Mice and rats were found to possess class II alcohol dehydrogenases with novel enzymatic and structural properties. A cDNA was isolated from mouse liver and the encoded alcohol dehydrogenase showed high identity (93.1%) with the rat class II alcohol dehydrogenase which stands in contrast to the pronounced overall variability of the class II line. The two heterologously expressed rodent class II enzymes exhibited over 100-fold lower catalytic efficiency ($k_{cat}/K_m$) for oxidation of alcohols as compared with other alcohol dehydrogenases and were not saturated with ethanol. Hydride transfer limited the rate of octanol oxidation as indicated by a deuterium isotope effect of 4.8. The mutation P47H improved hydride transfer and turnover rates were increased to the same level as for the human class II enzyme. Michaelis constants for alcohols and aldehydes were decreased while they were increased for the coenzyme. The rodent class II enzymes catalyzed reduction of p-benzoquinone with about the same maximal turnover as for the human form. This activity was not affected by the P47H mutation while a S182T mutation increased the $K_m$ value for benzoquinone 10-fold. ω-Hydroxy fatty acids were catalyzed extremely slow but functioned as potent inhibitors by binding to the enzyme-NAD$^+$ complex. All these data indicate that the mammalian class II alcohol dehydrogenase line is divided into two structurally and functionally distinct subgroups.

The family of alcohol dehydrogenases (ADH) has a well-documented ability to metabolize various alcohols and aldehydes and on the basis of these enzymatic properties one can assign potential functions for these enzymes in the metabolism of steroids, biogenic amines, lipid peroxidation products, retinoids, as well as xenobiotics (1–6). For assessment of the physiological role of ADHs, knowledge of the whole ADH system is of an old origin with a split into at least seven vertebrate classes of which six have been identified in mammals (6, 7). Within the murine ADH system, three different enzymes were early identified, and their corresponding genes were named Adh-1, -2, and -3 (8) encoding classes I, III, and IV, respectively (9–11). Neither class II nor class V/VI have, as yet, been isolated or cloned and it has been suggested that mice either lack these genes or possess forms with very low identity relative to the human variants (11).

The human form was the first class II ADH to be identified. This enzyme is predominantly found in liver and contributes to the metabolism of ethanol (12–14). It has a preference for unsaturated hydrophobic aldehydes and has been suggested a redox specific role in the noradrenaline metabolism (2, 13). Furthermore, this form is particularly effective in the reduction of the lipid peroxidation derived 4-hydroxyalkenals (4). Although the physiological implication is not clear, class II ADH also catalyzes the reduction of some benzoquinones and benzoquinone imines (15). At the structural level an extreme variability of the class II line is well established from characterization of species variants (16–18).

This paper reports on the existence of an ADH of class II type in mouse which together with the rat counterpart form a conserved subgroup of class II ADHs that exhibits low catalytic efficiency as a consequence of slow hydride transfer. The enzymatic characteristics and the consequences of the unique coenzyme binding residues Pro47 and Ser182 are investigated.

**EXPERIMENTAL PROCEDURES**

Isolation of a cDNA Coding for Mouse Class II ADH—A cDNA coding for an ADH of class II type was isolated from an adaptor-ligated mouse cDNA library (Marathon-Ready™ cDNA, CLONTECH), by PCR amplification utilizing $F_7$ polymerase (Stratagene). Multiple sequence alignments of characterized class II ADH sequences were used to localize regions with a high degree of positional identity and two primers were designed: 5'-TGGCCAGAAGTGAACA-3' and 5'-AGTCAGTG-GCTCCAGGGC-3' (Fig. 1). Cycling conditions were: 30 cycles of 95 °C, 45 s; 68 °C, 1 min; 72 °C, 1.5 min. PCR amplification yielded a 500-bp fragment which was subsequently ligated into the vector pCR II (Invitrogen) according to the TA-cloning protocol (19) for sequence analysis.

The full-length cDNA was obtained by using a rapid amplification of cDNA ends technique. An adaptor-specific primer, AP2 (CLONTECH), together with class II cDNA-specific primers: 5'-GAGCCCTCTCTCAAACCTCTTTG-3' and 5'-CTACACACCCCCGCCAACG-3', were used for the 5' and 3' rapid amplification of cDNA ends reactions, respectively. The PCR protocol was as follows: 33 cycles of 95 °C, 45 s; 66 °C, 1 min; 72 °C, 3 min. Sequence Analysis—DNA sequence analysis was performed with the dideoxy method (20) on both strands with sequence-specific primers using T7 DNA polymerase (Amersham Pharmacia Biotech), [α-32P]dATP (Amersham Pharmacia Biotech), and alkali-denatured plasmids. Deduced sequences were analyzed with the computer program GC/MS (21) and compared with EMBL data banks. The program ClustalW 1.7 (22) was used to create the sequence alignments and in combination with TreeView (Win16) (23) to investigate phylogenies.

Expression Plasmids and Site-directed Mutagenesis—The entire coding region of mouse class II ADH cDNA was PCR amplified with...
primers introducing restriction sites NdeI and BamHI, respectively, which facilitated subcloning into the unique restriction sites NdeI/BamHI of the pET29 expression vector (Novagen). In the same manner, the coding region of rat class II cDNA (17) was subcloned into the Ncol and BamHI restriction sites of pET3d (Novagen). Both plasmid constructs were verified by sequence analysis throughout the entire coding region.

Double-stranded plasmid was prepared with the flexiprep kit (Amersham Pharmacia Biotech) for mutagenesis of mouse class II cDNA. Reagents in the U.S.E. mutagenesis kit (Amersham Pharmacia Biotech) and mutagenesis primers: ACGTGTGTGCCA/G/C/TACTGACTC/CA/T/AC/CT/CAC/GC/AC/GCGA/GC/AC/CG/TCCGCCTGGT-3 were used to alter the codons and subsequently replacing Pro47 for His, Asn84 for His, and Ser182 for Thr (nucleotides corresponding to the changed codons are underlined). Selection was based on the elimination of the unique XbaI site of the pET29 vector according to the method described by Deng and Nickoloff (24). Sequence analysis was performed to confirm the presence of the correct mutations and the absence of any unexpected mutations in the cDNA.

Expression and Isolation of Class II ADH—Recombinant protein was expressed in 1-liter LB cultures of Escherichia coli strain BL21(DE3) at 29 °C and induced with a burst of 0.8 mM isopropyl-thio-β-D-galactosidase at an OD595 of about 1. Cells were harvested 4 h later and disrupted which facilitated subcloning into the unique restriction sites NdeI/BamHI. The coding region of rat class II cDNA (17) was subcloned into the NdeI/BamHI site of pET29 which had been modified by the deletion of the 200-bp 3′ noncoding region, a poly(A) signal, and a 200-bp spacer. Selection was performed according to the method described by Deng and Nickoloff (24). Sequence analysis was performed to confirm the presence of the correct mutations and the absence of any unexpected mutations in the cDNA.

Enzyme Assays—Enzyme activity was monitored with a Hitachi U-3000 spectrophotometer by following the conversion of NAD+ (εmax 6.22 mm2 cm−1) with exception for oxidation of all-trans-retinol (εmax 29.5 mm2 cm−1) (25) and menadione where activity was monitored with 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyloxazol bromide (εmax 11.3 mm2 cm−1) as described previously for DT-diaphorase (26). Alcohol dehydrogenase activity was measured in potassium phosphate, pH 7.5, and in 0.1 mM potassium phosphate, pH 7.5, before elution with 2.5 mM NAD+ in 10 mM potassium phosphate, pH 7.5, with 0.3 mM dithiothreitol and 10 mM β-mercaptoethanol and 10 μM ZnSO4. Isolated class II ADH was subjected to buffer change to 10 mM Hepes, pH 7.5, by gel filtration on Sephadex G-25 columns (Amersham Pharmacia Biotech), concentration (Microsep 30K, Pall Filtron) and purity analysis by SDS-polyacrylamide gel electrophoresis. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as standard complemented with amino acid analysis on a U-3000 spectrophotometer by following the conversion of NAD+ (εmax 6.22 mm2 cm−1) with exception for oxidation of all-trans-retinol (εmax 29.5 mm2 cm−1) (25) and menadione where activity was monitored with 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyloxazol bromide (εmax 11.3 mm2 cm−1) as described previously for DT-diaphorase (26). Alcohol dehydrogenase activity was measured in potassium phosphate, pH 7.5, and in 0.1 mM glycine/NaOH, pH 10.0, at 25 °C with 2.4 mM NAD+ while reductase activity was assayed in 0.1 mM potassium phosphate, pH 7.5, with NADH concentrations of 0.2 mM. For quinone reduction, 0.1 mM NADH was used instead. If not else stated, reagents were from Sigma and of the highest purity readily available. Octanol and [1,1-2H2]octanol was determined to 98%. Benzaldehyde and acetaldehyde were distilled before use. 4-Hydroxyoctenal, kindly supplied by Dr. Sacchi (29). Poly(A)-enriched RNA was obtained by mRNA purification with the Oligotex™ mRNA kit (Qiagen). 0.5 μg from each tissue was subjected to electrophoresis in 1% agarose and blotted to a nylon transfer membrane (Hybond-N™, Amersham Pharmacia Biotech). Class II mRNA was probed with a 311-bp cDNA fragment chosen for its low similarity with other ADH cDNAs (bp 337–648 in the cDNA), and a 2-kilobase fragment of human β-actin cDNA (CLONTECH) was used as a control. The probes were labeled with [α-32P]dCTP to a specific activity of 5 × 108 cpm/μg (megaprime DNA labeling system, Amersham Pharmacia Biotech). Hybridizations were performed at 42 °C overnight in 50% formamide, 5 × SSPE (1 × = 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 10 × Denhardts solution (1 × = 0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 2% SDS, and 1 mg/ml salmon sperm DNA. Filters were washed with high stringency in 0.1 × SSC (1 × = 0.15 M NaCl, 15 mM sodium citrate, pH 7.6), 0.1% SDS before exposure to Kodak X-Omat films for 1–7 days using intensifying screens.

RESULTS

Structural and Evolutionary Characteristics of Rodent Class II ADHs—A cloning strategy based on the high sequence identity of earlier characterized class II ADHs in the regions around amino acid residues 79 and 241 was used to PCR amplify a 500-bp fragment of the mouse class II ADH cDNA. The full-length cDNA was thereafter isolated from an adapter ligated mouse liver cDNA library using the rapid amplification of cDNA ends technique. The entire cDNA sequence, covering 1554 bp, included a 1131-bp coding region, a 25-bp 5′ noncoding region, a 200-bp 3′ noncoding region, a poly(A) tail, and a poly(A) tail (Fig. 1). The region around the translation start codon was similar to that of the corresponding human form but lacked similarities with the consensus sequence, CCRC-CATGR, except for ATG and the purines at position +3 and +4 (30). The cDNA translated into a 376-amino acid polypeptide that had a sequence identity of 93.1% with rat class II ADH. A relative rate test of divergence versus ostrich class II ADH showed higher positional identities with ostrich for the human and rabbit isoforms (68–70%) than for the mouse and rat forms (65–66%). Still, it could not be excluded that differences in identities might be explained by the natural higher rates of nucleotide substitutions in rodents than in man (31). The phylogenetic tree of class I-IV ADHs showed generally longer branches in the class II line indicating faster divergence than for class I, III, and IV (Fig. 2). The short separation distance between rat and mouse class II as compared with the other
class II forms is not compatible with the general assumption of a uniform divergence rate for the same enzyme in different species.

Alignments of all structurally characterized class II ADHs revealed three variable regions around positions 60, 120, and 300 with insertions and deletions as compared with the ADH consensus sequence. Within these regions there are two deletions specific for the rodent class II ADHs and a 4-residue insertion at position 289–300. Deviations from the consensus sequence of ADHs were found at three coenzyme binding positions, 47, 51, and 182. The latter corresponds to 178 in the class I ADH numbering system, a residue positioned on the opposite side of the nicotinamide ring as compared with the substrate. The alcohol or octanol and gave turnover numbers were 5–10-fold lower and saturation was reached at far higher concentrations. In the case of ethanol, none of the rodent class II forms were saturated with substrate. The reduction of aldehydes was more efficiently catalyzed by the rodent forms than alcohol oxidation, still turnover numbers were 5–10-fold lower than for the human form. In contrast, benzoquinone reduction proceeded with about the same turnover rate for all class II forms as compared with the human form. Turnover numbers were more than 10-fold lower and saturation was reached at far higher concentrations. In the case of ethanol, none of the rodent class II forms were saturated with substrate. The reduction of aldehydes was more efficiently catalyzed by the rodent forms than alcohol oxidation, still turnover numbers were 5–10-fold lower than for the human form. In contrast, benzoquinone reduction proceeded with about the same turnover rate for all class II forms as compared with the human form. Turnover numbers were more than 10-fold lower and saturation was reached at far higher concentrations. In the case of ethanol, none of the rodent class II forms were saturated with substrate. The reduction of aldehydes was more efficiently catalyzed by the rodent forms than alcohol oxidation, still turnover numbers were 5–10-fold lower than for the human form. In contrast, benzoquinone reduction proceeded with about the same turnover rate for all class II forms as compared with the human form.
pH 10.0 and at least 200-fold at pH 7.5 as compared with the wild-type enzyme. An increase was seen for aldehyde reduction as well, but less pronounced (10–50-fold). The N51H mutation did not significantly effect the catalytic activity of the enzyme with the exception for a 3-fold decrease in octanol and octanal $K_m$ values. The S182T mutation increased $k_{cat}/K_m$ values for alcohol oxidation (5–10-fold) while the corresponding values for aldehyde reduction decreased 2-fold. The most striking characteristic of this mutant was a 10-fold increased $K_m$ value for benzoquinone (Table II).

Coenzyme saturation was studied for human class II, mouse class II, and the P47H mutant and the Michaelis constants were determined (Table IV). The wild-type mouse enzyme had lower $K_m$ values for NAD$^+$ and NADH as compared with the human form, 10- and >30-fold, respectively. Introduction of the P47H mutation gave an increase in the $K_m$ values to a level comparable with that of the human form. Furthermore, NADPH could not serve as a coenzyme for mouse class II ADH.

Isotope effects were determined using octanol and [1,1-$^2$H$_2$]octanol as substrates (Table V). A large isotope effect was seen on $k_{cat}$ and $k_{cat}/K_m$ for mouse wild-type enzyme showing that the hydride transfer step was rate-limiting for the oxidation of octanol. Isotope effects were seen for the P47H and S182T enzymes as well, indicating that, although catalytic efficiency was increased, hydride transfer was at least partially rate-limiting. The lack of isotope effect on $k_{cat}$ for human class II ADH is compatible with coenzyme release being rate-limiting which has been proposed previously (13).

The effect of ammonia on the benzyl alcohol and octanol activity of mouse class II ADH was studied at pH 9.4. Addition of 1–20 mM ammonia did not significantly effect the specificity constants for oxidation of these alcohols suggesting that introduction of exogenous amines as proton acceptors do not increase the activity of the enzyme.

**DISCUSSION**

*Structure and Function Indicates a Novel Subgroup of Class II ADH—In the vertebrate ADH family seven distinct classes*
A Novel Subtype of Class II Alcohol Dehydrogenase in Rodents

Steady-state kinetic constants for alcohol oxidation catalyzed by mouse, rat, human, and mutant forms of mouse class II ADHs

| Enzyme   | Ethanol  | Octanol  | Benzyl alcohol |
|----------|----------|----------|----------------|
|          | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ |
| pH 10.0  |
| Rat wt   | 0.04     | 0.04     | 0.12           | 0.12     | 0.45     | 0.027        |
| Mouse wt | 0.11     | 0.22     | 0.65           | 0.65     | 2.7      | 0.24         |
| N51H     | 0.09     | 0.076    | 0.85           | 0.85     | 1.7      | 0.5          |
| S/2T     | 0.61     | 0.25     | 2.9            | 2.9      | 1.6      | 1.3          |
| P47H     | 2.2      | 0.070    | 8.5            | 8.5      | 0.85     | 8.9          |
| Human wt | 8.3      | 0.007    | 9.2            | 9.2      | 0.007    | 1300         |
| pH 7.5   |
| Mouse wt | 0.001    | 0.48     | 0.002          | 0.012    | 3.5      | 0.003        |
| N51H     | 0.004    | 0.052    | 0.077          | 0.020    | 1.7      | 0.012        |
| S182T    | 0.008    | 0.52     | 0.015          | 0.033    | 2.5      | 0.013        |
| P47H     | 0.22     | 0.13     | 1.7            | 0.67     | 1.4      | 0.48         |
| Human wt | 0.019    | 2.4      | 0.003          | 0.12     | 0.007    | 17           |

$*The enzyme was not possible to saturate with ethanol. The activity was directly proportional to ethanol concentrations up to 1.7 mM and the $k_{cat}/K_m$ value was derived from the slope in the velocity versus ethanol graph.

Steady-state kinetic constants for aldehyde and p-benzoquinone reduction at pH 7.5 catalyzed by mouse, rat, human, and mutant forms of mouse class II ADHs

| Enzyme   | Acetaldehyde  | Octanal  | Benzaldehyde | p-Benzoquinone |
|----------|---------------|----------|--------------|----------------|
|          | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ |
| Rat wt   | 0.28     | 41       | 0.0068       | 1.1      | 0.67     | 1.6         | 0.055     | 1.3       | 0.42       |
| Mouse wt | 0.82     | 0.022    | 0.22          | 1.7      | 0.20     | 8.5         | 1.0       | 0.45      | 2.9        |
| N51H     | 0.55     | 1.3      | 0.42          | 1.3      | 0.45     | 2.9         | 9.0       | 0.24      | 37         |
| S182T    | 0.0077   | 23       | 0.11          | 1.1      | 0.27     | 4.1         | 8.3       | 0.23      | 36         |
| P47H     | 12       | 0.060    | 0.47          | 12       | 0.17     | 28          | 7.5       | 0.27      | 28         |
| Human wt | 9.5      | 0.007    | 1400          | 0.14     | 0.088    | 160         |          |           |            |

$*ND, not determined.

have been identified which share about 65% positional identity. In the mouse only three of these classes have been described earlier: class I, III, and IV. With the aim to further investigate the ADH repertoire we found an ADH of class II type with novel structural and functional characteristics. The class II branch of the ADH family show extreme divergence, and the rate for introduction of nonsynonymous substitutions are 2- and 6-fold higher as compared with the class I and III ADHs, respectively. The low identity between the human and mouse class II cDNAs probably explains why cross-hybridization at the mRNA level has been unsuccessful for this class of ADH (11). With this in mind, the high identity between mouse and rat class II ADH (93.1%, at the protein level) is surprising. The corresponding value for the overall more conserved class I ADH is 89.6% and for the highly conserved class III ADH is 96.5%. The deviation from a uniform divergence in species variants could be indicative of a different function for this enzyme in rodents or even the existence of two subtypes of class II ADH that are related by gene duplication, i.e., they are paralogous rather than orthologous. Thus far, gene duplications of class II ADH have only been detected in rabbit, and although one variant shares some enzymatic characteristics with the rodent class II ADHs, structural identities indicate a more recent gene duplication for the two rabbit isoforms (18).

By immunoblotting of tissue homogenates, rodent class II were found to be predominantly expressed in liver, a pattern resembling that of the human variant. Immunoreactive signals were also found for kidney homogenates from both rat and mouse. Class II mRNA has previously been found absent in rat kidney (34) which evidently also was the case for mouse kidney (Fig. 3). We conclude that our immune serum cross-reacts with a protein in kidney with a subunit mass of the same size as mouse class II ADH (40 kDa) which possibly could be an uncharacterized ADH expressed in the kidney (35). Most enzymes catalyze reactions with turnover numbers between 1 and 1000 s$^{-1}$ (36) and the human class II ADH oxidizes alcohols at a maximal rate of 4–9 s$^{-1}$ at pH 10.0 and more than 10-fold lower at physiological pH (13, 37). The kinetic constants determined for the human class II ADH in this study were in agreement with these previous results. In comparison, the rodent forms had over 10-fold lower turnover for alcohol oxidation and this difference was observed at both pH 7.5 and 10.0. In addition, $K_m$ values were higher for the alcohol/aldehyde pairs used for comparison of species variants (Tables I and II). While the human form contributes to the metabolism of ethanol in the liver, the rodent forms were not possible to saturate with this alcohol. Taken into account, the rodent forms of class II ADH can be considered as low activity ADHs, with presumably no significance in general alcohol detoxification. The low activity can further explain why this form of ADH has not previously been described in studies on liver ADH activity in these species (38, 39). The characterization of the mouse class II ADH indicated no overlap in activity for endogenous substrates with other classes within the ADH family (Table III). Moreover, class II characteristic substrates such as 4-hydroxynonenal and benzaldehyde derivatives were metabolized with low efficiency. In consequence, the reductive metabolism of 4-hydroxynonenal in rat liver homogenates, attributed to class I ADH only, is low
compared with the biocconversion via the glutathione S-transferase pathway (40). Since human class II ADH is far more efficient than class I for this reaction, it is possible that reductive metabolism of 4-hydroxynonenal is more pronounced in homogenates of human liver. Furthermore, mouse class II ADH oxidized ω-hydroxyfatty acids at an extremely slow rate. Still they were potent inhibitors against octanol oxidation (Fig. 5). Since octanoic acid also inhibited octanol oxidation competitively, it is likely that the carboxylic group coordinates to the catalytic zinc in the enzyme-NAD$^+$ complex where the charged nicotinamide ring also can contribute with electrostatic interactions. The special substrate repertoire can be explained by a number of exchanges at residue positions suggested to be important for substrate binding (Table VI). Notably, while the residues are identical for mouse and rat class II ADH at all these positions, the residue identity between mouse and human class II ADH is only 54% which is even lower than the positional identity between the entire sequences (72%).

"The Role of Pro$^{47}$ and Ser$^{182}$ for Alcohol Dehydrogenase Activity—The ADH activity of the rodent class II forms could be drastically improved by replacing Pro with His at position 47. The effects of residue exchanges at this position have been studied extensively and explains, e.g. the activity differences between the allelic variants of the human ADH class I β isoform and the resistance to allyl alcohol poisoning of mutant yeast strains (41–43). The guanidino group of Arg$^{47}$ stabilizes the enzyme-coenzyme complex by the formation of a salt bridge with the pyrophosphoryl moiety of the coenzyme (44–46) and subsequently, coenzyme dissociation rates are in general

table iii

| Substrate                  | $K_m$ | $k_{cat}$/$K_m$ | $K_i$ |
|---------------------------|-------|----------------|-------|
|                           | mM    | s$^{-1}$ m$^3$M$^{-1}$ | µM   |
| **Alcohols**              |       |                |       |
| Ethanol                   | 0.000019 | ND           | ND    |
| Octanol                   | 0.22  | 0.5            | ND    |
| Benzy alcohol             | 2.7   | 0.24           | ND    |
| 4-Hydroxybenzyl alcohol   | 0.051 | 0.78           | ND    |
| 4-Hydroxy-3-methoxybenzyl alcohol | 1.9  | 0.34           | ND    |
| (R)(−)-t-Phenyl-1,2 ethanediol | No activity | NI    |
| 5-Hydroxycyclopentanol    | No activity | NI    |
| All-trans-retinol         | No activity | NI    |
| 5α-Androstan-3β-ol-17-one | No activity | NI    |
| 5β-Androstan-3β-ol-17-one | No activity | NI    |
| Cyclohexanol              | Low activity | 700 ± 100 |
| **Fatty acids**           |       |                |       |
| Acetaldehyde              | 37    | 0.022          | ND    |
| Octan-1-ol                | 0.20  | 8.5            | ND    |
| 4-Hydroxyoctenal          | 0.13  | 1.3            | ND    |
| Benzy aldehyde            | 0.45  | 2.9            | ND    |
| **Pyrazoles**             |       |                |       |
| Pyrazole                  | No activity | 5400 ± 1600 |
| 4-Methylpyrazole          | No activity | 2400 ± 370 |
| 4-Bromopyrazole           | No activity | 33 ± 2  |
| **Quinones**              |       |                |       |
| p-Benzoquinone            | 0.24  | 37             | ND    |
| Q$_1$                     | No activity | NI    |
| Menadione                 | No activity | NI    |
| a ND, not determined.     |       |                |       |
| b Detection limit was 0.0002 s$^{-1}$. |       |                |       |
| c No detectable inhibition of benzyl alcohol oxidation. |       |                |       |
| d The maximal activity, 0.004 s$^{-1}$ at pH 10.0 was too low for determination of kinetic constants. |       |                |       |
| e The maximal activity, 0.602 s$^{-1}$ at pH 10.0 was too low for determination of kinetic constants. |       |                |       |

FIG. 5. Inhibition patterns for mouse class II ADH with 12- HDA. Concentrations of the varied substrate is indicated on the graphs while the concentration of 12-HDA increase from the bottom line to top. A, inhibition by 0, 1.9, 3.8, and 7.7 µM 12-HDA at 2.4 mM NAD$^+$. The $K_m$ was 6.0 ± 0.7 µM. B, inhibition by 0, 4, and 8 µM 12-HDA at 40 µM 4-hydroxybenzyl alcohol. The $K_m$ was 5.0 ± 0.4 µM.

Table IV

| Enzyme   | $K_m$ | $K_m$ | µM |
|----------|-------|-------|----|
| wt mouse | 9     | <0.5  |    |
| S182T    | 43    | 4     |    |
| P47H     | 30    | 14    |    |
| wt human | 85    | 15    |    |

Table V

| Enzyme   | $K_m$ | $K_m$ | µM |
|----------|-------|-------|----|
| wt mouse | 4.8   | 4.9   |    |
| S182T    | 4.1   | 5.0   |    |
| P47H     | 3.5   | 4.5   |    |
| wt human | 1.0   | 1.2   |    |
slower with stronger bases than with neutral or mild bases at this position (43). Substitutions at position 47 also affects hydride transfer (43). In addition to His47 and Arg47, Gly47 are found in a few ADHs whereas Pro47 is unique for the rodent forms of class II ADH. Local structural rearrangements and/or alternative coenzyme binding seems to compensate for the lack of salt bridge formation with Gly47 and results in coenzyme dissociation constants comparable to that of Arg47 variants (47). In analogy, the P47H replacement could result in weaker coenzyme binding and increased turnover rates. Still, the pronounced isotope effect found for mouse class II ADH showed that hydride transfer is rate-limiting for alcohol oxidation. This implies that the two mutations P47H and S182T, both increasing the catalytic efficiency more than 1 order of magnitude, are slightly different than for aldehyde reduction. This could be a consequence of the longer distance between hydride transfer and proton donation for quinone reduction since the hydride is not transferred to the α-carbon in this case, but to the oxygen at the opposite side of the quinone ring, yielding hydroquinones.

The residue replacements discussed are shared by both rodent class II ADHs and make them biased for quinone reductase rather than alcohol oxidation and thus makes us suggest that this enzyme is primarily involved in reductive metabolism. Although derivatives of benzoquinone are naturally occurring, the physiological significance of this activity is not clear and must be studied further. Mouse class II did not catalyze reductit of the naptoquinone menadione. Neither was Q10, the functional part of the ubiquitous coenzyme Q10, a substrate for the enzyme. For the human enzyme, lack of activity for bulkier quinones have been suggested to be the result of steric hindrance due to an assumed narrow substrate pocket (15). In conclusion, the special functional characteristics together with the structural conservation between the mouse and rat class II enzymes as opposed to the divergence in the class II line in general, strongly indicates that the rodent enzymes form a class II subgroup within the ADH family.

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