible to formaldehyde toxicity, and ADH4 deficient mice were shown to metabolize retinol less effectively (Deltour et al. 1999). Although these studies confirmed the role ADH1 isoenzymes play in ethanol metabolism in mice, they have not yet identified potential substrates affected by the presence of ethanol. In addition, these studies cannot address the larger question of why humans possess three distinct class I isoenzymes and why these isoenzymes should show differences in expression during development. In humans, the product of the *ADH1A* gene is the sole isoenzyme expressed in fetal liver, with the expression of the *ADH1B* and *ADH1C* genes rising just before and after birth, respectively (Smith et al. 1972).

The basic functional characteristics of class I ADH isoenzymes are a low K_M for ethanol and a high sensitivity for inhibition by pyrazole and its 4-substituted derivatives (Edenberg and Bosron 1997). Indeed, the three human class I isoenzymes show K_M values for ethanol ranging between 0.05 mM and 6 mM and K_i values between 0.2 μ M and 2 μ M for 4-methylpyrazole (Bosron and Li 1987). Overall, the human class I isoenzymes show pairwise sequence identities of ~93%, but only an average of 60% identity among residues lining the substrate-binding pocket. Not surprisingly, the substrate preferences of these three isoenzymes, while overlapping for some substrates, display distinct characteristics. For instance, the ADH1C*1 and ADH1C*2 isoenzymes are the only human isoenzymes known to be active toward steroid substrates (McEvily et al. 1988; Höög et al. 1992). The ADH1A isoenzyme is the most active toward secondary alcohols, and this isoenzyme's stereospecificity for secondary alcohol oxidation is reversed relative to the ADH1B*1 and ADH1C*1 enzymes (Stone et al. 1989; Hur-

ley and Bosron 1992). Both the ADH1B*2 and the ADH1B*3 allelic variants are a result of different single amino acid substitutions within the coenzyme-binding site of ADH1B*1 (Jörnvall et al. 1984; Burnell et al. 1987). Three-dimensional structures for all three allelic variants of ADH1B are known, and the structural perturbations resulting from these single amino acid substitutions are limited and are consistent with the known kinetic behavior of the isoenzymes (Hurley et al. 1994; Davis et al. 1996). Three-dimensional structures are also available for the human class III and class IV isoenzymes (Yang et al. 1997; Xie et al. 1997), and together with the available class I structures have provided a framework from which to examine the divergence of function resulting from gene duplication over long evolutionary time scales. The combination of structure and sequence analysis has defined constant and variable regions of ADH enzyme structure with respect to these diverse enzymatic functions (Jörnvall et al. 1996). However, much less is known concerning the structural and functional underpinning of the more recent duplications and triplications of the class I genes.

Here, we report the three-dimensional structure of the class I ADH1A as a pseudoternary complex between NAD(H) and 4-iodopyrazole and the ADH1C*2 structure in a binary complex with NAD(H). We also report a new higher-resolution structure of an ADH1B*1 binary complex with NAD(H). All structures were obtained from frozen crystals at -165°C and are at resolutions between 2.5 Å and 2.0 Å. The structures enable a detailed comparison of the divergence of substrate-binding site residues and provide an understanding of the substrate preferences for these highly related, but functionally distinct, isoenzymes.

Results

Structures of the individual class I ADH isoenzymes

The refined structures show good overall model quality as evidenced by the statistics presented in Table 1 and by the fact that no nonglycine residues were found in the disallowed regions of their respective Ramachandran plots (data not shown). The ADH1A structure contains four zinc ions, two bound NAD(H) molecules, two 4-iodopyrazole molecules, and 362 water molecules. The ADH1B*1 structure contains four zinc ions, two bound NAD(H) molecules, and 416 water molecules.

The ADH1C*2 structure contains four zinc ions, two bound NAD(H) molecules, and a total of 650 water molecules. All structures show the so-called closed conformation characteristic of class I ADH binary and ternary complexes. Not surprisingly, considering the 93% pairwise sequence identity, all the structures reported here show a high degree of overall structural similarity. The α -carbon positions of the individual subunits show ~0.35 Å r.m.s.d. in

Table 1. Data collection and refinement statistics

	ADH1B*1 (β_1)	ADH1A (α)	ADH1C*2 (γ_2)
Diffraction data statistics			
Space group	P1	P2 ₁	P2 ₁
Cell dimensions			
a, b, c Å	43.9, 53.4, 90.2	55.7, 100.2, 69.1	56.4, 71.5, 92.1
α , β , γ°	79.9, 89.5, 69.3	90, 104.9, 90	90, 102.9, 90
Resolution range	44–2.2 Å	44–2.5 Å	44–2.0 Å
Total observations	66,027	48,374	188,548
Unique reflections	33,051	23,704	47,641
Completeness ^a (%)	87.5 (64.5)	92.9 (83.0)	97.9 (86.8)
R _{merge} ^a (%)	3.4 (12.0)	9.2 (29.4)	8.2 (21.6)
I/ σ ^a	21.9 (7.4)	10.5 (3.2)	21.3 (6.7)
Refinement statistics			
Test set size (% of total)	6	5	6
R _{work} /R _{free}	0.193/0.257	0.191/0.248	0.193/0.231
Average B-value	19.6	21.6	21.0
Rms deviation from ideal bond lengths (Å)	0.009	0.008	0.009
Rms deviation from ideal bond angles (°)	1.56	1.58	1.50
Noncrystallographic symmetry rms deviation			
all atoms (Å)	0.60	0.41	0.64
Main chain (Å)	0.18	0.16	0.32

^a The statistics for the high-resolution shells within each data set are given in parentheses. The high-resolution shells for each data set are as follows: 2.27–2.20 Å for ADH1B*1, 2.59–2.50 Å for ADH1A, and 2.07–2.00 Å for ADH1C*2.

pairwise comparisons (Fig. 1A). Superposition of the dimeric ADH1B*1 and ADH1C*2 structures yielded overall r.m.s.d. values of 0.38 Å. In contrast, the superposition of the ADH1A dimer with either the ADH1B*1 or the ADH1C*2 dimers showed distinct differences (0.95 Å r.m.s.d.).

Alignments of individual coenzyme or catalytic domains yielded r.m.s.d. values between 0.23 Å and 0.31 Å. The r.m.s.d. values obtained for the aligned domains were independent of the structures used for the superimposition. The ADH1A structure shows a slightly more closed conformation when compared with either other human class I structure, which accounts for the elevated r.m.s.d. values when whole dimers were aligned. We estimate that, relative to the other class I closed complexes, the catalytic domain is rotated an additional 1.5 degrees toward the coenzyme-binding cleft in the ADH1A structure (Fig. 1B).

The ADH1B*1 and the ADH1C*2 structures are binary complexes between enzyme and coenzyme, and both show the fourth coordination site of the catalytic zinc atom to be occupied by a solvent water molecule (Fig. 2A). We have not, however, confirmed the oxidation state of the cofactor in any of these complexes by spectroscopic means; therefore, we will refer to all coenzymes with the ambiguous abbreviation NAD(H). The ADH1A structure appears to represent an intermediate complex trapped by the cryogenic freezing procedure. The enzyme was cocrystallized with NAD⁺ and 4-iodopyrazole. However, it appears that the cryoprotectant solution perturbed the binding of the inhibi-

tor molecule, and consequently 4-iodopyrazole is found at reduced occupancy (~50%) near the entrance to the substrate-binding pocket and not directly coordinated to the active site zinc ion and the C4 position of NAD⁺ (Fig. 2B). The occupancy of 4-iodopyrazole was estimated by applying alternative rounds of occupancy and temperature factor refinement to the iodine atom. We observed the direct coordination of 4-iodopyrazole to the catalytic zinc ion and the C4 position of the nicotinamide ring in a lower-resolution (2.8 Å) structure of ADH1A collected under room temperature conditions (Hurley et al. 1996). As a consequence, it is likely that this aberrant binding mode is not inherent to ADH1A but is a result of the freezing procedure.

Discussion

The three alcohol dehydrogenase isoenzyme structures reported here possess identical sequence lengths to the prototypical class I isoenzyme, horse EE ADH, with which they share ~86% sequence identity. Thus, all elements of secondary structure are highly similar in span and location. Both from sequence analysis and by functional characteristics, they are all class I ADH isoenzymes and each of the human isoenzymes possess overall molecular architecture that is identical to the horse EE isoenzyme (Eklund et al. 1976). The structural similarity of the human isoenzymes extends to the dimer interface region and accounts for their ability to form the heterodimers observed in human liver preparations. The heterodimers will possess substrate pref-

Fig. 1. Ribbon diagrams of the aligned human class I dimeric isoenzymes. (A) The ADH1C*2 isoenzyme (blue) was aligned with the ADH1B*1 isoenzyme (green) by using the α -carbon atoms in their respective coenzyme-binding domains (residues 176–322 in both subunits). (B) The ADH1A isoenzyme (red) was aligned with the ADH1B*1 isoenzyme (green) by using the same procedure as for the ADH1C*2 isoenzyme. The ribbon diagrams were produced using the programs MOLSCRIPT and Raster3D (Bacon and Anderson 1988; Kraulis 1991; Merrit and Murphy 1994).

ferences identical to the individual homodimers, because residues in the substrate-binding pocket contributed from the second subunit, positions 306 and 309, are identical in each isoenzyme. This structural observation coincides with enzyme kinetic data in which the kinetic properties of a particular heterodimer are completely explained by the summation of the individual isoenzyme's kinetic expressions (Bosron et al. 1983; Yang et al. 1994). The ADH1C*1 and ADH1C*2 isoenzyme were reported to show subunit communication in the form of negative cooperativity (Bosron et al. 1983). However, subsequent reevaluation of these data combined with additional work has shown that the nonlinear behavior in double-reciprocal plots was due to a kinetically significant pathway for dead-end complex formation (Charlier and Plapp 2000). As a consequence of this independence of function, this discussion will focus on unique aspects of the ADH1A and ADH1C*2 structures in comparison with the well-characterized ADH1B*1 isoenzyme.

The ADH1A isoenzyme is found only in humans and certain primates, such as the rhesus monkey (Light et al. 1992). Although the ADH1A and ADH1B*1 isoenzymes differ by 23 amino acid substitutions, the unique structural and functional features of ADH1A can be reduced to two

amino acid replacements, Arg 47 $\rightarrow$ Gly and Phe 93 $\rightarrow$ Ala. We will focus here on the substitution at position 47 and discuss position 93 later in the context of substrate binding. One sequence characteristic of most class I isoenzymes is an arginine at position 47. Arg 47 interacts electrostatically with, and donates two hydrogen bonds to, the adenosine phosphate of the bound cofactor NAD(H). The exchange of His for Arg 47 in the ADH1B*2 isoenzyme is associated with large changes in coenzyme-binding kinetics (Yin et al. 1984). Thus, it was somewhat surprising that the less conservative substitution of Gly for Arg 47 has much less of an effect on coenzyme-binding kinetics. Direct structure determination on the ADH1B*1, ADH1B*2 and an ADH1B*1 variant with Gly at position 47, showed that a greater extent of domain closure in the Gly 47 enzyme appeared to somehow compensate for the loss of a basic amino acid at position 47 (Hurley et al. 1994). An almost identical extent of domain closure is observed in the ADH1A structure reported here (Fig. 1B). Similar to what was observed in the Gly 47 variant of ADH1B*1, the space occupied by the side chain at position 47 is occupied by three ordered water molecules (Fig. 3A). The extent of domain closure has been observed to vary when amino acid replacements are made in the vicinity of the coenzyme-binding cleft. Substitution of Ala for Val 203 in the horse EE isoenzyme, like the Gly for Arg 47 in ADH1B*1 appears to increase the extent of domain closure, whereas substitution of Trp for Phe 93 appears to limit domain closure (Colby et al. 1998). In addition, substitutions on the coenzyme-binding domain side of the cleft at positions 293 and 295 also appear to limit the extent, or alter the equilibrium, of domain closure (Ramaswamy et al. 1999).

The ADH1C*2 isoenzyme shows an identical amount of domain closure as observed for both the ADH1B*1 and the horse EE isoenzyme. This is perhaps not surprising in that all the residues in direct contact with the coenzyme, except for Gln 271, are conserved between the horse EE and human ADH1C*2 isoenzymes. Gln 271 is one of two amino acid differences between the ADH1C*1 (Arg 271) and ADH1C*2 isoenzymes, the other being Val 349 versus Ile in ADH1C*1. Because of its position on the surface of the structure and its relative remoteness from the coenzyme- or substrate-binding sites, the latter substitution is not expected to have a significant effect on structure or function. The substitution of Gln for Arg 271 does alter the interactions between the adenine ring of NAD(H) and the enzyme. Arg 271 in both the ADH1A and ADH1B*1 structures interacts with the adenine ring strictly through van der Waals contacts and the guanidinium group is held away from the ring by forming two hydrogen bond interactions with Asp 273. However, Gln 271 interacts directly with the adenine ring by donating a hydrogen bond from its side chain amide nitrogen to the N9 atom of the adenine ring and accepting a hydrogen bond from the N6' atom of the adenine ring to its

Fig. 2. Stereodiagrams of the substrate-binding sites of the human class I isoenzymes. The identical alignment procedure used in Fig. 1 was used here to minimize differences in the cofactor positioning between the aligned substrate-binding sites. (A) The substrate-binding site in ADH1B*1 (green) overlaid on the corresponding region of the ADH1C*2 substrate-binding site. (Zn) Active site zinc atom; (Wat) zinc-bound water. Individual residues are labeled with their sequence identifiers. (B) The substrate-binding site of ADH1A (red) overlaid on the substrate-binding site of ADH1B*1 (green). The active site zinc is labeled identically as in Fig. 2A, and the position of the partially occupied 4-iodopyrazole molecule is shown in blue and labeled 4IP. The figure was generated using the program MOLSCRIPT (Kraulis 1991).

side chain carbonyl oxygen atom (Fig. 3B). Coenzyme release is apparently rate limiting for both ADH1C isoenzymes, but whether or not these additional hydrogen-bonding interactions are responsible for the twofold reduction in turnover rate observed for the ADH1C*2 versus ADH1C*1 isoenzyme remains to be determined.

Alcohol dehydrogenase isoenzymes are believed to function as broad detoxication enzymes for both biogenic and dietary substrates, and their relatively low k_{cat}/K_m values are compensated by their abundance in tissue and their overlapping specificity for substrates (Edenberg and Bosron 1997). Thus, it is likely that more than one ADH participates in the metabolism of any single substrate. This is most clearly shown by the knockout studies in mice in which removal of one ADH locus diminishes but does not elimi-

nate metabolism of any single substrate (e.g., ethanol or retinol) (Deltour et al. 1999). Alcohol dehydrogenase has been used as a type of "reverse paradigm" for studies on enzyme structure and function (Danielsson and Jörnvall 1992; Grimshaw 1992). In contrast with most highly conserved enzymes found across species, alcohol dehydrogenase isoenzymes tend to show higher conservation of sequence away from the substrate-binding site, suggesting that different environmental conditions have shaped different and overlapping substrate preferences, while maintaining similar structural architectures (Jörnvall et al. 1996). The structures we report in this article further support this idea. The three structures reported here share >90% pairwise sequence identity, but their substrate-binding residues share between 50% (ADH1A vs. ADH1B*1 or ADH1C*2)

Fig. 3. Stereodiagrams of the coenzyme-binding site residues in ADH1A and ADH1C*2 near the positions or residues 47 and 271, respectively. (A) The structure of the ADH1A isoenzyme (red) in the vicinity of Gly 47 overlaid onto the same region of the ADH1B*1 isoenzyme (green). (Wat1, Wat2, Wat3) Positions of the three water molecules recruited to take the place of Arg 47. Three additional ordered water molecules (not labeled) that are common between all three human ADH1 isoenzymes also are shown. The alignment procedure was identical to the one used in Fig. 1. (B) The structure of the ADH1C*2 isoenzyme (blue) in the vicinity of Gln 271 overlaid onto the similar region of the ADH1B*1 isoenzyme. Three water molecules common to these two structures are shown near Lys 228 and the N6' atom of the adenine ring. In addition, an ordered water molecule unique to this region of the ADH1C*2 also is shown positioned between Asp 273 and Gln 271. The figures were generated using MOLSCRIPT (Kraulis 1991).

and 69% (ADH1B*1 vs. ADH1C*2) sequence identity. Their overall structural similarity is consistent with their high overall sequence similarity, yet as a consequence of localized sequence variation, their substrate-binding pockets have distinctly different topologies. These different topologies explain their different substrate preferences but do not necessarily prevent the oxidation any single substrate (Table 2). For example, whereas cyclohexanol is a substrate for all the class I isoenzymes, the $V_{\max}/K_M$ values for this substrate vary by 2700-fold between the isoenzymes (Table

2). In contrast, the catalytic efficiencies for 1-butanol only vary by eightfold. Clearly, the shape and flexibility of the substrate influences its ability to be oxidized by the different human ADH1 isoenzymes.

The substrate-binding pocket of alcohol dehydrogenase has been described as a cylinder, 7–10 Å in diameter, that extends some 15 Å from the catalytic zinc ion to the surface of the enzyme (Eklund et al. 1982). As a consequence of this shape and length, secondary alcohols are generally poor substrates and primary alcohols with chain lengths of >10 carbons extend out of the substrate-binding pocket into solution. The residues lining this cylinder can be divided into three regions (inner, middle, and outer), based on their proximity to the catalytic zinc ion (Eklund et al. 1987). The inner part of the substrate-binding site is composed of residues 48 and 93, as well as the catalytic face of the nicotinamide ring. The middle region is composed of residues 94, 140, 141, 294, and 318 and residue 309 from the opposite subunit of the dimer. The outer region is composed of residues 57, 58, 110, 116, 117, and 297 and residue 306 from the other subunit (Fig. 2). Of these 15 residues, only five residues,

Table 2. $V_{\max}/K_M$ values for alcohol substrates at pH 7.5^a

Substrate	ADH1A	ADH1B*1	ADH1C*1
Ethanol	0.67	42	37
1-butanol	160	59	460
Cyclohexanol	530	0.19	150
R-3-methyl-2-butanol	45	0.02	0.23
S-3-methyl-2-butanol	2.7	0.07	1.2

^a Data obtained from Stone et al. (1989).

110, 140, 294, 306, and 309, are identical among the three isoenzymes discussed here. The remaining 10 residues, and their context within the substrate-binding site, determine the differences in substrate and inhibitor specificity shown by these isoenzymes.

The most substantial changes in substrate specificity are effected through substitutions within the inner region of the substrate-binding site, residues 48 and 93. Residue 48 participates in the reaction mechanism by facilitating the transfer of the alcohol proton to bulk solvent as part of a "proton relay" (Eklund et al. 1982). Active forms of alcohol dehydrogenase possess either a Ser or a Thr at this position, and the hydroxyl moiety of the side chain participates in the proton relay mechanism. However, enzymes that possess a Thr rather than a Ser at this position generally possess a more severely restricted specificity for secondary alcohols. It appears that the additional methyl group in the Thr side chain interferes with the binding of alcohols with substituents other than two hydrogens at the C1 position (Fig. 2A). This is most clearly seen with the ADH1C isoenzymes in which it has been shown that the Ser at position 48 is almost exclusively responsible for this enzyme's ability to oxidize 3 β -hydroxy-5 β -steroids (Höög et al. 1992). The position occupied by amino acid 93 is directly opposite of residue 48 and may have a greater impact on substrate specificity. In most forms of the medium chain alcohol dehydrogenase superfamily, the position equivalent to amino acid 93 generally contains an aromatic residue (Phe, Tyr, Trp; Sun and Plapp 1992). This aromatic residue forms the floor of the substrate-binding site and creates a severe steric restriction to the binding of secondary alcohols with the R configuration (Stone et al. 1989). As a result, most alcohol dehydrogenases generally prefer S over R enantiomers of secondary alcohols as substrates (Stone et al. 1989). The ADH1A isoenzyme differs from either the ADH1B or the ADH1C isoenzymes in that ADH1A has an Ala instead of Phe at position 93 (Fig. 2B). This substitution was predicted to permit relatively efficient oxidation of secondary alcohols by the ADH1A isoenzyme (Eklund et al. 1987). This prediction was later borne out by enzymatic characterization of the liver purified isoenzyme and through site-directed mutagenesis studies (Stone et al. 1989; Hurley and Bosron 1992). Even for nonchiral substrates, such as cyclohexanol, the substitution of Ala at position 93 creates a more favorable environment for secondary alcohol binding than a Ser at position 48, as illustrated by the ~3.5-fold difference in the $V_{\max}/K_M$ between ADH1A and ADH1C*1 (Table 2). Model-building studies indicate that this difference in $V_{\max}/K_M$ is due to the ability of the ADH1A isoenzyme to productively bind both the axial and equatorial conformations of cyclohexanol. In contrast, the ADH1C*1 and ADH1C*2 isoenzymes appear to be able to productively bind only the axial conformation, which comprises <10% of the solution conformations.

The middle region of the substrate-binding pocket is composed of residues 140, 141, 294, 318, and 319 and residue 309 (Fig. 2). Residue 309 is contributed by the neighboring subunit of the dimer. Among the class I ADH isoenzymes, only residues 141, 318, and 319 vary in the identity of the residues located at these positions. There are very little direct data on how changes to these residues in the class I isoenzymes might affect their substrate specificity or their inhibitor selectivity. However, data from studies on the class IV isoenzyme might shed light on the influence of position 141 on substrate binding. We previously reported that Met at position 141 interfered with the methyl substituent at the 4-position and was primarily responsible for the class IV-specific characteristic of low affinity for 4-methyl-pyrazole (Xie and Hurley 1999). The ADH1C*2 and ADH1C*1 isoenzymes both possess Val at position 141 whereas the ADH1A and ADH1B*1 isoenzymes possess Leu at this position. There is no direct evidence to indicate that Val 141 is responsible for the greater binding affinity shown by the ADH1C isoenzymes for 4-methylpyrazole (Bosron and Li 1987), but we would suggest that Val at position 141 would provide a more favorable environment than the corresponding Leu residue (Fig. 2A). Conversely, the additional domain rotation present in the ADH1A isoenzyme moves Leu 141 further into the substrate-binding pocket thus narrowing the middle region more than might be expected based solely on the amino acids present (Fig. 2B). This positioning of Leu 141 in ADH1A, combined with the loss of Phe 93 as the "floor" to the pyrazole-binding site, may explain this enzyme's 10-fold lower affinity for pyrazole-based inhibitors when compared with the other human ADH1 isoenzymes (Bosron and Li 1987).

The outer region of the substrate-binding pocket is composed of residues 57, 58, 110, 116, 117, 297, and 306 (Fig. 2). As with residue 309 in the middle region, residue 306 is contributed by the second subunit of the dimer. Of these residues, only positions 57, 116, 117, and 297 vary in their amino acid identity between the human ADH1 isoenzymes. Residues 57, 116, 117, and 306 form a 'bottleneck' for substrates entering the substrate-binding site from solvent that is roughly 6 Å from one side to the other. This further narrows to ~5 Å in the ADH1B*1 isoenzyme depending on the side chain rotamer conformation of Leu 116. Leu 116 generally exists in one of two common side chain conformations, one that partially occludes access to the substrate-binding site and another that does not (Hurley et al. 1994). Figure 2B shows the latter of the two conformations. Leu 116 has been suggested to play a role in substrate access to and retention in the active site of the ADH1B*1 isoenzyme and mutagenesis results confirm this hypothesis (Hurley and Vessell 1995). The ADH1A isoenzyme has a Val at position 116 and its position in the substrate-binding site, and the amount of steric constraint it provides to substrate binding is intermediate between the two conformations for Leu 116 at

this position. The occurrence of Val at position 116 appears to be compensated for by the presence of Met at position 57. The Met 57/Val 116 combination in ADH1A yields a similar accessible volume at this restriction point, as the Leu 57/Leu 116 combination in both the ADH1B*1 and ADH1C*2 isoenzymes (Fig. 2). Residues 58, 110, 117, and 297 would only interact with particularly long alcohol substrates (>10 atoms) that possess hydrophilic substituents at the opposite end of the substrate. In one subunit, an additional hydrogen bond is present between Asp 297 and the peptide nitrogen of Val 58, whereas in the other subunit this interaction is water mediated (not shown). In either case, this interaction is unique among the three human class I ADH isoenzymes and may help to stabilize the more closed domain conformation observed for the ADH1A isoenzyme.

A recently published study examined the ability of *N*-substituted formamides to selectively inhibit the human ADH isoenzymes (Schindler et al. 1998). The most potent inhibitors were *N*-cyclopentyl-*N*-cyclobutylformamide, *N*-benzylformamide, and *N*-1-methylheptylformamide for the ADH1A, ADH1B*1, and ADH1C*2 isoenzymes, respectively. We can better understand the selective binding of these inhibitors based on the active site topologies now observed in our structures. The easiest to interpret is the inhibition of ADH1A by *N*-cyclopentyl-*N*-cyclobutylformamide. This disubstituted compound undoubtedly takes advantage of the space available by the Phe to Ala 93 substitution (Fig. 2B), as predicted by Schindler et al. (1998). We see no additional information in our structure that would require revision of the original interpretation. The tight binding of benzylformamide to the ADH1B*1 isoenzyme is not necessarily surprising, but the 15-fold difference between the binding to ADH1B*1 and ADH1C*2 is somewhat surprising, as even horse EE ADH binds this compound with an affinity comparable to ADH1B*1. Because both horse EE ADH and ADH1C*2 possess Ser 48, the lack of the methyl group in Thr 48 of the ADH1B*1 enzyme would not appear to affect the binding of the inhibitor. The most likely explanation for the relatively poor binding of benzylformamide to the ADH1C*2 isoenzyme is the replacement of Leu 141 with Val and the replacement of Val 318 with Ile. The former substitution would weaken a favorable contact between position 141 and the benzyl ring, whereas the latter substitution would likely create a steric constraint to the binding of the benzyl ring. The additional methyl group of the Ile side chain would be forced to point toward the benzyl ring as a consequence of other side chains nearby (Fig. 2A). Last, the selectivity of *N*-1-methylheptylformamide for the ADH1C*2 isoenzyme was suggested to be due to the Ser for Thr 48 substitution near the active site zinc atom. This is undoubtedly true, but we suggest that the substitution of Val for Leu 141 also would create the necessary room for this substituent and lead to the favorable binding of this compound (Fig. 2A). These human class I

ADH structures should lead to the development of even more selective compounds after direct structure determination with these known selective agents. Ultimately, selective inhibition of human ADH isoenzymes may be useful in determining which substrates these distinct ADH isoenzymes metabolize in vivo and how the consumption of ethanol affects the metabolism of these endogenous substrates.

Materials and methods

Expression and purification of recombinant ADH isoenzymes

The three class I isoenzymes were expressed in *Escherichia coli* by using the vector pKK223-3 (Pharmacia-Biotech) as previously described for the ADH1B*1 isoenzyme (Hurley et al. 1990). Briefly, the transformed cells were grown at 37°C in 10 liters of TB media (1L contains 12 g Peptone 140, 12 g yeast extract, 4 mL glycerol, 0.017 M potassium phosphate monobasic, 0.072 M potassium phosphate dibasic) containing 50 µg/mL ampicillin until the culture achieved an optical density of 0.8 at 595 nm. Expression of the ADH isoenzyme was induced by the addition of 0.1 mM IPTG and 10 µM zinc sulfate. Immediately after induction, the temperature of the culture was reduced to 16°C, and the cells were incubated for an additional 16 h. The cells were pelleted and resuspended in 10 mM Tris-HCl at pH 8.5, 4 mM DTT, 1 mM benzamidine, 10 µM zinc sulfate. The cells were lysed using a French pressure cell operated at 900 pounds per square inch. The resulting lysate was clarified by centrifugation at 35,000 rpm in a Beckman Ti45 rotor, and the clarified lysate supernatant was passed over a 300-mL bed of DEAE-cellulose (Whatman International, Ltd.) and collected in the column wash. The flowthrough from the DEAE column was buffer-exchanged into 10 mM Na-Hepes at pH 8.0 (ADH1B*1) or pH 7.2 (ADH1A and ADH1C*2), 2 mM DTT, 1 mM benzamidine, 10 µM zinc sulfate and then applied to a 5 × 10-cm column of S-Sepharose equilibrated with the same buffer. The column was washed to remove unbound protein and then eluted in a single step with 120 mM sodium chloride in column buffer. The active fractions were pooled and dialyzed into 50 mM Tris-HCl at pH 7.5, 1 mM DTT, 10 µM zinc sulfate. The ADH1A and ADH1B*1 isoenzymes were loaded onto a 2.5 × 10-cm column of Affi-Gel Blue (Bio-Rad Laboratories). The column then was washed to remove unbound proteins and then step eluted with 800 mM sodium chloride in column buffer. The ADH1C*2 enzyme does not bind to the Affi-Gel Blue column. As a final purification step, NAD⁺ at a concentration of 2 mM was added to the pooled ADH1C*2 isoenzyme, and this pooled enzyme was loaded onto a 2.5 × 10-cm column of caproyl-γ-aminopropylpyrazole (Lange and Vallee 1976). The column was washed to remove unbound protein and eluted in a single step with 100 mM Tris-HCl at pH 7.5, 1 mM DTT containing 0.5 M ethanol. The active fractions from both affinity columns were dialyzed into 10 mM sodium phosphate at pH 7.5, 0.5 mM DTT, concentrated to ~2 mg/mL, and stored as 50% glycerol stocks at -20°C.

Crystallization and data collection

Recombinant ADH1A was crystallized at 24°C from solutions containing 14% PEG 6000, 100 mM Na-ACES at pH 7.2, 2 mM NAD⁺, 2 mM 4-iodopyrazole, 10 mg/mL protein. The recombinant ADH1B*1 isoenzyme was crystallized at 4°C from solutions containing 14% PEG 8000, 50 mM sodium phosphate at pH 7.5, 2 mM NAD⁺, 14 mg/mL protein. The recombinant ADH1C*2 isoenzyme

was crystallized at 4°C from solutions containing 18% PEG 6000, 50 mM Tris at pH 9.0, 4 mM NAD⁺, 10 mg/mL protein. Before data collection, all crystals were transferred to solutions containing the crystallization medium supplemented with 20% PEG 200 in a two-step procedure. The crystals then were flash-frozen at -165°C in the cold nitrogen gas stream. All data were collected on a Rigaku 200HB rotating anode generator operating at 50 kV and 100 mA equipped with Yale focusing mirrors and a Rigaku RAXIS IIC image plate detector (Molecular Structure Corporation). All data were indexed, integrated, merged, and scaled using the HKL/SCALEPACK program suite (Otwinowski and Minor 1997).

Molecular replacement and refinement

The Protein Data Bank entry number 1DEH (Davis et al. 1996), without the solvent and 4-iodopyrazole molecules, served as the starting model for all structures. For historical reasons, previous work had used a nonstandard triclinic indexing solution for the ADH1B*1 diffraction data (Hurley et al. 1994; Davis et al. 1996). However, the new indexing solution now conforms to the standard convention for triclinic cells, thus necessitating a new molecular replacement solution. All molecular replacement calculations were performed using the program AMoRe (Navaza 1994) and diffraction data between 15 Å and 4 Å. The properly oriented model then was refined using XPLOR-3.845 (Brünger and Rice 1997) to the full resolution of each individual data set. Throughout the refinement of the ADH1A structure, noncrystallographic symmetry restraints of 100 kcal/mole were applied to the main chain atoms in the dimer and restraints of 25 kcal/mole for the side chain atoms. Initially, noncrystallographic symmetry restraints of 100 kcal/mole were applied to the main-chain atoms during the refinement of the ADH1C*2 structure. These restraints were reduced to 10 kcal/mole during the final cycles of refinement. Similarly, noncrystallographic symmetry restraints of 100 kcal/mole were applied during the refinement of the ADH1B*1 model until the last cycle of refinement, when these were relaxed to 10 kcal/mole. All models were refined against all available data by applying a bulk-solvent correction to the low-resolution data and individual restrained isotropic temperature factors for all atoms. Stereochemical restraints developed in our laboratory for the active site and structural zinc atoms were applied throughout refinement to maintain ligand geometry observed in the high-resolution structures of the horse liver EE isoenzyme. After the initial round of rigid-body and positional refinement, the correct amino acid sequence was introduced into the ADH1A and ADH1C*2 models by using the program CHAIN (Sack 1988). Subsequent rounds of refinement and visual inspection of the resulting models were accomplished using XPLOR-3.845 and the program O (Jones et al. 1991). Solvent molecules were added as indicated by the presence of strong electron density peaks in both 2Fo-Fc and Fo-Fc maps within hydrogen-bonding distance to protein atoms.

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Bank under the codes 1HSO, 1HSZ, 1HT0. This work was supported by PHS grants R29-AA10399, R37-AA07117, and P50-AA07611.

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