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Mammalian Alcohol Dehydrogenase – Functional and Structural Implications

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Key Words

Alcohol dehydrogenase · Enzyme kinetics · Protein expression

Abstract

Mammalian alcohol dehydrogenase (ADH) constitutes a complex system with different forms and extensive multiplicity (ADH1–ADH6) that catalyze the oxidation and reduction of a wide variety of alcohols and aldehydes. The ADH1 enzymes, the classical liver forms, are involved in several metabolic pathways beside the oxidation of ethanol, e.g. norepinephrine, dopamine, serotonin and bile acid metabolism. This class is also able to further oxidize aldehydes into the corresponding carboxylic acids, i.e. dismutation. ADH2, can be divided into two subgroups, one group consisting of the human enzyme together with a rabbit form and another consisting of the rodent forms. The rodent enzymes almost lack ethanol-oxidizing capacity in contrast to the human form, indicating that rodents are poor model systems for human ethanol metabolism. ADH3 (identical to glutathione-dependent formaldehyde dehydrogenase) is clearly the ancestral ADH form and S-hydroxymethylglutathione is the main physiological substrate, but the enzyme can still oxidize ethanol at high concentrations. ADH4 is solely extrahepatically expressed and is probably involved in first pass metabolism of ethanol beside its role in retinol metabolism. The higher classes, ADH5 and ADH6, have been poorly investigated and their substrate repertoire is unknown. The entire ADH system can be seen as a general detoxifying system for alcohols and aldehydes without generating toxic radicals in contrast to the cytochrome P450 system.

The mammalian alcohol dehydrogenase (ADH) system is divided into six classes, ADH1–ADH6, whereof five have been identified in man [4, 9]. These dimeric enzymes belong to the protein superfamily of medium-chain dehydrogenases/reductases [13] and are further divided into subgroups (ADH2), isoenzymes (ADH1 and ADH2) and allelic forms (ADH1). All ADH classes catalyze the reversible oxidation of alcohols to aldehydes/ketones using NAD^+/NADH as electron acceptor and donor, respectively, and have a broad but only partially overlapping substrate repertoire [5]. Apart from the formaldehyde scavenging of ADH3, functional roles of the other ADHs are not fully established. However, based on their catalytic activities they could play roles in the metabolism of steroids, retinoids, biogenic amines, lipid peroxidation products, ω-hydroxy fatty acids as well as xenobiotic alcohols and aldehydes.

The structure determinations for ADH1–ADH4 have provided a structural basis for the understanding of their different properties. All ADH subunits consist of one catalytic and one coenzyme-binding domain, and both

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coenzyme and substrate bind in a cleft between the two domains [6, 15]. Low positional identities between the classes are especially observed in three segments, constituting parts of the substrate-binding pocket and subunit interaction areas [15]. This results in large differences in substrate pocket topology although the overall positional identity is high (~65%) and acceptance of residue exchanges at the substrate-binding site is likely to reflect the functional divergence of the classes.

ADH3, identical to glutathione-dependent formaldehyde dehydrogenase, is clearly the ancestral form of all mammalian ADHs and has been traced in all living species investigated [9, 10]. Further, this is the only ADH that has been ascribed a physiological substrate, S-hydroxymethylglutathione (HMGSH) and is identified as a functional formaldehyde scavenger [7]. ADH1, the major enzyme in the metabolism of ingested ethanol is the only human class where more than one isoenzymic form exists (α-, β- and γ-subunits, ADH1A, ADH1B and ADH1C in the new nomenclature system [4]). Mainly, rodents have been used to study the contributions of various ADHs in the metabolism of ethanol. The rodents do not show a setup of ADH1 isoenzymes, but the set of different classes is present (ADH1–ADH4 and ADH6). The ADH1 enzymes are involved in several metabolic pathways beside the oxidation of ethanol, e.g. norepinephrine, dopamine, serotonin and as recently shown bile acid metabolism [5, 12, 17]. This class is able to further oxidize aldehydes into the corresponding carboxylic acids, i.e. dismutation. The γγ-isoenzyme (ADH1C) shows the highest capacity for dismutation among the human ADHs and it seems to be a common theme that this form is involved in several specific pathways beside its high capacity for ethanol oxidation [12, 16].

Human ADH2 was isolated as a liver enzyme with a high K_m for ethanol [11] that was reinterpreted for the recombinantly isolated enzyme [18]. Reductions of the intermediate aldehydes in serotonin and norepinephrine catabolism are efficiently catalyzed [17] and it has further been shown that the human ADH2 is fairly efficient in retinoid metabolism [3]. For all these reactions, ADH1 and the extrahepatically expressed ADH4 have overlapping activities, and the ADH4 enzyme has been suggested to be the main bioactivator of retinoids [3]. ADH2 can be divided into two subgroups, one group consisting of the human enzyme together with a rabbit form and another consisting of the rodent forms. The ADH2 forms are found almost exclusively in the liver where the rodent forms almost lack ethanol dehydrogenase activity [18].

The higher classes, ADH5 and ADH6, have been poorly investigated, and nothing is known about their substrate repertoire. They are however liver expressed, and at least the human form, ADH5, shows an alternative splicing pattern.

In a further attempt to understand the interactions between different ADHs (classes and isoenzymes) as well as between different substrates, we here focus on the complex interplay between the participants in oxidoreductive cell defense.

Materials and Methods

Enzyme Preparations

Enzymes were recombinantly expressed in *Escherichia coli*, mainly using pET expression vectors for subcloning of mammalian ADH cDNAs. The recombinant proteins were purified to homogeneity essentially in a three-step procedure including ion exchange, affinity and gel permeation chromatography as described earlier [16, 18]. Protein concentrations were determined with the Bio-Rad protein assay with bovine serum albumin as standard and enzymatic activity was determined spectrophotometrically at pH 10 in glycine-NaOH buffer [16].

Kinetic Analysis

Ethanol and all-trans retinol oxidation were determined at pH 7.5 with a Hitachi U-3000 spectrophotometer, by monitoring the formation of NADH at 340 nm for ethanol oxidation and by the formation of retinal at 400 nm for retinol oxidation. Oxidation of HMGSH, spontaneously formed by formaldehyde and glutathione, was determined at pH 8.0 by monitoring the NADH formation. Reactions with serotonin metabolites were quenched by addition of perchloric acid, and metabolites were separated by HPLC and detected electrochemically [17].

Cloning and Expression

Human ADH5 and rat ADH6 were cloned with conventional techniques from liver cDNA libraries and the PCR technique with oligonucleotides designed after a published cDNA sequence [19]. Genomic DNA was prepared from blood samples and the ADH5 3′-end region was amplified with the PCR technique for sequence analysis. For *in vitro* translation the cDNA coding for the ADH6 was subcloned into transcription vector pTRIkan. This cDNA was further cloned into pEGFP adjacent to the coding sequence for green fluorescence protein for transfection into COS cells. The harvested cells were fixed and analyzed for protein expression.

Data Analysis

To fit lines to kinetic data points and to calculate kinetic parameters, a weighted non-linear-regression analysis program was used (Fig.P for Windows). DNA and deduced protein sequences were analyzed using the University of Wisconsin Genetics Computer Group Program and compared with EMBL data banks. For phylogenetic calculations, the programs Clustal W and Tree View were used.

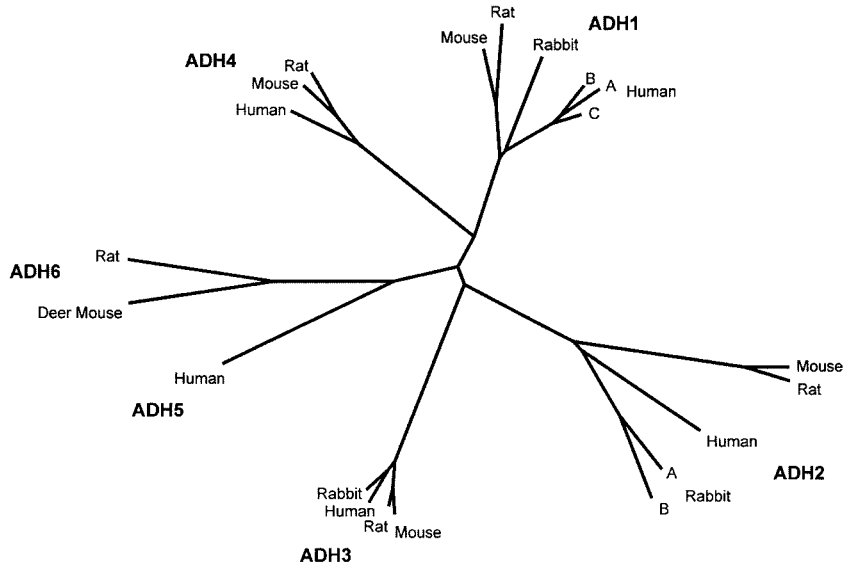


Fig. 1. Unrooted phylogenetic tree, relating the human, mouse, rabbit and rat ADH1–ADH6 enzymes. Isoenzymic forms are denoted A, B and C. Sequence data were from data banks, and line lengths are proportional to separation distances.

Results and Discussion

Mammalian ADH forms a setup of enzymes, isoenzymes and allelic variants with ADH3 as the ancestral form (fig. 1). All the different ADHs participate to some extent in the cell defense towards exogenous alcohols and aldehydes. ADH3 is the only enzyme, so far, within the system that has been ascribed a physiological substrate, HMGSH. All enzymes are capable of metabolizing ingested ethanol where the highly expressed ADH1 is established as the main ethanol-metabolizing enzyme. Several of the ADHs participate in specific metabolic pathways and metabolism of ethanol will therefore interact with these specific pathways (fig. 2). For example, ethanol oxidation affects human serotonin, bile acid and retinoid metabolism by increasing the relative formation of alcohol products, while decreasing the formation of carboxylic acid products [3, 12, 17]. These interactions are often underestimated and the contribution to the total ethanol metabolism of the higher classes is poorly understood. However, a direct effect of ethanol metabolism is a change in redox state within the cell, where the NADH/NAD^+ ratio has been shown to increase one order of magnitude in rat hepatocytes [2].

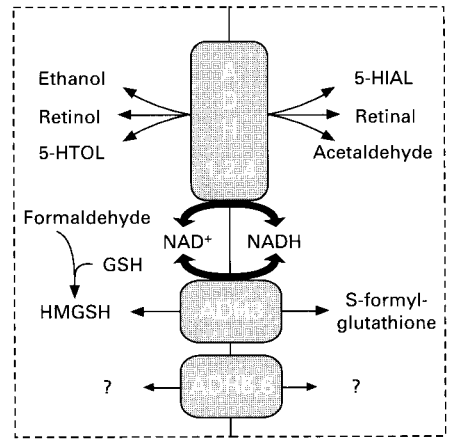


Fig. 2. Interaction of metabolic pathways where ADH is involved. 5-HTOL = 5-Hydroxytryptophol; 5-HIAL = 5-hydroxyindole-3-acetaldehyde; GSH = glutathione; HMGSH = S-hydroxymethylglutathione.

Ethanol Metabolism

The main function associated with mammalian ADH, ethanol oxidation, has been thoroughly described in the literature [5 and references therein]. All ADHs can participate in this metabolism; however, ADH3 is not able to saturate with ethanol and the rodent ADH2 enzymes show only traces of ethanol dehydrogenase activity. Furthermore, aldehyde oxidation catalyzed by ADH1 can be as efficient as the classical alcohol oxidation [16]. By sequential oxidation and reduction of an aldehyde the dismutation circumvents the slow step of coenzyme dissociation. Human ADH2 has a higher K_m value for ethanol

Table 1. Kinetic constants for human and rat ADHs

	EtOH		5-HTOL		All-trans-retinol		HMGSH	
	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}
ADH1								
Human (AA)	4.2	6.7			0.056	92	–	NA
Human (BB)	0.05	29			0.045	20	–	NA
Human (CC)	0.52	100	0.22	350	0.29	19	–	NA
Rat	1.4	28			0.047	250	–	NA
ADH2								
Human	8	1.4	0.053	180	0.014	650	–	NA
Rat	NS	0.0006			0.0015	85	–	NA
ADH3								
Human	NS	0.045	–	NA	–	NA	0.004	50,000
Rat	ND	ND					0.00092	235,000
ADH4								
Human	28	65	1.3	80	0.031	190	–	NA
Rat	5,000	24					–	NA

All values are determined at pH 7.5, 25 °C, except the values for HMGSH that are determined at pH 8.0.
Human ADH1: AA = αα-isoenzyme; BB = ββ-isoenzyme; CC = γγ-isoenzyme. Neither human ADH5 nor rat ADH6 have been isolated as active proteins. Therefore they are excluded from the table. Values are from the authors' laboratory or from Duester [3]. NA = No activity; NS = not possible to saturate.

than the ADH1 isoforms and therefore makes a significant contribution to ethanol metabolism only at high ethanol concentration [11]. Results from our determinations, however, show a fairly low K_m for ethanol, 8.0 m*M* at pH 7.5 (table 1) [18], as compared to literature values that are in the range from 34 to 120 m*M* [5, 11]. Not only acetate and 5-hydroxyindole acetate, but also chloride ions competitively inhibit ADH2 and this sensitivity for anions might explain the K_m anomaly. The contribution of the higher classes, ADH5 and ADH6, is unknown since they have not been isolated at the protein level. Both show amino acid residues at the substrate-binding pocket that suggests low ethanol dehydrogenase activity. In a large European study, two allelic ADH1 forms, ADH1B2 and ADH1C1, have been suggested to protect from alcoholism [1]. Notably, these two alleles are associated and are the most active ADH forms in ethanol metabolism.

Serotonin Metabolism

Ethanol intake significantly changes the cytosolic redox potential by increasing the NADH/NAD^+ ratio. This makes reductive ADH metabolism more favorable, which may explain the increased turnover of 5-hydroxyindole-3-acetaldehyde (5-HIAL) to 5-hydroxytryptophol (5-HTOL), observed after ethanol intake [17]. We have shown that 5-HIAL reduction is efficiently catalyzed by ADH1 (γγ-isoenzyme/ADH1C) and that 5-HIAL reduction is roughly 40-fold more efficient than 5-HTOL oxidation. The higher efficiency is due to higher k_{cat} for aldehyde reduction than for alcohol oxidation as observed for most alcohol/aldehyde pairs [17, 18]. In addition, a comparison of specificity constants shows that ethanol would at high concentrations competitively inhibit oxidation of 5-HTOL. ADH2 can partly contribute to this serotonin metabolism, where ADH3 lacks and ADH4 shows traces of this capability (table 1).

Retinol Metabolism

ADH is probably one of the main enzymes in the conversion of different isomers of retinol to the corresponding retinals. The extrahepatically distributed ADH4 has the highest specific activity for retinols (table 1). However, ADH1 and ADH2, both found in large amounts in the liver, can convert retinols into retinals. In rodents, the ADH2 cannot use all-trans-retinol as a substrate but 9-cis-retinol seems to be a substrate for rat ADH2 [14]. ADH4 involvement in cell differentiation has been established in several investigations, which is verified of the

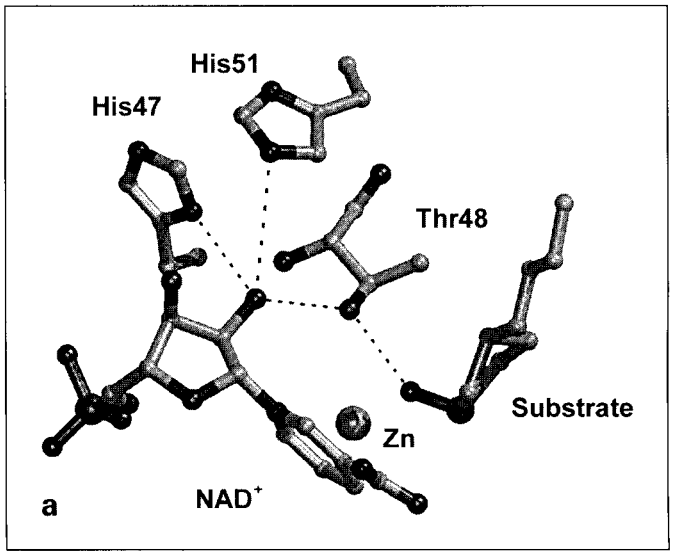
colocalization of ADH4 expression and conversion of retinols into retinoic acid [3].

Formaldehyde Metabolism

ADH3 is the only ADH that can participate in cell detoxification of formaldehydes. Formaldehyde spontaneously forms HMGSH with glutathione that is converted into S-formylglutathione by ADH3, an activity that can be traced in all living species that contain glutathione [10]. The mechanism for this reaction is identical to any alcohol oxidation by ADH and produces NADH, which thereby changes the redox state in the cell. Furthermore, recently it has been shown that ADH3 is able to reduce GSNO, a conjugation product between glutathione and NO, but the physiological relevance of this reaction is not known [8].

The above activities show the interference between different ADH substrates that competitively inhibit each other. Furthermore, ethanol oxidation will heavily change the NADH/NAD^+ ratio that directly disturbs other metabolic pathways (fig. 2). These examples of interactions can be one molecular explanation to fetal alcohol syndrome.

The higher classes, ADH5 and ADH6, showed unexpected gene arrangements. Notably, ADH5 was reported to be truncated at the C-terminus due to a deletion of the last exon [19]. We have shown, however, that the last exon is present in the ADH5 gene, that yields a shorter 'truncated' and a longer 'full-length' message, which can be explained by a complex transcription pattern. Rat ADH6, only 65% identical to ADH5, was isolated as a cDNA with an open reading frame that codes for 389 amino acid residues which yields an elongated N-terminus as compared to the other mammalian ADHs. Both these ADHs show residues at the active-site pocket that deviate from other mammalian ADHs. The coenzyme interacting residue at position 47 is Gly, in contrast to Arg and His in most mammalian forms. Thr at position 48 is identical with ADHs without steroid dehydrogenase activity; Lys51 corresponds to His in ADH1, which is proposed to interact in a charge relay system (fig. 3) [5]. Concomitantly, this amino acid residue setup suggests a low alcohol dehydrogenase activity. In vitro translation of the ADH6 mRNA yielded a protein product slightly larger than the product of ADH1 mRNA (43 kD as compared to 40 kD) and expression of ADH6 in COS cells, as judged from a fluorophore of ADH6 fused to green fluorescence protein, suggests a correctly folded protein. However, so far no active protein has been isolated when conventional substrates have been used.



Enzyme/Position	47	51
ADH1	Arg	His
Hu ADH2	His	Thr
Ra ADH2	Pro	Asn
ADH3	His	Lys
Hu ADH4	Arg	His
Ra ADH4	Gly	His
ADH5/ADH6	Gly	Lys

b

Fig. 3. Active site of mammalian ADH with His47 and His51. **a** A histidine at either position 47 or 51 has been shown to be a prerequisite for ethanol dehydrogenase activity to maintain the charge relay system. The active site zinc, NAD^+ and substrate are shown together with residues involved in the hydrogen-bonding network. **b** Amino acid residues at positions 47 and 51 in different mammalian ADHs.

For ADH2, the variability around the substrate-binding pocket is profound, with residue insertions and deletions as compared to the other classes of ADH. Several species variants of ADH2 have been described, and the rodent ones form a functionally distinct subgroup with interesting catalytic properties (fig. 1). Rodent ADH2s are by several orders of magnitude less efficient in alcohol oxidation as compared to other ADHs and are not able to saturate with ethanol (table 1). By replacing Pro47 with His, the ADH activity can be restored (fig. 3). In addition to alcohol/aldehyde oxidation/reduction, the ADH2 en-

zymes are capable of catalyzing the reduction of benzoquinones [18]. Notably, this activity is not affected by the Pro47His mutation. The structures provide new information on the generation of functional diversity between the ADH classes (fig. 1, 2) and give insights into the function of this particular ADH class. In a comparative study of human and rodent forms, the rodent ADH2 enzymes seem to be involved in reductive rather than in the oxidative catalysis.

The entire ADH system works as a general detoxification system that protects the cell from toxic alcohols and aldehydes. In many respects, the system can be compared to other detoxifying systems, e.g. the cytochrome P450 system, but without generation of cell-toxic radicals. However, the overall mechanism for the entire ADH system has as yet to be established.

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