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Identification and Characterization of Novel Mammalian Alcohol Dehydrogenases

by

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ABSTRACT

The vertebrate medium chain alcohol dehydrogenases are dimeric zinc metalloenzymes that catalyze the reversible oxidation/reduction of alcohols/aldehydes using NAD^+/NADH as coenzyme. In mammals, six classes of ADH have been defined (ADH1-ADH6) whereof five have been identified in humans. These enzymes are further divided into isozymes, and the presence of allelozymes, which have been detected for a number of gene *loci*, adds additional multiplicity to this enzyme system. Only a few ADHs have been assigned specific metabolic functions, e.g. glutathione dependent formaldehyde oxidation by ADH3, whereas other ADHs seem to contribute in the general defense against xenobiotics and endogenously formed alcohols and aldehydes. This thesis aims to provide insights into ADH structure and function, with emphasis on the identification and characterization of novel ADH forms.

A cDNA coding for a mouse ADH2 was cloned, which showed high structural similarity with the rat ortholog but lower identity with other species variants. Interestingly, the mouse and rat ADH2 forms share the same residue replacements, as compared to other ADHs, at positions important for catalysis and substrate specificity. Kinetic measurements displayed that rodent ADH2s are low activity enzymes, but the activity could be restored by substituting a unique Pro47 for His. Large substrate isotope effects for octanol oxidation showed that hydride transfer is rate-limiting for turnover. Altogether, these results indicate that the rodent enzymes form an ADH2 subgroup within the ADH family. The mechanisms of mammalian ADH2 enzymes were studied further and an ordered mechanism with coenzyme binding as the first reactant was suggested for both human and mouse ADH2. The enzymes display strong pH dependence with pK values for k_{cat} and k_{cat}/K_m several pH units higher than fot the ADH1 enzymes. Additional replacements at position 47 in the mouse enzyme (Pro47Ala and Pro47Gln) also increased oxidative activity and we propose that the rigid ring structure of Pro47 causes coenzyme binding that is unfavorable for efficient catalysis. This also shows that a His at position 47 does not act as a catalytic base in the deprotonation of the alcohol substrate. Experiments with deuterated substrates and transient kinetic measurements indicate that dissociation of coenzyme is rate-limiting for human ADH2 whereas hydride transfer is rate-limiting for all mouse ADH2 forms.

Polymorphisms in both the *ADH2* and the *ADH3* genes were found by single strand conformational polymorphism (SSCP) analysis. The *ADH2* polymorphism affects the coding region and results in an Ile308Val substitution. Homology modeling located position 308 in

the subunit interface of the molecule and in the vicinity of the active site pocket entrance. Characterization of the two allelozymes showed that the 308Val substitution decreases protein stability as compared to the 308Ile variant and that K_m-values, for a number of model substrates, were higher for the 308Val enzyme. The *ADH3* polymorphisms were detected within the 5'-flanking region. A reporter gene assay showed a significant reduction in promoter activity for a rare $C_{+9} \rightarrow T_{+9}$ transition, which could arise from decreased binding of nuclear proteins, as indicated by electrophoretic mobility shift assays.

ADH5 and ADH6 have been identified only at the nucleic acid level in human and deermouse, respectively. These enzymes share 67% structural identity and could possibly belong to the same diverged class. Previous reports indicated that human ADH5 differs from other ADHs in exon/intron organization, lacking the last exon and thus should be expressed in a truncated form. However, we were able to identify full-length ADH5 transcripts and the presence of a ninth exon was also confirmed by sequencing of genomic DNA. Northern blot analyses established the full-length variant as the major transcript with the strongest signal from adult liver. We conclude that the *ADH5* gene contains a composite internal/terminal exon, which can be differentially processed as a result of competition between polyadenylation and splicing. In addition, a new ADH was identified in rat which showed 78% identity with the deermouse ADH6 variant. No soluble ADH5 or ADH6 protein could be recombinantly expressed in *E. coli*, but using a protocol of refolding insoluble proteins from inclusion bodies, milligram amounts of ADH-GST fusion proteins of both human ADH5 and rat ADH6 could be isolated. The purified proteins were not stable without the GST tag and no activity was observed with a number substrates, which could be a result of an incorrect fold of the ADH part of the fusion protein. *In vitro* translation experiments and expression of ADH-GFP fusion protein in COS cells indicate that soluble ADH5 and ADH6 protein could be produced in mammalian cells.

CONTENTS

LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, printed in the appendix, which will be referred to in the text by their roman numerals:

I Svensson, S*., Strömberg, P*., and Höög, J.-O. (1999) A novel subtype of class II alcohol dehydrogenase in rodents - Unique Pro^{47} and Ser^{182} modulates hydride transfer in the mouse enzyme. *J. Biol. Chem.* **274**(42), 29712-29719

II Strömberg, P., Svensson, S., Berst, K., Plapp, B.V., and Höög, J.-O. (2002) Enzymatic properties of mammalian alcohol dehydrogenase 2 (ADH2). *Manuscript*

III Strömberg, P., Svensson, S., Hedberg, J.J., Nordling, E., and Höög, J.-O. (2001) Identification and characterisation of two allelic forms of human alcohol dehydrogenase 2. *Cell. Mol. Life Sci.* **59**, 552-559

IV Hedberg, J.J., Backlund, M., Strömberg, P., Lönn, S., Dahl, M.-L., Ingelman-Sundberg, M., and Höög, J.-O. (2001) Functional polymorphism in the alcohol dehydrogenase 3 (*ADH3*) promoter. *Pharmacogenetics* **11**, 815-824

V Strömberg, P., and Höög, J.-O. (2000) Human class V alcohol dehydrogenase (ADH5) – A complex transcription unit generates C-terminal multiplicity. *Biochem. Biophys. Res. Commun.* **278**, 544-549

VI Strömberg P., Hedberg J.J., Brandt M., Hirschberg D., Estonius M., and Höög J.-O. (2002) Analyses of mammalian ADH5 and ADH6. *Manuscript*

* Contributed equally to the results of this article

ABBREVIATIONS

ADH	alcohol dehydrogenase
DNA	deoxyribonucleic acid
EST	expressed sequence tag
HLADH	horse liver alcohol dehydrogenase
GFP	green fluorescent protein
GSH/GSSG	glutathione (reduced/oxidized)
GSNO	*S*-nitrosoglutathione
GST	glutathione S-transferase
12-HDA	12-hydroxydodecanoic acid
HMGSH	*S*-hydroxymethylglutathione
MDR	medium-chain dehydrogenases/reductases
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate(oxidized/reduced)
NO	nitric oxide
ORF	open reading frame
PCR	polymerase chain reaction
PD	Parkinson´s disease
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
SDR	short-chain dehydrogenases/reductases
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
UTR	untranslated region

Three and one letter codes for amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

INTRODUCTION

Historical Perspective

With no disrespect to the ancient Greek or other great civilisations in the past, I would like to start this short historical résumé of alcohol dehydrogenase (ADH) research in the nineteenth century when scientist began to comprehend the action of biological catalysts. This phenomenon was mainly studied on the microbial fermentation of carbohydrates to alcohol and carbon dioxide and what was to become the term *enzyme* was initially called *ferment* in accordance with this process. Jöns Jakob Berzelius, one of the founders of the Karolinska Institutet, coined the words *catalysis* and *protein* in the 1830s (Berzelius, 1836). However, the fact that enzymes were proteins was not proven until the late 1920s when J. B. Sumner isolated the enzyme urease in pure crystalline form and suggested, contrary to the prevailing opinion, that the molecule was a protein (Sumner, 1926). Models for how enzymes act were proposed early. Emil Fischer, in 1890, used the metaphor of "lock and key" to describe how enzyme and substrate combine (Fischer, 1894), and a simple and still very useful mathematical model for the kinetics of enzyme catalysis was proposed by Michaelis and Menten in 1913 (Michaelis and Menten, 1913).

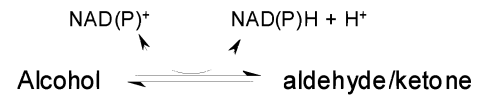
The first ADH to be isolated was the form of *Saccharomyces cereviciae* (Bakers Yeast) in 1937 (Negelein and Wulff, 1937), and about ten years later the first liver ADH was isolated from horse (HLADH, Bonnichsen and Wassén, 1948). These enzymes can be regarded as model enzymes in biochemical research and thorough kinetic studies were performed to deduce the mechanism of the ADH reaction (see below, Theorell and Chance, 1951; Wratten and Cleland, 1963). Both the primary and tertiary structures of HLADH were elucidated early on (Brändén *et al.*, 1973; Eklund *et al.*, 1976; Jörnvall, 1970b) and a corresponding human liver form was isolated in 1964 (von Wartburg *et al.*, 1964). Since the first ADH was defined in yeast, numerous ADH enzymes have been isolated and characterized from an impressive variety of organisms and species. Early methods for detecting novel enzymes included starch gel electrophoresis followed by activity staining, purification and direct protein sequencing. Moreover, optimization of purification methods as well as developments of specific affinity media enabled yet additional forms of ADHs to be isolated (Andersson *et al.*, 1974; Lange and Vallee, 1976). However, with the advances in DNA technology and the recent explorations of complete genomes, many more ADH forms have now been identified at the

nucleic acid level and characterizations have been made with recombinantly expressed proteins.

In 1955 Hugo Theorell, professor at what was to become this department, received the Nobel Prize in Physiology or Medicine for his discoveries concerning the nature and mode of action of oxidation enzymes.

Multiplicity of ADH

Nature has provided cells with mechanisms to allow genes to be duplicated, modified and recombined during the course of evolution. Furthermore, continuously accumulated point-mutations lead to gradually changed functions of individual genes and once a functional motif has evolved it may be incorporated into pre-existing structures or duplicated to produce additional related proteins. This has resulted in the occurrence of large families of related proteins which have evolved as a result of duplications and modifications from single ancestral genes. Different levels of multiplicity within these families can be related to certain genetic events during evolution.



Scheme I. The ADH reaction

The NAD(P)-dependent interconversion of alcohols and aldehydes/ketones catalysed by alcohol dehydrogenase (Alcohol:NAD oxidoreductase, EC 1.1.1.1), is essential in both prokaryotic and eukaryotic systems (scheme I). These alcohol dehydrogenases comprise a complex system of different enzymes and enzyme families. Following the line leading down to the mammalian ADHs, covered in this thesis, at least four levels of multiplicity can be traced. The basis for the divergence at each level appears to correspond mainly to a number of gene duplications with the exception of the first level where larger rearrangements and recombinatory events seem to have occured. The first level of ADH multiplicity constitutes a number of very distantly related enzyme systems, principally the short-chain dehydrogenases/reductases family (SDR), the medium-chain dehydrogenases/reductases family (MDR) and the "iron-activated" alcohol dehydrogenases (Jörnvall *et al*., 1987; Jörnvall *et al*., 1995; Jörnvall *et al*., 2001; Scopes, 1983). These enzyme families display completely different catalytic solutions and reaction mechanisms. SDR forms are numerous in all

kingdoms and e.g. the main alcohol dehydrogenase form of *Drosophila* belongs to this family (Jörnvall *et al.*, 1995; Jörnvall *et al.*, 1999; Schwartz and Jörnvall, 1976; Thatcher, 1980). Iron-activated alcohol dehydrogenases have been identified in different bacteria and yeast but these are not as well characterized as the SDR and MDR forms (Reid and Fewson, 1994; Scopes, 1983). The vertebrate line of ADH belongs to the MDR protein family (Jörnvall *et al.*, 1987; Jörnvall *et al.*, 2001). However, MDRs have also been found in bacteria, plants, insects and yeast (Danielsson *et al.*, 1994a; Jörnvall *et al.*, 1987; Nordling *et al.*, 2002a; Nordling *et al.*, 2002b). Since this thesis focuses on mammalian alcohol dehydrogenase, the term ADH from now on refers to the zinc-containing MDR forms. A second level of multiplicity is traced in the dimer/tetramer divergence and constitutes the different enzyme types of the MDR line, which in addition to the ADHs are e.g. sorbitol dehydrogenase, threonine dehydrogenase, xylitol dehydrogenase and ζ-crystallin. The MDR ADHs are then further divided into different enzymes or classes beginning with the ADH3/non-ADH3 duplication, representing the third level of multiplicity, and the last level constitutes different isozymes within a class. Moreover, additional complexity arises from the presence of polymorphisms in certain ADH genes giving rise to allelozymes or differences in gene regulation and expression (se below).

The vertebrate ADH family

As mentioned above, the vertebrate ADHs belong to the MDR protein family. These enzymes are characterized by the length of the polypeptide chain, which is 350 to 400 amino acid residues as compared to the SDR chain-length of approximately 250. Vertebrate ADHs are dimeric zinc metalloenzymes, for which at least eight structurally distinct classes can be defined (Duester *et al.*, 1999; Peralba *et al.*, 1999). Initially this division was made according to chemical and functional features; e.g. electrophoretic properties, ability to form heterodimers, substrate repertoires and sensitivity to inhibition by pyrazole derivates (Vallee and Bazzone, 1983). At present, since structural information is available, structural identity and evolutionary relationships are the main criteria for classification, where the different enzyme classes fall within separate branches when phylogenies are examined (fig. 1).

Mammalian ADH genes seem to be clustered on single chromosomes as a result of repeated tandem gene duplications (Edenberg, 2000). The human genes localize on chromosome 4 q21-25 in the order (from the centromere) *ADH4* → *ADH1C* → *ADH1B* → *ADH1A* → *ADH5* → *ADH2* → *ADH3*, and the mouse genes are located on chromosome 3 in a region syntenic to human chromosome 4 (Edenberg, 2000; Holmes *et al.*, 1982; Holmes *et*

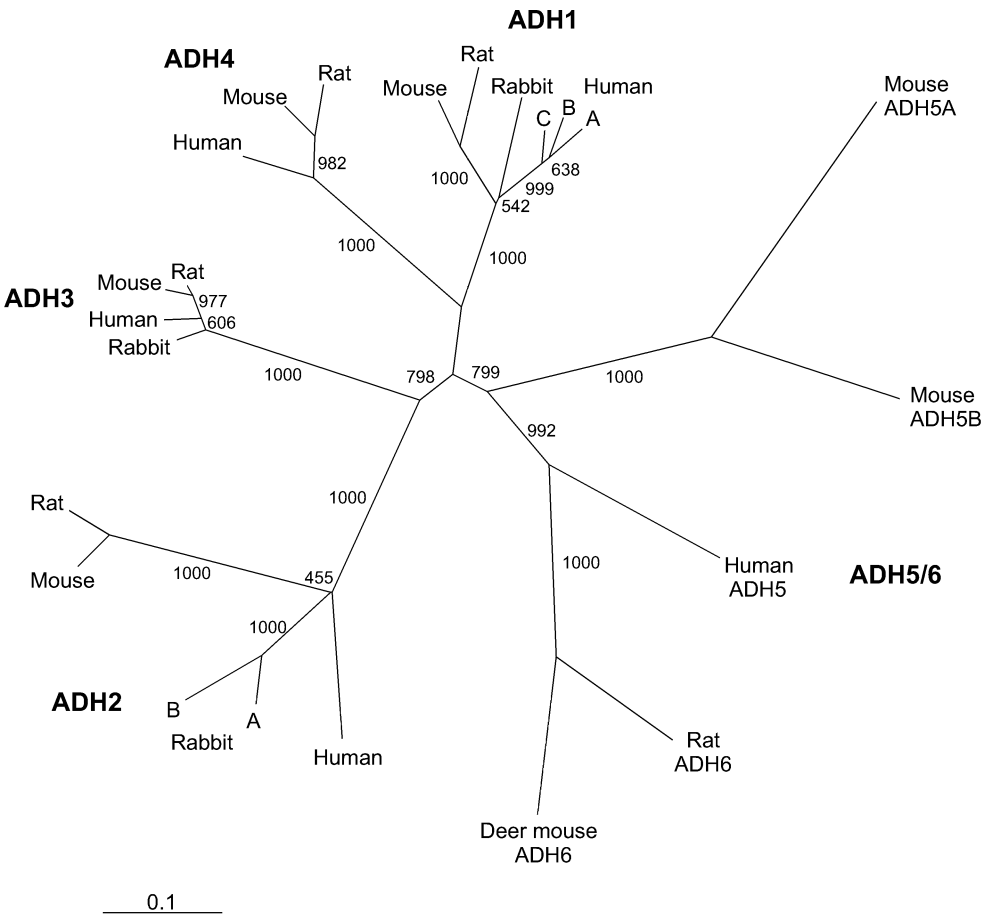


Figure 1. Unrooted phylogenetic tree relating different ADH forms from a number of mammalian species. Line lengths are proportional to separation distances. The initial alignment was made using Clustal W (shown in fig. 5) with parameters set to exclude gaps and phylogenies were investigated using the neighbor-joining method. The tree was created with TreeView and numbers on branches represent the result of bootstrap analysis with 1000 replicates. Line length are proportional to separation distances.

al., 1986; Nordling *et al*., 2002a; Nordling *et al*., 2002b; Szalai *et al*., 2002; Yasunami *et al*., 1990). The gene structures are very conserved with regard to intron/exon boundaries and consist of 9 exons and 8 introns (Edenberg, 2000; cf paper V). It should be pointed out that the terminology of the ADH system is still a matter of debate and that the recently revised nomenclature presented by Duester *et al*. (1999), which is used in this thesis, is not universal. Consequently, in the reference list and in original articles, the names of ADH enzymes, subunits and genes will differ depending on which nomenclature is used (Duester *et al*., 1999; Jörnvall and Höög, 1995). Table I lists the classical and "new" nomenclature for comparison.

Table I. Nomenclature of the Human Alcohol Dehydrogenases[a]

	New Nomenclature[b]		Old Nomenclature[c]	
Class	Protein	Gene	Protein	Gene
I	ADH1A	*ADH1A*	α	*ADH1*
	ADH1B	*ADH1B*	β	*ADH2*
	ADH1C	*ADH1C*	γ	*ADH3*
II	ADH2	*ADH2*	π	*ADH4*
III	ADH3	*ADH3*	χ	*ADH5*
IV	ADH4	*ADH4*	σ or μ	*ADH6 (ADH7)*[d]
V	-	*ADH5*	-	*ADH7 (ADH6)*[d]

[a] It is recommended that the new nomenclature system also is applied for other species where the original terminology is often confusing. E.g. the mouse proteins were originally denoted A_2, B_2 and C_2 for ADH1, ADH3 and ADH4, respectively.
[b] Duester *et al.* (1999).
[c] Jörnvall and Höög (1995).
[d] According to the original publications describing the order of discovery.

ADH1 enzymes

ADH1 or class I ADH is the classical ethanol active liver enzyme which is abundant in all vertebrate livers. Human ADH1 is comprised of homo- and heterodimeric forms of three isozymic subunits: ADH1A-C (previously denoted α, β and γ, Hempel *et al.*, 1985; Smith *et al.*, 1971). The subunits are encoded by the genes *ADH1A-C* (*ADH1-3* in the old nomenclature) which have all been structurally characterized (Carr *et al.*, 1989; Duester *et al.*, 1986; Matsuo *et al.*, 1989; Matsuo and Yokoyama, 1989; Stewart *et al.*, 1990). The ADH1 enzymes from horse and human have been extensively studied over the last 50 years (Bonnichsen and Wassén, 1948; Duester *et al.*, 1984; Höög *et al.*, 1986; Jörnvall, 1970a; Park and Plapp, 1991; von Bahr-Lindström *et al.*, 1986; von Wartburg *et al.*, 1964). However, ADH1 enzymes/genes/cDNAs have also been isolated and characterized from numerous other species: mouse (Edenberg *et al.*, 1985), rat (Crabb and Edenberg, 1986), deermouse (Zheng *et al.*, 1993), rabbit (Höög *et al.*, 1993), gopher (Bradley *et al.*, 1993), rhesus monkey (Light *et al.*, 1992), baboon (Trezise *et al.*, 1989), chicken (Estonius *et al.*, 1990), quail (Kaiser *et al.*, 1990), ostrich (Estonius *et al.*, 1994a), kiwi (Hjelmqvist *et al.*, 1995b), alligator (Persson *et al.*, 1993), cobra (Shafqat *et al.*, 1996), lizard (Hjelmqvist *et al.*, 1996), frog (Cederlund *et al.*, 1991; Hoffmann *et al.*, 1998) and codfish (Danielsson *et al.*, 1992). The presence of ADH1 enzymes in all these vertebrates together with lack of ADH1 in invertebrates pinpoints the time of expansion of the vertebrate ADH family, i.e. the ADH3/ADH1 duplication, to early

vertebrate radiation approximately 500 million years ago (Canestro *et al.*, 2002; Canestro *et al.*, 2000; Jörnvall *et al.*, 2001).

ADH1 constitutes the major activity for metabolizing ingested ethanol (Crabb *et al.*, 1987). However, other enzyme systems also contribute, e.g. microsomal cytochrome P450 (CYP2E1) and to a lesser extent peroxisomal catalase (Crabb *et al.*, 1987). K_m-values for ADH1-catalyzed ethanol oxidation vary greatly between the different isozymes and also between different alleles (see below under the polymorphism section) but are generally lower than for other ADH classes. The ADH1B1 (β_1) variant displays a K_m of 50 μM, which is the lowest within the mammalian ADH family (Hurley *et al.*, 1990). ADH1 is further characterized by sensitivity to 4-methylpyrazole inhibition (Edenberg and Bosron, 1997; Wagner *et al.*, 1983; von Wartburg *et al.*, 1964). The substrate specificity of ADH1 is broad and besides the role in ethanol metabolism, ADH1 has been implicated in the conversions of many other physiological compounds. Activity for 3β-hydroxy 5β-steroids was detected early on for one of the horse isozymes (Waller *et al.*, 1965), and this and other sterol activities have in subsequent studies been shown for other ADH1 enzymes (Björkhem *et al.*, 1973; Cronholm *et al.*, 1975; Frey and Vallee, 1980; Marschall *et al.*, 2000; McEvily *et al.*, 1988; Okuda and Okuda, 1983). Reduction of aldehydes formed in lipid peroxidation, such as 4-hydroxynonenal (Boleda *et al.*, 1993; Sellin *et al.*, 1991), and oxidation of ω-hydroxylated fatty acids from fatty acid and eicosanoid metabolism (Björkhem *et al.*, 1973; Boleda *et al.*, 1993; Wagner *et al.*, 1983), can also be catalyzed by ADH1. Moreover, ADH1 enzymes have been shown to catalyze the conversion of intermediates from dopamine-, noradrenaline- and serotonin metabolism (Consalvi *et al.*, 1986; Mårdh *et al.*, 1985; Mårdh and Vallee, 1986; Svensson *et al.*, 1999). Finally, retinoids are substrates for ADH1 (Yang *et al.*, 1994). The role of ADH1 in ethanol as well as retinoid metabolism has further been demonstrated in ADH-deficient "knock-out" mice (Deltour *et al.*, 1999b; Molotkov *et al.*, 2002a; Molotkov and Duester, 2002; Molotkov *et al.*, 2002c). These mice are viable but extra sensitive for ethanol and retinol intoxication. In addition, a naturally occurring strain of deermouse with a deleted *adh1* gene has been characterized (Burnett and Felder, 1978a; Burnett and Felder, 1978b; Zheng *et al.*, 1993).

ADH represents about 3% of the soluble protein in human liver, of which ADH1 is the most abundant form (Edenberg and Bosron, 1997). However, ADH1 is also present in significant amounts in other organs. In rodents, ADH1 protein has been detected in liver, kidney, adrenals, intestine, lung, testis and uterus (Boleda *et al.*, 1989; Holmes, 1978; Julià *et*

al., 1987). Northern blot analyses of ADH1 mRNA levels in human tissues gave analogous results (Estonius *et al*., 1996). Human ADH1 is also developmentally regulated with ADH1A being the most abundant form in fetal liver (Ikuta and Yoshida, 1986; Smith *et al*., 1971; Smith *et al*., 1972). The ADH1 genes contain a number of cis-acting elements in the promoter region and hormones, e.g. androgens, glucocorticoids and growth hormone have been shown to affect gene expression (Edenberg *et al*., 1997). Furthermore, retinoic acid response elements have been found in some ADH1 genes and the levels of rat ADH1 has been shown to fluctuate in response to infusions of ethanol (Badger *et al*., 2000; Edenberg *et al*., 1997).

ADH2 enzymes

Electrophoresis with subsequent activity staining revealed that human livers contain an additional more anodic ethanol dehydrogenase activity (Li and Magnes, 1975). Later this form, denoted π or class II ADH, was isolated and characterized as a variant of liver ADH exhibiting a high K_m for ethanol and insensitivity to pyrazole inhibition (Bosron *et al*., 1977; Bosron *et al*., 1979; Ditlow *et al*., 1984; Li *et al*., 1977). It was proposed that ADH2 would have a great impact on ethanol metabolism at intoxicating concentrations, which could implicate ADH2 as a determinant of alcoholism (Li *et al*., 1977). Notably, differences in stability were also observed from different preparations of enzyme (Bosron *et al*., 1977; Bosron *et al*., 1979; Ditlow *et al*., 1984; Li and Magnes, 1975; cf paper III). ADH2 primary structures have thus far been characterized from human (Höög *et al*., 1987), rat (Höög, 1995), rabbit (two isozymic forms, Svensson *et al*., 1998), ostrich (Hjelmqvist *et al*., 1995a) and mouse (paper I), and the identification of species variants have established ADH2 as a structurally very divergent form of ADH. The divergence of ADH2 is also seen in a functional perspective where activities and substrate specificities vary between different ADH2 forms (Hjelmqvist *et al*., 1995a; Höög and Svensson, 1997; Höög *et al*., 1999; Svensson *et al*., 1998; paper I; paper II). Generally, ADH2 shows a preference for hydrophobic alcohols and aldehydes (Deetz *et al*., 1984; Ditlow *et al*., 1984). Physiologically relevant substrates include: retinoids (Popescu and Napoli, 2000; Yang *et al*., 1994), 4-hydroxyalkenals (Sellin *et al*., 1991), ω-hydroxy fatty acids (Ditlow *et al*., 1984),

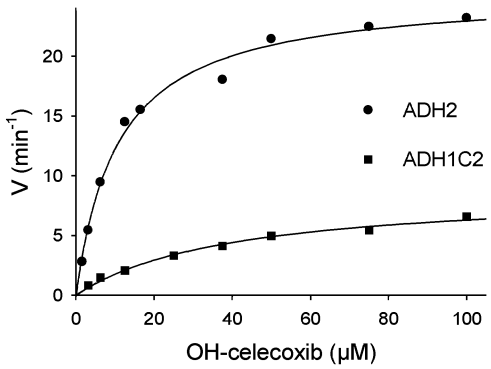


Figure 2. Oxidation of OH-celecoxib by human ADH1C2 and ADH2. Initial velocity measurements were conducted spectrophotometrically at pH 10, with fixed concentration of NAD^+ (2.4 mM) and varied concentrations of substrate. Data were fitted to the Michaelis-Menten equation using the computer program Sigma Plot.

intermediates in the metabolism of noradrenaline and serotonin (Consalvi *et al.*, 1986; Mårdh *et al.*, 1986; Svensson *et al.*, 1999) and recently, ADH2 was also implicated in the metabolism of the cyclooxygenase-2 inhibitor celecoxib (fig. 2, Sandberg *et al.*, 2002). In addition, reduction of *p*-benzoquinone and *p*-benzoquinoneimine seems to be ADH2 specific within the ADH family, but the physiological relevance of these reactions is not clear (Maskos and Winston, 1994; Svensson *et al.*, 1998; Paper I). ADH2 expression is restricted mainly to the liver but small amounts can also be detected in e.g. intestine and skin (Cheung *et al.*, 1999; Estonius *et al.*, 1993; Estonius *et al.*, 1996; Li *et al.*, 1977). The human *ADH2* gene, including the promoter region, has been characterized and the high expression in liver could be explained by stimulatory binding of C/EBP proteins, which are abundant in adult liver, to cis-acting elements within the promoter (Li and Edenberg, 1998; von Bahr-Lindström *et al.*, 1991).

ADH3 - Glutathione-dependent formaldehyde dehydrogenase

New electrophoretic forms of ADH, which were initially overlooked in ethanol activity screens, were detected by their ability to readily stain with pentanol (Parés and Vallee, 1981). These variants, jointly denominated class III or χ-ADH (now ADH3), were isolated as one homogenous form which exhibited unique kinetic properties and substrate specificity (Kaiser *et al.*, 1991; Parés and Vallee, 1981; Wagner *et al.*, 1984). Later it was shown that ADH3 is identical to glutathione (GSH)-dependent formaldehyde dehydrogenase in both rat and human (Holmquist and Vallee, 1991; Koivusalo *et al.*, 1989). GSH-dependent formaldehyde dehydrogenase, catalyzing the oxidation of formaldehyde to formate in the presence of GSH, was isolated already in 1955 from beef and chicken (Strittmatter and Ball, 1955), and the human form was purified some twenty years later (Uotila and Koivusalo, 1974a). The enzyme

is not active with formaldehyde *per se.* However, *S*-hydroxymethyl-glutathione (HMGSH), a thiohemiacetal spontaneously formed from formaldehyde and GSH, is efficiently oxidized by ADH3 to the product *S*-formylglutathione (Uotila and Koivusalo, 1974a), which is further hydrolyzed to formate and GSH by a second enzyme, *S*-formylglutathione hydrolase (identical to esterase D, Uotila and Koivusalo, 1974b). Hence, the overall reaction proceeds without no net consumption of GSH.

Activities for the oxidation of formaldehyde coupled to thiols have been found in all organisms examined including prokaryotes, yeasts and plants, and with the exception of some gram-positive bacteria where formaldehyde is conjugated to mycothiol (Norin *et al.*, 1997), GSH and ADH3 are the thiol and enzyme responsible, respectively (Jörnvall *et al.*, 2000). Primary structures of vertebrate ADH3 have been determined from human (Giri *et al.*, 1989a; Kaiser *et al.*, 1991; Sharma *et al.*, 1989), mouse (Hur *et al.*, 1992), rat (Julià *et al.*, 1988), horse (Kaiser *et al.*, 1989), rabbit (Svensson *et al.*, 1998), chicken (partial, Kedishvili *et al.*, 1997b), lizard (Hjelmqvist *et al.*, 1995c), codfish (Danielsson *et al.*, 1996), shark (Shafqat et al., unpublished) and hagfish (Danielsson *et al.*, 1994b). These structures and the functional properties of ADH3 show that this enzyme can be regarded as a constant form of ADH, as characterized by the ubiquitous formaldehyde activity, with a high degree of conservation among structures and low evolutionary speed (Danielsson and Jörnvall, 1992; Jörnvall *et al.*, 2001; Kaiser *et al.*, 1989). Evidently, ADH3 represents the ancient form of ADH from which all other vertebrate ADHs have evolved (Danielsson and Jörnvall, 1992; Jörnvall *et al.*, 2001).

In addition to the GSH-dependent formaldehyde dehydrogenase activity of ADH3, it was recently shown that reduction of *S*-nitrosoglutathione (GSNO), the conjugation product of nitric oxide (NO) and GSH, could be catalyzed by rat ADH3 with high efficiency (Jensen *et al.*, 1998). This has later been shown for the human, mouse, plant, yeast and *E. coli* ADH3 as well (Liu *et al.*, 2001; Sakamoto *et al.*, 2002; Hedberg *et al.*, unpublished). ADH3 has also been shown to be active with long-chain primary alcohols and aldehydes including 20-hydroxy-leukotriene B_4 and other ω-hydroxy fatty acids, however with much less efficiency than HMGSH and GSNO (Giri *et al.*, 1989b; Gotoh *et al.*, 1989; Gotoh *et al.*, 1990; Parés and Vallee, 1981; Sanghani *et al.*, 2000; Wagner *et al.*, 1984). Short-chain alcohol oxidation can also be achieved by activation by fatty acids (Moulis *et al.*, 1991). Notably, although ADH3 has been shown to have no or very low activity for retinoids (Yang *et al.*, 1994), studies on *Adh3* -/- mice have implicated a function of this enzyme in retinoid signalling under physiological conditions as well as for vitamin A toxicity (Molotkov *et al.*, 2002b; Molotkov *et al.*, 2002c).

ADH3 seems to be constitutively expressed since activity, protein or transcripts have been detected in all tissues examined, including brain where it is probably the only ADH expressed (Adinolfi *et al.*, 1984; Duley *et al.*, 1985; Estonius *et al.*, 1993; Estonius *et al.*, 1996; Julià *et al.*, 1987). The *ADH3* gene has been characterized and the promoter region differs from other ADH genes in e.g. absence of a TATA box and presence of a GpC island, which are common features of housekeeping genes (Edenberg, 2000; Hur and Edenberg, 1992; Hur and Edenberg, 1995). However, the promoter also displays tissue specific cis-acting elements which is in line with differential expression of ADH3 in different cell types and tissues (Edenberg, 2000; Estonius *et al.*, 1993; Estonius *et al.*, 1996; Hedberg *et al.*, 2000; Hur and Edenberg, 1995; Uotila and Koivusalo, 1997).

ADH4 enzymes

A novel human ADH form with specific kinetic characteristics was isolated from stomach in two different studies and named μ-ADH or σ-ADH (Moreno and Parés, 1991; Yin *et al.*, 1990). Stomach ADH forms had been isolated earlier in rodents but they were initially mistaken for being of the class II type (Algar *et al.*, 1983; Boleda *et al.*, 1989; Julià *et al.*, 1987). However, partial structural data from the rat, and later also for the human, confirmed that this enzyme belonged to a new fourth class of mammalian ADH (Parés *et al.*, 1990; Stone *et al.*, 1993b). At present, primary structures for ADH4 have been determined from human (Farrés *et al.*, 1994; Satre *et al.*, 1994; Yokoyama *et al.*, 1994a), rat (Crosas *et al.*, 2000; Parés *et al.*, 1994) and mouse (Zgombic-Knight *et al.*, 1995a). In addition, variants with ADH4 type properties have been identified in two species of frog, *Rana perezi* and *Xenopus laevis* (Hoffmann *et al.*, 1998; Peralba *et al.*, 1999), but based on the level of structural identity, it is dubious if they represent orthologs of the mammalian ADH4 form (see below). The mammalian ADH4 structures display intermediate evolutionary speed as compared to ADH3 and ADH1.

Functionally, ADH4 is characterized by a high K_m and k_{cat} for ethanol and being less sensitive to 4-methylpyrazole inhibition than ADH1 (Algar *et al.*, 1983; Julià *et al.*, 1987; Moreno and Parés, 1991; Yin *et al.*, 1990). Furthermore, 4-hydroxyalkenals and ω-hydroxy fatty acids are readily catalyzed by ADH4 (Allali-Hassani *et al.*, 1998; Boleda *et al.*, 1993; Moreno and Parés, 1991). Of particular interest is that ADH4 has the highest activity for retinoids among the mammalian ADHs (Boleda *et al.*, 1993; Chou *et al.*, 2002; Kedishvili *et al.*, 1995; Yang *et al.*, 1994). ADH4 is expressed in epithelial and endothelial tissues (Ang *et al.*, 1996b; Boleda *et al.*, 1989; Zgombic-Knight *et al.*, 1995a) but not in liver (Moreno and

Parés, 1991; Yokoyama *et al.*, 1995; Zgombic-Knight *et al.*, 1995b). The lack of ADH4 in liver could possibly be attributed to an unexpected negative effect by C/EBP transcription factors on cis-acting elements in the promoter region of the *ADH4* gene (Edenberg, 2000; Kotagiri and Edenberg, 1998; Satre *et al.*, 1994; Yokoyama *et al.*, 1995; Yokoyama *et al.*, 1994b). Moreover, the expression of ADH4 has been shown to coincide with retinoid target tissues and retinoic acid production at different developmental stages during embyogenesis, further implicating the role of ADHs in the regulation of this morphogen (Ang *et al.*, 1996a; Ang *et al.*, 1996b; Ang and Duester, 1997; Haselbeck *et al.*, 1997; Haselbeck and Duester, 1997). Experiments on ADH deficient mice suggest that ADH4 and ADH3 are important for retinol utilization and during vitamin A deficiency, whereas ADH1 and to a lesser extent ADH3 provide protection from vitamin A toxicity (Deltour *et al.*, 1999a; Molotkov *et al.*, 2002a; Molotkov *et al.*, 2002c). Still, it should be noted that the role of ADHs in retinoid signaling and metabolism is currently under debate, and that there are conflicting results on the enzymatic activation of retinol (Duester, 2000; Kedishvili *et al.*, 1998; Napoli, 1999a; Napoli, 1999b). The high activity for ethanol in combination with the abundance of ADH4 in stomach have implicated this enzyme also in the first-pass metabolism of ingested alcohol in humans (Frezza *et al.*, 1990; Haber *et al.*, 1996; Moreno and Parés, 1991; Seitz *et al.*, 1993; Yin *et al.*, 1990), but this seems not to be a conserved function since rat ADH4 displays different ethanol kinetics (Crosas *et al.*, 2000).

ADHs of higher classes

Class V ADH or ADH5 has thus far only been detected at gene- and mRNA level in humans and no other species variant has been isolated (Yasunami *et al.*, 1991; paper V). Recombinant expression in *E. coli* yielded no soluble protein but limited kinetic data are available from *in vitro* translated ADH5 (Chen and Yoshida, 1991). Based on these data it was proposed that the previously characterized stomach ADH (ADH4) was the product of the *ADH5* gene, which was eventually proven wrong when the protein and nucleic acid sequences of ADH4 were determined (Chen and Yoshida, 1991; Farrés *et al.*, 1994; Satre *et al.*, 1994; Stone *et al.*, 1993b; Yokoyama *et al.*, 1994a). Notably, the *ADH5* gene was initially proposed to deviate from the highly conserved intron/exon organization of ADH genes in lacking the ninth terminal exon (Yasunami *et al.*, 1991). However, the presence of this exon has now been shown (paper V). ADH5 transcripts have been detected preferentially in liver, both adult and fetal, but low levels were also observed in intestine and kidney (Estonius *et al.*, 1996; Yasunami *et al.*, 1991; paper V). Support for the limited expression pattern of ADH5 is

further seen from the identification of tissue specific regulatory elements within the promoter region of the *ADH5* gene (Yasunami *et al.*, 1991; Zhi *et al.*, 2000). Recently, two mouse genes were identified and proposed to be of ADH5 origin (Szalai *et al.*, 2002), but low positional identities between these mouse genes and human ADH5 indicate that they are not orthologs (paper VI).

During the characterization of the genomic basis for the ADH-negative deermouse (Burnett and Felder, 1978a; Burnett and Felder, 1980), an additional cDNA was identified encoding a novel class of ADH, presently called ADH6 (Zheng *et al.*, 1993). No ADH6 protein has as yet been detected or characterized but correlating ADH6 transcription with activity-stained starch gels, indicates a broad substrate specificity for this enzyme (Zheng *et al.*, 1993). Furthermore, an orthologous form of ADH6 has been identified in rat (Höög and Brandt, 1995; Höög *et al.*, 2001; paper VI). The ADH6 sequences show the highest resemblance to human ADH5 but since the level of identity is relatively low (65-67%), these forms have been divided into separate classes (Duester *et al.*, 1999). However, the positional identity between the two rodent ADH6 forms is also low, as compared to other classes of ADH, which suggests that the ADH5 and ADH6 forms could in fact be members of the same very diverged class (Höög and Brandt, 1995; Höög *et al.*, 2001) (paper VI).

Two more vertebrate classes, ADH7 and ADH8, have been defined. ADH7 has only been detected in avian species (Kedishvili *et al.*, 1997a) (Hjelmqvist *et al.*, unpublished). This enzyme is characterized as an ethanol active form with additional activity for retinoids and steroids, indicating physiological roles in the metabolism of these compounds (Kedishvili *et al.*, 1997a). ADH8 has thus far only been isolated from frog (*Rana perezi*) and it is the sole vertebrate ADH with $NADP^+$/NADPH as preferred coenzyme (Peralba *et al.*, 1999). This enzyme is highly aldehyde active and probably functions as a reductase, which is further supported by the favorable ratio of NADPH/$NADP^+$ in the cell for the reductive direction (Peralba *et al.*, 1999). Possible physiological substrates are different retinal species, indicating a role in the storage of vitamin A derived from provitamin A sources (Duester, 2000; Peralba *et al.*, 1999).

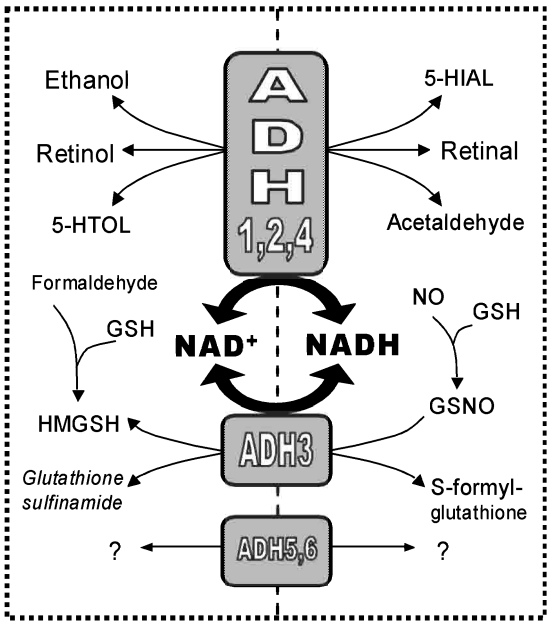


Figure 3. Schematic representation of some important ADH-catalyzed reactions indicating interaction of different metabolic pathways where ADH is involved. 5-HTOL, 5-hydroxytryptophol; 5-HIAL, 5-hyroxyindole-3-acetaldehyde; GSH, glutathione; HMGSH, *S*-hydroxymethyl-glutathione; NO, nitric oxide; GSNO, *S*-nitrosoglutathione. It should be noted that recently also ADH3 has been implicated in the metabolism of retinoids.

Structure and Mechanism

MDR enzymes are composed of subunits of approximately 350-400 amino acid residues, arranged as either tetramers, exemplified by yeast ADH, or dimers as for all vertebrate ADHs. The zinc-content varies from two per subunit (e.g. vertebrate ADH), one per subunit (e.g. sorbitol dehydrogenase) to no zinc at all (e.g. ζ-crystallin). Most studies concerning both the structure and mechanism of ADHs have been conducted with HLADH (Brändén *et al.*, 1973; Eklund *et al.*, 1976; Theorell and Chance, 1951; Wratten and Cleland, 1963). Additional structural information for HLADH coenzyme- and substrate binding was obtained from binary and ternary complexes with both inhibitors and substrates (Cedergren-Zeppezauer *et al.*, 1982; Eklund *et al.*, 1982a; Eklund *et al.*, 1982b; Ramaswamy *et al.*, 1994). Furthermore, at present three dimensional structure data are also available from human ADH1 isozymes (Hurley *et al.*, 1991; Hurley *et al.*, 1994; Niederhut *et al.*, 2001), ADH3 (Yang *et al.*, 1997) and ADH4 (Xie *et al.*, 1997) as well as mouse ADH2 (Svensson *et al.*, 2000; Svensson *et al.*, 2001) and cod ADH1 (Ramaswamy *et al.*, 1996).

Overall structure of ADH

All structurally determined ADHs display a similar overall architecture. Each ADH subunit is folded into one catalytic domain and one coenzyme binding domain, and in between a crevice

containing the active site is formed (Brändén *et al.*, 1975). In the dimer, the two catalytic domains are located centrally within the molecule with the catalytic domains flanking on either side (fig 4). The dimerization interface is formed as an extension of the β-pleated sheet region of one coenzyme domain with the same region in the second subunit, forming a 12-stranded β-sheet structure (Brändén *et al.*, 1975). The structure of the coenzyme binding domain, i.e. the Rossman-fold, is similar to nucleotide binding domains of other dehydrogenases (Brändén *et al.*, 1975; Rossmann *et al.*, 1974), whereas the catalytic domain conformation is unique for MDR structures (Brändén *et al.*, 1975). The catalytic domain holds the two zinc atoms, of which one is buried deep within the molecule and participates in catalysis, and the other is located in a superficial loop region and is essential for maintaining structure and stability (Brändén *et al.*, 1975; Jelokova *et al.*, 1994). The zinc ligands are conserved within the vertebrate ADHs. The catalytic zinc is coordinated to Cys46, His67 and Cys174, with the fourth ligand being either water, hydride ion or substrate. An exception is seen in the ADH3 structure where Glu68 has been observed to be directly coordinated to the zinc (Yang *et al.*, 1997). The structural zinc ligands are Cys97, Cys100, Cys103 and Cys111 (Brändén *et al.*, 1975).

The adenine part of the coenzyme binds with hydrophobic interactions in a crevice within the coenzyme-binding domain, whereas the pyrophosphate bridge and the nicotinamide moiety have interactions with both domains (Eklund *et al.*, 1984). Coenzyme binding induces an isomerization, accomplished by a rotation of the catalytic domain by approximately 10° for HLADH, from an open conformation of the apo-enzyme to a more closed conformation of the holo-enzyme (Colonna-Cesari *et al.*, 1986; Eklund and Bränden, 1979). This essentially narrows the cleft between the two domains, thus forming the substrate binding pocket. The subsequent binding of substrate occurs by replacing the zinc-bound water at the bottom of the active site. A probable proton relay system in the HLADH structure involving the hydroxyl group of Ser48, the 2' hydroxyl of the nicotinamide ribose and the imidazole of His51 has been proposed to transfer the hydroxyl proton of the alcohol substrate to the bulk solvent (Eklund *et al.*, 1982a). This is further facilitated by the need of a compensatory negative charge for the positive charge brought on by NAD^+ (Eklund *et al.*, 1982a; Eklund *et al.*, 1984; Kvassman *et al.*, 1981). The subsequent catalysis step is a hydride transfer from the C1 carbon of the alcohol substrate to C4 in the nicotinamide ring of the coenzyme.

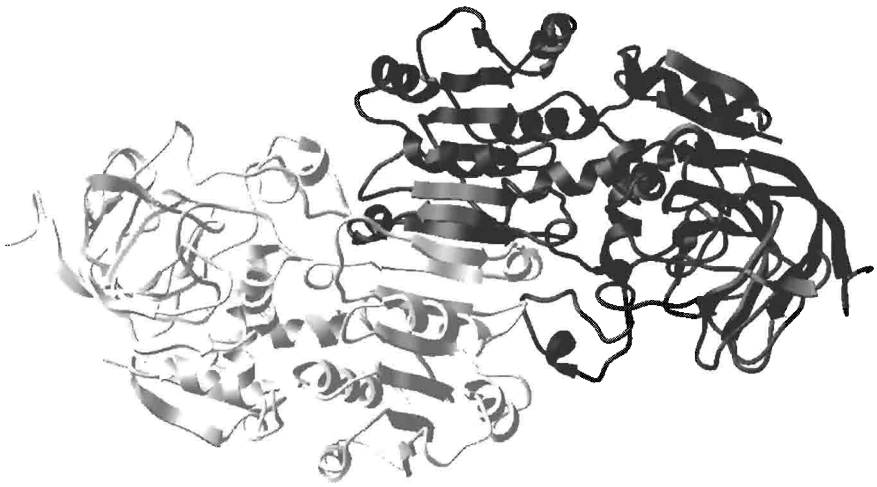


Figure 4a. Ribbon structure of the dimeric HLADH molecule. The two subunits are shown in different shades of gray. The figure was created with the ICM package (Molsoft LLC).

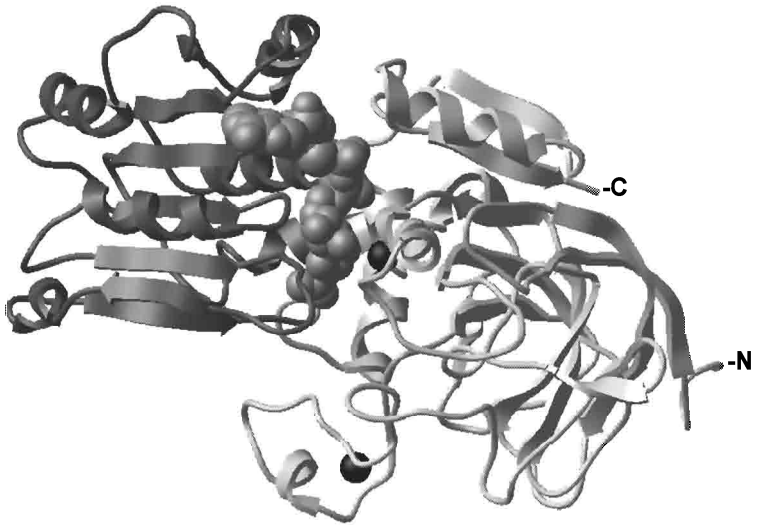
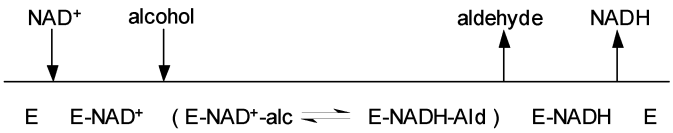


Figure 4b. One subunit of the HLADH molecule. The coenzyme binding domain is shown in dark gray and the catalytic domain in light gray. Also shown are the two zinc ions (black spheres), the coenzyme (space filling model) and the N- and C-termini. The figure was created with the ICM package (Molsoft LLC).

Structural determinants for the unique properties of different ADHs can be attributed to the high degree of substitutions at positions in the substrate- and coenzyme-binding pockets. The three human ADH1 subunits for example, which share ~93% overall positional identity, display only about 60% identity among the residues lining the substrate-binding pocket (Eklund *et al.*, 1987; Niederhut *et al.*, 2001). In addition, the extent of domain closure as well as the existence of functional proton transfer routes differ among the members of the ADH family, and may be critical parameters for substrate specificity and catalysis (Colby *et al.*, 1998; Hurley *et al.*, 1997; Niederhut *et al.*, 2001; Ramaswamy *et al.*, 1996; Ramaswamy *et al.*, 1999). Principally, residues determining coenzyme affinity, particularly positions 47 (Arg in HLADH), 369 (invariant for Arg in all mammalian ADHs except for the allelozyme ADH1C3) and 228 (Lys in most structures), have major impact on turnover since coenzyme dissociation is rate-limiting for most ADHs (Davis *et al.*, 1996; Hurley *et al.*, 1990; Jörnvall *et al.*, 1984; Plapp *et al.*, 1983; Stone *et al.*, 1993a). In addition, coenzyme specificity, i.e. the preference for NAD to NADP, seem to be determined by an aspartate residue at position 223 (Brändén *et al.*, 1975; Fan *et al.*, 1991; Peralba *et al.*, 1999). Specific amino acid residues in the active site regulate substrate specificity and, especially positions 48 (occupied by either Thr or Ser) and 93 (Phe in HLADH) located in the inner part of the substrate binding pocket, have been shown to determine the selectivity for secondary alcohols and steroids (Eklund *et al.*, 1987; Eklund *et al.*, 1990; Hurley and Bosron, 1992; Höög *et al.*, 1992). In addition, ADH3 has a prerequisite for a negatively charged Asp57 and a positively charged Arg115 for efficient HMGSH binding (Engeland *et al.*, 1993; Estonius *et al.*, 1994b; Hedberg *et al.*, 1998; Yang *et al.*, 1997).

Reaction mechanism

The classical ADH reaction, i.e. alcohol oxidation by HLADH, has been thoroughly investigated by steady-state and transient kinetic techniques. This has shown that, for most substrates, the kinetic mechanism is ordered Bi Bi, i.e. the two substrates bind in an obligatory order to form a ternary complex which breaks down with release of products in an obligatory order (Dalziel, 1963; Theorell and Chance, 1951; Wratten and Cleland, 1963) (scheme II). Kinetic isomerization was early shown to take place for the enzyme-NAD^+ complex, which is probably associated with the rotation of the catalytic domain as shown by x-ray crystallography (Eklund and Bränden, 1979; Shore *et al.*, 1975; Wratten and Cleland, 1963; Wratten and Cleland, 1965). The conformational change and the fact that the 2' hydroxyl of the nicotinamide ribose is required for the proposed proton relay system provide structural



Scheme II. Ordered Bi Bi kinetic mechanism of HLADH

explanations for the ordered binding of substrates (Eklund *et al.*, 1982a; Fersht, 1999). However, the mechanisms of other ADHs and substrates do not necessary follow the same mechanism as for HLADH with aliphatic alcohols/aldehydes. Interestingly, the kinetic mechanism for ADH3 with the natural substrate HMGSH has been shown to be random Bi Bi (Sanghani *et al.*, 2000; Uotila and Mannervik, 1980).

For HLADH, coenzyme dissociation is the rate-limiting step in the overall reaction for both ethanol oxidation and acetaldehyde reduction (Brändén *et al.*, 1975). With transient measurements of ethanol oxidation, characterized by a burst phase before steady-state is reached, the rate of interconversions of the ternary complexes can be determined (Shore and Gutfreund, 1970). Such experiments in combination with coenzyme binding studies and simulations have been used to explain the rates and limiting factors for the different steps in the catalytic mechanism. Coenzyme binding involves two separate steps with different rates. The binding of the adenosine part, representing the first step, is fast, whereas the subsequent isomerization and positioning of the nicotinamide moiety is limited to approximately 500 s^{-1} and 1200 s^{-1} for the E-NAD^+ and E-NADH complexes, respectively (Adolph *et al.*, 1997; Sekhar and Plapp, 1988). Another isomerization step has been proposed for the enzyme-aldehyde-NADH ternary complex, since transient aldehyde reduction does not display isotope effects (with NADD) and is slower than the isomerization of the binary enzyme-NADH complex (Sekhar and Plapp, 1990). Other steps in the reaction mechanism affecting the interconversion of ternary complexes are the actual transfer of a hydride ion and the deprotonation of the substrate. In addition, pH dependencies for the different steps in HLADH catalysis have also been widely studied and important ionizable groups in the active site are the zinc bound water or alcohol, His51 of the proton shuttle and Lys228, interacting with the coenzyme. NAD^+ association displays a bell-shaped pH dependence with p*K* values of 7 and 9 (Dalziel, 1963; Sekhar and Plapp, 1988). The p*K* of 9 can be ascribed Lys228, which in the protonated form electrostatically attracts the pyrophosphate moiety of the coenzyme, whereas the p*K* of 7 seems to arise from the proton relay system (including His51 and the zinc bound water, LeBrun and Plapp, 1999). The pH dependency of the hydride transfer displays a p*K* of 6.4 with ethanol as substrate (Brooks and Shore, 1972). This probably reflects the

deprotonation of the zinc bound substrate, since formation of an alcoholate ion has been proposed to be obligatory for hydride transfer (Kvassman *et al.*, 1981; Kvassman and Pettersson, 1980). The p*K* values for the ionization of alcohols in the ternary complex varies for differently substituted alcohols, but essentially represent a decrease of about 9 units as compared to the p*K*s of the same alcohols in solution (Kvassman *et al.*, 1981). pH effects on the association of NADH are predominantly of electrostatic origin and probably not dependent on deprotonation of the zinc-bound water since no positive charge is present in the nicotinamide ring as in the case of NAD^+ (Adolph *et al.*, 1997; LeBrun and Plapp, 1999; Pettersson and Eklund, 1987). Scheme III summarizes the different steps and complexes in the catalytic mechanism of HLADH.

$$E \rightleftharpoons E\text{-}NAD^+ \rightleftharpoons {}^*E\text{-}NAD^+ \rightleftharpoons E\text{-}NAD^+\text{-}RCH_2OH \rightleftharpoons E\text{-}NAD^+\text{-}RCH_2O^- \rightleftharpoons$$

$$\rightleftharpoons {}^*E\text{-}NADH\text{-}RCHO \rightleftharpoons E\text{-}NADH\text{-}RCHO \rightleftharpoons {}^*E\text{-}NADH \rightleftharpoons E\text{-}NADH \rightleftharpoons E$$

Scheme III. Proposed sequential catalytic mechanism for HLADH including the isomerizations of both binary complexes and the ternary enzyme-NADH-aldehyde complex indicated by *E in the formula.

ADH and Disease

Polymorphisms in ADH genes

Genetic variation is a common phenomenon in nature and about 1.4 million single nucleotide polymorphisms (SNPs) have been described throughout the human genome (Sachidanandam *et al.*, 2001). It has been estimated that 60,000 of these polymorphisms fall within exons (Sachidanandam *et al.*, 2001), and thereby may result in polypeptides with altered properties. Polymorphisms have been detected in all seven human ADH genes. Allelozymes have been discovered and characterized from *ADH1B*, *ADH1C* and *ADH2*, and promoter variations have been analyzed for *ADH2* and *ADH3* (Table II, Burnell *et al.*, 1987; Edenberg *et al.*, 1999; Höög *et al.*, 1986; Jörnvall *et al.*, 1984; Smith *et al.*, 1971; Paper III; Paper IV). In addition, uncharacterized mutations in the 5'- and 3'-flanking regions of ADH genes, as well as numerous silent mutations and polymorphisms in introns have been detected (Buervenich *et al.*, 2000; Iida *et al.*, 2001; Iida *et al.*, 2002).

Three alleles have been identified for the *ADH1B* gene. The ADH1B2 (β_2) subunit differs from ADH1B1 at position 47 where Arg in ADH1B1 is substituted for His in ADH1B2 and the ADH1B3 subunit has a Cys at positing 369, a position occupied by Arg in

ADH1B1 (Bosron *et al.*, 1993). These positions are involved in coenzyme binding and the substitutions cause major effects on the kinetics of these allelozymes. K_m for ethanol, which is 50 μM for ADH1B1, is increased to 1 mM and 36 mM for ADH1B2 and B3, respectively, and turnover numbers are increased 30-fold for both ADH1B2 and ADH1B3, as compared to ADH1B1 (Bosron *et al.*, 1993). Two allelozymes are derived from the *ADH1C* gene locus and two residues differ between ADH1C1 and ADH1C2; at position 271 (Arg/Gln) and 349 (Ile/Val). ADH1C1 displays approximately 2-fold higher catalytic efficiency for ethanol oxidation, as compared to ADH1C2 (Bosron *et al.*, 1993). In addition, a rare nonsense mutation affecting the *ADH1C* gene was recently discovered (Buervenich *et al.*, unpublished). This mutation truncates the protein after only 78 amino acid residues, which intuitively would result in a non-functional protein.

For the human *ADH2* gene, polymorphisms have been detected in both the coding sequence and the promoter region (Edenberg *et al.*, 1999; Iida *et al.*, 2002; Paper III). The polymorphism affecting the coding region results in an Ile to Val substitution at position 308, the significance of which has been investigated in paper III (see further in the results and discussion section). Three polymorphisms were initially detected in the *ADH4* promoter, which were analyzed in a reporter gene system (Edenberg *et al.*, 1999). One of these, an A/C transition at position -75, was found to significantly affect transcription levels (Edenberg *et al.*, 1999). In addition to the three characterized polymorphisms in the promoter, five more variations in the 5'-flanking region have been detected in a Japanese population (Iida *et al.*, 2002). However, the same survey failed to detect the SNP in the coding region indicating that this polymorphism is rare in that population (Iida *et al.*, 2002; Paper III).

No polymorphisms have been found in the coding region of *ADH3*. However, paper IV describes the identification of a number of variations on the promoter sequence. A reporter gene assay showed a significant reduction in promoter activity for a $C_{+9} \rightarrow T_{+9}$ transition, which could arise from decreased binding of nuclear proteins, as indicated by electrophoretic mobility shift assays.

Association of ADH polymorphisms with disease

Alcoholism is a leading cause of morbidity and premature death, and the heritability of alcohol dependence has been estimated to be approximately 60% (Goate and Edenberg, 1998). Plausible candidates are e.g. genes involved in the reward systems of the central nervous system and enzymes involved in alcohol metabolism (Goate and Edenberg, 1998). Genome wide searches to identify candidate genes underlying the susceptibility for

alcoholism, e.g. the collaborative study on the genetics of alcoholism (COGA), are currently ongoing. Associations between alcoholism and ADH polymorphisms have been found. Notably, a protective role for the high activity variants of ADH1 (especially the *ADH1B*2* allele common in Asian populations) have been shown, presented as a significant overrepresentation of these alleles among non-alcoholics (Borras *et al.*, 2000; Chambers *et al.*, 2002; Hasin *et al.*, 2002; Thomasson *et al.*, 1993; Thomasson *et al.*, 1991). In addition, linkage disequilibrium has been found between the *ADH1B* and *ADH1C* loci associating the two high activity alleles (*ADH1B*2* and *ADH1C*1*, Borras *et al.*, 2000; Osier *et al.*, 1999). This suggests a possible additive effect, and show that the protective effect brought on by *ADH1C*1* probably can be accounted for by the disequilibrium with *ADH1B* (Borras *et al.*, 2000; Osier *et al.*, 1999).

Fetal alcohol syndrome (FAS), a common cause of mental and growth retardation in infants, has been proposed to be associated with ADH and retinoic acid signaling during development (Deltour *et al.*, 1996; Duester, 1991). The basis for this hypothetical mechanism would be that ethanol acts as a competitive inhibitor of the retinol dehydrogenase activity attributed to ADH. Both the *ADH1B*2* and *ADH1B*3* alleles have been correlated with decreased prevalence of FAS and other alcohol related birth defects (McCarver *et al.*, 1997; Viljoen *et al.*, 2001). However, it is dubious whether these associations are related to retinoid metabolism or not simply relates to the mothers drinking behavior. Furthermore, since chronic alcohol consumption is associated with an increased risk of cancer in the upper aerodigestive tract, the *ADH1B*1* and *ADH1C*1* alleles have been implicated as risk factors (Coutelle *et al.*, 1997; Harty *et al.*, 1997; Hori *et al.*, 1997; Yokoyama *et al.*, 2001). However, other studies have failed to detect similar associations (Bouchardy *et al.*, 2000; Olshan *et al.*, 2001). An additional association between ADH1 alleles and disease have been found for the beneficial effect of moderate alcohol consumption on myocardial infarction (Hines *et al.*, 2001). In this study moderate drinkers, homozygous for the *ADH1C2* allele, were found to have higher HDL levels and a significantly decreased risk of myocardial infarction (Hines *et al.*, 2001).

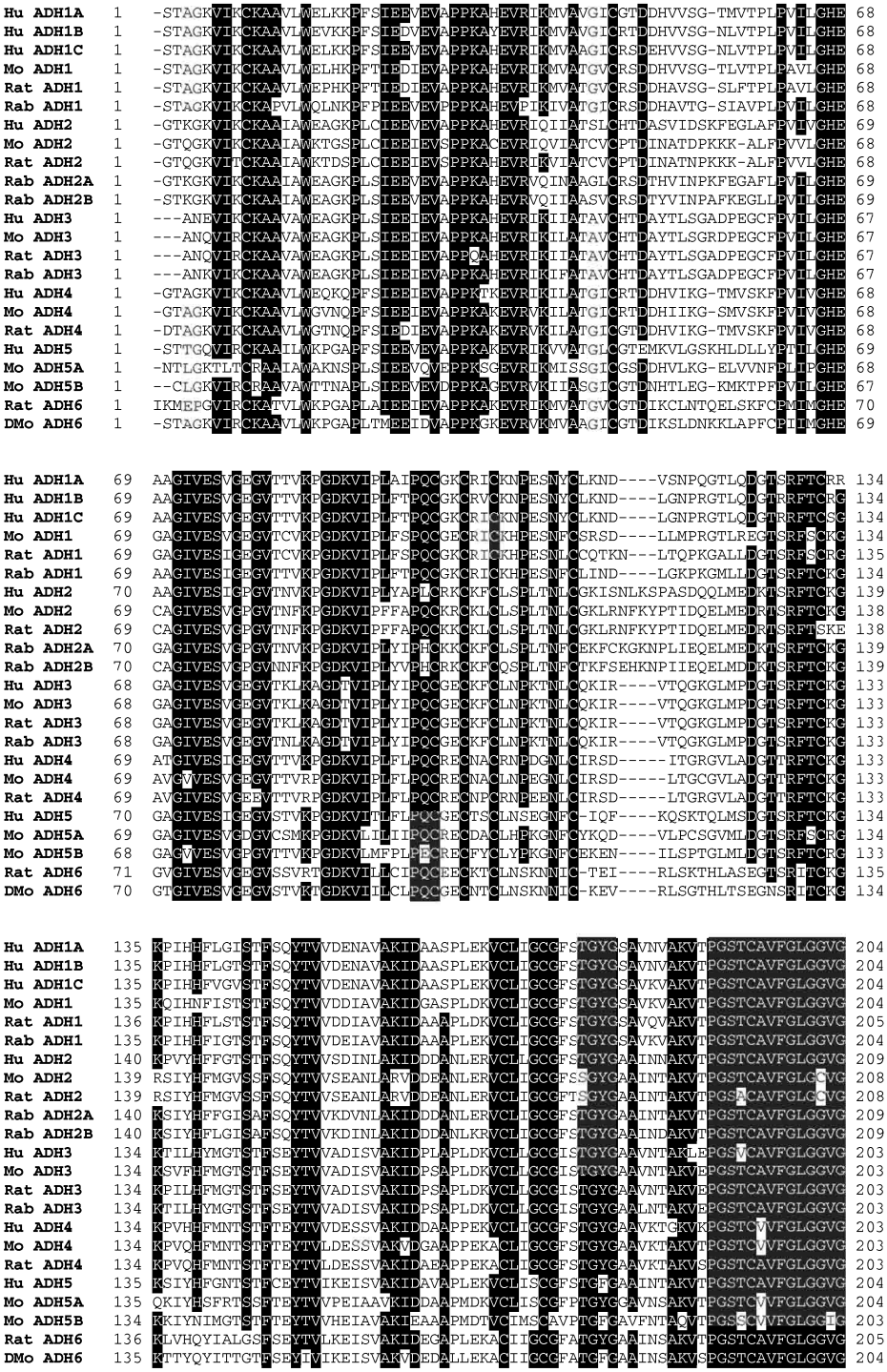
Genetic markers on chromosome 4q21-23 have been linked to a certain familiar form of Parkinson's disease (PD, Polymeropoulos *et al.*, 1996). This region coincides with the localization of the ADH cluster and could be interesting since dopamine metabolites as well as retinoids are substrates for ADH. In two recent studies, attempts were made to identify ADH polymorphisms associated with PD (Buervenich *et al.*, 2000; Buervenich *et al.*, unpublished). Interestingly, polymorphisms in the promoter and coding regions, as well as in

intron sequences of the *ADH4* gene were detected. One of these alleles was demonstrated to be significantly more frequent in patients with PD than in control subjects, whereas other known ADH polymorphisms could not be significantly linked to PD in the same samples (Buervenich *et al.*, 2000) (Buervenich *et al.*, unpublished). However, this association was not found to be significant in another study (Tan *et al.*, 2001).

Table II. Human ADH polymorphisms that affects the promoter region or give rise to allelozymes[a]

Gene	Location	Nuclotides[b]	Amino Acids	References
ADH1A	5'-flanking region	$C_{-55} \rightarrow A$		Iida *et al.*, 2001
*ADH1B*1*	exon 3, exon 9	G_{143}, C_{1108}	R_{47}, R_{369}	Hedén *et al.*, 1986
*ADH1B*2*	exon 3, exon 9	A_{143}, C_{1108}	H_{47}, R_{369}	Jörnvall *et al.*, 1984; Ehrig *et al.*, 1988
*ADH1B*3*	exon 3, exon 9	G_{143}, T_{1108}	R_{47}, C_{369}	Burnell *et al.*, 1987; Carr *et al.*, 1989
*ADH1C*1*	exon 6, exon 8	G_{815}, A_{1048}	R_{271}, I_{349}	Höög *et al.*, 1986
*ADH1C*2*	exon 6, exon 8	A_{815}, G_{1048}	Q_{271},V_{349}	Höög *et al.*, 1986
ADH1C	exon 3	$G_{232} \rightarrow T$	$G_{78} \rightarrow$ stop	rs#283413; Buervenich *et al.*, unpubl.
ADH1C	5'-flanking region	$C_{-254} \rightarrow G$		Iida *et al.*, 2002
ADH2	5'-flanking region	$T_{-1487} \rightarrow C$		Iida *et al.*, 2002
	5'-flanking region	$A_{-482} \rightarrow G$		Iida *et al.*, 2002
	5'-flanking region	$G_{-437} \rightarrow A$		Iida *et al.*, 2002
	5'-flanking region	$A_{-234} \rightarrow G$		Iida *et al.*, 2002
	5'-flanking region	$C_{-361} \rightarrow G$		Iida *et al.*, 2002
	5'-flanking region	$T_{-192} \rightarrow A$		Edenberg *et al.*, 1999
	5'-flanking region	$G_{-159} \rightarrow A$		Edenberg *et al.*, 1999
	5'-flanking region	$C_{-75} \rightarrow A$		Edenberg *et al.*, 1999
	exon 7	$A_{925} \rightarrow G$	$I_{308} \rightarrow V$	paper III
ADH3	5'-flanking region	$GG_{-197,-196} \rightarrow AA$		paper IV
	5'-flanking region	$G_{-79} \rightarrow A$		paper IV
	5'-UTR	$C_{+9} \rightarrow T$		paper IV
ADH4	5'-flanking region	$G_{-1081} \rightarrow T$		Iida *et al.*, 2001
	5'-flanking region	$T_{-528} \rightarrow C$		Iida *et al.*, 2001
	5'-flanking region	$T_{-94} \rightarrow C$		Buervenich *et al.*, 2000
	5'-UTR	$T_{+25} \rightarrow C$		Buervenich *et al.*, 2000
	exon 3	$G_{212} \rightarrow C$	$G_{79} \rightarrow A$	Buervenich *et al.*, 2000

[a] All detected polymorphisms within coding sequences and 2 kb of 5'-flanking regions are shown. In addition to the polymorphisms presented in this table, numerous silent mutations as well as polymorphisms in introns have been reported. [b] numbered according to the reference in the right column.



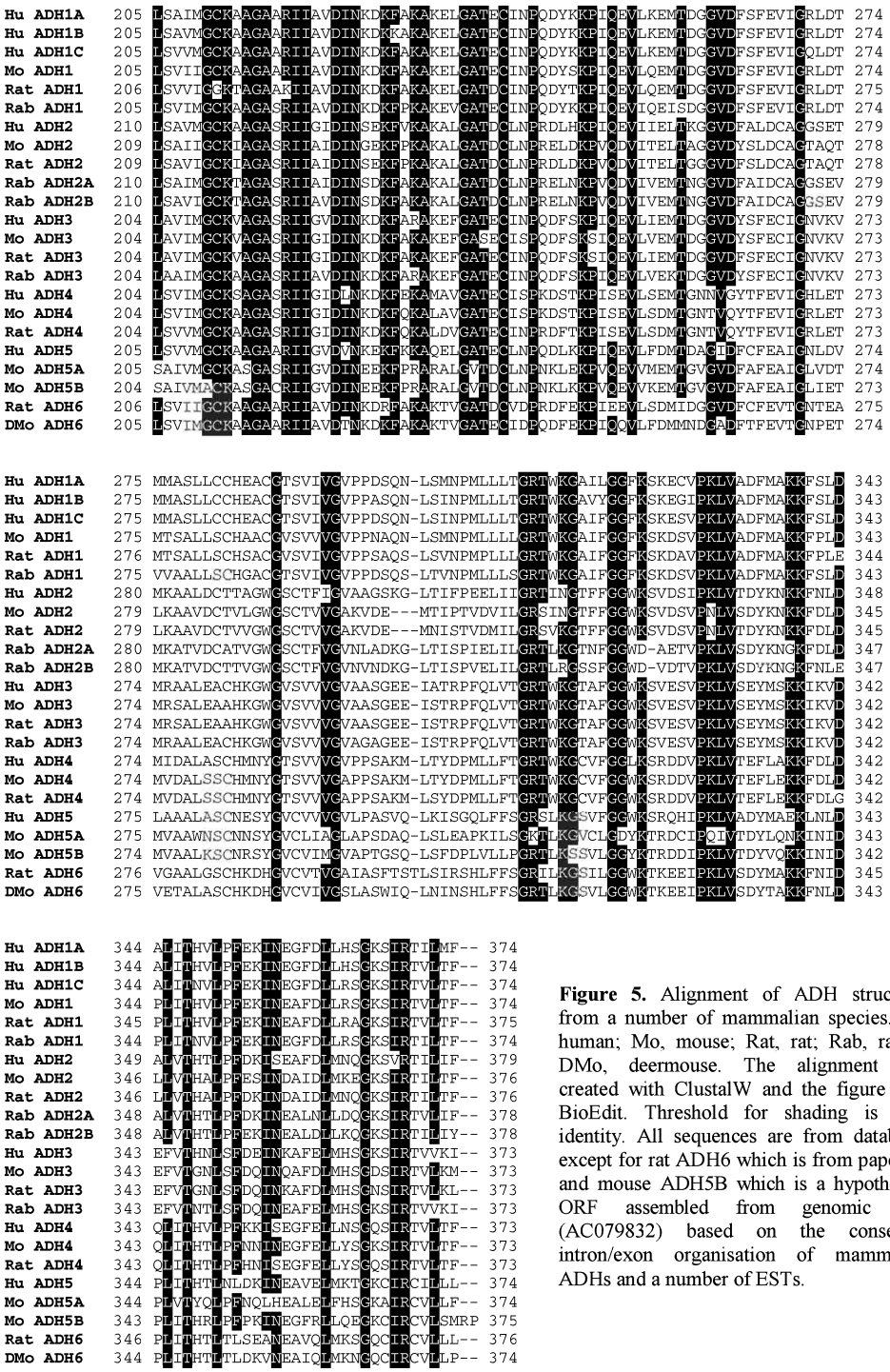


Figure 5. Alignment of ADH structures from a number of mammalian species. Hu, human; Mo, mouse; Rat, rat; Rab, rabbit; DMo, deermouse. The alignment was created with ClustalW and the figure with BioEdit. Threshold for shading is 80% identity. All sequences are from databases except for rat ADH6 which is from paper VI and mouse ADH5B which is a hypothetical ORF assembled from genomic data (AC079832) based on the conserved intron/exon organisation of mammalian ADHs and a number of ESTs.

AIMS OF THIS THESIS

With the recent advances in the fields of genomics and proteomics, many gene families have expanded to include proteins with still unknown functions, and the mammalian ADH system is no exception. Only a few ADHs have been assigned specific metabolic functions, e.g. glutathione dependent formaldehyde oxidation by ADH3, whereas other ADHs seem to contribute in the general defense against xenobiotics as well as endogenously formed alcohols and aldehydes. Many known ADH forms have not yet been fully characterized and new members will probably be added to the mammalian ADH family.

The aim of this thesis project has been to identify and investigate novel forms, i.e. new enzymes, species orthologs, allelozymes, splice variants or post-translational modifications of mammalian ADH, in order to gain additional knowledge of the entire system. Finding new forms will enable us to further understand structure/function relationships and evolutionary aspects of the ADH family. Moreover, this will provide clues to help identify novel physiological substrates and functions for the ADH enzymes.

The project specific aims has been:

- To identify and characterize an ADH2 species variant in mouse and further determine the functional features of mammalian ADH2 enzymes.

- To identify polymorphisms within human ADH genes and assess the possible functional role of these inter-individual differences.

- To investigate if the human *ADH5* gene deviates from the conserved genomic structure of mammalian ADH and perform additional analyses of ADHs of higher classes.

RESULTS AND DISCUSSION

Detailed information about the methods used to obtain the results described in this section is provided in the original articles and manuscripts in the materials and methods sections.

Structure and function of mammalian ADH2 enzymes

The cloning and characterization of a mouse ADH2 type enzyme, which together with the rat counterpart, forms a subgroup of the mammalian ADH2 branch is described in paper I. In paper II the kinetic features of the mouse ADH2 form are further enlightened and related to the human form.

A novel subgroup of ADH2 in rodents

ADH2 was first isolated from human liver and orthologs have been identified in rabbit, rat and ostrich (Bosron *et al.*, 1977; Hjelmqvist *et al.*, 1995a; Höög, 1995; Höög *et al.*, 1987; Svensson *et al.*, 1998). These studies have shown that the ADH2 line of vertebrate ADH2 is both structurally and functionally divergent, as compared to other ADH classes. It has been proposed that mice are devoid of this enzyme, since no ADH2 was detected neither during activity screening nor from probing genomic DNA and mRNA with the human ADH2 cDNA (Algar *et al.*, 1983; Zgombic-Knight *et al.*, 1995a). However, on the basis of the sequence conservation of ADH2 structures around positions 80 and 240, we were able to isolate an ADH2 cDNA by PCR cloning from a mouse liver cDNA library. The entire cDNA sequence, covering 1354 bp, included a 1131 bp coding region, a 23 bp 5´non-coding region, a 200 bp 3´non-coding region, a poly(A) signal and a poly(A) tail. The ORF translated into a 376 amino acid polypeptide that showed a sequence identity of 93% with the rat ADH2 variant, which is remarkably high considering the high overall variability of this class (cf fig. 1). In addition, whereas residue substitutions between different ADH2 forms are common at positions lining the substrate binding pocket, no such substitutions are seen between the rodent forms (table III). Alignments of different ADH structures reveals three variable regions around positions 60, 120 and 300, which display insertions and deletions as compared to the ADH consensus sequence. Within these regions there are two deletions specific for the rodent ADH2s (around positions 60 and 300) and a four-residue insertion around position 120, a feature common to all characterized ADH2 (fig. 5).

Table III. Amino acid residues lining the substrate binding pocket of HLADH and the corresponding residues in ADH2.

residue no.[a]	Horse ADH1	Human ADH1	Rat ADH2	Mouse ADH2
48	Ser	Thr	Thr	Thr
57	Leu	Phe	Δ/Lys[b]	Δ/Lys[b]
93	Phe	Tyr	Phe	Phe
110	Phe	Leu	Leu	Leu
116	Leu	Leu	Leu	Leu
117	Ser	Ser	Arg	Arg
140	Phe	Phe	Phe	Phe
141	Leu	Phe	Met	Met
143	Thr	Thr	Val	Val
294	Val	Val	Ala	Ala
306	Met	Glu	Val	Val
309	Leu	Ile	Ile	Ile
318	Ile	Phe	Phe	Phe

[a] Numbering refer to the ADH1 sequence.
[b] Equal alignment scores in multiple sequence alignment.

The ADH2 forms from mouse, rat and human were recombinantly expressed and purified for kinetic characterization. Ethanol, octanol, benzyl alcohol and their corresponding aldehydes, together with benzoquinone were used as model substrates for activity determinations. The alcohol oxidation was catalyzed dramatically less efficient by the rodent forms as compared to the human form. Turnover numbers were up to two orders of magnitude lower and saturation was reached at far higher concentrations. The reduction of aldehydes was more efficiently catalyzed by the rodent forms than alcohol oxidation but still turnover numbers were 5-10 fold lower than for the human form. In contrast, benzoquinone reduction proceeded with about the same turnover rate for all species variants, although the K_m-values were lower for the human form. No activity by mouse ADH2 was seen for a number of other substrates and ω-hydroxy fatty acids acted as inhibitors rather than substrates. In addition, coenzyme saturation was reached at much lower concentrations for the mouse enzyme, as compared to the human form. Large substrate isotope effects on k_{cat} and k_{cat}/K_m were seen for mouse ADH2, using octanol and [1,1-$^2H_2$]octanol, demonstrating that hydride transfer is a rate-limiting step in alcohol oxidation. This was not observed for the human form, which agrees with coenzyme release being rate-limiting as proposed previously (Ditlow *et al.*, 1984).

A structural explanation for the low activity of the rodent ADH2 forms was traced to a couple of unique residues in the rodent structures, i.e. at the coenzyme binding positions 47 and 182 (the latter corresponds to 178 in the ADH1 numbering system), which was studied by

site-directed mutagenesis. A mouse Pro47His mutant showed markedly increased k_{cat}-values and decreased K_m-values for alcohol oxidation and aldehyde reduction while benzoquinone reduction was unaffected, which indicates a different mechanism for this reaction. Catalytic efficiency for alcohol oxidations was increased about 50-fold at pH 10.0 and at least 200-fold at pH 7.5 as compared to the wild type enzyme. A Ser182Thr mutation also increased k_{cat}/K_m-values for alcohol oxidation, however to a lesser extent (5-10 fold). Both substitutions also increased the K_m-values for NAD^+ and NADH. Still, hydride transfer seemed to limit the reaction rate for the more active mutants, as deduced from substrate isotope effect experiments.

As mentioned previously, the effects of residue replacements at position 47 have been extensively studied, and explain e.g. the activity differences between two of the ADH1B allelic variants (ADH1B1, Arg47; ADH1B2, His47, Hurley *et al.*, 1990; Jörnvall *et al.*, 1984). In the ADH1 case, the high activity for the ADH1B2 variant can be assigned to dramatic differences in coenzyme dissociation, which is the rate-limiting step. Shifted coenzyme binding properties probably occur in the mouse ADH2 mutants as well (reflected in the K_m values for the coenzyme). However, since turnover is enhanced, which is limited by hydride transfer in mouse ADH2, the substitution at position 47 must also affect this step. By analogy, hydride transfer in ADH1B have been shown to be modulated by substitutions at position 47 (Stone *et al.*, 1993a). Although there are many structural and functional similarities between the two rodent ADH2 forms, there are also some differences e.g. in substrate specificity. In a recent study 9-cis retinol was found to be a substrate for rat ADH2 (Popescu and Napoli, 2000), and we have found that 9-cis retinal is an even better substrate for this enzyme (unpublished observations). In contrast, mouse ADH2 does not display any detectable activity for 9-cis retinoids (unpublished observations). However, within the ADH2 line of vertebrate ADH, the rodent enzymes clearly form a subgroup with significantly different properties as compared to the other ADH2 orthologs.

Structural and kinetic properties of mammalian ADH2

A number of crystal structures of mouse ADH2 were recently determined, including both binary and ternary complexes for the wild-type enzyme as well as a binary complex structure for the Pro47His mutant (Svensson *et al.*, 2000). The ADH2 structures display a semi-open conformation, as compared to the open apo- and closed holo structures of HLADH, which give rise to a more voluminous substrate binding pocket. The Pro47His mutant structure is somewhat more closed than the wild-type structure and the coenzyme is positioned closer to

the catalytic zinc, which possibly would decrease hydride transfer distances and thus increase turnover. The effect of position 47 on domain closure has been previously shown for the human ADH1 forms (Niederhut *et al.*, 2001). In addition, His47 is suitably positioned to act as a catalytic base in a proton relay system, analogous to His51 in ADH1 (fig. 6). This residue has previously been implicated in similar hydrogen bonding systems for human ADH2, benzyl alcohol dehydrogenase from *Pseudomonas putida*, and from the structures of human ADH3 and cod ADH1 (Ehrig *et al.*, 1991; Inoue *et al.*, 1998; Ramaswamy *et al.*, 1996; Yang *et al.*, 1997). For the mouse wild-type structure, having no His at either position 47 or 51, an alternative deprotonation route has been proposed which includes a water molecule and Asn51 as participants (fig. 6, Svensson *et al.*, 2000).

In order to further clarify the enzymatic properties of ADH2, we performed additional kinetic studies on both the human and the mouse forms. Steady-state and transient techniques were used, and pH dependencies as well as substrate isotope effects were investigated. In addition to the Pro47His mutant, we generated two additional substitutions at position 47 of the mouse enzyme to deduce the mechanistic basis for the low activity of the rodent ADH2 variants; Gln which resembles His in size, hydrophilicity and ability to form hydrogen bonds, and Ala which is smaller and neutral in its character.

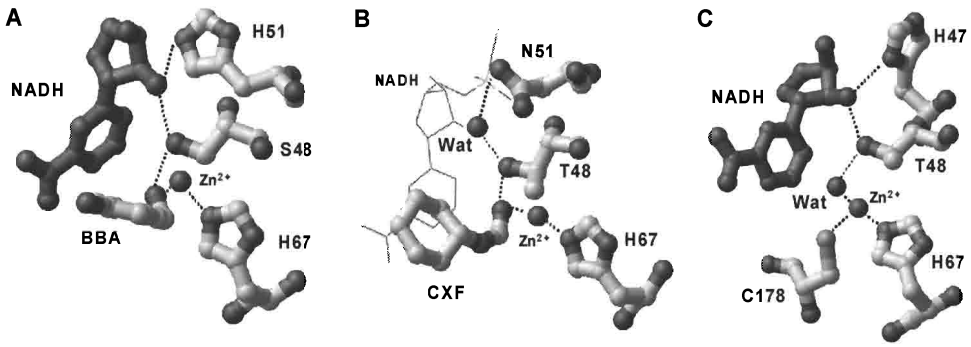


Figure 6. Proposed proton relay systems in HLADH **(A)** (Eklund *et al.*, 1982a), mouse wild-type ADH2 **(B)** and mouse Pro47His mutant ADH2 **(C)** (Svensson *et al*, 2000; Svensson *et al*, 2001). BBA, *p*-bromo-benzyl alcohol; CXF, *N*-cyclohexylformamide; Wat, water. The figure was created with the ICM package (Molsoft LLC).

Initial velocity experiments and product and dead-end inhibition studies suggest that both human and mouse ADH2 follow a sequential Bi Bi mechanism with coenzyme binding as first reactant. The effects of the additional substitutions at position 47 in the mouse enzyme were similar to the Pro47His mutant, with enhanced oxidative activity by approximately two orders of magnitude. Coenzyme affinities were also greatly decreased but still, hydride transfer limited the reaction rate for all mouse ADH2 forms as seen from large isotope effects for benzyl alcohol oxidation. Human ADH2 did not show any isotope effect in steady-state experiments. However, transient ethanol oxidation by human ADH2 seem to be, at least partly, limited by hydride transfer. This, together with a number of simulations and maximum steady-state velocities indicate that coenzyme dissociation is rate-limiting for turnover in the human ADH2 reaction. In addition, coenzyme binding rates for human ADH2 is much slower than for ADH1 enzymes, and rate constants were directly proportional to coenzyme concentration over the whole range used. The fact that no saturation was seen simplifies ADH2 coenzyme binding to a one-step process as compared to HLADH where coenzyme association is followed by a clearly detectable isomerization step (see above).

All ADH2 enzymes displayed strong pH dependence on k_{cat} and k_{cat}/K_m, with p*K*s above physiological pH. Interestingly, all mouse ADH2 mutants showed similar pH dependency patterns, indicating that no ionizable group at position 47 participates in catalysis. Hence, the activation achieved by His in position 47 described in paper I, is not due to the catalytic base activity of the His imidazole in a proton relay system. Rather it seems that substituting the rigid ring structure brought about by Pro47 for any other residue affects coenzyme binding and domain closure and as a result enhances hydride transfer (fig. 7). Still, deprotonation of substrate is inefficient in mammalian ADH2 enzymes, as seen from pH dependence studies. For HLADH the p*K* value of ethanol in solution of 15.7 is suppressed to about 6.4 when bound to the enzyme (Kvassman *et al.*, 1981). This decrease in p*K* has been assigned to the zinc, the positive charge brought on by NAD^+ and the proton relay system connecting the substrate with the bulk solvent (Eklund *et al.*, 1982a). For mouse ADH2 this p*K* is increased approximately 2 units as compared to other ADHs, thus decreasing the activity at physiological pH. Probably the low activity of the rodent enzymes is dependent on two separate steps. First, the positioning of the coenzyme is not optimal for hydride transfer to occur, which can be overcome by substitutions at position 47. Second, hydride transfer requires prior deprotonation of the zinc bound substrate which seems to be less efficient for ADH2 than for other ADHs, and for which substitutions at position 47 only have minor

effects. Since dissociation of coenzyme is rate-limiting for the human ADH2 enzyme the pH dependence of the hydride transfer needs to be analyzed by transient techniques.

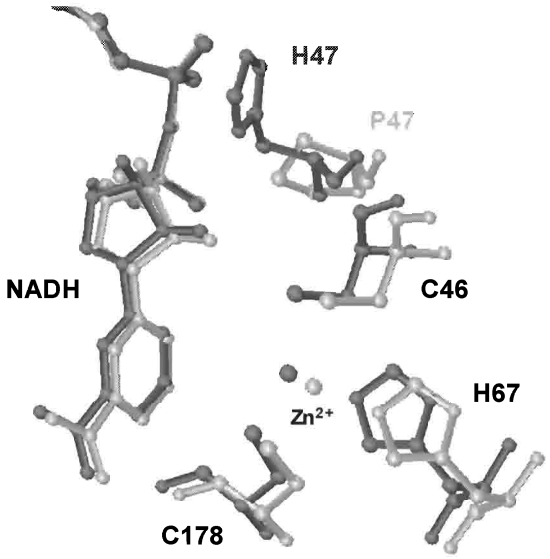


Figure 7. Comparison of the active sites of mouse ADH2 wild-type (light gray) and the Pro47His mutant (dark gray). The coenzyme-binding domains and NADH moieties of each structure are superimposed. The mutant structure is more closed as compared to the wild-type, and as a result the distance of hydrogen transfer is reduced. The superposition was created using the O program and the figure was made with computer program Ribbons.

In conclusion, the ADH2 forms of vertebrate alcohol dehydrogenase display many structural and functional differences. Notably, the overall high variability of ADH2 within the ADH family does not apply to the rodent forms, which are 93% identical at the protein level and display an almost identical active site (table III). This structural conservation, together with the unique kinetic features of this subgroup of ADH2 enzymes, indicates a specific function. The low activity of these enzymes is still puzzling. However, it is possible that the nature of the substrate could affect both hydride transfer rates as well as p*K* values; i.e., local rearrangements upon substrate binding may optimize hydride transfer distances, and alcohols with lower p*K* values may be the natural substrates. At present, the physiological role of ADH2 is to a great extent still unknown.

Identification and characterization of polymorphisms in human ADH genes

This section describes the findings of papers III and IV, in which we have screened for polymorphisms in the human *ADH2* and *ADH3* genes.

Human ADH2 allelozymes

Numerous polymorphisms have been detected within human ADH genes and, especially, the allelozymes of the *ADH1B* and *ADH1C* genes have been extensively investigated. The *ADH2* promoter was recently found to contain polymorphic sites that affected transcription levels (Edenberg *et al.*, 1999). In addition, sequence discrepancies were detected during the combined cDNA and protein sequence determination of human ADH2 (Höög *et al.*, 1987; Höög *et al.*, 1988). The affected positions were Ser/Thr 51, which however was invariant for Thr in a subsequent screen of 150 samples (Davis *et al.*, 1994), Ile/Val 308 (303 in ADH1 numbering), Arg/Lys 317 (312 in ADH1), Val/Ile 373 (368 in ADH1) and at the termination codon (resulting in an elongated peptide if present, Höög *et al.*, 1987; Höög *et al.*, 1988). In an attempt to confirm or dismiss these possible polymorphisms, we PCR amplified fragments of exons 7 and 9 for analysis by single strand conformation polymorphism analysis (SSCP). Sequence analysis of fragments with deviant migration patterns verified the Ile308Val substitution (an A→T transition at position 925 of the ORF counting the A of the ATG start codon as 1), whereas none of the other substitutions could be confirmed. The alleles were named *ADH2*1* (Ile308) and *ADH2*2* (Val308) in accordance with the recent nomenclature (Duester *et al.*, 1999). A screening of 99 DNA samples from a Swedish population showed that both alleles were relatively common in the samples, with the highest frequency (73%) for *ADH2*2*, i.e. valine at position 308.

Both allelozymes were recombinantly expressed and analyzed. Kinetic parameters were determined at pH 7.5 for the two allelozymes of ADH2 using ethanol, octanol, 12-HDA and all-trans retinol as model substrates. The two variants displayed similar overall kinetic characteristics, which can be expected since an Ile/Val exchange represents a conservative substitution. However, generally higher K_m values were obtained with the Val308 enzyme. Activity measurements and circular dichroism spectroscopy were used to detect differences in thermostability between the two allelozymes. The results of both analyses indicate that the 308Val substitution decreases thermostability slightly, as compared to the 308Ile allelozyme (fig. 8). In addition, stability was assayed after storage in cold room (6ºC) and at 37ºC in PBS. Similarly, the Ile308 was more stable than Val308 at both temperatures. This might

explain why differences in stability have been reported in studies with ADH2 obtained from different liver preparations (Bosron *et al.*, 1977; Bosron *et al.*, 1979; Ditlow *et al.*, 1984; Li and Magnes, 1975). A model of human ADH2, which was created using the mouse crystal structure as a template, located position 308 at the end of one of the β-strands in the subunit interface, adjacent to residues lining the substrate binding pocket. Removal of a methylene group within the hydrophobic core of a protein, as for an Ile to Val substitution, typically decreases the stability by 1.5±0.5 kcal/mol (Fersht, 1999) and this agrees with the calculated value for the Ile308Val substitution (1.2 kcal/mol) using the molecular model of human ADH2.

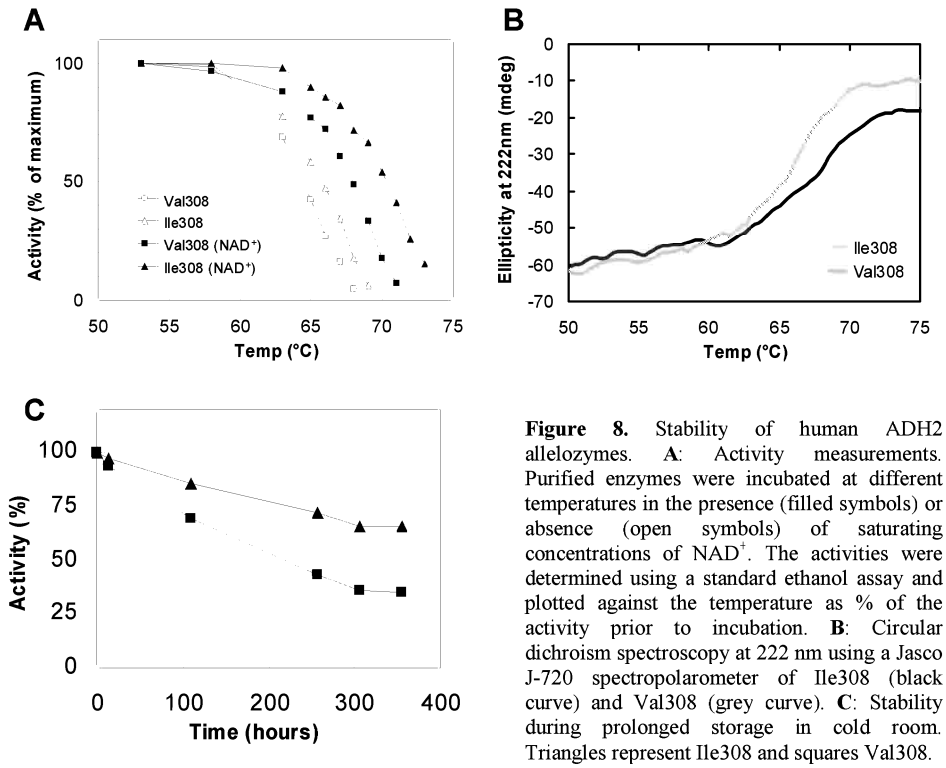


Figure 8. Stability of human ADH2 allelozymes. **A**: Activity measurements. Purified enzymes were incubated at different temperatures in the presence (filled symbols) or absence (open symbols) of saturating concentrations of NAD^+. The activities were determined using a standard ethanol assay and plotted against the temperature as % 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). **C**: Stability during prolonged storage in cold room. Triangles represent Ile308 and squares Val308.

The *ADH3* promoter is polymorphic

ADH3 is characterized by a constant function as a formaldehyde scavenger in cellular metabolism, a constitutive expression pattern and by a high degree of sequence conservation, even between distantly related species. We used a similar SSCP approach as for ADH2 to screen for mutations in three large exons as well as the promoter region (including exon 1) of the *ADH3* gene. No variation, that would result in amino acid substitutions, was detected in the 80 samples used, which could be representative for the functional constraints upon the ADH3 structure. However, a number of different migration patterns were observed for the promoter/exon1 samples. Sequence analysis revealed four polymorphic sites; three G→A transitions were detected before the transcriptional start site at positions -197, -196 and -79, and the fourth transition C→T was detected after the transcriptional start site at position +9 (fig. 9). The two base exchanges at -197 and -196 seem only to appear together and are probably connected with the same allele. The distribution of the different genotypes and allele frequencies was screened by allele specific PCR on samples from three different populations; Swedish, Spanish and Chinese. The polymorphism at -197/-196 was relatively common in all three populations. The G_{-79} allele, although quite frequent in the European samples, was absent in the Chinese population. The very rare exchange $C_{+9}\rightarrow T_{+9}$ was only detected in three Swedish heterozygots and not in any sample from the other populations.

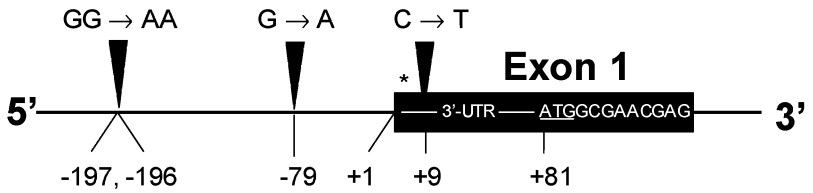


Figure 9. Polymorphisms detected in the *ADH3* promoter region. Schematic picture of the *ADH3* 5′-flanking region including exon 1 and parts of intron 1. ∗ indicates the transcription start site. The various polymorphisms are depicted above the line and their respective location are indicated below.

The effects of the different base exchanges in the *ADH3* promoter were assessed in a luciferase reporter gene system introducing the 5′-flanking region covering bases -323 to +80. Significant decreases in transcription were observed with the C → T exchange at position +9, alone or in combination with the $AA_{-197,-196}$ and A_{-79} alleles (a haplotype observed during the initial sequencing), in HeLa cells. A similar trend was also seen in HepG2 cells although the decrease was not statistically significant. The *ADH3* promoter displays characteristics of a housekeeping gene, i.e. high GC base pair content and absence of TATA or CCAAT boxes

(Hur *et al.*, 1992). However, several tissue specific elements have been located in the 5'-flanking region, which explains the differential expression observed in certain cells and tissues (Estonius *et al.*, 1996; Hedberg *et al.*, 2000; Hur and Edenberg, 1995; Uotila and Koivusalo, 1997). The region -34 to +61, denoted the minimal promoter, promotes high levels of transcription in multiple cell types, which is mainly achieved by binding of the Sp1 transcription factor (Hur and Edenberg, 1995; Kwon *et al.*, 1999). In addition, the structurally related transcription factors Sp3 and Sp4 repress the action of Sp1, demonstrating a complex interplay between various transcription factors in the regulation of *ADH3* (Kwon *et al.*, 1999). Interestingly, the $C_{+9}\rightarrow T_{+9}$ coincides with one of the proposed Sp1 binding sites in the minimal promoter of *ADH3*. Electrophoretic mobility shift assays with probes carrying the polymorphic base and nuclear extracts from HeLa cells confirmed the binding of nuclear proteins to that region, and further suggest that the C_{+9} variant exhibits higher affinity for both Sp1 and Sp3 as compared to the T_{+9} probe. In conclusion, the polymorphisms discovered in this study, especially the $C_{+9}\rightarrow T_{+9}$ transition, indicate that inter-individual differences in the capacity of metabolizing formaldehyde and possibly other ADH3 related toxic compounds are present within certain populations.

Analyses of ADH5 and ADH6

ADHs of higher classes, i.e. ADH5-ADH8, have thus far only been identified from a limited number of species and little is known about their functional properties. Of these enzymes ADH5 and ADH6 have been defined in mammals, whereas ADH7 and ADH8 seem to be avian and amphibian specific forms, respectively. In papers V and VI we have investigated the atypical genomic structure of the human *ADH5* gene and analyzed the molecular properties of that enzyme and a related form in rat previously denoted ADH6.

The complex transcription unit of human *ADH5*

When the structures of the gene and cDNA encoding ADH5 were presented, it was shown that this enzyme differs from other ADHs in exon/intron organisation (Yasunami *et al.*, 1991). The ORF of the isolated transcript was terminated prematurely as compared to other ADHs, but still the cDNA was complete since a 3'-UTR and a poly(A) tail were present. An explanation for this appeared in the gene structure where only eight exons were found and the eighth terminal exon displayed both a termination codon as well as a typical poly(A) signal. Additional screens were conducted to identify a possible ninth exon but yielded only negative

results (Yasunami *et al.*, 1991). In an attempt to further investigate the *ADH5* gene, 3'-ends of ADH5 transcripts were PCR amplified using the RACE technique on an adaptor-ligated human liver cDNA. With this procedure, three different polyadenylated ADH5 transcripts were isolated, of which one corresponded to the previously cloned cDNA. The other two transcripts, differing from each other only in the length of the 3'-UTR, were not truncated as the original ORF, but displayed typical ADH C-terminals with additional codons for eight amino acid residues prior to the stop codon. Sequencing of genomic DNA confirmed the presence of the "missing" ninth exon in the *ADH5* gene and probably the truncated form is a result of alternative polyadenylation by a poly(A)-signal located in intron eight. Figure 10 depicts a schematic representation of the *ADH5* gene and the proposed mechanism by which the different transcripts are generated. In a previously reported northern blot analysis two signals were detected (1.6 and 3.3 kb) after hybridization with an *ADH5* cDNA probe (Estonius *et al.*, 1996). We repeated this analysis with similar results and extended it with oligonucleotide and cDNA probes, specific for both the truncated and full-length transcripts. This enabled us to correlate each signal with the type of transcript it represented. ADH5 transcription is highest in liver, where both types of transcripts are readily detected. However, the now discovered full-length variant seems to be the predominant transcript generated from the *ADH5* gene (fig. 10).

It is tentative from our results to assume that the differential processing of the *ADH5* transcripts represent a regulatory event, since recent studies have shown that regulation of gene expression can be achieved at the level of 3'-end generation (Barabino and Keller, 1999; Edwalds-Gilbert *et al.*, 1997). All known alternative polyadenylation sites in the ADH genes, and the majority of other genes as well, are consequences of tandem arrays of poly(A)-signals located within a single 3'-UTR exon and as a result do not give rise to different polypeptides (Edwalds-Gilbert *et al.*, 1997). For the ADH5 variants reported in this study, together with a number of other genes, the alternative poly(A) site selections are more complex, displaying composite internal/terminal exons, which can be differentially processed (Edwalds-Gilbert *et al.*, 1997). A well known example of such a transcription unit is the immunoglobulin heavy chain that, depending on the cellular environment, produces either secretory-specific or membrane-bound antigen receptor mRNA (reviewed in Barabino and Keller, 1999; Edwalds-Gilbert *et al.*, 1997). In these cases, 3'-end generation is a result of competition between polyadenylation and splicing, where the strength of the poly(A)- and splice sites, the abundance of the components in the splicing and polyadenylation machinery as well as other regulatory elements probably are important factors.

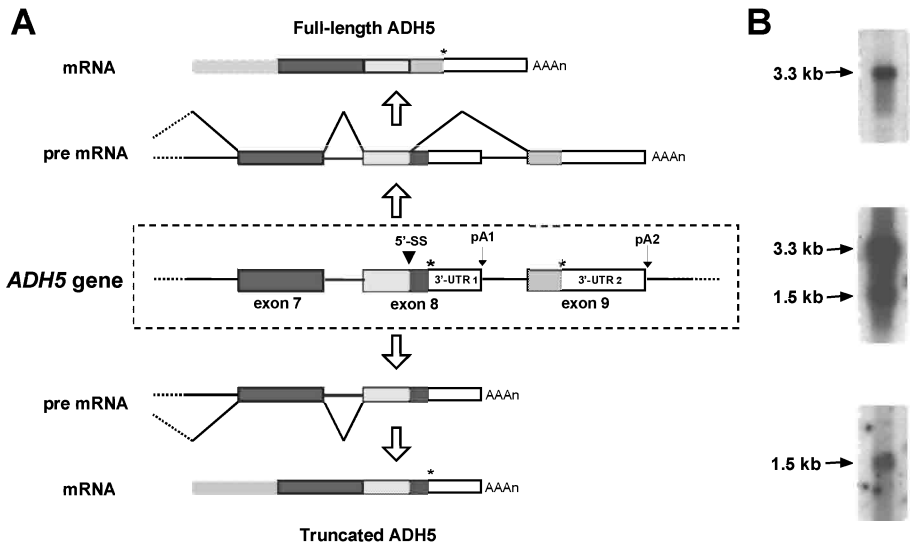


Figure 10. The complex transcription unit of human ADH5. **A**: the 3'-end of the *ADH5* gene is drawn in the centre of the figure and boxes and lines represent exons (and 3'-UTRs) and introns respectively. A 5'-splice site (5'-SS) is indicated in exon 8, which in other ADHs marks the end of this exon, and two different polyadenylation sites denoted pA1and pA2 are included. Asterisks indicate termination codons. **B**: Northern blot analysis of ADH5 transcripts. Top panel, oligonucleotide probe against the full-length variant of ADH5. Only the 3.3 kb band is clearly detected. Middle panel, cDNA probe covering the entire coding region of ADH5. Two transcripts, 3.3 kb and 1.5 kb, were detected in liver and kidney. Bottom panel, A 200 bp cDNA probe against the 3'-UTR of the truncated ADH5 form. Only the 1.5 kb fragment is detected.

Properties of ADH5 and ADH6

A rat cDNA, with the highest sequence identity with the deermouse ADH6 (previously Adh-2, Zheng *et al.*, 1993), was isolated by screening of a rat liver cDNA library with a human ADH5 probe. Total DNA sequence analysis yielded a sequence of 1335 bp, which harbored the entire coding region, 5'- and 3'-UTRs. The rat ADH6 cDNA displays an additional short ORF partly overlapping the main reading frame at the 5'-UTR, which was deduced to give a polypeptide of twelve amino acid residues, including the initiation Met, terminated by a double stop codon. By analogy, in deermouse ADH6 a similar but shorter ORF was observed (Zheng *et al.*, 1993). From alignments with other ADH cDNAs, homologous sequence is seen throughout the main ORF. However, upstream of the corresponding splicing junction of intron one, the ADH6 cDNAs showed insertions with no homology to any other ADH structure. Further upstream of this point, within the short ORF, a high degree of homology is found again. Interestingly, this short 5' ORF translates into amino acid residues identical to rat ADH2 until the point of the first splice site (Höög, 1995; von Bahr-Lindström *et al.*, 1991). The

unusual 5'-ends of the ADH6 cDNAs lead to the termination of the normal ADH ORF from exon one and introduces additional initiator ATG codons in the inserted sequences that elongate the deduced rat amino acid sequence of the main ORF by 13 residues, as compared to ADH1 forms. Without genomic data for rat ADH6 it is hard to know if the origin of these atypical cDNA structures is a result of different RNA processing or a recombination at the genomic level.

The ADH6 structures show the highest positional identity with the human ADH5 (65% and 67% for rat and deermouse, respectively), but the overall low identity classifies them as different classes according to the current nomenclature (Duester *et al.*, 1999; Höög *et al.*, 2001). The identity between the two ADH6 enzymes in contrast, is 79% and clearly indicates that they are of orthologous origin. However, the two rodents are closely related, and other rodent ADH orthologs generally display higher identity, often around 90%. This suggests that ADH6 is extremely divergent and that a human ADH6 structure would display relatively low positional identity with the rodent forms. Further similarities between the ADH5 and ADH6 forms were observed from alignments of different ADH structures (fig. 5). Compared to the ADH1 sequence, human ADH5 and the ADH6 structures contain an insertion around position 60 (corresponding to exon 3), which is also observed in ADH3, and one deletion around position 120 (corresponding to exon 5), also found in ADH4. Furthermore, rat ADH6 but not deermouse ADH6 or human ADH5, displays an additional insertion around position 300. In contrast, two additional ADHs that were recently presented within the mouse genome, named *Adh5a* and *Adh5b* since they were suggested to be of ADH5 type (Szalai *et al.*, 2002), clearly should not be included within the ADH5 class. The mouse "ADH5" sequences display only 49% to 59% positional identity with the other ADH5 and ADH6 structures and differ also with respect to the deletions/insertions common for the ADH5/ADH6 structures (fig. 1 & 5). Interestingly, we found a rat EST similar to the mouse *Adh5b* suggesting that rat expresses six distinct forms of ADH, which has not been shown for any other species.

In general, ADHs can easily be produced in large amounts as soluble heterologously expressed protein in *E. coli.* However, previous attempts to produce recombinant ADH5 (with the truncation at the C-terminal, see above) have been unsuccessful (Chen and Yoshida, 1991). By analogy, we experienced the same with both human ADH5 (truncated and full-length) and rat ADH6 using the same vectors and conditions as for other mammalian ADHs. However, fusing these ADHs with GST resulted in strong immunoreactive signals from the insoluble pellet. After refolding insoluble proteins from inclusion bodies, we were able to produce milligram amounts of ADH-GST fusion proteins of both human ADH5 and rat

ADH6, which were readily purified with GST affinity chromatography. The purified proteins were not stable without the GST tag and no activity could be monitored with ethanol, octanol or benzyl alcohol. The instability and lack of activity could be a result of an incorrect fold of the ADH part of the fusion protein. Nevertheless, the purified fusion proteins could be used to raise antiserum in rabbits against human ADH5 and rat ADH6 (fig. 11). Functional ADH5 protein is possible to produce in *in vitro* translation experiments using rabbit reticulocyte lysate (Chen and Yoshida, 1991). Accordingly, we were also able to produce rat ADH6 *in vitro*. No obvious interference of expression is seen from the short 5' ORF, as deduced from mutagenesis studies, and efficient translation is also seen if the first initiator codon of the main reading frame is deleted. In addition ADH6-GFP fusion proteins could be readily expressed in COS cells, which taken together indicate that soluble ADH5 and ADH6 protein could be produced in mammalian cells.

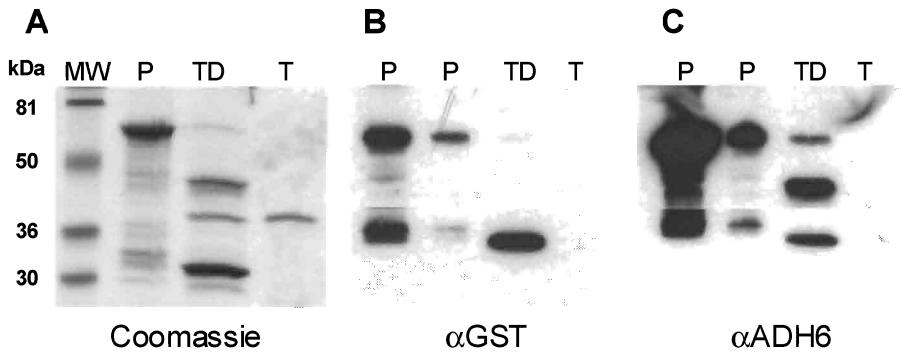


Figure 11. Purification of recombinant rat ADH6 and generation of antiserum. **A**: Coomassie brilliant blue stained SDS-PAGE; **B**: Immunoblot using antiserum against GST; **C**: Immunoblot using antiserum against ADH6-GST fusion protein. MW is a pre-stained molecular weight marker (molecular weights in kDa to the left), P is the ADH6-GST fusion protein purified on GSH-sepharose, TD is a thrombin digest of P, T is 5 units of thrombin.

In conclusion, our studies on ADH5 and ADH6 indicate that these enzymes are in fact members of the same class. This would order the mammalian ADH family as follows: ADH3, ADH4, ADH1, ADH2 and ADH5 by a gradual increase of divergence and evolutionary speed. This is interesting from a perspective of molecular evolution. Obviously, functional similarities are less common in the highly diverged classes, as previously shown for ADH2, than for the constant ADH3 enzymes. Still, the functional properties of the ADH5/ADH6 forms need to be analyzed in order to understand the physiological role of these enzymes.

Concluding remarks and outlook

The vertebrate ADH system displays extensive multiplicity at several levels. At the level of class divergence, six mammalian classes of ADH have been established, but current studies indicate that yet more ADH enzymes exist, e.g. the so called ADH5 forms in mice (Szalai *et al.*, 2002), and possibly also the additional vertebrate ADH classes, ADH7 and ADH8, are represented in mammals as well. Previously, no more than five structurally distinct forms have been identified within a single species. However, the presence of a rat EST mentioned above (paper VI), suggests that this species expresses six classes of ADH. The complete human genome surveys indicate that humans only possess seven functional ADH genes of five classes (Nordling *et al.*, 2002a). In addition to the separate classes, subgroups exist within some classes, such as the rodent forms of ADH2 (paper I and II). Late duplications have lead to the occurrence of isozymes in certain species. Principally, the horse E and S, and human ADH1A-C subunits of ADH1 have been thoroughly investigated, but recently, isozymic forms of ADH2 were also identified and characterized in rabbit (Svensson *et al.*, 1998). Inter-individual multiplicity is seen in polymorphic ADH genes. The "classical" ADH1 allelozymes have been associated with different pathological conditions and, in addition, novel polymorphisms are continuously being reported. We characterized allelic variants of the *ADH2* and *ADH3* genes that affected protein stability and gene expression, respectively (papers III and IV), and an interesting non-sense mutation in ADH1C has been reported but not yet evaluated (Buervenich *et al.*, unpublished). At the level of mRNA processing, we have presented the first evidence that alternative splicing occurs during the transcription of mammalian ADH genes (paper V), which opens up a new dimension of ADH multiplicity. Finally, with the recent advances in *proteomics*, one can expect that even further multiplicity will be detected also at the protein level.

With the discoveries of novel ADH forms, reported both here and elsewhere, the main bottleneck in the future will be to characterize and determine the physiological significance of these variants. At present, many ADHs are still "orphan" enzymes with respect to natural substrates and metabolic roles. Considering the perspectives in the post-genomic era where a whole genome can be sequence analyzed in a matter of weeks, the actual analysis of individual genes and gene products is still time-consuming and laborious. Most likely, novel high-throughput techniques will enable large samples to be screened at astonishing speed, but in order to get the complete picture, classical and more rigorous experiments need to be performed.

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