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Research Article

Identification and characterisation of two allelic forms of human alcohol dehydrogenase 2

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Abstract. The human alcohol dehydrogenase system is comprised of multiple forms that catalyse the oxidation/reduction of a large variety of alcohols and aldehydes. A transition that results in an Ile308Val substitution was identified in the human *ADH2* gene by single-strand conformation polymorphism analysis. Screening a Swedish population revealed that Val308 was the most frequent allele (73%), and site-directed mutagenesis was used to obtain both allelozymes, which were expressed in *Escherichia coli* for characterisation. Thermostability was assayed by activity measurements and circular dichroism spectroscopy. The results showed that the 308Val substitution decreases protein stability, as compared to the Ile308 variant, an effect also demonstrated during prolonged storage. Ethanol, octanol, 12-hydroxydodecanoic acid and all-trans retinol were used as model substrates and, generally, slightly higher K_m values were observed with Val at position 308. Finally, homology modelling, from mouse ADH2, further supported the decreased stability of the Val308 variant and located position 308 in the subunit interface of the molecule and in the vicinity of the active-site pocket entrance. In conclusion, the Ile308Val substitution represents a novel functional polymorphism within the human alcohol dehydrogenase gene cluster that may affect the metabolism of ethanol and other substrates.

Key words: Alcohol dehydrogenase; human; polymorphism; allelozyme; mutagenesis.

Genetic variation is a common phenomenon in nature and about 1.4 million single-nucleotide polymorphisms (SNPs) have been described throughout the human genome [1]. Around 60,000 of these polymorphisms are estimated to fall within exons [1], and may thereby result in polypeptides with altered properties. Within the human alcohol dehydrogenase system (ADH, EC 1.1.1.1), five genes out of seven have been shown to be polymorphic, i.e. *ADH1B*, *ADH1C*, and the promoters of *ADH2*, *ADH3* and *ADH4* [2–9][1]. The physiological significance of these SNPs is still a matter of debate. However, associations with various pathological processes have been suggested, including ethanol metabolism and drinking behaviour [10–14], carcinogenesis [15, 16], myocardial infarction [17] as well as the onset of neurodegenerative disorders [9].

The different ADHs display similar topology, broad substrate repertoires and have been proposed to play general protective roles against various compounds in humans and other organisms [11, 18]. ADH2 was originally iso-

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[1] The nomenclature of the mammalian ADH system is under revision. Consequently, in the reference list, the names of ADH enzymes, subunits and genes will differ depending on which nomenclature is being used. This paper uses the nomenclature of Duester et al. [2] in which the ADH proteins and genes have been designated the same number; e.g. for the enzyme formerly known as class II ADH for which the human gene was called *ADH4*, the protein is now denominated ADH2 and the gene *ADH2*.

lated from human liver as an anodic form with a high K_m for ethanol [19, 20]. The enzyme has a preference for hydrophobic substrates [21] and shows high specificity for unsaturated aldehydes, such as lipid-peroxidation-derived 4-hydroxy alkenals [22] and intermediary aldehydes in noradrenalin and serotonin metabolism [23, 24]. In addition, p-benzoquinone and p-benzoquinone imine reduction seem to be ADH2 specific within the ADH family [25]. Recently, a subgroup of ADH2 enzymes have been identified in rodents which display low oxidative activity owing to a reduced capacity to transfer hydride [26]. The crystal structure of the mouse enzyme, the only ADH2 structure that has been solved, shows a more voluminous substrate pocket than the ADH1 enzymes [27].

The *ADH2* promoter was found to contain three polymorphic sites that affected gene expression in a reporter gene assay [7]. Four additional polymorphisms have been proposed in the coding sequence of the human *ADH2* gene based on discrepancy between peptide and cDNA sequences [28]. Two of these possible polymorphisms were in exon 7, corresponding to substitutions at positions 308 and 317 in the polypeptide chain, whereas the other two substitutions were in the ninth terminal exon, position 373 and the termination codon. The polymorphism affecting the termination codon would give rise to an elongated peptide, if present. This study describes the identification and characterisation of an SNP in exon 7 that results in an Ile to Val substitution at position 308, in the subunit interface near the active-site cleft.

Materials and methods

PCR amplification of *ADH2* exons

DNA was isolated from leukocytes from Swedish volunteers (with the approval of the Karolinska Institutet ethical committee) who had declared themselves as healthy. Fifty to 150 ng of genomic DNA was used as template. Primers specific for the human ADH2 gene (formerly *ADH4*, [2, 29]), forward: 5′-ATCACAGAAAGCAGCCCTGG-3′ and reverse: 5′-ACCACCAAAGAATGTTCCATTTA-3′; forward: 5′-CGCTTTTTCGAAGCGTCCGA-3′ and reverse: 5′-AGCTCTTTTATGTTCCCATATTAAATG-3′ were used to amplify the entire exons 7 and 9, respectively. PCR reactions were carried out with 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP and 0.5 U Taq polymerase (Life Technologies) in 50-μl reaction volumes. Cycling conditions were 95 °C for 5 min, followed by 5 cycles of 95 °C 30 s 68 °C 30 s, and 30 cycles of 95 °C 30 s, 52 °C 20 s 72 °C 20 s, with a final extension at 72 °C for 10 min. For quality and size conformation, PCR amplification products were analysed on 1.5% agarose gels and visualised by ethidium bromide staining.

Single-strand conformation polymorphism analysis

PCR product (2.5 μl) was mixed with 8 μl loading buffer, 95% deionised formamide, 0.05% bromphenol blue, 0.05% xylene cyanol FF and 20 mM EDTA. Samples were denaturated at 95 °C for 5 min, chilled on ice for 5 min and loaded on 80 × 60 mm, 9.1% polyacrylamide gels (50:1 acrylamide: N,N′-methylene-bis-acrylamide) containing 10% glycerol and 2 × TBE buffer. Gels were run at 6 °C, 50 V for 15 h followed by fixation in 40% methanol/10% acetic acid, washed with water, oxidised in 3.4 mM potassium dichromate/3.2 mM HNO_3 and washed again. Gels were silver stained in 12 mM $AgNO_3$ and bands were visualised in 0.28 mM sodium carbonate/0.02% formaldehyde.

Sequence analysis

Sequence analysis of plasmid DNA and PCR fragments was performed with the BigDye terminator cycle sequencing kit (PE/Applied Biosystems) using fluorescent dNTPs and plasmid- or sequence-specific primers and analysed on an ABI Prism 377 DNA sequencer.

Plasmid constructs and site-directed mutagenesis

The coding region of human *ADH2* [28] was subcloned into the pET3d expression vector (Novagen) and site-directed mutagenesis was performed on double-stranded plasmid isolated with the flexiprep kit (Amersham Pharmacia Biotech). The transition resulting in the Ile/Val substitution at position 308 was introduced using the U.S.E. mutagenesis kit (Amersham Pharmacia Biotech) with mutagenesis primer, 5′-GCAAAGGATTGACT-GTTTTTCCAGAGG-3′, and selection primer, 5′-TGT-CAAACATGAGACTTCTTGAAGACGAA-3′. Sequence analysis was performed on both strands to confirm positive clones and to exclude the possibility of undesired mutations.

Expression and purification of ADH2

The allelozymes were expressed in *Escherichia coli* as described earlier for human ADH2 [30] and purified in two steps with DEAE cellulose (Whatman) and either AMP sepharose or blue sepharose (Amersham Pharmacia Biotech). Proteins were concentrated on 30 K cut-off filters in either centrifugal cells (centriplus YM–30; Millipore) or in a stirred ultrafiltration cell (Amicon/Millipore). Protein concentrations were determined with the Bio-Rad protein assay, standardised with bovine serum albumin.

Enzyme assays

Activity was determined with a Hitachi U-3000 spectrophotometer by monitoring the production of NADH at 340 nm using an NADH absorption coefficient (ε) of 6220 M^{-1} cm^{-1}, or the production of all-trans retinal (ε_{400}:

29,500 M^{-1} cm^{-1}). Specific activities (U/mg; 1 U is defined as 1 µmol NADH formed/min) for the ADH2 allelozymes were determined in a standard assay at 25 °C with 0.1 M glycine/NaOH, pH 10.0, 33 mM ethanol and 2.4 mM NAD^+.

Steady-state enzyme kinetics were performed at 25 °C in 0.1 M sodium phosphate or 0.1 M potassium phosphate, pH 7.5. NAD^+, all-trans retinol, 12-hydroxydodecanoic acid (12-HDA) and Tween-80 were purchased from Sigma; and 1-octanol was from Merck. 1-Octanol and 12-HDA were dissolved in methanol to yield a final concentration of 2% methanol in the photometric cell. For retinol, 0.02% Tween-80 was added to the reaction buffer and the substrate was prepared according to Parés and Julià [31]. The retinol concentration was determined using an absorption coefficient in the reaction buffer of ε_{328}: 39,500 M^{-1} cm^{-1} [32]. K_m values for NAD^+ were determined with 50 mM ethanol. To fit lines to data points and to calculate kinetic parameters, a weighted non-linear regression analysis program was used (fig. P for Windows; Biosoft). The velocity rates were calculated per subunit (40 kDa) and standard errors were less than 10% for k_{cat} values and less than 15% for K_m values.

Thermostability measurements

Purified enzymes were incubated at different temperatures for 5 min in the presence or absence of saturating concentrations of NAD^+. The tubes were centrifuged to pellet-denatured protein and the activities were determined using the standard assay. The same protocol was used to determine the stability under prolonged storage at 6 °C and at 37 °C in phosphate-buffered saline (PBS), pH 7.4.

Thermostability was further monitored by circular dichroism spectroscopy at 222 nm using a Jasco J-720 spectropolarometer. The protein concentration was 1 mg/ml and the temperature gradient was set from 5 to 80 °C at 0.5 °C/min. All experiments were performed in duplicate.

Structure modelling

A model of human ADH2 (Swissprot code ADHP_HUMAN) [28, 33] was created by homology modelling from the mouse enzyme (PDB code 1e3i) [27, 34]. The template and the target showed 72% sequence identity, which makes homology modelling feasible. The initial alignment was created using the Gonnet matrix [35] with gap open/extension penalties of 2.4/0.15 [36]. The placement of a two-residue insertion in the active site area was further investigated using the Modeller package [37] to create models with different placement of the insertion. Correct placement of the insertion was then deduced by investigating the different models. The final alignment was then fed into the ICM package [38] to create an all-atom model.

Results

Identification of a new *ADH2* allele

In an attempt to confirm or dismiss four previously proposed polymorphisms in the *ADH2* gene [28], PCR-amplified fragments of exons 7 and 9 (approximately 150 bp each) were analysed by single-strand conformation polymorphisms (SSCP) gel electrophoresis. For the exon 7 samples, three different migration patterns were readily detected (fig. 1) whereas no difference in SSCP gel mobility was observed for the exon 9 experiments (20 individual DNA samples, data not shown). Sequence analysis revealed that the different patterns for exon 7 corresponded to the different homozygous and heterozygous genotypes of the Ile308Val substitution, i.e. an A → T transition at position 925 of the open reading frame (ORF) (counting the A of the ATG start codon as 1; fig. 1). According to the nomenclature of Duester et al. [2], the Ile308 allele should be named *ADH2*1* and the Val308 allele *ADH2*2*. The fact that three of the potential polymorphisms were not detected indicated that they are absent or rare within the Swedish population. However SSCP analysis may fail to detect all mutations, although only rarely when short fragments (< 200 bp) are analysed [39].

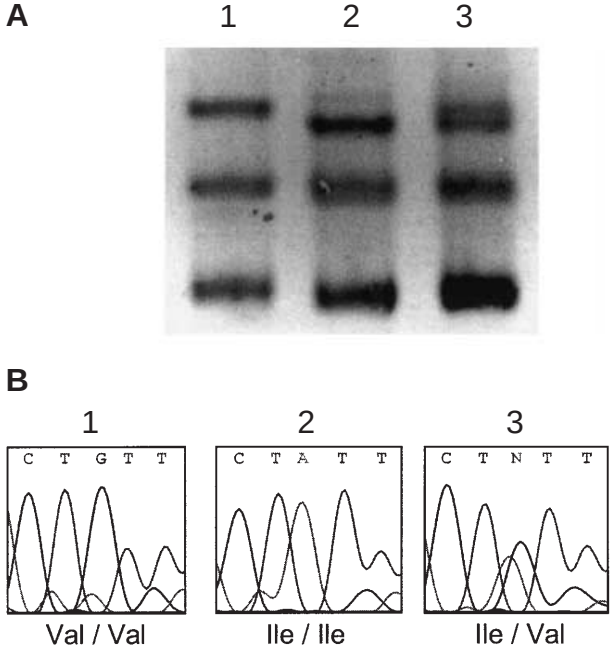

Figure 1. Detection of polymorphism in exon 7 of the human *ADH2* gene. (*A*) SSCP analysis. Silver-stained polyacrylamide gel showing the three different migration patterns detected for the exon 7 PCR products. Lanes 1 and 2 correspond to the two homozygotes whereas lane 3 shows the heterozygote. (*B*) Sequence analysis was used to deduce polymorphic sites in multiple samples with different migration patterns in SSCP analysis. Both G and A are visible in the heterozygous sample (3).

Table 1. Distribution and allele frequencies in 99 individuals from a Swedish population.

Genotypes, frequencies (%)						Alleles (%)	
Ile/Ile		Ile/Val		Val/Val		Ile308 *ADH2*1*	Val308 *ADH2*2*
observed	predicted	observed	predicted	observed	predicted		
7	7.4	40	39.6	53	52.8	27	73

Predicted genotype frequencies were calculated according to the Hardy-Weinberg distribution [40], i.e. $p^2 : 2pq : q^2$, where p and q are allele frequencies for either of the two alleles.

Allele frequencies

PCR-amplified fragments covering exon 7 of the *ADH2* gene from 99 DNA samples from a Swedish population were subjected to SSCP analysis as above. Both *ADH2*1* (A_{925}) and *ADH2*2* (G_{925}) alleles were relatively common in the sample, with the highest frequency (73%) for *ADH2*2*, i.e. valine at position 308. The allele frequencies followed the Hardy-Weinberg distribution and the observed genotypes did not deviate significantly from those predicted (table 1) [40].

Recombinant expression and kinetic properties of ADH2 allelozymes

Site-directed mutagenesis was performed to introduce Val308 into the human Ile308 ADH2 construct and both allelozymes were expressed in *E. coli*. Purification was conducted on both anion exchange and affinity columns, yielding pure protein as judged by Coomassie-brilliant-blue-stained SDS/polyacrylamide gels (fig. 2).

Specific activities were determined in a standard assay (33mM ethanol, 2.4mM NAD^+) to 1.4 U/mg for both allelozymes. The alcohols, ethanol, octanol 12-HDA and all-trans retinol were used as model substrates to characterise enzymatic activity (table 2). All measurements were carried out at 25 °C in 0.1 M phosphate buffer pH 7.5. The turn-over values (k_{cat}) were similar for both allelozymes and varied from 2 min^{-1} for 12-HDA to about 10 min^{-1} for ethanol (table 2). Generally, higher K_m values were obtained with Val compared to Ile at position 308 (table 2). K_m values for octanol and 12-HDA were increased about 2- and 1.5-fold, respectively, for the Val308 enzyme, while only small differences in K_m were detected for ethanol and retinol. In addition, coenzyme saturation was also reached at slightly higher concentration with Val at position 308.

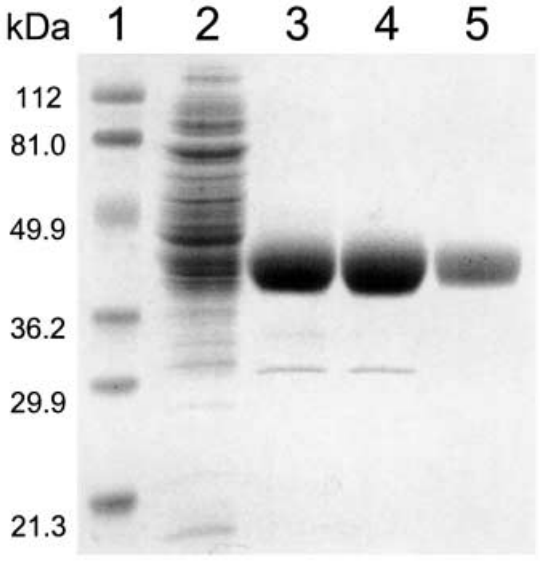

Figure 2. Coomassie-brilliant-blue-stained SDS/polyacrylamide gel of recombinantly expressed Val308 ADH2. Lane 1, protein markers with masses in kDa; lane 2, *E. coli* crude cell lysate; lane 3, after DE-52 anion exchange: lane 4, after desalting with PD-10 gel filtration columns; lane 5, pure ADH2 after blue sepharose.

Table 2. Kinetic parameters for the human ADH2 allelozymes assayed at pH 7.5.

Substrate	308Ile			308Val		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Ethanol	9	10	1.1	10.6	10.5	1.0
Octanol	0.009	2.3	250	0.016	2.4	150
12-HDA	0.062	1.5	24	0.091	1.8	20
Retinol	0.011	5.2	470	0.012	6.6	550
NAD^+	0.063	–	–	0.074	–	–

Octanol and 12-HDA were dissolved in methanol yielding a final concentration of 2% in the photometric cell. All-trans retinol was prepared according to Parés and Julia [31]. The K_m for NAD^+ was measured with 50 mM ethanol.

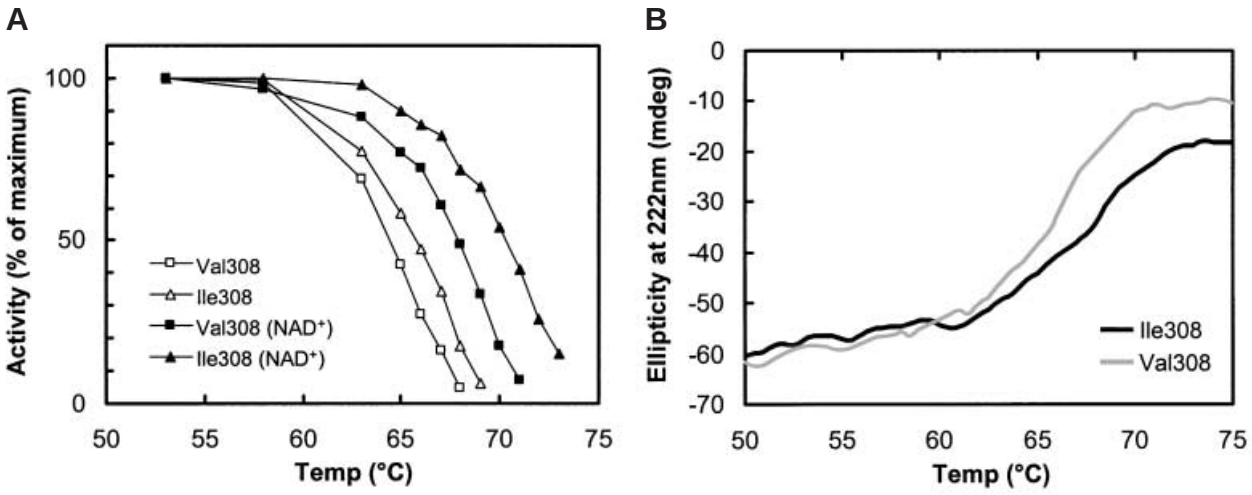

Figure 3. Thermostability of human ADH2 allelozymes. (*A*) Activity measurements. Purified enzymes were incubated at different temperatures in a PCR machine for 5 min in the presence (filled symbols) or absence (open symbols) of saturating concentrations of NAD^+. Activities were determined using the standard ethanol assay and plotted against the temperature as percent of the activity prior to incubation. (*B*) Circular dichroism spectroscopy at 222 nm using a Jasco J-720 spectropolarometer of Ile308 (black curve) and Val308 (grey curve). Protein concentration was 1 mg/ml and the temperature gradient was set from 5 to 80 °C at 0.5 °C/min.

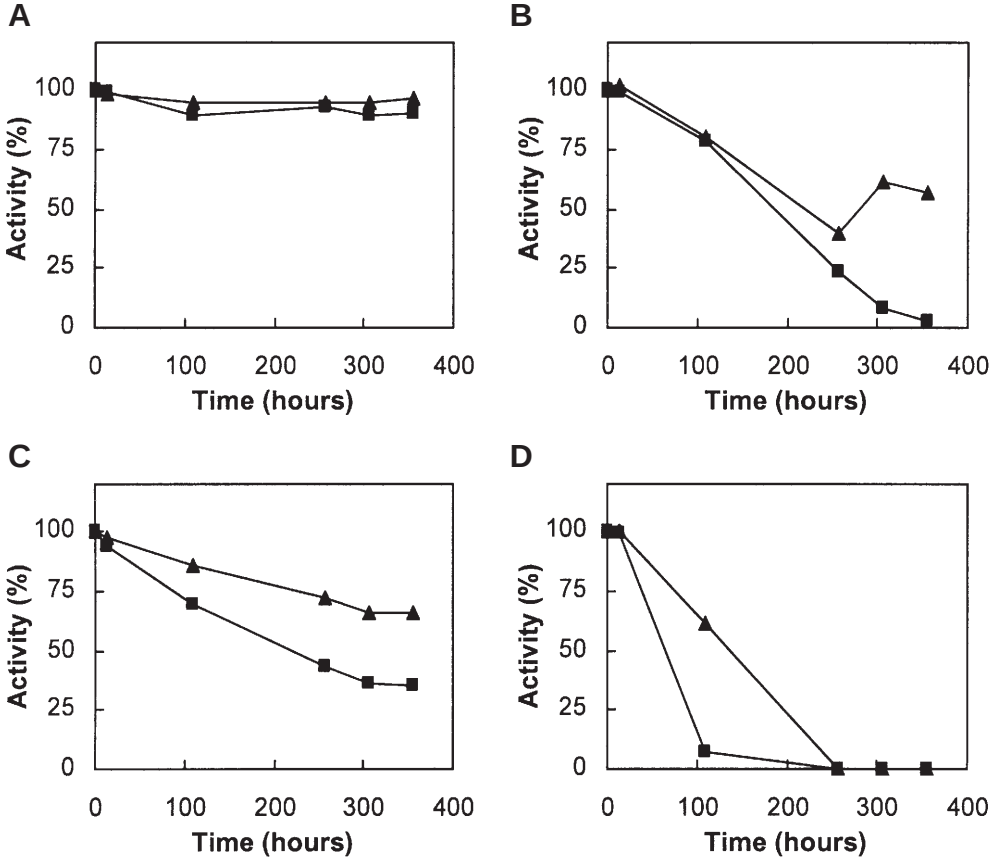

Figure 4. Stability of human ADH2 during prolonged storage. Pure ADH2 protein (0.25 mg/ml in PBS) was incubated at 6 and 37 °C in the presence or absence of saturating NAD^+. Activities were determined using the standard ethanol assay and plotted against time as percent of the activity for the first measurement. Triangles represent Ile308 and squares Val308. (*A*) 6 °C with NAD^+. (*B*) 37 °C with NAD^+. (*C*) 6 °C without NAD^+. (*D*) 37 °C without NAD^+.

Enzyme stability

Two methods were used to detect differences in thermostability between the two allelozymes, i.e. activity measurements and circular dichroism spectroscopy (fig. 3). The results of both analyses indicate that the Val308 substitution decreases thermostability slightly, compared to the Ile308 allelozyme. In addition, stability was assayed after storage in a cold room (6 °C) and at 37 °C in PBS. Again Ile308 was more stable than Val308 at both temperatures (fig. 4). Addition of coenzyme increased stability for both allelozymes, in accordance with previous studies [41, 42].

Modelling of human ADH2

The mouse ADH2 crystal structure [27] was used as template for making a structure model of the human form. The mouse and human ADH2 display 72% sequence identity, making homology modelling feasible. The overall structure of human ADH2 is consistent with the general ADH fold [43], which was expected due to the high sequence identity between the human and the mouse form. A two-residue insertion around position 305 located in a loop between two β strands makes the entrance to the active site narrower, compared to the mouse form. This together with a number of substitutions, e.g. non-conserved substrate-binding residues, may account for differences in substrate specificity or turn-over rate between the rodent and human enzymes. Position 308 is located at the end of one of the β strands in the subunit interface (fig. 5), adjacent to residues lining the substrate-binding pocket [27, 44]. The Ile308 to Val substitution decreases the solvation energy, calculated as the difference in transfer energy from octanol to water for the folded and the extended conformation, by 1.2 kcal/mol. In addition, the difference in stability between the dimer and the monomer in water is 0.6 kcal/mol higher for Ile308 than Val308 [38].

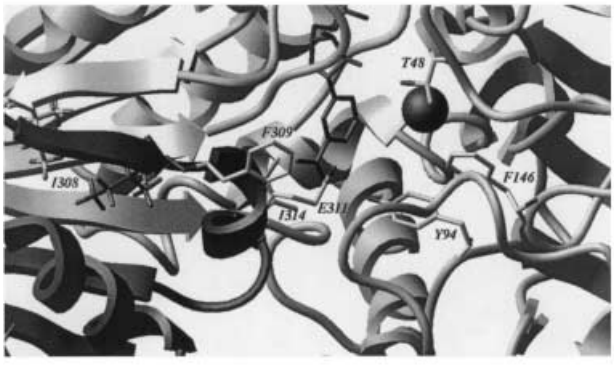

Figure 5. The substrate binding pocket of human ADH2. The mouse ADH2 three-dimensional structure was used as a template for making a structure model of the human form. The active site is depicted as a ribbon model with the two subunits in dark and light grey. The polymorphic residue Ile308 and a selection of amino acid residues are represented as stick models. Also shown are the catalytic zinc and the NAD molecule. Phe309 corresponds to Pro306 in the mouse orthologue where it lines the entrance of the substrate-binding pocket [27]. Residues Glu311 and Ile314 (corresponding to positions 306 and 309 in ADH1) have also been shown to be involved in substrate binding [44, 50]. Substituting Ile for Val at position 308 would remove a methylene group in the hydrophobic core of the protein which may, as a result, decrease protein stability and induce a shift in the fold around the active site. The figure was created using the ICM package (Molsoft LLC) [38].

Discussion

Polymorphisms in *ADH* genes

Polymorphism within the coding region at the human *ADH2* locus was indicated during cDNA cloning and peptide structure analysis. Four positions showed discrepancies between the directly determined and the deduced amino acid sequence, i.e. positions 308, 317, 373 and at the termination codon [28]. Polymorphisms are common in the human genome, on average one SNP per 1000–2000 bases, adding profound complexity when analysing the genetics behind human diseases [1]. For several of the *ADH* genes, polymorphisms have been reported, and recently, six of the human *ADH* genes, not including *ADH2*, were screened to create an archive of SNPs for drug-metabolising enzymes [45]. Variants of *ADH1B* and *ADH1C* are well characterised and their influence on the risk for alcoholism has been investigated [10–12, 14]. Previously, three polymorphic sites were detected in the *ADH2* promoter [7] and, in similarly *ADH3* and *ADH4* also display polymorphisms within their 5′-flanking regions [8, 9]. These polymorphisms may result in reduced transcriptional activity and, moreover, the polymorphism in *ADH4* has been proposed to be associated with Parkinson's disease [9]. Here we identified and characterised one of the four proposed SNPs in the coding region of the human *ADH2* gene, that results in an Ile to Val substitution at position 308. The Val308 variant, denoted *ADH2*2*, was the most common allele in a Swedish population, as deduced from screening roughly 100 individuals using SSCP analysis (table 1). In contrast, the mutation previously observed at the stop codon has never been detected in any DNA from other isolated cDNAs or genomic DNAs, and thus far the discrepancies at amino acid positions 317 and 373 have not been confirmed at the DNA level.

Substrate specificities

The substrate repertoires of ADHs are generally broad and overlapping. However, some reactions are class unique, e.g. S-hydroxymethylglutathinone oxidation and nitrosoglutathione reduction by ADH3 [41, 46] and, possibly, quinone reduction by ADH2 [25]. Several site-directed mutagenesis studies have been performed, which

have revealed certain residues as specificity determining [47, 48]. This implies that small changes in the substrate-binding pocket can cause major shifts in substrate specificity. Kinetic parameters were determined at pH 7.5 for the two allelozymes of ADH2 using a number of model substrates (table 2). Generally, the activities at pH 7.5 were 50- to 100-fold lower than previous studies performed at pH 10, corroborating the strong pH dependence of human ADH2 [21, 26]. In addition, turn-over numbers varied from about 2 to 10 min^{-1} indicating that coenzyme release might not be rate limiting in the overall reaction for certain substrates. Only small differences in kinetic data were monitored for the two allelozymes. The Val308 enzyme displayed a slightly higher K_m for all substrates tested, the most prominent being a two fold increase for octanol, when compared with Ile308 (table 2.). Altogether, this demonstrates that this substitution induces discrete changes in the catalytic properties of human ADH2, possibly due to subtle changes in the topography of the substrate-binding pocket that affects the binding affinities for some substrates, as in this case for octanol.

Structure/function relationships

Residue 308 is involved in subunit interactions and is positioned in juxtaposition with Ile319 in the opposite subunit. Substitutions in the subunit interface will conceivably affect the stability of the protein. Early liver purifications demonstrated that ADH2 could be a labile form [19, 42] and one can speculate whether this is associated with polymorphic variants of this enzyme. Notably, changes in stability between the two allelozymes were detected both during prolonged storage at 6 and 37 °C, as well as in thermostability measurements (cf. fig. 3, 4). This indicates that the ADH2 structure is more stabile with Ile than Val at position 308, possibly through weaker hydrophobic interactions for Val. Removal of a methylene group within the hydrophobic core of a protein typically decreases the stability by 1.5 ± 0.5 kcal/mol [49], which agrees with the calculated value for the Ile308Val substitution (1.2 kcal/mol) using the molecular model of human ADH2. Adjacent to position 308 in the folded protein (fig. 5) are several residues predicted to be involved in substrate binding, i.e. Phe309, Glu311 and Ile314 [27, 44, 50]. Substituting Ile for Val at position 308 would remove a methylene group which may, as a result, induce a shift in the fold around the active site (fig. 5).

In conclusion, the allelozymes described in this study display differences regarding protein stability, and minor changes in substrate specificity. Individual differences in alcohol (ethanol) metabolism have been widely discussed and the now characterised variants of ADH2 could contribute to individual differences in ethanol turnover as well as in the metabolism of other physiological substrates. In addition, since many of the earlier ADH preparations utilised purified human liver enzyme, lack of uniformity due to allelic variants could account for differences in data between studies.

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