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Function, Expression and Polymorphism of Human Alcohol Dehydrogenase 3/Glutathione-Dependent Formaldehyde Dehydrogenase

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To all members of my family, present and gone

Abstract

Alcohol dehydrogenase 3 (ADH3), identical to glutathione-dependent formaldehyde dehydrogenase, plays a vital role in the defense against formaldehyde through the activity for the spontaneously formed adduct between formaldehyde and glutathione, S-hydroxymethylglutathione (HMGSH). ADH3 may also participate in the metabolism of S-nitrosylated glutathione, GSNO.

To further investigate substrate enzyme interactions, Arg115 which has been shown to be crucial for HMGSH binding through charge interactions, was mutated into Ser or Lys. Both ADH3 mutants showed reduced activities for HMGSH and GSNO while only the ADH3-Arg115Ser mutant showed reduced activity for the model substrate 12-hydroxydodecanoate. Mainly, changes in activities were due to increased K_m values. The decreased HMGSH and GSNO activities of the ADH3-Arg115Lys mutant indicate that not only a positive charge but also an exact positioning of the substrate is necessary for efficient catalysis. In addition, an attempt was made to introduce ADH3 characteristics in an ADH1 enzyme through the generation of the double mutant ADH1C2-Leu57Asp/Asp115Arg. This was partly successful since ADH3-like kinetic features for 12-hydroxydodecanoate and ethanol was monitored. However, the ADH1C2-Leu57Asp/Asp115Arg displayed no HMGSH activity. GSNO was efficiently reduced by the human ADH3 with NAD^+ as preferred coenzyme. By electrospray tandem mass spectrometry the major products of GSNO reduction were identified as glutathionesulfinamide and GSSG. We speculate that ADH3 catalyzed GSNO reduction takes place *in vivo*.

The potential enzymatic defense by ADH3 was characterized in oral buccal tissue specimens and oral epithelial cell lines using RNA hybridization and immunological methods as well as enzyme activity measurements. From mRNA and protein distribution profiles in the intact tissue, substantiated by *in vitro* experiments, an association of ADH3 mRNA was observed primarily to proliferative keratinocytes while the protein was retained during the entire keratinocyte life span in oral mucosa. Substantial capacity for formaldehyde detoxification was shown from quantitative assessments of alcohol and aldehyde oxidizing activities including K_m-determinations, demonstrating that ADH3 is the major enzyme involved in formaldehyde oxidation in oral mucosa. The expression of ADH3 in epithelial *in vitro* model systems, i.e., monolayer cultures and regenerated epithelia with normal and transformed epithelial cell lines, were characterized. Similar

ADH3 expression levels among the various cell lines and tissue like protein distribution in regenerated epithelia indicate preservation of ADH3 during malignant transformation and functionality of the transformed cell lines as *in vitro* models for studies of formaldehyde metabolism in human oral mucosa.

A screen for allelic variants of ADH3 revealed four possible base pair exchanges in the promoter region: $GG_{-197,-196} \rightarrow AA$, $G_{-79} \rightarrow A$ and $C_{+9} \rightarrow T$. The $AA_{-197,-196}$ allele was relatively common among Chinese, Spaniards and Swedes while the presence of the A_{-79} allele was restricted to Spaniards and Swedes. The T_{+9} allele was found only among Swedes with a frequency of 1.5 %. Promoter activity assessments and electrophoretic mobility shift assays demonstrated that the $C_{+9} \rightarrow T$ exchange resulted in a significant transcriptional decrease in HeLa cells and possibly also a decreased binding of nuclear proteins.

In summary, the finely tuned substrate specificity of ADH3 offers an enzymatic defense against both formaldehyde and nitrosative stress. In human oral tissue as well as in human epithelial cell lines, ADH3 serves as the prime guardian against formaldehyde. Finally, the *ADH3* gene is polymorphic which might influence the protective capacity in certain individuals.

List of original papers

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 **Hedberg, J.J.**, Strömberg, P. & Höög, J.-O. (1998) An attempt to transform class characteristics within the alcohol dehydrogenase family. *FEBS Lett*. 436, 67-70

II **Hedberg, J.J.**, Griffiths, J.W., Nilsson, S.J.F. & Höög, J.-O. (2001) Reduction of S-nitrosoglutathione by human alcohol dehydrogenase 3. *Manuscript*

III **Hedberg, J.J.**, Höög, J.-O., Nilsson, J.A., Xi, Z., Elfwing, Å. & Grafström, R.C. (2000) Expression of alcohol dehydrogenase 3 in tissue and cultured cells from human oral mucosa. *Am. J. Path*. 157, 1745-1755

IV **Hedberg, J.J.**, Hansson, A., Nilsson, J.A., Höög, J.-O. & Grafström, R.C. (2001) Uniform expression of alcohol dehydrogenase 3 in epithelia regenerated with cultured normal, immortalized and malignant human oral keratinocytes. *ATLA* 29, in press

V **Hedberg, J.J.**, Backlund M., Strömberg, P., Lönn, S., Dahl, M.-L., Ingelman-Sundberg, M. & Höög, J.-O. (2001) Functional polymorphism in the alcohol dehydrogenase 3 (*ADH3*) promoter. *Submitted for publication*

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Contents

Abbreviations

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
DNA	deoxyribonucleic acid
FPLC	fast protein liquid chromatography
GSH	glutathione, reduced
GSNO	S-nitrosoglutathione
GSSG	glutathione, oxidized
12-HDA	12-hydroxydodecanoic acid
HMGSH	S-hydroxymethylglutathione
MCA	methylcrotyl alcohol
MDR	medium-chain dehydrogenases/reductases
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism

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

Background

General Introduction

The conversion of various metabolites and toxic compounds are essential processes in all life forms. Examples of such reactions are the enzymatic conversions of alcohols and aldehydes catalyzed by enzymes belonging to the medium-chain dehydrogenases/reductase family (MDR). These enzymes have evolved through a series of gene duplications resulting in a complex pattern of enzyme families, enzyme classes and isozymes (Jörnvall, 1985; Jörnvall *et al.*, 1987; Jörnvall *et al.*, 1999). The MDR family includes alcohol dehydrogenases, sorbitol dehydrogenases, glucose dehydrogenases, threonin dehydrogenases, quinone reductases, enoyl reductases and ζ-crystalins. The enzyme mainly covered in this thesis, alcohol dehydrogenase 3 (ADH3) also known as glutathione-dependent formaldehyde dehydrogenase, belongs to the alcohol dehydrogenase family (ADH; EC 1.1.1.1.), suggested to have protective metabolic functions (Danielsson & Jörnvall, 1992). In general, mammalian ADHs are cytosolic dimeric metalloenzymes with typically ~375 amino acids, two zinc atoms and a molecular mass of ~40 kDa per subunit. Each subunit is comprised of two domains, denoted the catalytic and the coenzyme binding domain, between which a hydrophobic cleft forms the active site pocket (Fig. 1; Eklund *et al.*, 1976; Yang *et al*., 1997).

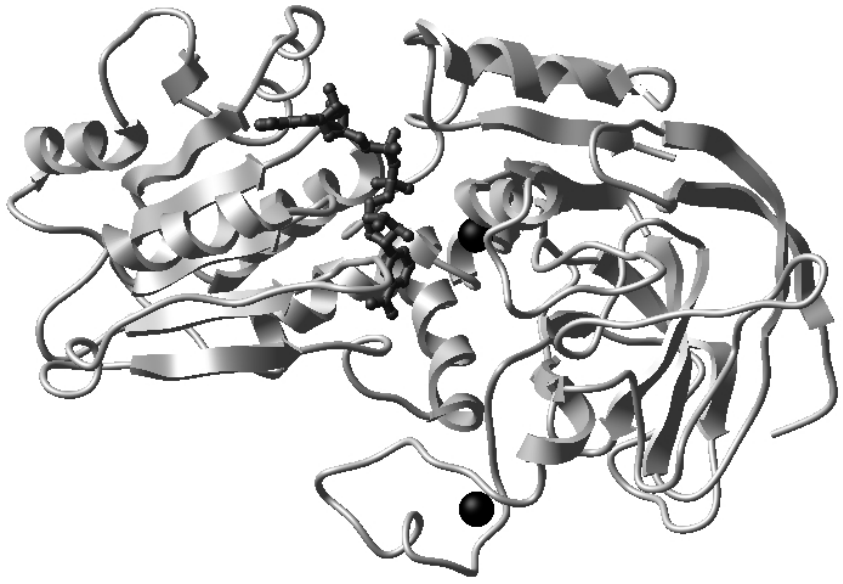
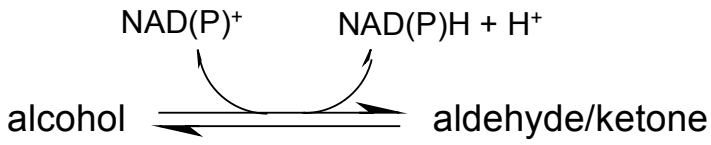


Figure 1. Three dimensional structure of one of the ADH3 subunits in its binary complex, i.e., with NAD^+ bound (Yang *et al.*, 1997). The coenzyme binding domain is to the left and the catalytic domain to the right. NAD^+ and the zinc atoms are depicted in black.

All ADHs are capable, with varying efficiencies, to oxidize or reduce alcohols or aldehydes, respectively, with the concomitant conversion of coenzyme, which commonly is NAD^+/NADH and less commonly $NADP^+$/NADPH (von Wartburg *et al.*, 1964; Uotila & Koivusalo, 1974a; Peralba *et al.*, 1999):



ADH3 display many biochemical and biophysical properties that are common within the ADH family although some clear differences are obvious. These are primarily an ubiquitous expression, highly conserved structure, unique reaction mechanism and the activity for formaldehyde in conjugation with glutathione (GSH), i.e., S-hydroxymethylglutathione (HMGSH) as well as the activity for the S-nitrosothiol of GSH, i.e., S-nitosoglutathione (GSNO).

The Nomenclature of Alcohol Dehydrogenases, a Subject Still Under Debate

Different but closely related forms of ADHs, characterized as isozymes, were early identified in human (Smith *et al.*, 1971). As the ADH family was further enlarged by new discoveries of additional forms of ADHs the concept of classes was introduced (Vallee & Bazzone, 1983). Criteria on which the classes have been based include substrate specificity, sensitivity to 4-methyl pyrazole inhibition, electrophoretic peptide mapping patterns, cross hybridization properties, sequence alignments, phylogenetic tree analysis, or simply order of discovery (Jörnvall *et al.*, 1987; Jörnvall & Höög, 1995; Duester *et al.*, 1999; Edenberg, 2000). Like all systems with multiple components, the complexity increased as new enzymes were discovered and different nomenclatures were applied by different authors. This thesis is written after the publishing of a new nomenclature proposal (Table 1; Duester *et al.*, 1999) but during a time period when the nomenclature is still under debate (for detailed information on the debate see http://www.gene.ucl.ac.uk/nomenclature/ADH.shtml).

Naturally, one problem with changing the nomenclature is that it may result in confusions when referring to earlier literature. Some authors argue that the old nomenclature is confusing since the names do not reflect to which class some ADH enzymes belong (cf.

ADH1, ADH2 and ADH3 versus ADH1A, ADH1B and ADH1C) and in addition, various species homologs do not have names that reflect their inter-species relationships. Therefore, the challenging task is to find a system that, correlates class belongings and species homologs in an understandable way and as well makes citations to 25 years of literature reasonably easy. However, since a consensus have not yet been reached, the nomenclature used in this thesis will follow the most recently published (Table 1; Duester *et al.*, 1999).

Table 1.Nomenclature of the Human[a] Alcohol Dehydrogenases

	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
		ADH3P1[d]		ADH5P1
IV	ADH4	*ADH4*	σ or μ	ADH7
V	-	*ADH5*	-	ADH6

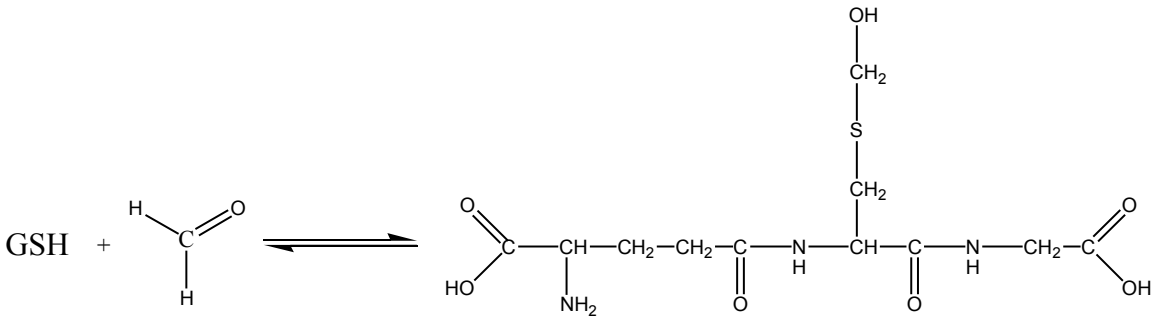
[a] Although ADHs from several other species are discussed in this thesis only the human ones are presented in this table.
[b] Based on nomenclature recommended in (Duester *et al.*, 1999).
[c] Based on nomenclature recommended in (Jörnvall & Höög, 1995).
[d] ADH3 related pseudogene.

Proteins from different classes display about 60% sequence identity at the protein level and isozymes display 90% identity or more (Jörnvall *et al.*, 1987; Duester *et al.*, 1999). Further multiplicity of the ADH family relies on the fact that several of the ADH genes are polymorphic (see later chapter in this thesis). Substrate repertoires for the various classes are in many cases overlapping although some activities are class unique.

ADH3 also known as Glutathione-Dependent Formaldehyde Dehydrogenase

An oxidative activity for formaldehyde, dependent on the presence of GSH, was early described in various tissue homogenates (Strittmatter & Ball, 1955). The enzyme responsible for this activity, GSH-dependent formaldehyde dehydrogenase was purified to homogeneity and characterized some twenty years later (Uotila & Koivusalo, 1974a). In contrast to the earlier assumption, that formate was the first and only product, it was

shown that the enzymatically formed product was S-formylglutathione, generated through the highly specific oxidation of the thiohemiacetal between formaldehyde and GSH, i.e., HMGSH. This molecule is spontaneously formed from formaldehyde and GSH (K_{eq}=1.77 mM at pH 7.5; Fig. 2; Uotila & Koivusalo, 1974a; Sanghani *et al.*, 2000).



Formaldehyde S-hydroxymethylglutathione

Figure 2. S-hydroxymethylglutathione (HMGSH) is spontaneously formed at physiological conditions from formaldehyde and glutathione (Uotila & Koivusalo, 1974a; Sanghani *et al.*, 2000).

During the purification procedure, a previously undetected enzyme, S-formylglutathione hydrolase was detected, capable of catalyzing the hydrolysis of S-formylglutathione to formate and GSH (Uotila & Koivusalo, 1974b). Thus, it was concluded that formaldehyde can be oxidized in two steps, catalyzed by two separate enzymes with no net consumption of GSH.

Parallel to the characterizations of GSH-dependent formaldehyde dehydrogenase, additional classes of alcohol dehydrogenases were discovered, in particular a third form of human alcohol dehydrogenase defined as class III ADH (Parés & Vallee, 1981). This ADH displayed similar overall domain properties as other ADHs but differed in having a "peculiar" substrate repertoire (Wagner *et al.*, 1984). The enzyme was insensitive to 4-methylpyrazole inhibition, unsaturable with ethanol but oxidized alcohols with longer carbon chains relatively efficiently. Similar enzymes were purified from other species (Dafeldecker *et al.*, 1981; Julia *et al.*, 1987). It was suggested that the human enzyme could function as an ω-hydroxy fatty acid dehydrogenase (Wagner *et al.*, 1984; Giri *et al.*, 1989b) but the exact physiological role remained an enigma. In addition, this ADH was shown to be highly conserved implying an important physiological role (Kaiser *et*

al., 1989). Later investigations revealed that GSH-dependent formaldehyde dehydrogenase and class III ADH were identical in both rat and human (to date known as ADH3; Koivusalo *et al.*, 1989; Holmquist & Vallee, 1991), and the enigma of the alcohol dehydrogenase "without natural substrate" was thereby solved. The stability of both activity and structure through evolution was further delineated to cover the entire vertebrate line and ADH3 was concluded to be the ancestral form of ADH, from which all other vertebrate ADHs have evolved (Danielsson & Jörnvall, 1992; Jörnvall *et al.*, 2001). The importance of ADH3 is further illustrated by its existence in all species investigated including simian (Dafeldecker *et al.*, 1981), bovine (Strittmatter & Ball, 1955; Pourmotabbed *et al.*, 1989), rabbit (Keung *et al.*, 1995), rat (Julia *et al.*, 1987), hamster (Keung, 1996), chicken (Strittmatter & Ball, 1955), amphioxus (Canestro *et al.*, 2000), cyclostome (Danielsson *et al.*, 1994b), drosophila (Danielsson *et al.*, 1994a), arabidopsis (Dolferus *et al.*, 1997), pea (Uotila & Koivusalo, 1979), rice (Dolferus *et al.*, 1997), baker´s yeast (Wehner *et al.*, 1993) and *E. coli* (Gutheil *et al.*, 1992). A recent scan of completed genome sequences also confers the ubiquitous existence of ADH3 (Jörnvall *et al.*, 1999).

Several residues lining the active site pocket of ADH3 are highly conserved including, Asp57 and Arg115. These two residues have been shown to have central roles in the binding of charged substrates and activators, e.g., HMGSH, 12-hydroxydodecanoic acid (12-HDA) and pentanoate (Engeland *et al.*, 1993; Holmquist *et al.*, 1993; Estonius *et al.*, 1994). Arg115 interacts electrostatically with the carboxylate group of the glycine residue in the GSH moiety and Asp57 interacts with the α-amino group of the γ-glutamyl residue (Yang *et al.*, 1997). The sub-optimal oxidation of short-chain alcohols such as ethanol or methylcrotyl alcohol (MCA), have been shown to be activated by the addition of hydrophobic anions, e.g., octanoate or pentanoate (Moulis *et al.*, 1991). Most likely, these hydrophobic anions electrostatically bind to Arg115 positioned at the substrate binding pocket. Thereby, the active site pocket becomes smaller and more hydrophobic and substrate binding is substantially enhanced. Optimal activation is obtained when the additive length of substrate and activator is 9-10 carbons.

Recently, the S-nitrosothiol of GSH, GSNO (Fig. 2), was shown to be reduced by rat ADH3 with NADH or NADPH as coenzyme (Jensen & Belka, 1997; Jensen *et al.*, 1998). Notably, this reaction is in the reverse direction as compared to the oxidation of HMGSH.

The activity for GSNO have been extended to include ADH3 homologs found in mouse, yeast and *E. coli* (Liu *et al.*, 2001) as well as in humans (paper II). Furthermore, deletion of the *ADH3* gene in yeast and mice demonstrates that ADH3 may also regulate the intracellular level of protein S-nitrosothiols.

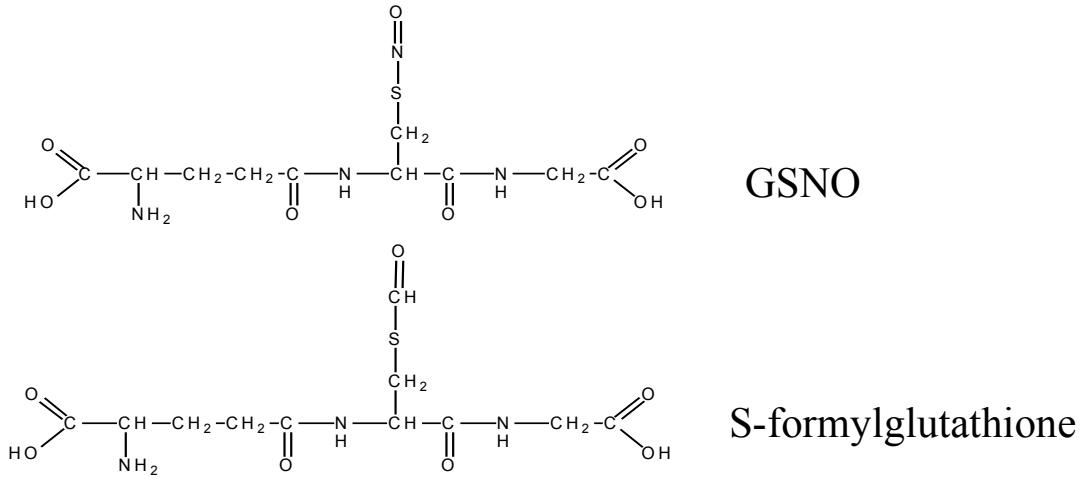


Figure 3. The similarities between the structures of GSNO and S-formylglutathione are striking.

In addition to the high activity for HMGSH and GSNO, ADH3 also display activity for primary alcohols (Wagner *et al.*, 1984). Methylglyoxal and some α-ketoaldehydes, e.g., hydroxypyruvaldehyde and ketoxal have been shown to be substrates for ADH3 in a GSH-dependent manner (Uotila & Koivusalo, 1983). Further, 20-hydroxy-leukotriene B^4 and ω-hydroxy fatty acids have been shown to be a substrates for ADH3 (Giri *et al.*, 1989b; Gotoh *et al.*, 1989; Gotoh *et al.*, 1990). Additional thiols such as Cys-Gly and Cys can function as alternatives to GSH (Holmquist & Vallee, 1991). ADH3 has even been proposed to participate in the formation of certain thiol ester products (Uotila & Koivusalo, 1983). The catalytic efficiencies for these substrates are however much lower than for both HMGSH and GSNO and the physiological relevance of these activities is unclear.

Other Classes of Alcohol Dehydrogenase

ADH1 is often referred to as the "classical" liver ADH. It was the first human ADH to be purified (von Wartburg *et al.*, 1964) and on the basis of a high abundance in liver together with a high activity for ethanol, ADH1 constitutes the major activity for metabolizing ingested ethanol. In human, three separate gene loci encodes the isozymes

ADH1A-C (Table 1; earlier named α-, β- and γ-subunits) which readily forms heterodimers (Smith *et al.*, 1971; Hempel *et al.*, 1985). The ADH1 isozymes generally display K_m values for ethanol ranging from 0.05 to 4 mM (except for the allelic variant ADH1B3, K_m: 36 mM) and they are sensitive to 4-methylpyrazole inhibition (von Wartburg *et al.*, 1964; Wagner *et al.*, 1983; Edenberg & Bosron, 1997). Besides a protective role through the activity for primary and secondary alcohols, the various ADH1 isozymes have activity for several endogenous compounds including ω-hydroxylated fatty acids (Björkhem, 1972), norepinephrine and dopamine metabolites (Mårdh *et al.*, 1985; Mårdh & Vallee, 1986), serotonin metabolites (Consalvi *et al.*, 1986), retinoids (Yang *et al.*, 1994), bile acids (Okuda & Okuda, 1983; Marschall *et al.*, 2000) and steroids (McEvily *et al.*, 1988).

ADH2 was first purified from human liver as an enzyme with high K_m for ethanol and insensitivity to pyrazole inhibition (Li *et al.*, 1977). These kinetic parameters were refined for the recombinantly expressed and characterized enzyme (Svensson *et al.*, 1999). ADH2 also harbors activity for serotonin metabolites (Consalvi *et al.*, 1986) and norepinephrine metabolites (Mårdh *et al.*, 1986) as well as retinoids (Yang *et al.*, 1994). ADH2 shows high evolutionary divergence as compared to the other ADHs (Svensson *et al.*, 1998; Svensson *et al.*, 1999) and considering its relatively high reductive activity for aldehydes and *p*-bensoquinones, the latter being a class specific feature, it has been suggested that ADH2 might have evolved for these purposes (Svensson *et al.*, 1999).

ADH4 is commonly known as the “stomach ADH”. It was purified as an enzyme with high K_m and k_{cat} for ethanol (Yin *et al.*, 1990; Moreno & Parés, 1991) and on the basis of its high expression in epithelia it has been postulated to contribute to first-pass metabolism of ethanol (Seitz *et al.*, 1993). ADH4 has the highest activity for retinoids among the ADHs (Yang *et al.*, 1994) and its expression pattern coincides with retinoic acid production implying a role in the production of this morphogen during embryogenesis (Ang *et al.*, 1996a; Haselbeck *et al.*, 1997a; Haselbeck *et al.*, 1997b). Furthermore, a lowered retinoic acid production and increased number of stillbirths during vitamin A deficiency in ADH4 -/- mice, demonstrates the importance of ADH4 when vitamin A is low in the diet (Deltour *et al.*, 1999b; Deltour *et al.*, 1999a). The role of ADH4 as a retinol dehydrogenase in the adult animal, under “normal” conditions, is however left to be revealed.

ADH5 has so far not been isolated at the protein level. At cDNA/RNA level, it was first identified as a truncated variant of ADH, missing the C-terminal eight amino acids encoded by the ninth and last exon (Yasunami *et al.*, 1991), as compared to the otherwise conserved gene structure among ADHs. Later, a ninth exon was discovered and it was shown that the enzyme has a complex transcription pattern resulting in three different mRNA species, two forms coding for a "full length" protein harboring all nine exons and one truncated form coding for exons one to eight (Strömberg & Höög, 2000). There is limited information on the activity of ADH5, however *in vitro* translation yielded an enzyme with relatively high K_m for ethanol (Chen & Yoshida, 1991).

ADH6 has only been identified in rodents (Zheng *et al.*, 1993; Höög & Brandt, 1995) and separation of tissue homogenates in native gels followed by activity staining suggests a broad substrate specificity (Zheng *et al.*, 1993). ADH7 has only been detected in chick (Kedishvili *et al.*, 1997). In addition to ethanol oxidizing capacity, this enzyme also displays activity for retinoids and steroids indicating physiological roles in the metabolism of these compounds (Kedishvili *et al.*, 1997; Duester, 2000). ADH8 has up to date only been isolated from frog and it is the sole vertebrate ADH with $NADP^+$/NADPH as preferred coenzyme (Peralba *et al.*, 1999). The enzyme displasy reductive capacity for retinal and has been proposed to participate in the uptake and storage of vitamin A (Peralba *et al.*, 1999; Duester, 2000).

Formaldehyde, an Ubiquitous Chemical with both Endogenous and Exogenous Origin

Formaldehyde is an ubiquitous compound generated from a number of both endogenous and exogenous sources. Hence, humans are continuously exposed to this reactive agent which forms reversible adducts with amides, amines, hydroxyls and thiols (IARC, 1995). The largest contributor to endogenous formaldehyde appears to be the metabolism of serine and glycine as well as nucleotide metabolism, since glycine is a direct precursor in the synthesis of purines (Neuberger, 1981; Uotila & Koivusalo, 1989). These metabolic reactions involve N^5,N^{10}-methylene-tetrahydrofolate as cofactor which is in nonenzymatic equilibrium with free formaldehyde. N^5,N^{10}-methylene-tetrahydrofolate is also known as "active formaldehyde" (Neuberger, 1981). Conversions of other metabolites may also contribute to the generation of formaldehyde, these include sarcosine, choline,

methionine and homoserine (Uotila & Koivusalo, 1989 and references therein). Cytochrome P450 dependent demethylation of glycerol have also been shown to produce formaldehyde *in vitro* (Winters *et al.*, 1988; Winters & Cederbaum, 1990; Rashba-Step *et al.*, 1994). Deamination of epinephrine produces methylamine, which can be converted to formaldehyde and hydrogen peroxide by semicarbazide-sensitive amine oxidase in endothelial cells (Yu & Zuo, 1996; Yu *et al.*, 1997). Hypothetically, chronic stress may result in elevated levels of circulating formaldehyde. Further, treatment with various xenobiotics, including antitumor drugs, have been shown to generate formaldehyde, primarily through demethylation reactions by cytochrome P450 (Gillette, 1966; Kato *et al.*, 2000).

As our world have become more industrialized the number of exogenous sources of formaldehyde have increased dramatically. The most prominent factors that formaldehyde emanates from are automotive emissions and tobacco smoke (IARC, 1995), the latter also containing nitrosamines which in turn can be demethylated to yield additional formaldehyde (IARC, 1986). Other sources include tissue fixatives, food and cosmetic preservatives as well as certain dental materials. Methanol poisoning is known to result in the production of formaldehyde through oxidation by both hepatic and extra hepatic ADHs.

Detection of free formaldehyde have proven difficult. However, estimates of total formaldehyde content in biological fluids have demonstrated levels of 0.05-0.5 μmol/g wet weight (corresponding to up to 500 μM; Heck *et al.*, 1982), a surprisingly high value. The larger part of this formaldehyde is presumed to be in the form of thiol adducts, a significantly smaller part as circulating free formaldehyde and a minor part as “active formaldehyde” (Uotila & Koivusalo, 1989).

Formaldehyde is a Toxic Compound

Since formaldehyde is a highly electrophilic compound, exogenous formaldehyde will be absorbed and metabolized at the first site of contact. Several studies have demonstrated that formaldehyde exposure induces cytogenic effects in rats, manifested as DNA-protein cross links (Casanova & Heck, 1987; Heck & Casanova, 1987) and squamous cell carcinoma (Swenberg *et al.*, 1980) in the nasal epithelium. Humans exposed to formaldehyde, notably well below permissible limits, show many-fold increased genetic

damage in the form of micro nuclei in both nasal and olfactory mucosa (Ballarin *et al.*, 1992; Suruda *et al.*, 1993). Evidently, the target tissue of formaldehyde exposure is dependent on the rout of inhalation. *In vitro* assessments of formaldehyde toxicity have demonstrated loss of membrane integrity, decreased colony forming efficiency, decreased clonal growth rate, DNA-protein cross-links, DNA single-strand breaks and impaired DNA repair mechanisms (Grafström *et al.*, 1983; Ku & Billings, 1984; Grafström, 1990; Nilsson *et al.*, 1998). Furthermore, it has been shown that other chemical and physical mutagenic agents in combination with non-genotoxic concentrations of formaldehyde result in synergistic genotoxic effects (Grafström *et al.*, 1985; Grafström *et al.*, 1993). Based on the above findings, including epidemiological studies (IARC, 1995), formaldehyde is considered as a probable human carcinogen. The defense against this reactive agent is clearly of great importance.

Metabolism of Formaldehyde

Primarily, formaldehyde can be metabolized by two different enzymes systems (Fig. 4). These are ADH3 and low-K_m aldehyde dehydrogenases (ALDHs) of class 1 ("classical" cytosolic ALDH) and class 2 (mitochondrial ALDH), the latter two having affinity for free formaldehyde. Purified low-K_m ALDHs display K_m values in the range of 0.5 mM (Mukerjee & Pietruszko, 1992). Notably, this value is much higher than the K_m displayed by ADH3 for HMGSH, 4 µM (Uotila & Koivusalo, 1974a). The expression levels of ADH3 and low-K_m ALDHs have been demonstrated to vary significantly between tissues (Casanova-Schmitz *et al.*, 1984) indicating that the two pathways may contribute to different degrees in different tissues. Accordingly, there are some contradicting studies on the contributions of ADH3 and low-K_m ALDHs to formaldehyde metabolism. In rat hepatocyte lysates, ADH3 and low-K_m ALDHs have been proposed to equally contribute to formaldehyde metabolism (Dicker & Cederbaum, 1984b; Dicker & Cederbaum, 1984a). Notably, these experiments were conducted with high formaldehyde concentrations, ranging from 0.1 to 1 mM. Since GSH concentrations is known to be high in most tissues (1-10 mM; Kosower & Kosower, 1978), even small amounts of formaldehyde will be effectively bound as HMGSH, favoring the ADH3 pathway. Furthermore, GSH depletion have been shown to impair the formaldehyde metabolism (Jones *et al.*, 1978) and studies on ADH3-/- mice demonstrate a significant decrease in

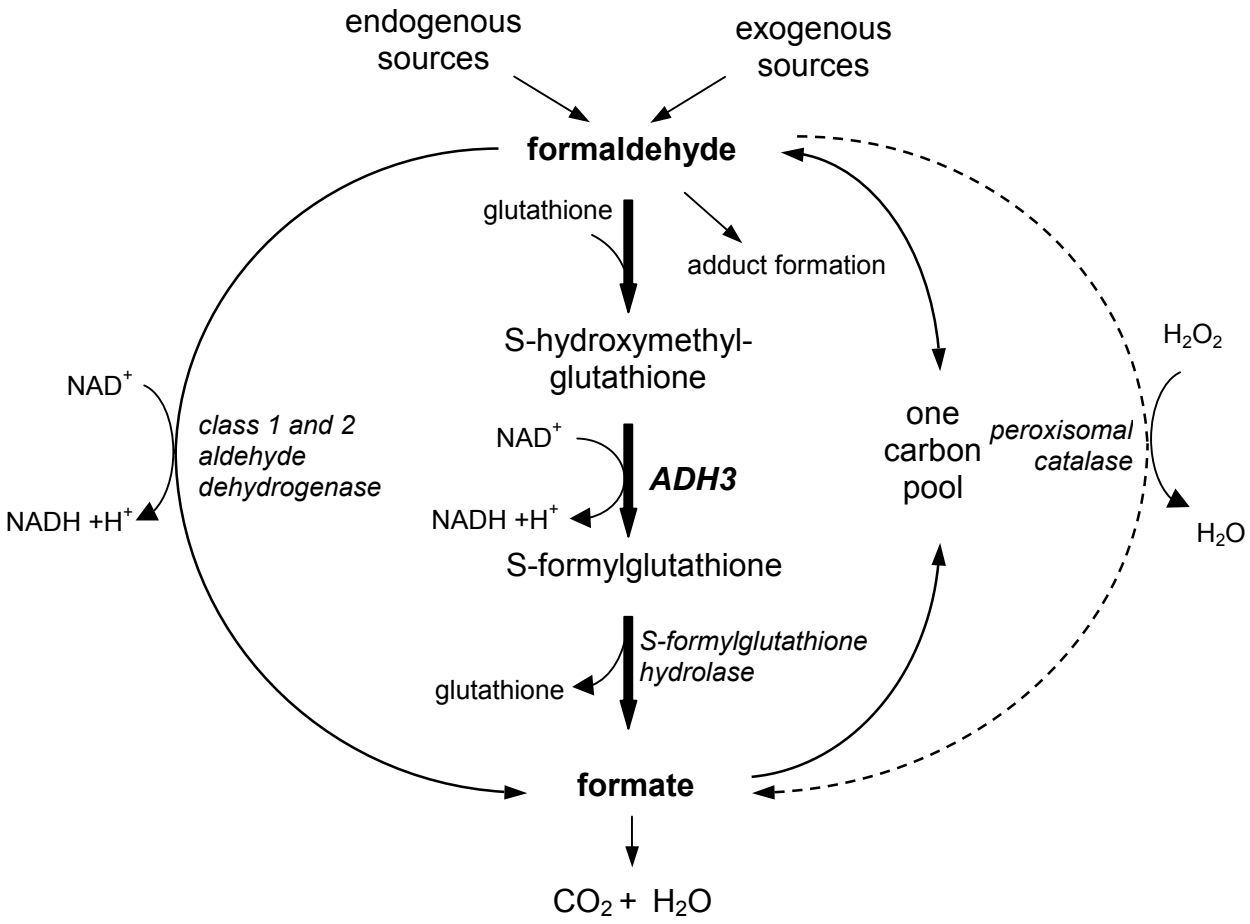


Figure 4. Fate and metabolism of formaldehyde.

LD_{50} values for formaldehyde exposure (Deltour *et al.*, 1999b). Formaldehyde oxidation as well as the induction of DNA-protein cross links display non-linear characteristics dependent on concentrations, in a manner possible to correlate to a saturation profile for ADH3 (Casanova & Heck Hd, 1987; Heck Hd & Casanova, 1987). Dicker *et al.*, suggested the idea that in tissues where both enzyme systems are present, ADH3 is the predominant enzyme responsible for formaldehyde oxidation at low to moderate levels while low-K_m ALDHs contribute increasingly as formaldehyde concentrations are elevated (Dicker & Cederbaum, 1986). Hence, excluding extreme exposure levels, ADH3 is probably the prime guardian against formaldehyde. Catalase have also been postulated to contribute to formaldehyde metabolism (Uotila & Koivusalo, 1989). However this pathway is probably of minor importance due to the need for a continuous supply of H_2O_2. Activity measurements in various tissues have demonstrated that S-formylglutathione hydrolase activities are generally 2-3 orders of magnitude higher than

the ADH3 activities (Uotila & Koivusalo, 1997), resulting in that the activity of ADH3 is most likely the rate limiting step in the oxidation of HMGSH.

GSNO is Probably Formed Endogenously but is Rapidly Metabolized

The biological action and storage of NO commonly involves nitrosylation of different thiols (Stamler *et al.*, 1992; Stamler, 1994). Since GSH is by far the most abundant thiol within cells, GSNO have been postulated to be produced under physiological conditions (Kharitonov *et al.*, 1995; Gow *et al.*, 1997; Tsikas *et al.*, 2000). GSNO have indeed been detected in human airways (Gaston *et al.*, 1993) but otherwise the detection of this compound have proven difficult under "normal" conditions (Jensen *et al.*, 1998; Chatterjee *et al.*, 2000; Messana *et al.*, 2000; Liu *et al.*, 2001). In order to raise GSNO levels to the detection limit, nitric oxide synthase must be induced or alternatively, various NO donors must be administered (Jensen & Belka, 1997; Hour *et al.*, 1999; Chatterjee *et al.*, 2000; Messana *et al.*, 2000). Notably, when ADH3-/- yeast or mice cells where challenged with GSNO, intracellular levels of both GSNO and protein S-nitrosothiols were significantly increased as compared to ADH3 positive cells (Liu *et al.*, 2001). Therefore, the fact that GSNO is hard to detect is possibly due to the rapid metabolism by ADH3 which is present in all tissues (Estonius *et al.*, 1993; Estonius *et al.*, 1996). GSH may act as a protector against nitrosative stress or alternatively, function as a modulator of the action of NO.

ADH3 is Ubiquitously Expressed but Levels May Vary Among Tissues

Tissue specific expression of ADHs have been studied extensively in laboratory animals and to lesser extent in humans. Generally, humans and animals show similarities in expression patterns for the various ADHs although differences are evident when "taking a closer look". Further, numerous studies have been performed at both mRNA and protein levels but rarely at the same time in a comparative manner. Often, the presence of mRNA and protein coincides (Deltour *et al.*, 1997; Haselbeck *et al.*, 1997b), however, there are examples where this assumption no longer is applicable (paper III).

ADH3 have been detected in all tissues and species examined, including brain where it is the only ADH expressed (Holmes, 1978; Adinolfi *et al.*, 1984; Duley *et al.*, 1985; Holmes *et al.*, 1986; Julia *et al.*, 1987; Uotila & Koivusalo, 1987a; Giri *et al.*, 1989b; Keller *et al.*, 1990; Rout, 1992; Estonius *et al.*, 1993; Estonius *et al.*, 1996; Haselbeck &

Duester, 1997; Mori *et al.*, 2000). This ubiquitous expression is in line with the presumed role as a formaldehyde scavenger. Although the protein and activity is found basically everywhere, expression levels vary many-fold both at mRNA and protein levels among tissues and species (Estonius *et al.*, 1996; Uotila & Koivusalo, 1997; paper III). In human tissues, highest mRNA levels have been found in testis, colon and adrenal cortex and lowest levels in brain and placenta (Engeland & Maret, 1993; Estonius *et al.*, 1996). Rat ADH3 mRNA is most abundant in colon and epididymis and lowest in spleen and small intestine (Estonius *et al.*, 1993), while the mRNA distribution seems to be less differentiated in mice (Zgombic-Knight *et al.*, 1995a; Ang *et al.*, 1996b). At the activity level, comparative measurements on ADH3 are sparsely investigated in humans. In rat, ADH3 activity measurements in lysates from various tissues have demonstrated up to 10-fold differences in expression with the highest levels found in liver (5 U/g total protein where one U is defined as 1μmol NADH formed per minute) and colon (1.94 U/g total protein) and lowest levels in rectum, skin, and esophagus (<0.3 U/g total protein; Casanova-Schmitz *et al.*, 1984; Uotila & Koivusalo, 1987a). These differences in expression levels are supported by histochemical localization of ADH3 in rat tissues, where a wide variation in staining intensities, ranging from strong to non-detectable, were observed (Keller *et al.*, 1990). Furthermore, ADH3 activity measurements in tissue lysates prepared from dogs, demonstrate that ADH3 activity may vary as much as 4-fold within respiratory epithelial structures with the highest activity found in the nose and gradually lower activities in more distal parts of the respiratory tract (Maier *et al.*, 1999). Notably, with respect to the metabolism of formaldehyde, ratios between ADH3 and low-K_m ALDHs may also vary between tissues (Casanova-Schmitz *et al.*, 1984; Uotila & Koivusalo, 1997). Estimates in rat tissues have demonstrated that the ratio between formaldehyde oxidizing activity of ADH3 versus low-K_m ALDHs is 6.6 in colon while it is 0.03 in brown fat tissue (Uotila & Koivusalo, 1997). Moreover, ADH3 is the sole ADH detected in a cellular compartment other than the cytosol, i.e., the nucleus, where it is presumed to directly protect the DNA from damage (Keller *et al.*, 1990; Iborra *et al.*, 1992). Nuclear localization seems to be most prominent in differentiated nerve cells.

Expression of Other Alcohol Dehydrogenases

All ADHs, except ADH4, are expressed in the liver. ADH2 and ADH5 seem to be almost exclusively hepatically expressed, although ADH5 have only been detected at mRNA level (Estonius *et al.*, 1996; Strömberg & Höög, 2000). Investigators have demonstrated

that up to 3% of the total protein content in the liver may be ADH protein (Edenberg & Bosron, 1997 and references therein). ADH1A-C and ADH4 are expressed in a number of extra hepatic tissues. Human ADH1A-C and the rodent forms of ADH1 show similar expression patterns (Estonius *et al.*, 1993; Zgombic-Knight *et al.*, 1995a; Ang *et al.*, 1996b; Estonius *et al.*, 1996). In addition to the liver, these enzymes are moderately expressed in kidney, intestine, stomach, lung, adrenals, heart, and lower expression levels are observed in pancreas, spleen and lymph nodes. ADH1C have also been detected in human blood vessels (Allali-Hassani *et al.*, 1997). ADH4 is almost exclusively expressed extrahepatically, mainly restricted to epithelial structures (Zgombic-Knight *et al.*, 1995b; Estonius *et al.*, 1996). High levels of ADH4 is found in the upper gastrointestinal tract in both humans and rodents (Moreno & Parés, 1991; Ang *et al.*, 1996b; Dong *et al.*, 1996; Haselbeck & Duester, 1997; Yin *et al.*, 1997; Seitz & Oneta, 1998). In addition, ADH4 is also found in mouse testis, epidermis, adrenals and cornea as well as in rat blood vessels (Zgombic-Knight *et al.*, 1995a; Ang *et al.*, 1996b; Allali-Hassani *et al.*, 1997; Haselbeck & Duester, 1997).

In vitro Model Systems and their Expression of Alcohol Dehydrogenases

The literature on ADH expression and function in various model systems such as *in vitro* cultured cell lines are limited in comparison to reports from humans and laboratory animals. Many cell lines lack ethanol metabolizing activity (Koivisto & Salaspuro, 1997; Olivares *et al.*, 1997). This phenomenon is probably due to the rapid decrease in expression of ethanol metabolizing ADHs during prolonged cell growth *in vitro*, a feature commonly observed for phase I enzymes (Garle & Fry, 1996; Mace´ *et al.*, 1996). However, there are reports on cell lines that express ethanol metabolizing enzymes, primarily ADH1 (Thirion & Talbot, 1978; Ganey & Thurman, 1991; Flisiak *et al.*, 1993; Mapoles *et al.*, 1994; Efthivoulou & Berry, 1996; Casini *et al.*, 1998) as well as one ADH4 expressing rat hepatoma cell line (Plapp *et al.*, 1987). These cell lines have proven useful in studies on ethanol metabolism in relation to a variety of cellular processes including drug, prostaglandin and carbohydrate metabolism as well as cell viability studies. ADH1 expression have also been shown to be upregulated by dexamethazone in cultured hepatoma cells (Wolfla *et al.*, 1988; Garle & Fry, 1996) and interestingly, regenerated epithelia with hamster buccal epithelial cells have been shown to express an ethanol metabolizing enzyme (Tavakoli-Saberi & Audus, 1989). Studies on ADH3 expression in model systems are even more limited. Giri et al. reported that several

unidentified cell lines expressed ADH3 and the studies on the nuclear localization of ADH3 were performed in rat hepatocyte and astrocyte cell lines (Giri *et al.*, 1989a; Iborra *et al.*, 1992).

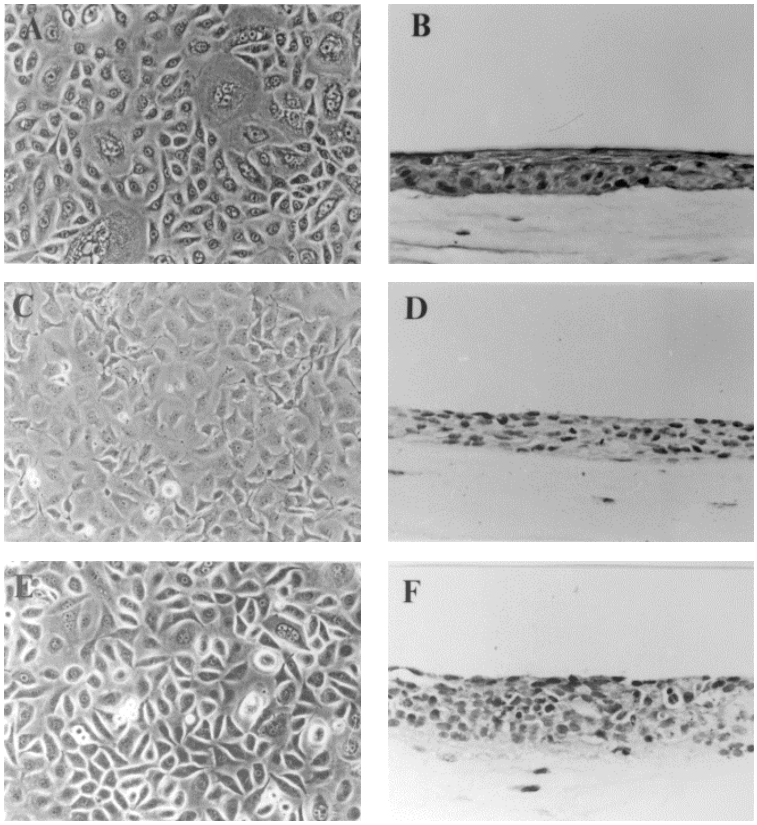


Figure 5. Normal, SV40-Immortalized (SVpgC2a) and malignant (SqCC/Y1) human buccal keratinocytes in monolayer (A, C & E) and *in vitro* regenerated epithelia (B, D & F). Normal keratinocytes as regenerated epithelia form a multi-layered stratified epithelium that mimics non-keratinized buccal epithelium *in vivo*. The regenerated epithelia with SVpgC2a forms a multilayered epithelium with decreased differentiation and regenerated epithelia with SqCC/Y1 shows lack of organization similar to carcinoma *in situ* (Hansson *et al.*, 2001). Original magnification x200. Hematoxylin and eosin stain. The picture was kindly provided by Ms A Hansson and Prof RC Grafström.

The *in vitro* grown cells used as model systems in this thesis include replicative cultures of normal and transformed keratinocytes as well as fibroblasts. Normal keratinocytes and fibroblasts can be established from human oral mucosal tissue (Grafström *et al.*, 1997). The serum-free method developed for the normal keratinocytes are also applicable to the transformed oral keratinocyte lines SVpgC2a (SV40 T antigen-immortalized) and SqCC/Y1 (squamous carcinoma cell line) including both monolayer cultures and *in vitro* regenerated epithelia (Fig. 5; Sundqvist *et al.*, 1991; Kulkarni *et al.*, 1995; Hansson *et al.*,

2001). The transformed keratinocyte lines model the step-wise development of oral cancer on the basis that they reflect acquisition of immortality (SVpgC2a), loss of p53 tumor suppressor function and eventually gain a tumorigenic phenotype (SqCC/Y1) (Grafström *et al.*, 1997).

Polymorphisms in Alcohol Dehydrogenase Genes

Genetic variation in the human genome is a common phenomenon. Five out of the seven known human ADH genes have been shown to be polymorphic, these include *ADH1B*, *ADH1C*, *ADH2*, *ADH3* and *ADH4* (Table 2; Smith *et al.*, 1971; Jörnvall *et al.*, 1984; Höög *et al.*, 1986; Burnell *et al.*, 1987; Edenberg *et al.*, 1999; Buervenich *et al.*, 2000; paper V). The various allelic variants of ADH1 display differences in activity for ethanol. The most striking difference is between ADH1B1 and ADH1B3 (unique to African Americans) which display K_m values for ethanol of 0.05 and 34 mM, respectively (Wagner *et al.*, 1983). The various allelic variants of ADH1B and ADH1C and their role in ethanol metabolism, drinking behavior, carcinogenesis and other pathological processes have been the subject of numerous investigations. It is believed that individuals expressing either of the high activity variants of ADH1, i.e, ADH1B2 (the "atypical oriental variant") or ADH1C1 are less prone to develop alcoholism (Thomasson *et al.*, 1991; Thomasson *et al.*, 1993; Edenberg & Bosron, 1997; Shen *et al.*, 1997). Notably, the second step in ethanol metabolism, i.e., the oxidation of acetaldehyde, is mainly catalyzed by the mitochondrial ALDH (class 2) of which an inactive variant is common among oriental populations (Petersen & Lindahl, 1997). This allele also protects against development of alcoholism. The role of the various *ADH1* genotypes in susceptibility to various forms of head and neck cancer have been debated. Increased risk for esophageal cancer have been associated with both the *ADH1B1* (Hori *et al.*, 1997) and *ADH1C1* alleles (Coutelle *et al.*, 1997; Harty *et al.*, 1997) although, other investigators have failed to see an increased risk of head and neck cancer associated with the ADH1C1 allele (Bouchardy *et al.*, 2000; Olshan *et al.*, 2001). Moreover, the *ADH1B3* allele has been shown to protect against ethanol related birth defects (McCarver *et al.*, 1997) and the *ADH1C2* allele, together with a moderate ethanol consumption, has been shown to protect against myocardial infarction (Hines *et al.*, 2001). For the *ADH2* gene, polymorphisms have been observed both in the promoter and the coding region (Edenberg *et al.*, 1999; Strömberg, unpublished). The A_{-75} allele, has been shown to

Table 2. Human ADH Polymorphisms

Gene	Location	Nucleotides	Amino Acids	References
*ADH1B*1*	coding region	G_{143}[c], C_{1108}[c]	R_{47}, R_{369}	(Heden *et al.*, 1986)
*ADH1B*2*	coding region	A_{143}[c], C_{1108}[c]	H_{47}, R_{369}	(Jörnvall *et al.*, 1984; Ehrig *et al.*, 1988)
*ADH1B*3*[a]	coding region	G_{143}[c], T_{1108}[c]	R_{47}, C_{369}	(Burnell *et al.*, 1987; Carr *et al.*, 1989)
*ADH1C*1*	coding region	G_{815}[b], A_{1048}[b]	I_{349}, R_{271}	(Höög *et al.*, 1986)
*ADH1C*2*[b]	coding region	A_{815}[b], G_{1048}[b]	V_{349}, Q_{271}	(Höög *et al.*, 1986)
ADH2	promoter region	$T_{-192} \rightarrow A$[d]		(Edenberg *et al.*, 1999)
	promoter region	$G_{-159} \rightarrow A$[d]		(Edenberg *et al.*, 1999)
	promoter region	$C_{-75} \rightarrow A$[d]		(Edenberg *et al.*, 1999)
	coding region	$A_{102} \rightarrow G$[e]	$I_{303} \rightarrow V$	Strömberg, unpublished
ADH3[b]	promoter region	$GG_{-197,-196} \rightarrow AA$[f]		paper V
	promoter region	$G_{-79} \rightarrow A$[f]		paper V
	promoter region	$C_{+9} \rightarrow T$[f]		paper V
ADH4[c]	promoter region	$T_{-94} \rightarrow C$[g]		(Buervenich *et al.*, 2000)
	promoter region	$T_{25} \rightarrow C$[g]		(Buervenich *et al.*, 2000)
	second intron	2 bp insertion $GG_{421,422}$[h]		(Buervenich *et al.*, 2000)
	second intron	$A_{88} \rightarrow G$[h]		(Buervenich *et al.*, 2000)
	coding region	$G_{212} \rightarrow C$[i]	$G_{79} \rightarrow A$	(Buervenich *et al.*, 2000)
	fourth intron	$T_{280} \rightarrow G$[j]		(Buervenich *et al.*, 2000)
	coding region	$G_{188} \rightarrow A$[k]	silent mutation	(Buervenich *et al.*, 2000)

[a] For *ADH1B*3* there are two additional silent mutations as compared to *ADH1B*1* in exon 6: $C_{67} \rightarrow T$ and $C_{208} \rightarrow T$ numbered according to (Carr *et al.*, 1989). [b] For *ADH1C*2* there are two additional silent mutations as compared to *ADH1C*1* $T_{312} \rightarrow C$ and $G_{474} \rightarrow A$ numbered according to (Höög *et al.*, 1986). [c] Numbered according to (Heden *et al.*, 1986), [d] Numbered according to (Li & Edenberg, 1998). [e] Numbered according to Genebank no. X56414. [f] Numbered according to (Hur & Edenberg, 1995). [g] Numbered according to (Zgombic-Knight *et al.*, 1995b). [h] Numbered according to Genebank no. U16287. [i] numbered according to Genebank no. U16288. [j] Numbered according to Genebank no. U16289. [k] Numbered according to Genebank no. U16290.

result in increased promoter activity in a hepatoma cell line as compared to the C_{-75} allele (Edenberg *et al.*, 1999) and the $I_{303} \rightarrow V$ exchange has been shown to affect protein stability (Strömberg, unpublished). Hypothetically, the *ADH2* polymorphisms may alter ethanol metabolism and possibly also influence the risk for alcoholism or alcohol related liver disease (Edenberg *et al.*, 1999). Fruitless attempts have been made to screen for variants of ADH3 among several hundred Australian, Caucasian, Chinese, Finish and Indian individuals (Castle & Board, 1982; Uotila & Koivusalo, 1987b). However, these screens were performed by staining for ADH3 activity in non-denaturing gels, a procedure not directly detecting differences within the DNA sequence. In paper V, we

describe the first polymorphisms observed within the *ADH3* gene. The effect of these polymorphisms will be discussed in greater detail below (see Results and Discussion). One form of parkinsonism have been mapped to chromosome 4q21-23 (Polymeropoulos *et al.*, 1996). On the basis that all ADH genes reside within this segment together with a possible role of retinoic acid in the dopamine system, screens for polymorphisms within *ADH4* have been performed (Buervenich *et al.*, 2000). Seven different polymorphisms in the promoter and coding regions as well as in intron sequences were detected. Interestingly, the allele harboring the $T_{-94} \rightarrow C$ exchange was demonstrated to be significantly more frequent in patients with Parkinson´s disease than in control subjects, indicative of that mutations in ADHs genes, particularly *ADH4*, may affect the onset of neurodegenerative disorders (Buervenich *et al.*, 2000).

Present Study

Aims

Although ADH3/glutahione-dependent formaldehyde dehydrogenase and its functions have been thoroughly investigated, several aspects of its function remains to be elucidated. For instance, the substrate repertoire is continuously growing, revealing novel and potentially important roles in the cellular metabolism. Furthermore, information on the expression of the enzyme in human tissues and how this expression may be modulated by various cellular processes or genetic variation is limited. Therefore, the overall aim of this thesis have been to further characterize the role of ADH3 in cellular metabolism and to put the enzyme and its functions in a biological perspective.

In more detail, the aims of this thesis have been:

- to characterize the activity and specificity for HMGSH and other model substrates of recombinantly expressed and purified ADH3 as well as in detail study the conversion of the new substrate GSNO.

- to assess the expression and distribution of ADH3 at mRNA, protein and activity level in human oral epithelium and various cell lines used as epithelial *in vitro* model systems including both monolayer cultures and regenerated epithelia of normal and transformed epithelial cell lines.

- to investigate possible inter-individual genetic differences within the *ADH3* gene and to assess whether these differences may effect the expression of the enzyme.

Results and Discussion

A number of different methods have been used. For a more detailed information on these methods I would like to refer to the appended original papers. The present section is devoted to three major issues. First, the specificity of ADH3 (paper I and II) second, the expression of ADH3 in human oral epithelium and various model systems (paper III and IV) and third, the genetic variation among individuals in the *ADH3* gene (paper V).

The Substrate Specificity of ADH3 is "Finely Tuned"

The various ADHs have evolved to metabolize different alcohols and aldehydes. ADH3 is regarded as the ancestral form of ADH from which the other ADHs have originated (Danielsson & Jörnvall, 1992). Accordingly, ADH3 shows structural and functional conservation while the other ADHs have gained specificities for other substrates, e.g., ethanol. As discussed earlier, Asp57 and Arg115 play crucial roles in the binding of HMGSH (Fig. 6.) The replacement of Arg115 for Ser or Lys in ADH3 followed by kinetic characterization further delineated the importance of Arg115.

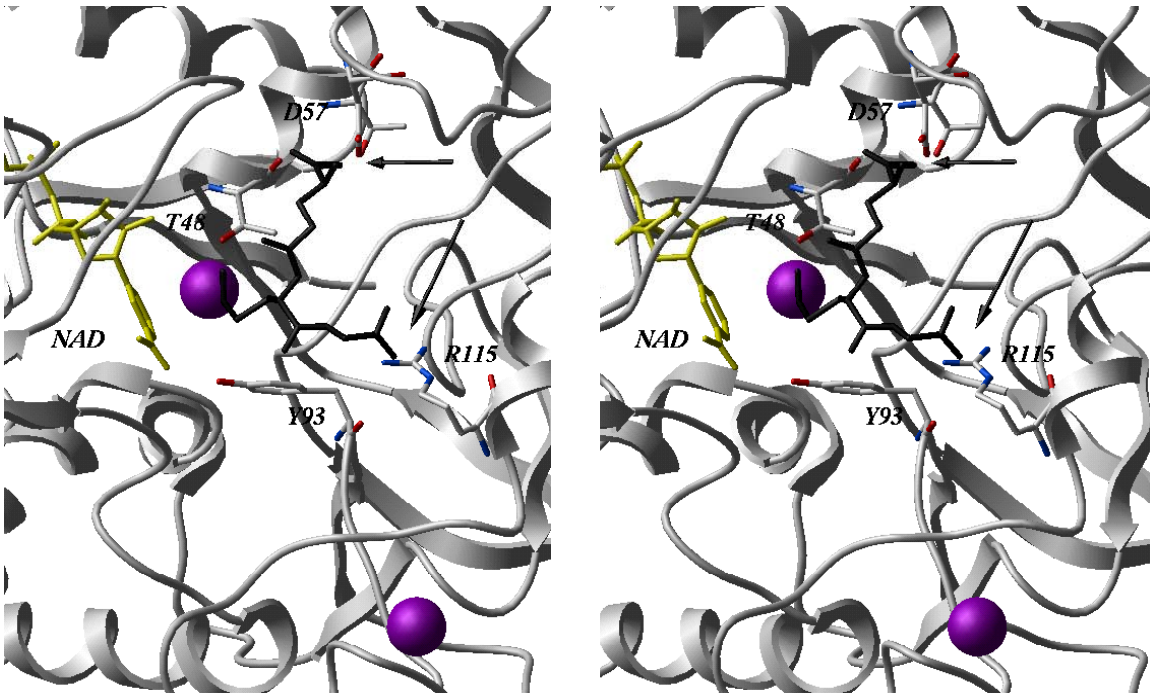


Figure 6. Stereo view of the substrate pocket of ADH3. HMGSH (black) interacts electrostatically with Arg115 and Asp57 (arrows).

Table 3. Kinetic Constants for Recombinant Wild-Type and Mutated forms of ADH3 and ADH1

	HMGSH[a]		GSNO[b]	
enzyme	K_m (μM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)	K_m (μM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
ADH3-wt[c]	4	58 000	27	90 000
ADH3-R115S[c]	580	500	700[g]	3900
ADH3-R115K[c]	290	1000	140	24 000
ADH1-wt[d]		n.a.[h]		
ADH1C2-L57D/D115R[d]		n.a.[h]		
ADH3-R115A[e]	280	350		
ADH3-R115D[e]	910	40		
ADH3-D57L[f]	600	90		
ADH3-D57L/Y93F[f]	500	40		
ADH3-T48A[f]	4400	7		
ADH3-Y93F[f]	4	10 000		

[a] Determined at pH 8. [b] Determined at pH 7.5. [c] Values are from paper I and II. [d] Values are from paper I. [e] Values are from (Engeland *et al.*, 1993). [f] Values are from (Estonius *et al.*, 1994). [g] Determined at non-saturating conditions. [h] no activity.

Not surprisingly, and in agreement with studies on the ADH3-Arg115Ala and ADH3-Arg155Asp mutants (Engeland *et al.*, 1993), the ADH3-Arg115Ser mutant displayed significantly lower catalytic efficiency for HMGSH as compared to the wild-type enzyme, primarily through increased K_m values (Table 3). A finding which was also reproducible for the structurally similar substrate GSNO. This is explained by a loss of charge interaction between the glycine carboxylate group of HMGSH or GSNO and Arg115. The ADH3-Arg115Lys mutant also displayed significantly reduced activity for HMGSH and GSNO. This was unexpected since an electrostatic interaction between HMGSH or GSNO and the Lys residue was hypothetically possible due to the chemical similarities between Arg and Lys. Notably, electrostatic interaction was evidently present between 12-HDA and Lys115 in the ADH3-Arg115Lys mutant since only small changes in kinetic constants were observed as compared to the wild-type enzyme at pH 7.5 (Table 4). Further evidence for this charge interaction was the reduced activity for 12-HDA at pH 10, i.e., at a pH when the Lys residue is not fully charged, and it was possible to activate ADH3-Arg115Lys mediated oxidation of methylcrotyl alcohol (MCA) by the addition of pentanoate at pH 7.5 but not at pH 10. Evidently, the activity for HMGSH and

GSNO have been extensively refined through evolution since not only a positive charge but also an exact positioning is of great importance for correct docking of HMGSH or GSNO.

Compared to other ADHs, ADH3 has a markedly larger and completely solvent exposed active site pocket (Eklund *et al.*, 1990; Yang *et al.*, 1997). This is due to several smaller and more hydrophilic amino acids lining the pocket allowing accommodation of more water. Only longer or more hydrophilic substrates which can displace these water molecules are substrates for ADH3 resulting in higher activity for alcohols with longer carbon chains through decreasing K_m values with increasing chain length (Wagner *et al.*, 1984). In ADH1, residues 57 and 115 corresponds to Leu and Asp, respectively. Due to the close relationship of ADHs a previous "transformation" of enzyme properties have been successful (Estonius *et al.*, 1994).

Table 4. Kinetic Constants for Recombinant Wild-Type and Mutated forms of ADH3 and ADH1

	12-HDA		Octanol		Ethanol	
enzyme	K_m (μM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)	K_m (μM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)	K_m (μM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
pH 7.5						
ADH3-wt[a]	80	125	900	11		
ADH3-R115S[a]	200	45	750	16		
ADH3-R115K[a]	80	65	700	6		
pH 10						
ADH3-wt[a]	50	1200	550	140		0.045
ADH3-R115S[a]	380	210	470	190		
ADH3-R115K[a]	270	440	550	180		
ADH1-wt[a]	55	700	5	10 000	560	85
ADH1C2-L57D/D115R[a]	15	13 300	5	7000	2500	14
ADH3-R115A[b]	680	180	1200	125		0.015
ADH3-R115D[b]	1300	75	2200	110		0.015
ADH3-D57L[c]	60	2700	300	530		0.045
ADH3-D57L/Y93F[c]	20	1250	100	300		0.045
ADH3-T48A[c]	n.a.[d]		n.a.[d]		n.a.[d]	
ADH3-Y93F[c]	40	750	500	70		0.045

[a] Values are from paper I. [b] Values are from (Engeland *et al.*, 1993). [c] Values are from (Estonius *et al.*, 1994). [d] no activity.

In this study, ADH3 was "transformed" to an enzyme with ADH1 characteristics through mutagenesis. To further investigate which factors may promote class properties, an attempted class transformation through the generation and kinetic characterization of the double mutant ADH1-Leu57Asp/Asp115Arg was performed. This mutagenesis did indeed result in an enzyme with increased K_m for ethanol and decreased K_m for 12-HDA (Table 4). The increased K_m for ethanol may be a result of an overall widening of the substrate pocket of the ADH1 double mutant and the decreased K_m for 12-HDA may reflect a charge interaction with Arg115. These features can be regarded as ADH3-specific. However, the ADH1 double mutant lacked HMGSH activity (Table 4) and it was impossible to increase the efficiency of MCA oxidation by the addition of pentanoate. Taken jointly, the active site pocket in ADH1-Leu57Asp/Asp115Arg is probably wider than in the wild type ADH1 but not large enough to accommodate a HMGSH molecule. The previous class transformation of ADH3, concluded that few residues lining the ADH3 active site pocket, including Asp57, Tyr93 and Arg115, were important for class differentiation. With the findings for the ADH1 double mutant taken into account, this does not apply to the same extent for ADH1. These findings are in line with ADH1 being a "variable" enzyme (Danielsson *et al.*, 1994a), with several residue exchanges at important sites, as compared to ADH3.

As mentioned above, the spontaneous formation of HMGSH from GSH and formaldehyde has a K_{eq} of 1.77 mM at pH 7.5 (Sanghani *et al.*, 2000). Upon experimental determinations of the K_m for GSH-dependent formaldehyde oxidation by ADH3, this has not always been taken into account (Engeland *et al.*, 1993; Estonius *et al.*, 1994; paper I; paper III). In these studies, the K_m has been determined only with respect to formaldehyde concentrations in the reaction mixture. It should be noted that these determinations does not reflect the K_m for the actual substrate, HMGSH, but rather reflects a K_m for free formaldehyde (4 μM) and should be viewed as an apparent K_m. This constant is naturally dependent on the concentration of GSH which commonly is 1 mM in the used reaction mixtures (Engeland *et al.*, 1993; Estonius *et al.*, 1994; paper I; paper III). However, a more strict determination of the K_m for HMGSH should include the calculation of the actual HMGSH concentrations in the reaction mixtures which results, by necessity, in a lower K_m value (2 μM) (Uotila & Koivusalo, 1974a; Uotila & Koivusalo, 1989; Sanghani *et al.*, 2000; paper II).

The Protective Role of ADH3 May be Double-edged

The discovery that GSNO is an excellent substrate for human ADH3 has expanded the potential role of ADH3 (paper II). This activity, as well as the activity for HMGSH, is apparently conserved throughout the kingdoms ranging from bacteria to humans with K_m values in the same range as the estimated concentrations for the respective substrates, i.e., in the micro molar range. For the human enzyme, k_{cat}/K_m values for HMGSH and GSNO are 58 000 $min^{-1}mM^{-1}$ and 90 000 $min^{-1}mM^{-1}$, respectively (Table 3). These values are at least one order of magnitude higher than for any other ADH3 substrate.

The production and biological function of NO have received considerable attention and it is a well known fact that S-nitrosothiols play central roles in the biological action of NO which includes vasodilation, inhibition of platelet aggregation and neurotransmission (Stamler *et al.*, 1992; Moncada, 1994; Gow *et al.*, 1997). In addition to the endogenous production, NO is also a common air pollutant (IARC, 1986; Graedel, 1988). It has been shown that under aerobic conditions, the formation of S-nitrosothiols is kinetically feasible (Gow *et al.*, 1997) and since GSH is the major intracellular thiol, GSNO is one of the prime candidates to be produced by nitrosative stress (Kharitonov *et al.*, 1995; Tsikas *et al.*, 2000). Up till now, the elimination of excess NO has been attributed to spontaneous reactions with other thiols, ascorbate or metal ions (Singh *et al.*, 1996; Wong *et al.*, 1998; Xu *et al.*, 2000). Alternatively, formed S-nitosothiols may be metabolized by various enzymes including thioredoxin (Nikitovic & Holmgren, 1996), γ-glutamyl transpeptidase (Hogg *et al.*, 1997) or glutathione peroxidase (Hou *et al.*, 1996). However, ADH3 is the first enzyme shown to regulate intracellular levels of both GSNO and other S-nitrosothiols (Liu *et al.*, 2001) and therefore, GSNO is most likely a natural substrate for ADH3.

The activity of ADH is generally dependent on the NAD^+/NADH ratio which normally is relatively high in the cytoplasm (500 or above; Bücher, 1970). For instance, the rate of ethanol elimination seems to be dependent on the regeneration of NAD^+ from NADH (Cronholm, 1987). Applying this rational, HMGSH oxidation is an obvious and favored reaction while the reduction of GSNO would be less favored. ADH3 mediated GSNO reduction yields a product which probably is rapidly rearranged to either GSSG or more likely glutathionesulfinamide, both of which were detected and identified by electrospray

tandem mass spectrometry. The exact origin of the detected GSSG is at the time unclear. GSH and GSNO have been shown to spontaneously form GSSG under physiological conditions (Singh *et al.*, 1996) but GSSG has also been proposed to be formed from the reaction between GSH and the presumed "intermediate product", i.e., the semimercaptale (Scheme 1 in paper II)(Kazanis & McClelland, 1992; Jensen *et al.*, 1998). In any event, non of the two products are ADH3 substrate candidates resulting in that the reaction is probably more or less irreversible. Hence, an equilibrium between substrate (GSNO) and product (GSSG or glutathionesulfinamide) is probably not formed but rather shifted towards the product. This notion is further substantiated by the observation that GSNO is rapidly metabolized in "normal" cells while disruption of the ADH3 gene in various organisms results in significantly reduced GSNO metabolism (Liu *et al.*, 2001). With the above findings as a base, ADH3 is probably not only a guardian against formaldehyde but also function as a protector against nitrosative stress.

ADH3 mRNA and Protein Show Differential Expression Pattern in Oral Epithelium

Since the oral mucosa is a documented target for formaldehyde genotoxicity, the enzymatic defense was assessed in human oral mucosa and human oral epithelial cell lines grown both in monolayer and as regenerated epithelia. The expression of ADH3 was characterized at mRNA, protein and activity levels. *In situ* hybridization revealed that in the intact oral epithelial tissue, ADH3 mRNA was confined to the basal and parabasal cell layers and as cells migrate towards the surface, ADH3 mRNA synthesis is significantly decreased. In contrast, ADH3 protein was detected throughout the epithelium except for the outmost keratinized layers demonstrating a differentiated expression pattern for mRNA and protein (Fig. 7). Certain keratins have also been shown to display differential mRNA and protein expression patterns (Bloor *et al.*, 1998). In expression analyses, it is often taken for granted that mRNA and protein coincide. Evidently, this assumption is not always applicable within epithelial structures and possibly also in other tissues. It would be wise to take this knowledge into account in future expression studies. For instance, the development and application of powerful RNA-hybridization methods such as the cDNA-chip techniques can be seductive by their immense output of data. However, these techniques should, at least for the most interesting gene products, be accompanied with the detection of protein.

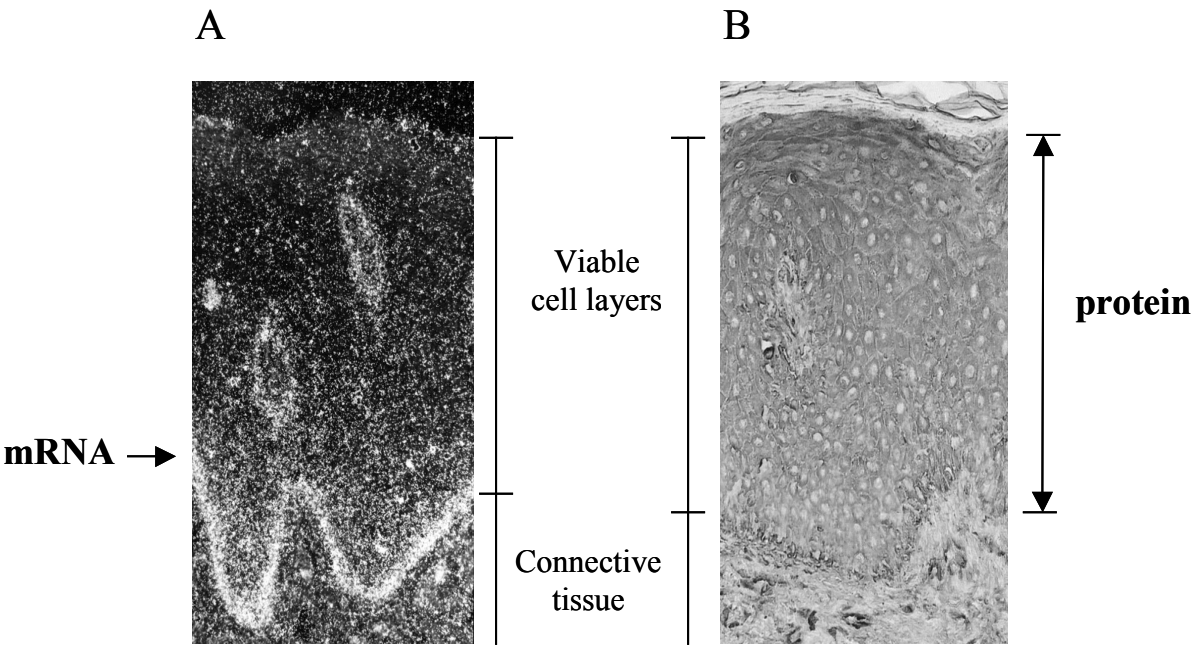


Figure 7. Localization of ADH3 mRNA (A) and protein (B) in human oral epithelium. mRNA is evenly distributed along the epithelium and preferentially localized in the basal and parabasal cell layers (arrow). Protein is distributed along and throughout the entire epithelium except for in the outmost non-viable cell layers. Original magnifications x100.

The expression pattern for ADH3 mRNA and protein found for the intact tissue were substantiated with *in vitro* experiments using the epithelial model systems. Growth inhibited normal keratinocytes displayed significantly lower ADH3 mRNA levels after 10 and 15 days at confluency in monolayer culture. Notably, the decrease in ADH3 mRNA abundance was not due to decreased cell viability as assessed by morphological appearance and the expression of certain differentiation markers, e.g., involucrin (unpublished). Further, pulse-chase experiments demonstrated that the ADH3 protein display an exceptionally high stability in keratinocytes, i.e., well above the expected life span of a keratinocyte. With the above findings as a base, ADH3 mRNA and protein is most likely synthesized when the cells are in their proliferative state and the protein is then retained during the entire life span of the keratinocyte. This notion is also supported by the finding that, as compared to normal keratinocytes, ADH3 mRNA levels seems to be higher in the transformed cell lines and as well in fibroblasts, all of which have been shown to have significantly higher clonal efficiency than normal keratinocytes.

Functionality of the *in vitro* Model Systems

ADH3 protein and activity levels showed marked correlation in cell and tissue lysates using purified enzyme as reference (pure human ADH3 has a specific activity of 4 U/mg; Uotila & Koivusalo, 1974a; Estonius *et al.*, 1994). This indicated that the ADH3 protein was metabolically active in the lysates and furthermore, asserted an immunohistochemical approach as applicable when assessing ADH3 mediated formaldehyde metabolism. When grown in monolayer, the three cell types showed similar levels of ADH3 protein and corresponding activity. As judged by immunohistochemical staining intensities, the various cell types also showed similar levels of ADH3 protein when grown as *in vitro* regenerated epithelia and as well in comparison to intact tissue. Although regenerated epithelia with normal keratinocytes have tissue like features, they have been reported to consist of ”modified” basal cells rather than of basal and suprabasal compartments (Oda *et al.*, 1998; Hansson *et al.*, 2001). Moreover, regenerated epithelia with SVpgC2a and SqCC/Y1 both show inclusively disturbed differentiation and proliferation features (Hansson *et al.*, 2001). Possibly, the regulation of ADH3 mRNA and protein synthesis, in the various regenerated epithelia, particularly in the ones with transformed cells, may differ from the regulation in tissue. Nevertheless, ADH3 protein is evenly distributed throughout the cell layers and in this manner all three kinds of regenerated epithelia resemble the tissue. Therefore, with respect to formaldehyde metabolism, the transformed cell lines may variably serve as alternative models to laboratory animals, in some instances involving studies of normal cell function, or alternatively, as models for the multi-step development of cancer. Moreover, the increased degree of complexity of *in vitro* regenerated epithelia as compared to monolayer cultures, may enable new perspectives on formaldehyde metabolism and pathology. For example, the observed nonlinearity of formaldehyde toxicology and/or the influence of mesechymal interactions may in the future be further delineated with the use of these model systems as a base.

ADH3 is the Prime Guardian Against Formaldehyde in Human Oral Epithelial Cells

Since both ADH3 and low-K_m ALDHs have been proposed to contribute to formaldehyde metabolism (Dicker & Cederbaum, 1984a), efforts were made to assess the respective roles of these enzymes in formaldehyde metabolism in human oral mucosa. Presence of low-K_m ALDH in lysates from tissue and the various cell lines was indicated from

oxidative activity for propanal and to lesser extent for free formaldehyde. However, markedly lower metabolic rates were observed for these substrates as compared to HMGSH. This is in line with findings that indicate that levels of low-K_m ALDHs are low in oral mucosa (Dong *et al.*, 1996). Furthermore, in agreement with K_m values reported for the respective purified enzymes (Mukerjee & Pietruszko, 1992; paper I), kinetic assessments demonstrated that ADH3 is metabolically active at significantly lower formaldehyde concentrations, i.e., in the low micro molar range, than low-K_m ALDHs. ADH3 is therefore the major guardian against formaldehyde in oral mucosa and as well in the various cell lines. Moreover, the *in vitro* analysis of different cell types, i.e., the normal versus the transformed and the principle of short-term versus extended culture, underline the essential role of ADH3 in basic cell metabolism as well as the preservation of ADH3 expression during malignant transformation.

The ADH3 Promoter is Polymorphic

Considering the essential role of ADH3 in cellular metabolism, an altered expression and/or activity of ADH3 presumably has an impact on the capacity of the cell to withstand formaldehyde or NO toxicity. Therefore, screens for allelic variants of the gene were performed using single-strand conformation polymorphism analysis (SSCP) on 80 samples of PCR amplified DNA from a Swedish population. Four different base exchanges as compare to the published sequence (Hur & Edenberg, 1992) were found in the promoter region while no base exchanges were detected within the investigated coding parts of the gene, i.e., exons 5, 6 and 7 (Table 2 & Fig. 8).

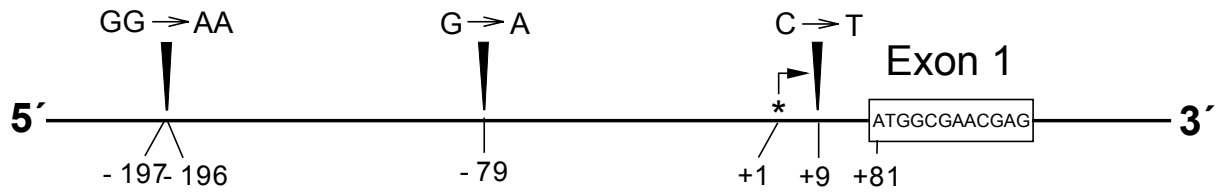


Figure 8 Schematic picture of *ADH3* 5´-flanking region including exon 1 and parts of intron 1 numbered with the transcription start site (*) as +1. The polymorphisms are depicted above and their respective position below the line.

Three out of the four base exchanges (G → A) were detected before the transcriptional start site at positions -197, -196 and -79 and the fourth exchange (C → T) was detected after and close to the transcriptional start site at position +9. The polymorphisms at positions -197 and -196 seemed to appear in pairs since the individual base exchanges were never detected. Development of allele specific PCR methods enabled determination of the allele frequencies in three populations, i.e., Chinese, Spaniards and Swedes. The $AA_{-197,-196}$ and $GG_{-197,-196}$ alleles were relatively common in all three populations with the lowest frequency for $AA_{-197,-196}$ among Chinese (22 %), and the highest frequency among Swedes (47 %). The G_{-79} allele was common among Spaniards and Swedes, with allele frequencies of 38 % and 33 % respectively, while this allele was absent within the Chinese population. The T_{+9} was by far the most rare allele, only detected within the Swedish population, with a frequency of 1.5 %. This frequency is above the limit at which a base exchange is defined as a polymorphism, i.e., 1 % (Meyer, 1991). Approximately 2 individuals in 10 000 are potentially homozygots for T_{+9} applying an allele distribution that follows the Hardy-Weinberg law, i.e., that the genotype distribution follows the equation $p^2 + 2pq + q^2$ where p and q are the chances (between 0 and 1) of picking either of the two alleles (Strachan & Read, 1996). Since the sample number was to low, only heterozygots for the T_{+9} allele were observed. The various distributions of the alleles indicate that the origin of $AA_{-197,-196}/GG_{-197,-196}$ is an older event than the Caucasoid/Asian split 35,000 years ago while the origin of A_{-79}/G_{-79} and C_{+9}/T_{+9} are later events (Cavalli-Sforza *et al.*, 1988).

The Base Exchanges May Affect the Expression

The promoter activity of the various polymorphic alleles were assessed in a reporter gene system introducing the 5´-flanking region covering bases -323 to +80. The fragments harboring the polymorphisms at positions -197,-196 and -79 failed to show any significant difference in promoter activity as compared to the published sequence. However, the C → T exchange at position +9 resulted in a 2-fold decrease in promoter activity when combined with the $AA_{-197,-196}$ and A_{-79} alleles in HeLa cells. A similar trend was also seen in HepG2 cells although the decrease was not statistically significant.

ADH3 has a GC-rich promoter found in a number of housekeeping genes (Gardiner-Garden & Frommer, 1987). Sp1 and other structurally related transcription factors

including Sp3 and Sp4, have been shown to regulate the transcription of thousands of GC-rich promoters, and thereby modulate the expression of proteins involved in a number of vital cellular processes including metabolic functions (Cook *et al.*, 1999). The minimal promoter extending from –34 to +61 is essential for transcriptional activation of *ADH3* through the binding of Sp1 (Hur & Edenberg, 1992; Hur & Edenberg, 1995). Mutation of residues at position +13 and +14 (CC → AA) have demonstrated that the binding of Sp1 in the region ranging from +8 to +16 is especially important in transcriptional activation (Hur & Edenberg, 1995). Furthermore, 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* (Hur & Edenberg, 1995). Electrophoretic mobility shift assays indicated that the $C_{+9} \rightarrow T_{+9}$ exchange affected the binding of Sp1 negatively (paper V). Possibly, this is the reason for the reduced promoter activity seen for the T_{+9} allele. However, how this exchange affects the binding of Sp3 and Sp4 remains to be further elucidated.

Considering the high activity for HMGSH and GSNO, a decreased expression of ADH3 would probably have an impact on the defense against formaldehyde and/or nitrsosative stress. For example, in a tissue such as the oral epithelium, which is a target for formaldehyde toxicity, a decreased synthesis of the ADH3 protein could increase the risk for genetic damage during exposure. Non-genotoxic levels of formaldehyde has been shown to synergistically potentiate the genotoxic effects of other agents including x-ray irradiation and nitrosamines. Since formaldehyde and nitrosamines both are components of tobacco smoke (IARC, 1986), a decreased ADH3 abundance in the oral epithelium would potentially increase the risk of cancer in the oral cavity of smokers. Interestingly, allelic variants of S-formylglutathione hydrolase have been detected among Finish individuals (Uotila, 1984). Since this enzyme rapidly catalyses the second step in the major metabolic path of formaldehyde metabolism, this indicates an additional level of complexity in the potential inter-individual variability in formaldehyde metabolism.

Conclusions

ADH3 displays high activity for the two substrates HMGSH and GSNO at physiologically relevant concentrations. These activities are highly specific and conserved throughout evolution. Since formaldehyde and NO are both known as common chemicals of endogenous and exogenous origin, ADH3 plays a vital role in the detoxification of these compounds through their respective GSH adducts. The characterization of ADH3 expression in human oral epithelial cells, *in vivo* and *in vitro*, demonstrates a novel solution to gene expression among ADHs, i.e., differential distribution of mRNA and protein, and indicates that ADH3 is the prime enzyme protecting the cell from formaldehyde. Possibly, the role of ADH3 in this tissue may be extended to include protection against nitrosative stress as well. Expression of ADH3 is preserved during malignant transformation and therefore, immortalized cells are functional as *in vitro* models for formaldehyde toxicity. Finally, the ADH3 gene is polymorphic and the inter-individual genetic differences may affect the capacity for formaldehyde or NO metabolism.

The Future

The relatively recent and novel insight that GSNO is metabolized by ADH3 deserves further investigative efforts. At the present, it is unclear how this activity is related to the formaldehyde oxidizing activity of ADH3 and also to other GSNO "metablolizers", both of enzymatic and spontaneous chemical character. Such information might be obtained by investigations of these activity relationships in cell systems and/or ADH3 -/- mice. To enable further assessments of the ADH3 activities to the *in vivo* situation in humans, a detailed picture of the expression is necessary. Therefore, further studies on the distribution of ADH3 in various tissues and cells, down to "single cell level", is required. The identification of allelic variants of *ADH3*, presented in paper V, should be viewed as an initial screen since the hole gene was not covered and the method used, i.e., SSCP, may result in that there are "false negative" samples. For a more complete screening in the future, it would be wise to use direct sequencing of the entire *ADH3* genomic sequence from a number of individuals. Further, a phenotype associated to the *ADH3* genotype is desirable and would ultimately delineate the effect of these genetic differences on expression. Such knowledge, combined with allele frequency

determinations in various populations and patient groups would yield a valuable base for future studies of formaldehyde and/or NO metabolism in humans.

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