Article I

Contribució de B.Crosas en el treball:

- Clonació del cDNA de l'ADH4 humana:
    - Triatge d'una biblioteca de cDNA d'estómac humà en el vector λgt10
    - Aïllament dels clons i clonatge a pBluescript
- Anàlisi de la seqüència i comparació amb les bases de dades de seqüències

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Alcohol dehydrogenase of class IV ($\sigma\sigma$-ADH) from human stomach
cDNA sequence and structure/function relationships

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Human stomach mucosa contains a characteristic alcohol dehydrogenase (ADH) enzyme, $\sigma\sigma$-ADH. Its cDNA has been cloned from a human stomach library and sequenced. The deduced amino acid sequence shows 59–70% identities with the other human ADH classes, demonstrating that the stomach enzyme represents a distinct structure, constituting class IV, coded by a separate gene, *ADH7*. The amino acid identity with the rat stomach class IV ADH is 88%, which is intermediate between constant and variable dehydrogenases. This value reflects higher conservation than for the classical liver enzymes of class I, compatible with a separate functional significance of the class IV enzyme. Its enzymic features can be correlated with its structural characteristics. The residues lining the substrate-binding cleft are bulky and hydrophobic, similar to those of the class I enzyme; this explains the similar specificity of both classes, compatible with the origin of class IV from class I. Position 47 has Arg, in contrast to Gly in the rat class IV enzyme, but this Arg is still associated with an extremely high activity (k_{cat} = 1510 min^{-1}) and weak coenzyme binding (K_mNAD$^+$ = 1.6 mM). Thus, the strong interaction with coenzyme imposed by Arg47 in class I is probably compensated for in class IV by changes that may negatively affect coenzyme binding: Glu230, His271, Asn260, Asn261, Asn363. The still higher activity and weaker coenzyme binding of rat class IV (k_{cat} = 2600 min^{-1}, K_mNAD = 4 mM) can be correlated to the exchanges to Gly47, Gln230 and Tyr363. An important change at position 294, with Val in human and Ala in rat class IV, is probably responsible for the dramatic difference in K_m values for ethanol between human (37 mM) and rat (2.4 M) class IV enzymes.

Human alcohol dehydrogenase (ADH) is a complex enzymic system composed of multiple molecular forms, which have been grouped into classes according to their enzymic and structural characteristics. Three classes (I–III) were recognized (Vallee and Bazzone, 1983) and have been thoroughly studied at the enzymic and structural level (Persson et al., 1993; Danielsson et al., 1994). The cDNA structures of the corresponding enzymes are also known (Hedén et al., 1986; Höög et al., 1987; Sharma et al., 1989). Class I, with isozymes of α, β and γ subunits and class II, with π subunits, are mainly hepatic enzymes and play a key role in ethanol metabolism. Class III has a wide distribution in most organs (Estonius et al., 1993) and is a glutathione-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989). More recently, an additional ADH form with σ subunits and different enzymic (Moreno and Parés, 1991; Yin et al., 1990) and structural characteristics (Parés et al., 1992; Stone et al., 1993), has been isolated from human stomach. This $\sigma\sigma$-ADH form has been recognized as class IV, because of its distinct features and its protein sequence similarity with the rat stomach class IV enzyme (Parés et al., 1990, 1992, 1994). A class V enzyme is known at the cDNA level, although the corresponding protein has not been detected in human tissues (Yasunami et al., 1991). One further class (class VI) has been recently characterized, thus far only from deermouse cDNA (Zheng et al., 1993). The separate class nomenclatures have been reviewed (Jörnvall and Höög, 1994).

Sequence identities between human ADH classes range from 59% to 64%, while isozymes within the same class exhibit 93–94% positional identity. Structural similarity at the level of about 80% is shown by members of the same class of different mammalian lines. A property of the classes supporting the present classification of the ADH enzymes is the lack of hybridization between monomers of different classes. In contrast, hybrids can be naturally formed between monomers of the same class.

Human class IV has been detected in the mucosa of the upper digestive tract, i.e., mouth, esophagus and stomach (Moreno et al., 1994) and in the cornea (Holmes, 1988), but not in liver. This specific distribution suggests a distinct role for the class IV enzyme, different from that of the hepatic ADH. We have recently demonstrated that rat class IV is an efficient enzyme in the transformation of ω-hydroxyfatty

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Abbreviation. ADH, alcohol dehydrogenase.
Enzyme. Alcohol dehydrogenase (EC 1.1.1.1).
Note. The novel nucleotide sequence data reported here have been submitted to the EMBL/GenBank Data Banks and is available under the accession number X76342.

acids, retinoids and lipid peroxidation-derived aldehydes (Boleda et al., 1993). In addition, class IV may contribute to the first-pass metabolism of ethanol that may be significant during some physiological conditions (Frezza et al., 1990). Knowledge of the primary structure is of interest for full understanding of the physiological function of human class IV ADH, its relationship with the other human classes and its position in the alcohol dehydrogenase system.

Only segments of the primary structure were known before at the protein level (Parés et al., 1992; Stone et al., 1993). In the present work, we report the sequence of the human class IV cDNA from stomach, comprising the full coding region and the non-coding regions at the 5′ and 3′ ends. From the deduced amino acid sequence it is possible to correlate specific structural features with the enzymic properties of human class IV. In addition, comparison with the homologous rat class IV enzyme establishes the structural changes that explain the kinetic differences between the class IV enzymes of the two species.

MATERIALS AND METHODS

Isolation of poly(A)-rich RNA and first-strand cDNA synthesis

Total RNA was isolated from 5 g rat stomach mucosa by the guanidinium isothiocyanate/CsCl centrifugation method (Sambrook et al., 1989). Poly(A)-rich RNA (15 μg) from two cycles of oligo(dT)-cellulose (Boehringer Mannheim) chromatography was used with protocols supplied for first-strand cDNA synthesis using a RiboClone kit and the *Not*I primer-adaptor (Promega). The cDNA synthesis was followed by labelling a reaction aliquot with 5 μCi [α-^{32}P]dCTP (>400 Ci/mmol, Amersham) and checking the denatured reaction products on 1% agarose gel electrophoresis.

Preparation of a class-IV-ADH-specific probe

Two oligonucleotide primers were based on the amino acid sequence of rat class IV ADH (Parés et al., 1994): primer 1, 5′-CARCAYTTYATGAAYAC-3′, coding for amino acid residues 138–143 and primer 2, 5′-CCRTARTT-CATRTGRCA-3′, complementary to the sequence coding for amino acid residues 287–282. The first-strand cDNA mixture (a 1-μl aliquot) was used as a template in a PCR mixture (100 μl) containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (mass/vol.) gelatin, 0.2 mM of each dNTP, with 5 μM each primers 1 and 2 and 2.5 U *Taq* polymerase (Perkin-Elmer). The reaction mixture was overlaid with 100 μl light mineral oil and the reactions were carried out for 35 cycles and followed by a final extension step at 72°C for 5 min, using a DNA thermal cycler (Perkin-Elmer 480). Each cycle included a heat-denaturation step at 95°C for 1 min, followed by an annealing step at 46°C for 1 min and an extension step at 72°C for 2 min. The PCR products were separated on a 1% agarose gel. The DNA with the expected size was eluted, phosphorylated, end-repaired and cloned into the *Sma*I site of pBluescript II SK(+) vector (Stratagene) for nucleotide sequence analysis and preparation of a PCR probe.

Screening of a human stomach cDNA library

The PCR probe (50 ng) was labeled with 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) by a random-priming method (Prime-a-Gene labeling system, Promega). The labeled probe was used in a plaque hybridization procedure to screen a human stomach cDNA library constructed in the λgt10 vector (Clontech Laboratories). The first screening was carried out with a heterologous DNA probe and low-stringency conditions (Maniatis et al., 1982). Human class I (Hedén et al., 1986) and class III (Sharma et al., 1989) ADH cDNA probes were used as negative controls. Subsequent screenings were performed with a homologous DNA probe under more stringent conditions (Maniatis et al., 1982). For each screening, three rounds were carried out in order to purify positive clones.

Characterization of isolated positive cDNA clones

λ DNA was prepared by a plate lysate method (Benson and Taylor, 1984), the cDNA inserts were excised by *Eco*RI digestion and analyzed by 1% agarose gel electrophoresis. The inserts from four independent positive phage clones (λ1, λ2, λ3 and λ4) were subcloned into the *Eco*RI site of pBluescript II SK(+). DNA sequence determination of double-stranded DNA was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977), using fluorescently labeled T7 and T3 primers with the *Taq* dye primer cycle sequencing kit (Applied Biosystems), in an Applied Biosystems 373A DNA sequencer.

Isolation of clones containing the 5′ end of the cDNA

To prepare the 5′ end of the human class IV ADH cDNA, direct amplification from the λgt10 cDNA library (Friedman et al., 1988) was performed, combined with the use of two sets of nested λgt10 and class-IV-specific primers. A 1-μl library aliquot (5×10^9 plaque-forming units/ml) was added to the PCR mixture. PCR conditions were the same as described above, but with a reaction volume of 50 μl and 1 μM primers, for 35 cycles followed by a final extension at 72°C for 7 min. Each cycle included steps at 94°C for 45 s, 60°C for 45 s and 72°C for 2 min. In the first PCR, the left primer was the λgt10 primer S-37 (Oligos etc.; 5′-AGCAAGTT-CAGCCTGGTTAAG-3′, complementary to λgt10 DNA 10–30 nucleotides upstream from the *Eco*RI site) and the right primer was the class-IV-specific primer 5, 5′-ACAGCAGC-GCCATATCCAGTGGAAA-3′, complementary to the 5′ to 3′ strand of human class IV cDNA at nucleotide positions 611–587 (Fig. 2). Alternatively, to allow amplification of reverse-oriented inserts, primer 5 was used together with λgt10 primer S-39 (Oligos etc.; 5′-CTTATGAGTATTTCTTCCA-GGGTA-3′, complementary to λgt10 DNA 14–34 nucleotides downstream from the *Eco*RI site). Specific PCR products, in the 500–600-bp range, were only obtained with primer S-37 and were identified by Southern blotting, excised from a 1% agarose gel, eluted and used as templates (100 pg) in a second PCR. The left primer was then the λgt10 primer 3, 5′-AGCCTGGTTAAGTCCAAGCTG-3′, complementary to λgt10 DNA 1–21 nucleotides upstream from the *Eco*RI site and the right primer was the class-IV-specific primer 4, 5′-GATCGAATTCCATCCACCACTGTGTACTC-GGTAAA-3′, complementary to the 5′ to 3′ strand of human class IV cDNA at nucleotide positions 520–496 (Fig. 2) and including an *Eco*RI site at its 5′ end. The resulting PCR product was excised from a 1% agarose gel, eluted, digested with *Eco*RI and subcloned into the *Eco*RI site of pBluescript II SK(+).

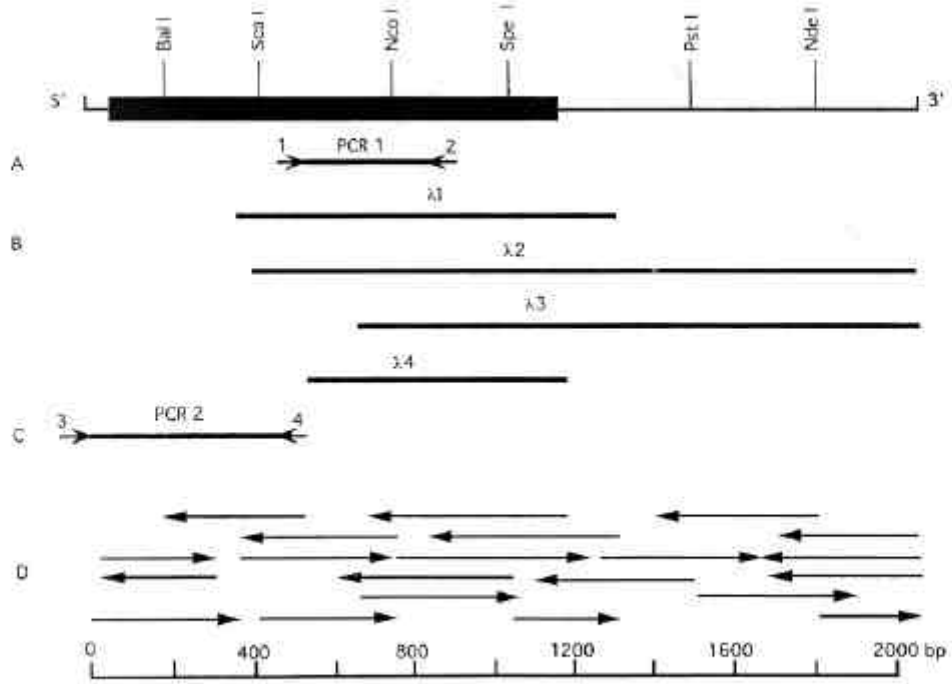

Fig. 1. Schematic representation of the restriction map, PCR probe, cDNA clones and sequencing strategy of human class IV ADH cDNA. The closed bar designates the coding sequence oriented in the 5′ to 3′ direction. Restriction enzymes used for subcloning are indicated. (A) Rat-derived PCR product (PCR 1) used as a probe for screening the library is aligned with its corresponding human sequence at the top. The arrows represent the PCR primers 1 and 2. (B) Sequences corresponding to the four clones isolated from the human stomach library in λgt10 are aligned. (C) PCR product (PCR 2) including the 5′ end. The arrows represent the primers 3 and 4. (D) Sequencing strategy, where arrows indicate the direction and extent of the sequencing.

Data bank analysis

The nucleotide and deduced amino acid sequences were compared against the EMBL, GenBank (release 79.0), Swiss-Prot (release 26.0) and PIR (release 38.0) data banks using the FASTA program (Pearson and Lipman, 1988). Other programs used for sequence analysis included BESTFIT, GAP, PILEUP and PUBLISH from the Genetics Computer Group Package (Devereux et al., 1984), version 7.

Kinetic constants

Human and rat class IV enzymes were purified and assayed as reported (Moreno and Parés, 1991; Parés et al., 1994; Julià et al., 1987). The dissociation constant for NAD^+ (K_{ia}) was calculated from the plot of $1/v$ versus $1/[NAD^+]$ at different alcohol concentrations, assuming an ordered bi-bi mechanism (Segel, 1975). NAD^+ concentrations ranged from 0.25 mM to 2 mM for the human enzyme and from 0.12 to 2.4 mM for the rat enzyme. Ethanol (25–200 mM) was used for the human enzyme and octanol (0.2–1 mM) for the rat enzyme.

RESULTS

Isolation and characterization of human class IV ADH cDNA

From the amino acid sequence of the rat class IV ADH (Parés et al., 1994), several degenerate oligonucleotides were designed to be used as PCR primers with a first-strand cDNA mixture, obtained from rat mRNA. Primers 1 and 2 amplified a cDNA sequence of 449 bp (PCR 1, Fig. 1), coding for amino acid residues 138–287 of rat stomach ADH.

The 449-bp PCR product was used as a heterologous probe to screen a human stomach cDNA library (3×10^5 clones) under low-stringency conditions. Out of eight positive clones, seven showed a weak hybridization signal with the class IV probe but a strong signal with a human-class-I-specific probe, reflecting the relatively low abundance of class IV ADH mRNA and the structural relationship between classes I and IV (Parés et al., 1994). None of these clones hybridized with a human-class-III-specific probe though, reflecting the more distant relationship between classes III and IV. One single clone (λ1), showing a strong hybridization signal with the class IV probe only, was selected and purified. This clone contained a 1.8-kb insert which, upon digestion with *Eco*RI, yielded two fragments of approximately 0.8 kb and 939 bp. The 939-bp fragment (Fig. 1) included nucleotide positions 358–1296 of human class IV ADH cDNA (Fig. 2), while the 0.8-kb fragment contained a sequence that could not be matched with any sequence found in the data banks. Presumably, this fragment was artifactually joined to ADH cDNA during library construction.

The 939-bp fragment was used as a homologous DNA probe for subsequent screenings under more stringent conditions. The class-I-specific probe did not then cross-hybridize with class IV clones. From a total of 6×10^5 clones screened, 3 independent positive clones (λ2, λ3 and λ4, Fig. 1) were isolated and their cDNA inserts were sequenced. None of the clones contained the 5′ end of the cDNA.

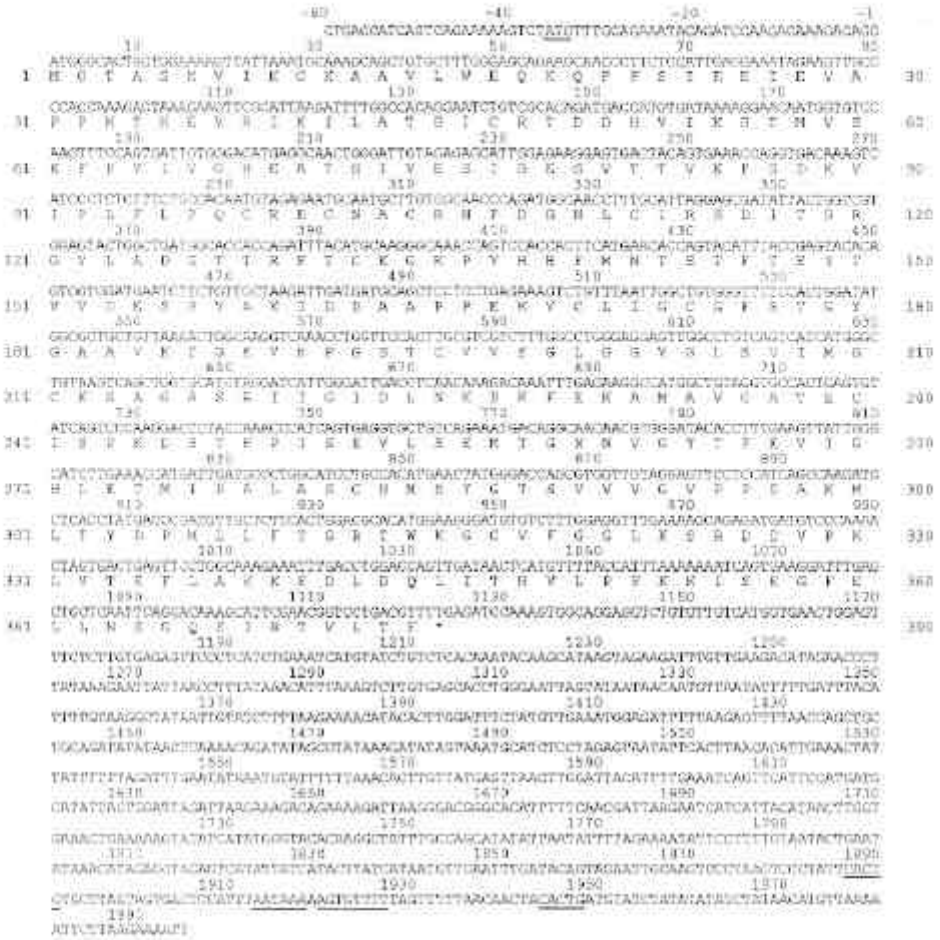

Fig. 2. Nucleotide sequence of human class IV ADH cDNA and its deduced amino acid sequence. The nucleotides are numbered in the 5′ to 3′ direction; nucleotide 1 has been assigned to the A of the putative ATG initiation codon; negative numbers refer to the 5′ flanking region. An upstream in-frame ATG codon at −36 is underlined. (*) The stop codon. The putative polyadenylation signal, and other consensus sequences in the 3′ untranslated region are underlined.

A 520-bp fragment (PCR 2, Fig. 1), covering the 5′ end of the human class IV cDNA, was isolated from the same library by using PCR with nested λgt10 and class-IV-specific primers, as described in Materials and Methods.

Altogether the overlapping cDNA fragments defined a 2055-bp sequence (Fig. 2), coding for human class IV ADH. The sequence included an open reading frame of 1158 bp, 24 bp in the 5′ flanking region and 873 bp in the 3′ untranslated region. A putative polyadenylation signal sequence, AATAAA (Proudfoot and Brownlee, 1976), was found starting at position 1913 and two CAYTG (Berget, 1984) and one YGTGTTYY (McLauchlan et al., 1985) sequences were found starting at positions 1887, 1944 and 1920, respectively, but not a poly(A) tail.

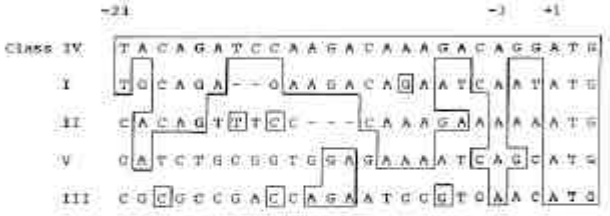

Fig. 3. Sequence alignment of 5′ flanking regions of different human ADH cDNA. Nucleotides found both in class IV cDNA and at least another cDNA are boxed. Some gaps (−) are introduced to maximize similarity.

Novel features of human class IV ADH

The proximal region of the 5′ flanking sequence (positions −23 to −1) of human class IV ADH cDNA is purine rich and could be aligned with those of other human ADH classes (Fig. 3). Class I ADH cDNA displayed 61% similarity, reflecting the closer evolutionary relationship of class IV with class I than with any other ADH class.

One remarkable feature of the human class IV cDNA sequence is that two in-frame ATG codons are present in the 5′ region. In eukaryotes, the first ATG is usually the one used as an initiation codon (Kozak, 1987), but in this case it is located in a non-optimal sequence context. In contrast, the ATG at +1 is found in a proper sequence for function as the initiation codon, with an A at −3 and a G at +4 (Fig. 2). The presence of an additional, upstream ATG codon might

decrease the translation efficiency of the class IV ADH mRNA out of the ATG at +1, as suggested for other mRNAs (Kozak, 1987). This could represent a system for post-transcriptional regulation of class IV ADH expression.

Assuming that the second ATG is used as the initiation codon, the cDNA would code for a protein of 374 amino acid residues (Fig. 2), typical of mammalian ADH and, in particular, of rat class IV ADH. The 88% identity between human and rat class IV ADH at the amino acid level was unexpected since it is exactly in between those known for the corresponding species variants of the variable class I and constant class III ADH. Hence, the variable/constant distinction, based on evolutionary properties, may be more complex than previously realized. Positional identities between human class IV and the other human ADH classes range from 59% (class II) to 70% (class I) (Table 1).

Kinetic constants of ADH class IV

Table 2 shows several kinetic constants for human ADH classes and for rat class IV. Dissociation constants for NAD^+ (K_{ia}) were determined for the human and rat class IV enzymes as indicated in Materials and Methods. Double-reciprocal plots of alternate substrate and product inhibition kinetics (data not shown) were compatible with an ordered bi-bi mechanism. Within the human enzyme, class IV shows the highest K_m and K_{ia} values for NAD^+ and, except for class III, class IV also shows the highest K_m and k_{cat} values for alcohols. K_m, K_{ia} and k_{cat} values for the rat enzyme are even higher than those for the human class IV protein. These are therefore concluded to be distinctive features of mammalian class IV ADH in general.

Table 1. Structural relationships among human and rat ADH classes. A class VI also exists (Zheng et al., 1993) but is not fully characterized from human or rat, and hence not included in this comparison. Parenthesis indicates a tentative value from an incompletely determined variant.

Human class	Interclass identity (identity toward human class IV) at the		Intraclass identity (human/rat) at the amino acid level
	nucleotide level	amino acid level	
	%		
I	72–73	69–70	80–82
II	65	59	(77)
III	66	61	94
IV			88
V	69	60	

DISCUSSION

Analysis of the structure

The present cDNA analysis establishes a gene of the human ADH family, *ADH7*, coding for human class IV ADH (Parés et al., 1992). This gene is clearly different from *ADH6* which was initially suggested to be for the stomach enzyme (Yasunami et al., 1991; Chen and Yoshida, 1991).

With the second ATG as an initiation codon, the human class IV cDNA would code for a protein of 374 amino acid residues (Fig. 2). In analogy with other vertebrate ADH, the N-terminal methionine is expected to be removed (Flinta et al., 1986; Egestad et al., 1990), leaving the next residue, glycine (Figs 2 and 4), to constitute the acetylated N-terminus. The coding sequence of human class IV ADH cDNA shows 65–73% identity at the nucleotide level with those of other human ADH classes, corresponding to 59–70% identity at the protein level (Table 1, Fig. 4). Class I ADH shows the highest degree of similarity (72–73% and 69–70% at the nucleotide and protein levels, respectively), in agreement with an origin of class IV from class I, suggested from analysis of the structure of rat class IV ADH (Parés et al., 1994).

The species difference between the human and rat forms of class IV is smaller than previously suspected (Parés et al., 1992). The human/rat class IV residue difference is now found to be 12%, or approximately half-way between the variable class I (≈18% divergence) and constant class III (≈6% divergence) previously distinguished (Yin et al., 1991; Jörnvall et al., 1993).

The deduced amino acid sequence includes the partial sequences previously reported for human class IV ADH, but

Table 2. Kinetic constants of ADH enzymes. Constants were determined in 0.1 M sodium phosphate/NaOH, at pH 7.5, 25°C (0.1 M glycine/NaOH, pH 10.0 within parentheses). K_m and K_{ia} for NAD^+ and K_i for 4-methylpyrazole have been measured with ethanol as a substrate, except for class III and rat class IV where octanol was used. Kinetic constants for classes I and II were taken form Bosron et al. (1983), Eklund et al. (1990) and Bosron et al. (1979). Class III data are from Farrés, J. and Parés, X., unpublished results, and class IV constants from Julià et al. (1987), Moreno and Parés (1991) and Parés et al. (1994), except for K_{ia} NAD^+ values and constants for ethanol which are from the present work. ns., no saturation.

Enzyme class	Enzyme form	K_m for ethanol	k_{cat} for ethanol	K_m for octanol	K_m for NAD^+	K_{ia} for NAD	K_i for 4-methyl-pyrazole
		mM	min^{-1}	μM			
Human class I	$\alpha\alpha$	4.2	54	–	13	32	1.1
	$\beta_1\beta_1$	0.05	18	(13)	7.4	90	0.13
	$\gamma_1\gamma_1$	1.0	170	(10)	7.9	–	0.1
Human class II	$\pi\pi$	34	40	(7)	14	86	2000
Human class III	$\chi\chi$	n.s.	n.s.	940	64	200	>10 000
Human class IV	$\sigma\sigma$	37 (11)	1510	27	180	1600	(10)
Rat class IV		2400 (340)	2600	500	300	4000	10 000 (20)

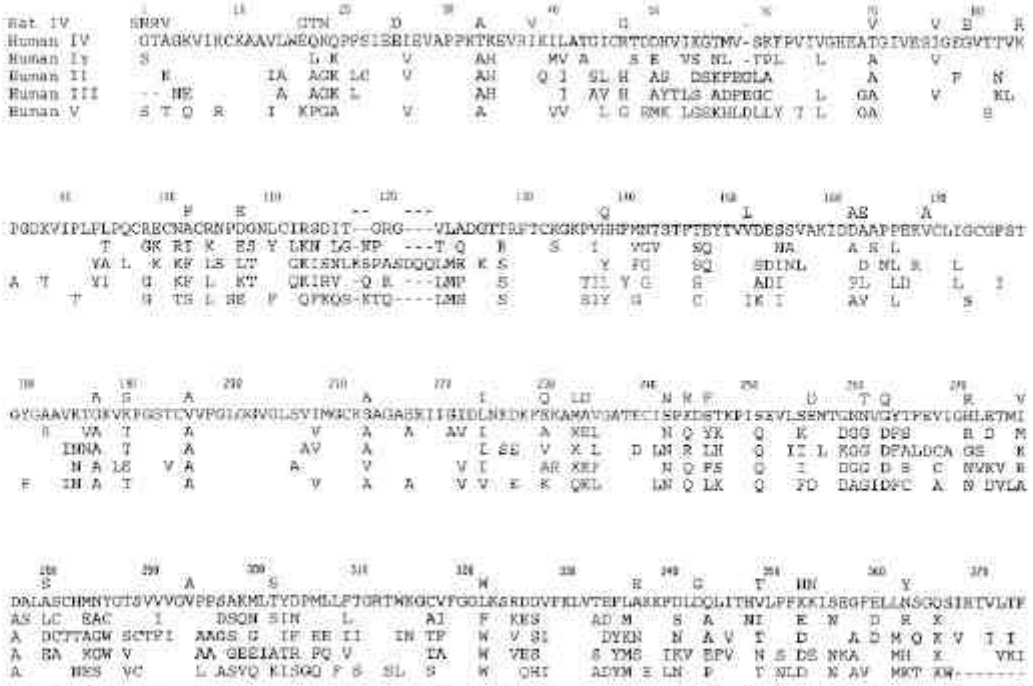

Fig. 4. Alignment of the amino acid sequence of human class IV ADH now determined with those of rat class IV and other human classes. In each of the discontinuous lines, only differences from human class IV are given, whereas all empty spaces indicate identities with the human class IV structure. The N-termini are acetylated (not finally established in human classes IV and V, although they, too, are given without their initiator methionine residues, since they are likely to be absent in the mature proteins). Amino acid residues are numbered according to the class I numbering system to allow for comparisons with previous structural data (with respect to class I, one additional gap has to be introduced after position 117). In human class IV ADH, amino acid residues 19, 70, 138, 139 and 143 differ from those reported by Parés et al. (1992), and residues 137, 158, 289, 290, 292 and 358 differ from those of Stone et al. (1993).

differs at several positions (Fig. 4). However, none of the differences involves a functionally critical residue and thus, even if reflecting polymorphism, they are unlikely to affect enzymic properties.

Enzymic features of human class IV

In general, the human class IV protein exhibits higher K_m values at pH 7.5 for all substrates than classes I and II proteins (Table 2). Class IV k_{cat} values are the highest for any human ADH, both with alcohols and aldehydes. It has been demonstrated for several ADH that the limiting step in ADH catalysis is coenzyme dissociation (Hurley et al., 1990) and kinetic data on class IV support this mechanism. Therefore, K_m and, mainly, K_m for NAD^+ should exhibit relationships with the k_{cat} values. As shown in Table 2, the two NAD^+ constants are indeed significantly higher for class IV. The k_{cat} and K_m values are even higher than those for the very active $\beta_2\beta_2$ variant (800 min^{-1} and 340 μM, respectively; Yin et al., 1984). All these constants are still larger for the kinetics of rat class IV ADH (Table 2). Thus, class IV enzymes exhibit distinctive kinetic features, with higher K_m values for substrates, higher k_{cat} and a lower strength of coenzyme binding than the other classes.

Structure/function correlations

As shown in Fig. 4, the overall conformation expected of class IV is typical of that of alcohol dehydrogenases of other classes in general, with conserved features of domain organization and functional segments (Eklund et al., 1990; Parés et al., 1994). Thus it should be possible to correlate specific amino acid replacements with the changes in enzymic properties, using the three-dimensional structures of the class I enzymes as models.

Out of 26 residues involved in subunit interactions (Eklund et al., 1990), 14 are different from those found in the class I enzymes, explaining the absence of class IV/class I hybrids in tissues where both classes are expressed, such as stomach mucosa (Moreno and Parés, 1991).

Similarly to rat class IV ADH (Parés et al., 1994), the substrate-binding cleft is lined with bulky hydrophobic residues, most of them also found in class I isozymes and therefore compatible with a substrate specificity similar to that of class I ADH.

In human class IV ADH (Fig. 4), most of the residues lining the coenzyme binding site are identical to those found in class I forms (Eklund et al., 1984; 1990). Specifically, Arg47 interacts in class I enzymes with the pyrophosphate moiety of the coenzyme and correlates with relatively low k_{cat} values (Hurley et al., 1990). However, human class IV, also with Arg47 (Table 3), is the human ADH form with the highest k_{cat} values, demonstrating that Arg47 is not always associated with high-affinity coenzyme binding and low k_{cat} values. Changes in other residues interacting with the coenzyme could compensate for Arg47. The negative charge of Glu230, absent in all other human ADH classes and the change at position 271 to His (Arg in all class I enzymes

Table 3. Important amino acid exchanges in class IV enzymes and their counterparts in classes I–III of the human enzymes.

Region	Residue	Amino acid of						
		human class						rat class IV
		I			II	III	IV	
		α	β_1	γ_1	π	χ	σ	
Substrate binding	294	Val	Val	Val	Val	Val	Val	Ala
Coenzyme binding	47	Gly	Arg	Arg	His	His	Arg	Gly
	230	Ala	Ala	Ala	Val	Ala	Glu	Gln
	271	Arg	Arg	Arg	Gly	Asn	His	Arg
	363	His	His	Arg	Asn	His	Asn	Tyr
Coenzyme-binding domain	259	Asp	Asp	Asp	Lys	Asp	Gly	Gly
	260	Gly	Gly	Gly	Gly	Gly	Asn	Asn
	261	Gly	Gly	Gly	Gly	Gly	Asn	Thr

except Gln in $\gamma_2\gamma_2$) may contribute to the compensatory effect.

Position 363, affecting the environment of Arg47 (Eklund et al., 1984; Hurley et al., 1991), has Asn in class IV (as in class II), in contrast to Arg or His in the class I enzymes (Table 3). This change could further contribute to a weakening of the coenzyme binding. A support for a distinct environment for Arg47 in class IV is the lack of activation by Cl^- in a concentration up to 100 mM NaCl, in contrast to the 2.7-fold increase of activity found for the Arg-containing class I ADH forms (Hurley et al., 1990). Rat class IV is also insensitive to Cl^-, as expected from the presence of Gly at position 47.

The highly conserved glycine residues at positions 260 and 261 correspond to larger residues in the human (two Asn) and rat (Asn and Thr) enzymes (Table 3). These positions constitute a reverse turn in class I and the structural consequences, although perhaps compensated for by the adjacent exchange of Asp259 to Gly, may contribute also to a weakening of the coenzyme binding.

Structural and functional relationships between human and rat class IV ADH

Kinetic and tissue distribution characteristics support an homologous function of class IV in both species. Thus, both enzymes exhibit preference for the oxidation of medium-chain and long-chain alcohols versus ethanol. They are efficient in the reduction of aromatic aldehydes, such as *m*-nitrobenzaldehyde and are detected in the upper digestive tract mucosa, cornea and other epithelia. A distinctive characteristic of the class IV enzymes is that they represent the most active ADH in each species, with a very high k_{cat} (Table 2). However, a remarkable difference exists between the two enzymes. In general, the K_m values for rat class IV are several fold higher than those for the human enzyme. The difference is extreme for ethanol, with a K_m of 2.4 M for rat class IV and only 37 mM for the human enzyme, at pH 7.5, indicating that contribution of class IV to ethanol metabolism is different in the two species. The inhibition constant for 4-methylpyrazole is also much higher for the rat enzyme (Table 2). These functional differences should be related to structural changes. The most characteristic difference in the coenzyme-binding site is at position 47, with Gly in the rat and Arg in the human class IV enzyme (Table 3). However, this exchange alone does not explain the 1.7-fold k_{cat} increase for the rat enzyme, since even an opposite change is observed when Arg47 in $\beta_1\beta_1$ is changed to Gly47 by site-directed mutagenesis (Hurley et al. 1990). Thus, additional exchanges like the one at position 271, with Arg in rat and His in the human enzyme, contribute to explain the higher k_{cat} for the rat class IV enzyme, in parallel to what occurs for the human $\gamma_1\gamma_1$ (Arg271, k_{cat} 174 min^{-1}) and $\gamma_2\gamma_2$ (Gln271, k_{cat}: 70 min^{-1}) forms (Bosron et al., 1983; Höög et al., 1986). Additional changes affect positions 230 and 363 (Table 3), which would also influence coenzyme binding.

With respect to the substrate-binding area, all residues are strictly conserved in the class IV enzymes of both species, except at position 294. While the human enzyme exhibits Val, like all other human ADH classes, the rat enzyme has Ala. This is an important position in the middle region of the substrate-binding cleft. The exchange to a smaller residue in this area yields an additional space at the active site. This is compatible with low affinity for small substrate molecules and can explain the large K_m for ethanol of the rat enzyme, approximately 60-fold higher than that of the human enzyme. Affinity for longer substrates would be affected to a lesser extent. Thus, K_m for octanol of rat class IV is 18-fold that of the human enzyme (Table 2). Furthermore, Val294 in class I is involved in the conformational change associated with the apoenzyme/holoenzyme transition and interacts with the nicotinamide moiety (Eklund et al., 1984). The change to Ala in the rat enzyme may result in a weaker coenzyme binding, contributing to the increased k_{cat}.

Important residues in the coenzyme-binding and substrate-binding sites (positions 47, 230, 271, 294, 363) are different in the two class IV enzymes (Table 3). These exchanges, as discussed above, can be related to the kinetic differences between the enzymes. As for class I enzymes, class IV variability in functionally important residues would modulate enzymic function, suggesting that the enzyme is co-evolving with the metabolic needs of each species, while maintaining the overall physiological function. This would probably be related to the metabolism of endogenous medium-chain and long-chain alcohols and aldehydes, such as those derived from lipid peroxidation, ω-hydroxyfatty acids and retinoids (Boleda et al., 1993). However, structural changes have resulted in a human class IV with more activity

toward ethanol under physiological conditions, supporting an increased role of class IV in ethanol metabolism in the human species.

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REFERENCES

Benson, S. A. & Taylor, R. K. (1984) A rapid small scale procedure for isolation of phage lambda DNA. *Biotechniques 2*, 126–127.

Berget, S. M. (1984) Are U4 small nuclear ribonucleoproteins involved in polyadenylation? *Nature 309*, 179–181.

Boleda, M. D., Saubi, N., Farrés, J. & Parés, X. (1993) Physiological substrates for rat alcohol dehydrogenase classes: Aldehydes of lipid peroxidation, ω-hydroxyfatty acids and retinoids. *Arch. Biochem. Biophys. 307*, 85–90.

Bosron, W. F., Li, T.-K., Dafeldecker, W. P. & Vallee, B. L. (1979) Human liver π-alcohol dehydrogenase: kinetic and molecular properties. *Biochemistry 18*, 1101–1105.

Bosron, W. F., Magnes, L. J. & Li, T.-K. (1983) Kinetic and electrophoretic properties of native and recombined isoenzymes of human liver alcohol dehydrogenase. *Biochemistry 22*, 1852–1857.

Chen, C.-S. & Yoshida, A. (1991) Enzymatic properties of the protein encoded by newly cloned human alcohol dehydrogenase *ADH6* gene. *Biochem. Biophys. Res. Commun. 181*, 743–747.

Danielsson, O., Atrian, S., Luque, T., Hjelmqvist, L., Gonzàlez-Duarte, R. & Jörnvall, H. (1994) Fundamental molecular differences between alcohol dehydrogenase classes. *Drosophila* octanol dehydrogenase is a class III alcohol dehydrogenase establishing molecular patterns and a continuous distribution of this zinc enzyme family. *Proc. Natl Acad. Sci. USA 91*, 4980–4984.

Devereux, J., Haeberli, P. & Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res. 12*, 387–395.

Egestad, B., Estonius, M., Danielsson, O., Persson, B., Cederlund, E., Kaiser, R., Holmquist, B., Vallee, B., Parés, X., Jeffery, J. & Jörnvall, H. (1990) Fast atom bombardment mass spectrometry and chemical analysis in determinations of acyl-blocked protein structures. *FEBS Lett. 269*, 194–196.

Eklund, H., Samama, J. P. & Jones, T. A. (1984) Crystallographic investigations of nicotinamide adenine dinucleotide binding to horse liver alcohol dehydrogenase. *Biochemistry 23*, 5982–5996.

Eklund, H., Müller-Wille, P., Horjales, E., Futer, O., Holmquist, B., Vallee, B. L., Höög, J.-O., Kaiser, R. & Jörnvall, H. (1990) Comparison of three classes of human liver alcohol dehydrogenase. Emphasis on different substrate-binding pockets. *Eur. J. Biochem. 193*, 303–310.

Estonius, M., Danielsson, O., Karlsson, C., Persson, H., Jörnvall, H. & Höög, J.-O. (1993) Distribution of alcohol and sorbitol dehydrogenases. Assessment of mRNA species in mammalian tissues. *Eur. J. Biochem. 215*, 497–503.

Flinta, C., Persson, B., Jörnvall, H. & von Heijne, G. (1986) Sequence determinants of cytosolic N-terminal protein processing. *Eur. J. Biochem. 154*, 193–196.

Frezza, M., di Padova, C., Pozzato, G., Terpin, M., Baraona, E. & Lieber, C. S. (1990) High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. *N. Engl. J. Med. 322*, 95–99.

Friedman, K. D., Rosen, N. L., Newman, P. J. & Montgomery, R. R. (1988) Enzymatic amplification of specific cDNA inserts from lambda gt11 libraries. *Nucleic Acids Res. 16*, 8718.

Hedén, L.-O., Höög, J.-O., Larsson, K., Lake, M., Lagerholm, E., Holmgren, A., Vallee, B. L., Jörnvall, H. & von Bahr-Lindström, H. (1986) cDNA clones coding for the β-subunit of human liver alcohol dehydrogenase have differently sized 3′-non-coding regions. *FEBS Lett. 194*, 327–332.

Holmes, R. S. (1988) Alcohol dehydrogenases and aldehyde dehydrogenases of anterior eye tissues from humans and other mammals, in *Biomedical and social aspects of alcohol and alcoholism* (Kuriyama, K., Takada, A. & Ishii, H., eds) pp. 51–57, Elsevier Science Publishers, New York.

Höög, J.-O., Hedén, L.-O., Larsson, K., Jörnvall, H. & von Bahr Lindström, H. (1986) The γ_1 and γ_2 subunits of human liver alcohol dehydrogenase. cDNA structures, two amino acid replacements and compatibility with changes in the enzymatic properties. *Eur. J. Biochem. 159*, 215–218.

Höög, J.-O., von Bahr-Lindström, H., Hedén, L.-O., Holmquist, B., Larsson, K., Hempel, J., Vallee, B. L. & Jörnvall, H. (1987) Structure of the class II enzyme of human liver alcohol dehydrogenase: Combined cDNA and protein sequence determination of the π subunit. *Biochemistry 26*, 1926–1932.

Hurley, T. D., Edenberg, H. J. & Bosron, W. F. (1990) Expression and kinetic characterization of variants of human $\beta_1\beta_1$ alcohol dehydrogenase containing substitutions at amino acid 47. *J. Biol. Chem. 265*, 16366–16372.

Hurley, T. D., Bosron, W. F., Hamilton, J. A. & Amzel, L. M. (1991) Structure of human $\beta_1\beta_1$ alcohol dehydrogenase: Catalytic effects of non-active-site substitutions. *Proc. Natl Acad. Sci. USA 88*, 8149–8153.

Jörnvall, H., Persson, B. & Jörnvall, H. (1993) Variability patterns of dehydrogenases versus peptide hormones and proteases/antiproteases. *FEBS Lett. 335*, 69–72.

Jörnvall, H. & Höög, J.-O. (1994) Nomenclature of alcohol dehydrogenases. *Alcohol Alcohol.*, in the press.

Juliá, P., Farrés, J. & Parés, X. (1987) Characterization of three isoenzymes of rat alcohol dehydrogenase. Tissue distribution and physical and enzymatic properties. *Eur. J. Biochem. 162*, 179–189.

Koivusalo, M., Baumann, M. & Uotila, L. (1989) Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. *FEBS Lett. 257*, 105–109.

Kozak, M. (1987) An analysis of 5′-non coding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res. 15*, 8125–8148.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

McLauchlan, J., Gaffney, D., Whitton, J. L. & Clements, J.-B. (1985) The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. *Nucleic Acids Res. 13*, 1347–1368.

Moreno, A. & Parés, X. (1991) Purification and characterization of a new alcohol dehydrogenase from human stomach. *J. Biol. Chem. 266*, 1128–1133.

Moreno, A., Parés, A., Ortiz, J., Enriquez, J. & Parés, X. (1994) Alcohol dehydrogenase from human stomach. Variability in normal mucosa and effect of age, sex, ADH3 phenotype and gastric region. *Alcohol Alcohol.*, in the press.

Parés, X., Moreno, A., Cederlund, E., Höög, J.-O. & Jörnvall, H. (1990) Class IV mammalian alcohol dehydrogenase. Structural data of the rat stomach enzyme reveal a new class well separated from those already characterized. *FEBS Lett. 277*, 115–118.

Parés, X., Cederlund, E., Moreno, A., Saubi, N., Höög, J.-O. & Jörnvall, H. (1992) Class IV alcohol dehydrogenase (the gastric enzyme). Structural analysis of human $\sigma\sigma$-ADH reveals class IV to be variable and confirms the presence of a fifth mammalian alcohol dehydrogenase class. *FEBS Lett. 303*, 69–72.

Parés, X., Cederlund, E., Moreno, A., Hjelmqvist, L., Farrés, J. & Jörnvall, H. (1994) Mammalian class IV alcohol dehydrogenase (stomach alcohol dehydrogenase): Structure, origin, and correlation with enzymology. *Proc. Natl Acad. Sci. USA 91*, 1893–1897.

Pearson, W. R. & Lipman, D. J. (1988) Improved tools for biological sequence analysis. *Proc. Natl Acad. Sci. USA 85*, 2444–2448.

Persson, B., Bergman, T., Keung, W. M., Waldenström, U., Holmquist, B., Vallee, B. L. & Jörnvall, H. (1993) Basic features of class-I alcohol dehydrogenase: variable and constant segments

coordinated by inter-class and intra-class variability. Conclusions from characterization of the alligator enzyme, *Eur. J. Biochem. 216*, 49–56.

Proudfoot, N. J. & Brownlee, G. G. (1976) 3′ Non-coding region sequences in eukaryotic messenger RNA, *Nature 263*, 211–214.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc. Natl Acad. Sci. USA 74*, 5463–5467.

Segel, I. H. (1975) *Enzyme kinetics*, John Wiley & Sons, New York.

Sharma, C. P., Fox, E. A., Holmquist, B., Jörnvall, H. & Vallee, B. L. (1989) cDNA sequence of human class III alcohol dehydrogenase, *Biochem. Biophys. Res. Commun. 164*, 631–637.

Stone, C. L., Thomasson, H. R., Bosron, W. F. & Li, T.-K. (1993) Purification and partial amino acid sequence of a high-activity human stomach alcohol dehydrogenase, *Alcohol. Clin. Exp. Res. 17*, 911–918.

Vallee, B. L. & Bazzone, T. J. (1983) Isozymes of human liver alcohol dehydrogenase, *Isozymes Curr. Top. Biol. Med. Res. 8*, 219–244.

Yasunami, M., Chen, C.-S. & Yoshida, A. (1991) A human alcohol dehydrogenase gene (*ADH 6*) encoding an additional class of isozyme, *Proc. Natl Acad. Sci. USA 88*, 7610–7614.

Yin, S. J., Bosron, W. F., Magnes, L. J. & Li, T.-K. (1984) Human liver alcohol dehydrogenase: purification and kinetic properties of the $\beta_1\beta_1$, $\beta_1\beta_2$, $\alpha\beta_2$, and $\beta_{2\gamma}$ 'Oriental' isoenzymes, *Biochemistry 23*, 5847–5853.

Yin, S.-J., Wang, M.-F., Liao, C.-S., Chen, C.-M. & Wu, C.-W. (1990) Identification of a human stomach alcohol dehydrogenase with distinctive kinetic properties, *Biochem. Int. 22*, 829–835.

Yin, S.-J., Vagelopoulos, N., Wang, S.-L. & Jörnvall, H. (1991) Structural features of stomach aldehyde dehydrogenase distinguish dimeric aldehyde dehydrogenase as a 'variable' enzyme. 'Variable' and 'constant' enzymes within the alcohol and aldehyde dehydrogenase families, *FEBS Lett. 283*, 85–88.

Zheng, Y.-W., Bey, M., Liu, H. & Felder, M. R. (1993) Molecular basis of the alcohol dehydrogenase-negative deer mouse. Evidence for deletion of the gene for class I enzyme and identification of a possible new enzyme class, *J. Biol. Chem. 268*, 24933–24939.