CMLS, Cell. Mol. Life Sci. 60 (2003) 2009–2016
1420-682X/03/092009-08
DOI 10.1007/s00018-003-3287-1

CMLS Cellular and Molecular Life Sciences

Research Article

Distinct but parallel evolutionary patterns between alcohol and aldehyde dehydrogenases: addition of fish/human betaine aldehyde dehydrogenase divergence

L. Hjelmqvist, A. Norin, M. El-Ahmad, W. Griffiths† and H. Jörnvall*

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm (Sweden),
Fax: + 46 8 33 74 62, e-mail: hans.jornvall@mbb.ki.se

Received 17 July 2003; accepted 23 July 2003

Abstract. Alcohol dehydrogenases (ADHs) of the MDR type (medium-chain dehydrogenases/reductases) have diverged into two evolutionary groups in eukaryotes: a set of 'constant' enzymes (class III)[1] typical of basal enzymes, and a set of 'variable' enzymes (remaining classes) suggesting 'evolving' forms. The variable set has larger overall variability, different segment variability, and variability also in functional segments. Using a major aldehyde dehydrogenase (ALDH) from cod liver[2] and fish ALDHs deduced from the draft genome sequence of *Fugu rubripes* (Japanese puffer fish), we found that ALDHs form more complex patterns than the ADHs. Nevertheless, ALDHs also group into 'constant' and 'variable' sets, have separate segment variabilities, and distinct functions. Betaine ALDH (class 9 ALDH) is 'constant,' has three segments of variability, all non-functional, and a limited fish/human divergence, reminiscent of the ADH class III pattern. Enzymatic properties of fish betaine ALDH were also determined. Although all ALDH patterns are still not known, overall patterns are related to those of ADH, and group separations may be distinguished. The results can be interpreted functionally, support ALDH isozyme distinctions, and assign properties to the multiplicities of the ADH and ALDH enzymes.

Key words. Alcohol dehydrogenase; aldehyde dehydrogenase; betaine aldehyde dehydrogenase; segment variability; constant/variable enzyme pairs; evolutionary pattern.

Aldehyde and alcohol dehydrogenases (ALDHs and ADHs) are metabolically linked in several pathways, but are structurally different, separate protein families. Nevertheless, both enzymes are highly multiple, with the major family in each case subdivided into different classes [1–5], derived from at least 17 and 7 functional genes, respectively, in the human genome [3, 6]. Furthermore, the two protein types exhibit extensive but similar spreads in evolutionary rate [7, 8]. Some of the classes evolve slowly (class III ADH [9] and class 2 ALDH [7]), others more rapidly (class I and II ADH [9–11] and class 1 ALDH [7]). For both systems, the spread in evolutionary rate is about three- to fivefold [7–12] and unrelated to subunit size or quaternary structure. In addition, ADH

* Corresponding author.
† Present address: Mass Spectrometry Unit, Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, University of London, 29/39 Brunswick Square, Bloomsbury, London, WC1N 1AX (UK)
[1] Throughout, ADH classes are given here with Roman numerals, and ALDH classes with Arabic numerals, in part to avoid confusion between the two enzyme types, and in part because of previous traditions [cf. refs 1–4].
[2] A supplement with additional data in the form of one table (supplement table 1. Purification of betaine aldehyde dehydrogenase from cod liver) and two figures [supplement fig. 1. Primary structure of cod liver betaine aldehyde dehydrogenase (ALDH9), and supplement fig. 2. Alignment of class 9 cod/human ALDH (A) and of class 1 human/*Fugu* ALDH (B) visualizing separate variant segments] are given at http://parellada.mbb.ki.se/aldh9

classes differ substantially in segment variability [9], and this is correlated with functions. The extent of evolutionary divergence therefore gives handles on the enzymes in interpreting their functional and metabolic roles. This has been best studied with ADHs, where the constant form (class III) has an early origin and defined metabolic role (formaldehyde dehydrogenase) with little active-site variability. In contrast, the variable ADH (class I, the major mammalian liver ADH) has a later origin, a less restricted metabolic role, and much segment variability at the active site [9].

The extent to which these properties also apply to ALDHs is less well known, especially for the forms beyond the initially assigned cytosolic (class 1) and mitochondrial (class 2) ALDHs [13]. We have therefore now studied class 9 ALDH (betaine ALDH). This class is known in humans, is active with short-chain aldehydes, and is involved in the metabolism of putrescine to γ-aminobutyric acid (GABA) and of choline to betaine [14–17]. Betaine is present intracellularly in the inner renal medulla and is protective against hyperosmotic stress, when it is synthesized in increased amounts [18–20]. The tertiary structure of cod liver betaine ALDH is known [21], and can be compared structurally and functionally with the corresponding human form. Classes of ADH are also known in cod, making possible a comparison of evolutionary divergence over wide distances, corresponding to the fish/mammal spread, for both ADHs and ALDHs. For the same reason, we also included relevant forms of suggested ADHs and ALDHs deduced from the draft sequence of the Japanese puffer fish (*Fugu rubripes*) genome [22]. We found that betaine ALDH is a constant enzyme. We also detected similarities in patterns between the ADH and ALDH systems regarding both the constant and the variable groups, suggesting general patterns of divergent evolution among isozyme pairs for many enzymes. In addition to the comparisons, we report data on the purification, enzymology, and polypeptide primary structure, not previously included in the conformational report [21]. Class 9 is a major ALDH in fish liver.

Materials and methods

Protein purification

Pooled livers from cod of Baltic origin (*Gadus morhua*) were homogenized in 20 mM bis-Tris/HCl, pH 7.1. After centrifugation and dialysis (the same buffer), the supernatant was applied to DEAE-Sepharose Fast Flow (Amersham Biosciences). The column was washed and eluted with a gradient of 0–0.3 M NaCl in the buffer. Active fractions were pooled, dialyzed against 20 mM sodium phosphate, pH 7.5, and applied to AMP-Sepharose (Amersham Biosciences). After washing, the column was eluted with a gradient of 0–2 mM NAD^+ in the buffer. The pooled active fractions were submitted to fast protein liquid chromatography (FPLC) on HiLoad Q-Sepharose in this buffer with a 0–0.5 M NaCl gradient. The active fraction was concentrated, washed with 20 mM bis-Tris/HCl, pH 7.1, and submitted to chromatography (Mono Q HR 5/5) in the bis-Tris buffer with a 0–0.5 M NaCl gradient. The entire purification was performed at 4 °C, with 0.1 mM dithiothreitol in all buffers, which were also bubbled with N_2 before use. Protein concentrations during purification were determined colorimetrically [23].

Enzymatic characterization

Enzyme activity was determined at 25 °C by monitoring NAD^+ reduction in 50 mM sodium phosphate, pH 7.5, measured at 340 nm. During purification, the activity was monitored with formaldehyde, and reduction of 1 μmol NAD^+/min was defined as one unit (1 U), but for substrate characterization of the pure enzyme, many aldehydes were used as given below. The K_m and k_{cat} values with aldehydes were determined in the same buffer at 2.4 mM NAD^+. Betaine aldehyde was from Sigma; formaldehyde, acetaldehyde, propionaldehyde, octanal, and benzaldehyde were from Aldrich (highest available quality). Octanal and benzaldehyde were dissolved in methanol, producing 2.5% methanol in the reaction mixture. The program ENZYME [24] for weighted non-linear regression analysis was used to calculate the kinetic parameters. The k_{cat} values are given per subunit.

Structural analysis

The protein was reduced with dithiothreitol (100 μg/mg protein; 2 h at 37 °C under N_2), and carboxymethylated with iodoacetate (2 μmol/mg protein; 2 h at 37 °C under N_2 in the dark). After removal of reagents and buffers with extensive dialysis or gel filtration, separate batches of the carboxymethylated protein were cleaved with CNBr (0.2 mg/ml in 70% formic acid at room temperature for 24 h), or digested with proteases Lys-C (Wako), Asp-N, and Glu-C (both Roche Diagnostics), chymotrypsin (Merck), and trypsin (Worthington) (4–20 h at 37 °C at protease:substrate ratios of 1:10–150, w/w, in 0.1 M ammonium bicarbonate, pH 8, with up to 2.2 M urea for solubilization). Peptides obtained were purified by reverse-phase HPLC on Vydac C_4 and C_{18} columns or on TSK ODS-120T C_{18} with linear gradients of acetonitrile in 0.1% trifluoroacetic acid. For N-terminal sequence analysis, peptides were degraded in N-terminal sequencers, and for C-terminal analysis with the Procise 494 C instrument [25]. Molecular ion masses were determined by MALDI-TOF mass spectrometry. The sequence of the blocked N-terminal peptide was determined by collision-induced dissociation mass spectrometry on an AutoSpec OA-TOF instrument (Micromass).

Structural comparisons

Alignments were performed with sequences from the SWISS-PROT database using the program CLUSTAL W [26]. Also included were deduced ADHs and ALDHs as interpreted from the known *F. rubripes* genome draft [22]. The same program was used to express relationships in a phylogenetic tree (plotted with the program NJPLOT [27]), with confidence limits evaluated using bootstrap sampling [28]. The cod ALDH 9 primary structure was further interpreted in the light of the tertiary structure [21] (figured with the program ICM [29]) to correlate the kinetic properties and residue conservations with the spatial positions.

Results

Enzyme purification

The major form of ALDH from cod liver was purified by four steps of chromatography, utilizing an initial ion-exchange, an affinity, and two additional ion-exchange steps, as detailed in supplement table 1. The final preparation showed a single, apparently homogeneous band upon SDS/polyacrylamide gel electrophoresis after a 260-fold purification, and had a specific activity of 0.94 U/mg with formaldehyde as substrate.

Kinetic properties

The enzyme was active with short-chain aliphatic aldehydes and benzaldehyde in addition to the betaine aldehyde reactivity. The kinetic parameters were examined at physiological pH, under conditions allowing comparisons with those obtained for the human forms of this [14, 15, 30] and other [31–34] ALDHs (table 1). The K_m value for betaine aldehyde resembles that of the human ALDH 9 form (0.14 mM vs 0.26 mM). The k_{cat} value is a magnitude lower (49 min^{-1} vs 350 min^{-1}) but still much larger than with the other substrates (ranging from 2.9 to 11 min^{-1}), confirming betaine aldehyde as the most active substrate. The cod enzyme has a broad substrate specificity with low K_m values for propionaldehyde, octanal, and benzaldehyde, compatible with a partly hydrophobic substrate-binding pocket [21]. The K_m values are much higher and the k_{cat} values lower than for the human class 1 and 2 enzymes, suggesting that the cod enzyme is a betaine aldehyde dehydrogenase proper.

Primary structure

Sequence information was obtained from all regions of the subunit by analysis of 109 overlapping fragments from six different cleavages of the carboxymethylated enzyme, as described in Materials and methods. The struc-

Table 1. Enzymatic properties of cod liver betaine aldehyde dehydrogenase (ALDH 9) compared with those of other aldehyde dehydrogenases.

Substrate	ALDH 9 cod	ALDH 9 human	ALDH 1 human	ALDH 2 human	ALDH 3 human
Betaine aldehyde					
K_m (mM)	0.14	0.26	NA	NA	ND
k_{cat} (min^{-1})	49	350			
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	360	1,300			
Formaldehyde					
K_m (mM)	0.44	ND	(9.2)	(0.32)	ND
k_{cat} (min^{-1})	2.9		ND	(1,000)	
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	6.6			(3,200)	
Acetaldehyde					
K_m (mM)	0.61	0.05	0.15	0.00059	85
k_{cat} (min^{-1})	9.7	15	77	25	1,300
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	16	300	510	42,000	15
Propionaldehyde					
K_m (mM)	0.062	0.008	0.021	0.00034	15
k_{cat} (min^{-1})	4.4	17	60	21	1,400
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	71	2,100	2 900	61,000	94
Octanal					
K_m (mM)	0.077	ND	< 0.00015	< 0.00015	0.0042
k_{cat} (min^{-1})	11		93	17	1,400
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	150		> 620,000	> 110,000	320,000
Benzaldehyde					
K_m (mM)	0.014	ND	ND	(0.000018)	(0.12)
k_{cat} (min^{-1})	3.7			(88)	(2,200)
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	260			(4,900,000)	(18,000)

Values are from this work (cod ALDH 9) or from the literature [14, 15, 30–34]. All values are at pH 7.5 (7.4 for human ALDH 9) except those within parenthesis (pH 8.5 for human ALDH 3 with benzaldehyde, pH 9.5 for all others). NA, no activity, ND, not determined. k_{cat} values are given per subunit and are in some cases calculated from V_{max} values in the literature.

ture obtained shows a 503-residue subunit with an acetylated N terminus (Ac-Ala, determined by mass spectrometry of the N-terminal heptapeptide) and with Phe503 proven as the C terminus by direct C-terminal sequence analysis [25]. This primary structure was given in relation to the crystallographic study of the enzyme [21], but without peptide proof, which is now supplied in supplement figure 1. An analytical problem was caused by the occurrence of four Asn-Gly structures, at positions 108–109, 299–300, 378–379, and 478–479 (marked in fig. 1B). These structures are labile, easily susceptible to β-aspartyl formation, and have in an ADH been ascribed possible roles in protein isoform formations [35]. These four positions were now found to give significant drops in yield during the analytical degradations. In particular, Asn378-Gly379 was sensitive to modification, thus giving Asp in several peptides covering position 378. The great susceptibility to deamidation of this residue is probably due to its position in a superficial loop of the subunit [21]. No other ambiguities or microheterogeneities were found.

Extent of variability of betaine ALDH

The enzymology (table 1) and sequence (supplement fig. 1) of the cod enzyme, together with its tertiary structure [21], make possible a comparison with the corresponding human enzyme. Sequence identities are 70%, and the functional emphasis on betaine is also conserved (table 1). The overall residue identity resembles that of the ADH 'constant' group [where fish ADH III_l ($_l$ for the low-activity subunit) has 76% residue identity to human ADH III [35]] rather than that of the ADH 'variable' group (where fish ADH I has 55% residue identity with the human ADH Iγ [36]). Hence, ALDH class 9 belongs to the constant group of enzymes with limited species variability and conserved functional properties.

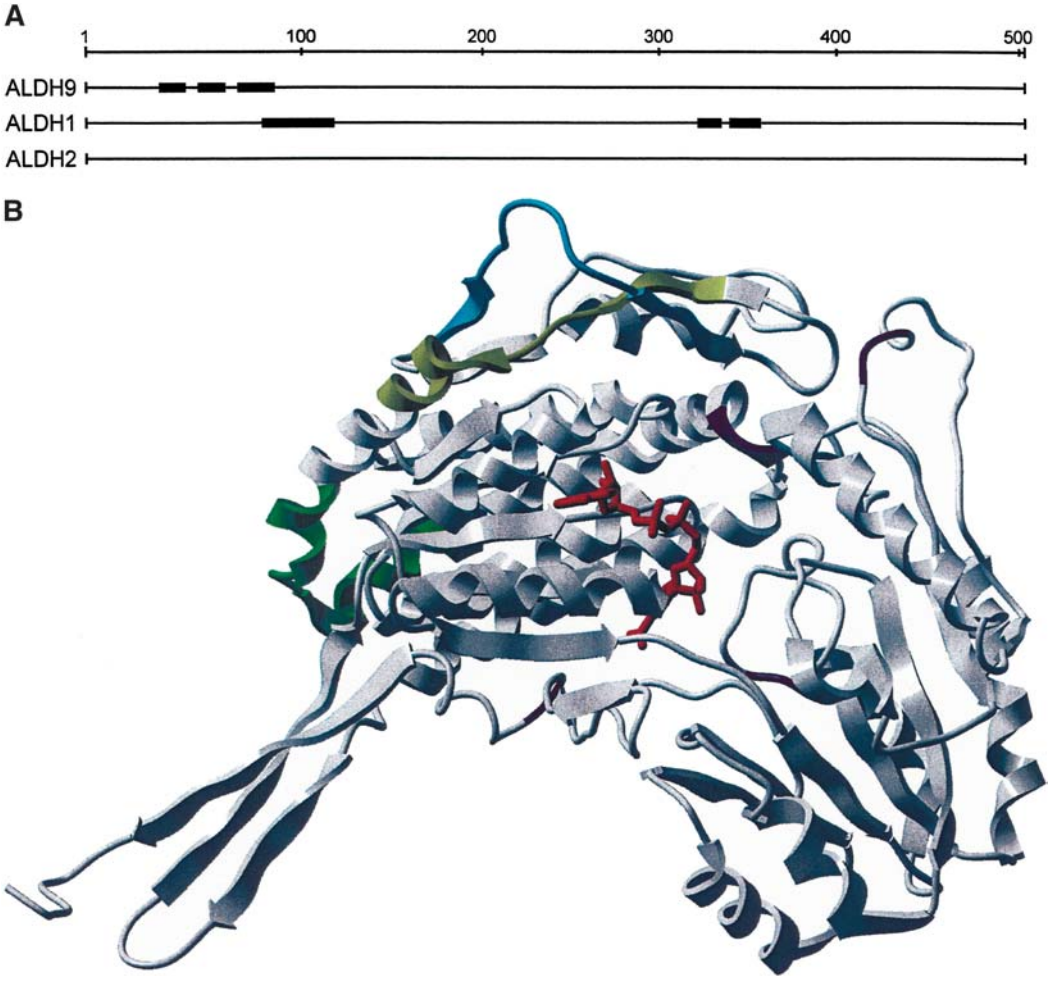

Figure 1. Residues and segments of particular structural and evolutionary interest in the subunit of betaine ALDH (ALDH 9). (*A*) The positions of the variable segments (colored in *B*) are shown with bold lines. For comparison, the differently localized variable segments in ALDH class 1 are also shown to highlight the fact that the variable segments are different in each class (in class 2, no variable segments were found). (*B*) The three variable segments are shown in light blue, yellow, and green, NAD^+ in red, and the four Asn-Gly structures in magenta. The figure was constructed with the program ICM [29]. The protruding part (lower left) is the oligomerization domain making hydrogen bonds to the catalytic domain (lower right) in a second monomer [21].

Monitoring the distribution of the ALDH class 9 identities with the tertiary structure of the enzyme [21] revealed that catalytically important residues and segments are highly conserved, as expected from the functional conservation, but that three segments elsewhere have great variability: positions 27–40, 49–62, and 71–86, with only 11 identities in 44 residues. In alignments, these three segments stand out against the rest of the highly conserved structure (supplement fig. 2A), are closely adjacent (fig. 1A), and occupy superficial positions in the tertiary structure (fig. 1B). For comparison, the different patterns of the segment variability in ALDH classes 1 and 2 are also shown in figure 1A, and detailed for ALDH 1 in supplement fig. 2B. The pattern with segment variability outside functionally important regions (fig. 1) and with limited species variability overall is typical of the conserved function of basal enzymes. One can conclude that among the multiple forms of ALDH, betaine ALDH is well conserved throughout all vertebrates, much like class III ADHs.

Screening of other characterized human and fish enzymes also picked up the other set of conserved enzymes: the variable type, with considerably greater variability and including the class I form of ADH (table 2). Hence, among these metabolic enzymes with multiple forms, the two groups, conserved and variable, are generally distinguishable, with fairly little spread (table 2) within each group (fish/human 70–78% for the constant and 54–56% for the variable enzymes).

ADH and ALDH forms deduced from the genome of *F. rubripes*

Screening the *F. rubripes* genome [22] for genes that may give rise to forms corresponding to the ADH and ALDH classes now discussed, i.e., classes I and III of ADH and 1, 2, and 9 of ALDH, we found three facts of particular relevance.

1) Equivalents of the previously [35] unexpected gene duplication in the cod class III ADH line appear to be present also in the *Fugu* structures: the cod class III enzyme was previously found in one high-activity form (ADH III_h) and one low-activity (ADH III_l) form [35]. Since these two forms were found to differ widely in the cod, they appeared likely to be of old occurrence, and it was

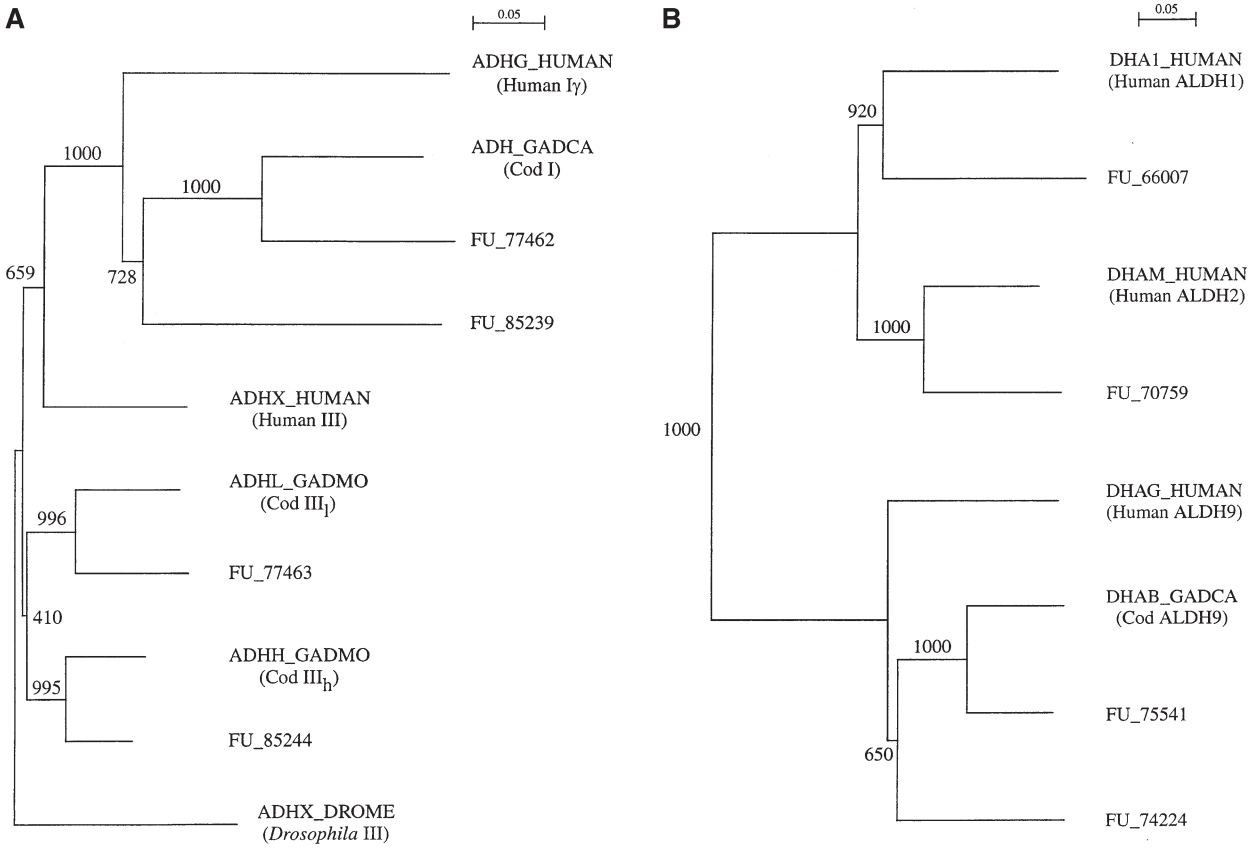

Figure 2. Unrooted phylogenetic trees, relating the fish/human ADH classes I and III (*A*), and the fish/human ALDH classes 1, 2, and 9 (*B*). Sequence data from databanks except for the present structure (supplement fig. 1). Uppercase designations at each branch indicate ID in SwissProt, or, for *Fugu*, abbreviation of the designation of the predicted polypeptide chain available at NCBI. Relationships shown are those obtained with the program CLUSTAL W [26] and visualized with NJPLOT [27]. Numbers indicate results from bootstrap analysis for evaluating confidence limits (1000 bootstrap replicates [28]).

Table 2. Comparisons between human and fish forms of metabolic enzymes defining two different sets of variability: 'constant' and 'variable'.

	Enzyme	Identities %
'Constant' (~75% identities)	betaine aldehyde dehydrogenase	70
	aldolase B	74
	alcohol dehydrogenase, class III_1	76
	glucose-6-phosphate dehydrogenase	76
	glycerol-3-phosphate dehydrogenase	76
	6-Phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 1	76
	aldehyde dehydrogenase, class 2	78
	lactate dehydrogenase B	78
'Variable' (~ 55% identities)	alcohol dehydrogenase, class I	54
	cytochrome P450, CYP1A1	56

therefore strange that they had not been found elsewhere. Now finding them in the *Fugu* line makes sense, and ascribes a wider occurrence to these two functionally different ADH class III forms, giving strength to conclusions on their separate functions.

2) The class gene duplications make sense also with the *Fugu* variants. In no case was an enzyme that was expected from previous gene duplication timings not found in the *Fugu* line. Two extra *Fugu* forms were detected. Their evolutionary tree positions (fig. 2) place them in the class I ADH line and the class 9 ALDH line. The former is positioned between the cod and human class I forms and makes sense in the manner that cod class I ADH has previously been found to be deviant, reflecting an additional gene duplication, or an altered evolutionary rate close to the time of the class I origin. The finding of an extra *Fugu* gene just here (fig. 2) may therefore be consistent and could support the alternative that there is one more duplication early in the fish class I ADH line. The other extra gene, in the class 9 ALDH line, is unexplained but could suggest extra duplications also in the ALDH line at early vertebrate times.

3) The evolutionary rates differ in the various ALDH lines. There are still too few species to give firm conclusions but the fish/mammal divergence in class 2 ALDH appears to be the smallest, and that in class 1 is large, while the branch lengths in class 9 are difficult to evaluate because of the extra forms (see above). In any event, the general pattern of the ADH line (fig. 2 A) is similar to that of the ALDH line, with repeated duplications, similar for cod versus *Fugu* and cod/*Fugu* versus human patterns. The spread between constant and variable forms appears larger and clearer in the ADH line than in the ALDH line, but to some extent also exists in the latter. Similar overall divergence patterns could have evolved in several multiple dehydrogenase enzymes.

Discussion

ALDH and ADH constitute complex enzyme families in vertebrates. All forms have not been functionally ascribed, not even in humans, where several ALDH forms are little characterized [2] and where even one ADH form is not yet ascribed (class V [6]). For understanding all dehydrogenases from genome projects, crucial forms must be isolated and characterized rather than just dealing with them as deduced forms, to assign the evolutionary properties, and to have correct orthologs in each case. In the present study, we purified a major fish ALDH from cod liver, determined its properties and related it to the fish/human spread of divergence for several classes of both ALDH and ADH. The tertiary structure of this ALDH has been determined [21], showing its overall relationship to the other crystallographically analyzed forms of ALDH, i. e., the enzyme of classes 3 [37], 2 [38], and 1 [39]. We now find three additional properties for this major form of ALDH.

1) Purification of the apparently major fish liver ALDH gives a class 9 form, whereas in the human, initial purifications yielded the class 1 and 2 ALDH forms [13]. Although the relative abundance of all classes has not been directly determined in any species, the spontaneous purification of different classes from separate species suggests the possibility that relative class occurrences may differ between species. Interestingly, similar variability is also apparent in the ADH lines. Thus, recent purifications of ADH from avian species gave unexpected ADH forms, an ADH of a new family assignment (AKR, for the aldo-keto-reductase family) in chicken [40], and a new class of ADH as a major form in pigeon [L. Hjelmqvist and A. P. Jonsson, unpublished data]. Hence, not only multiplicity per se, but also actual, relative abundance of all forms may differ between species for both ADH and ALDH. This highlights the difficulty with species cross-comparisons in conclusions on functional and metabolic effects of ADH and ALDH.

2) Betaine ALDH (class 9 ALDH) is enzymatically similar in fish and humans (table 1) and evolutionarily has segment variability patterns (fig. 1) like a constant enzyme with basal functional properties. Combined, this suggests a basic functional importance of betaine in vertebrate metabolism.

3) Spreads in evolutionary properties are noticed for both ADH and ALDH. Both families are highly multiple, have 'constant' and 'variable' forms, and although of completely different origin, appear to have some similarities in evolutionary patterns when the vertebrate systems are considered over a wide species spread (fig. 2). Hence, these multiple-enzyme families may in part evolve in a parallel manner and exhibit patterns that can be useful to discern distinctions of gene products of multiple enzyme forms from genome projects.

Acknowledgements. This work was supported by grants from the Swedish Research Council (Project 13X-3532), the Swedish Society of Medicine, the foundations of Magn. Bergvall, the foundations in memory of Clas Groschinsky, and in memory of Lars Hierta, and the Foundations of Fredrik and Ingrid Thuring, Åke Wiberg, and Berth von Kantzow (fellowship to L. H.).

1 Yoshida A., Rzhetsky A., Hsu L. C. and Chang C. (1998) Human aldehyde dehydrogenase gene family. Eur. J. Biochem. **251:** 549–557
2 Sophos N. A. and Vasiliou V. (2003) Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem. Biol. Interact. **143-144:** 5–22
3 Vasiliou V., and Sophos N. A. (2002) Human ALDH genes. http://www.uchsc.edu/sp/sp/alcdbase/ humaldhgenes.html
4 Jörnvall H. and Höög J.-O. (1995) Nomenclature of alcohol dehydrogenases. Alcohol Alcoholism **30:** 153–161
5 Duester G., Farrés J., Felder M. R., Holmes R. S., Höög J.-O., Parés X. et al. (1999) Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. Biochem. Pharmacol. **58:** 389–395
6 Nordling E., Persson B. and Jörnvall H. (2002) Differential multiplicity of MDR alcohol dehydrogenases: enzyme genes in the genome versus those in organisms initially studied. Cell. Mol. Life Sci. **59:** 1070–1075
7 Yin S.-J., Vagelopoulos N., Wang S.-L. and 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
8 Jörnvall H., Persson B. and Jörnvall H. (1993) Variability patterns of dehydrogenases versus peptide hormones and proteases/antiproteases. FEBS Lett. **335:** 69–72
9 Danielsson O., Atrian S., Luque T., Hjelmqvist L., Gonzàlez-Duarte R. and Jörnvall H. (1994) Fundamental molecular differences between alcohol dehydrogenase classes. Proc. Natl. Acad. Sci. USA **91:** 4980–4984
10 Hjelmqvist L., Estonius M. and Jörnvall H. (1995) The vertebrate alcohol dehydrogenase system: variable class II type form elucidates separate stages of enzymogenesis. Proc. Natl. Acad. Sci. USA **92:** 10905–10909
11 Svensson S., Hedberg J. J. and Höög J.-O. (1998) Structural and functional divergence of class II alcohol dehydrogenase: cloning and characterization of rabbit liver isoforms of the enzyme. Eur. J. Biochem. **251:** 236–243
12 Cañestro C., Albalat R., Hjelmqvist L., Godoy L., Jörnvall H. and Gonzàlez-Duarte R. (2002) Ascidian and Amphioxus *Adh* genes correlate functional and molecular features of the ADH family expansion during vertebrate evolution. J. Mol. Evol. **54:** 81–89
13 Weiner H. (1979) Aldehyde dehydrogenase: mechanism of action and possible physiological roles. In Biochemistry and Pharmacology of Ethanol, vol. 1, pp. 107–124, Majchrowicz E. and Noble E. P. (eds), Plenum, New York
14 Kurys G., Ambroziak W. and Pietruszko R. (1989) Human aldehyde dehydrogenase: purification and characterization of a third isozyme with low Km for gamma-aminobutyraldehyde. J. Biol. Chem. **264:** 4715–4721
15 Chern M.-K. and Pietruszko R. (1995) Human aldehyde dehydrogenase E3 isozyme is a betaine aldehyde dehydrogenase. Biochem. Biophys. Res. Commun. **213:** 561–568
16 Lin S. W., Chen J. C., Hsu L. C., Hsieh C.-L. and Yoshida A. (1996) Human gamma-aminobutyraldehyde dehydrogenase (ALDH9): cDNA sequence, genomic organization, polymorphism, chromosomal localization, and tissue expression. Genomics **34:** 376–380
17 Izaguirre G., Kikonyogo A. and Pietruszko R. (1997) Tissue distribution of human aldehyde dehydrogenase E3 (ALDH9): comparison of enzyme activity with E3 protein and mRNA distribution. Comp. Biochem. Physiol. **118B:** 59–64
18 Bagnasco S., Balaban R., Fales H. M., Yang Y.-M. and Burg M. (1986) Predominant osmotically active organic solutes in rat and rabbit renal medullas. J. Biol. Chem. **261:** 5872–5877
19 Petronini P. G., De Angelis E. M., Borghetti P., Borghetti A. F. and Wheeler K. P. (1992) Modulation by betaine of cellular responses to osmotic stress. Biochem. J. **282:** 69–73
20 Grunewald R. W. and Eckstein A. (1995) Osmotic regulation of the betaine metabolism in immortalized renal cells. Kidney Int. **48:** 1714–1720
21 Johansson K., El-Ahmad M., Ramaswamy S., Hjelmqvist L., Jörnvall H. and Eklund H. (1998) Structure of betaine aldehyde dehydrogenase at 2.1 Å resolution. Protein Sci. **7:** 2106–2117
22 Aparicio S., Chapman J., Stupka E., Putnam N., Chia J.-M., Dehal P. et al. (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science **297:** 1301–1310
23 Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254
24 Lutz R. A., Bull C. and Rodbard D. (1986) Computer analysis of enzyme-substrate-inhibitor kinetic data with automatic model selection using IBM-PC compatible microcomputers. Enzyme **36:** 197–206
25 Bergman T., Cederlund E. and Jörnvall H. (2001) Chemical C-terminal protein sequence analysis: improved sensitivity, length of degradation, proline-passage, and combination with Edman degradation. Anal. Biochem. **290:** 74–82
26 Thompson J. D., Higgins D. G. and Gibson T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680
27 Perriere G. and Gouy M. (1996) WWW-query: an on-line retrieval system for biological sequence banks. Biochimie **78:** 364–369
28 Felsenstein J. (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution **39:** 783–791
29 Totrov M. and Abagyan R. (1994) Detailed *ab initio* prediction of lysozyme-antibody complex with 1.6 Å accuracy. Nat. Struct. Biol. **1:** 259–263
30 Pietruszko R., Kikonyogo A., Chern M.-K. and Izaguirre G. (1997) Human aldehyde dehydrogenase E3: further characterization. In: Enzymology and Molecular Biology of Carbonyl Metabolism 6, pp. 243–252, Weiner H., Lindahl R., Crabb D. W. and Flynn T. G. (eds), Plenum, New York
31 Keung W.-M. and Vallee B. L. (1993) Daidzin: a potent, selective inhibitor of human mitochondrial aldehyde dehydrogenase. Proc. Natl. Acad. Sci. USA **90:** 1247–1251
32 Klyosov A. A. (1996) Kinetics and specificity of human liver aldehyde dehydrogenases toward aliphatic, aromatic, and fused polycyclic aldehydes. Biochemistry **35:** 4457–4467
33 Wang S.-L., Wu C.-W., Cheng T.-C. and Yin S.-J. (1990) Isolation of high-Km aldehyde dehydrogenase isoenzymes from human gastric mucosa. Biochem. Int. **22:** 199–204
34 Yin S.-J., Wang M.-F., Han C.-L. and Wang S.-L. (1995) Substrate binding pocket structure of human aldehyde dehydrogenase: a substrate specificity approach. In: Enzymology and Molecular Biology of Carbonyl Metabolism 5, pp. 9–16, Weiner H., Holmes R. S. and Wermuth B. (eds), Plenum, New York
35 Danielsson O., Shafqat J., Estonius M., El-Ahmad M. and Jörnvall H. (1996) Isozyme multiplicity with anomalous dimer patterns in a class III alcohol dehydrogenase: effects on activity and quaternary structure of residue exchanges at "nonfunctional" sites in a native protein. Biochemistry **35:** 14561–14568
36 Danielsson O. and Jörnvall H. (1992) 'Enzymogenesis': classical liver alcohol dehydrogenase origin from the glutathione-de-

pendent formaldehyde dehydrogenase line. Proc. Natl. Acad. Sci. USA **89:** 9247–9251

37 Liu Z.-J., Sun Y.-J., Rose J., Chung Y.-J., Hsiao C.-D., Chang W.-R. et al. (1997) The first structure of an aldehyde dehydrogenase reveals novel interaction between NAD and Rossmann fold. Nat. Struct. Biol. **4:** 317–326

38 Steinmetz, C. G., Xie, P., Weiner, H. and Hurley, T. D. (1997) Structure of mitochondrial aldehyde dehydrogenase: the genetic component of ethanol aversion. Structure **5:** 701–711

39 Moore S. A., Baker H. M., Blythe T. J., Kitson K. E., Kitson T. M. and Baker E. N. (1998) Sheep liver cytosolic aldehyde dehydrogenase: the structure reveals the basis for the retinal specificity of class 1 aldehyde dehydrogenases. Structure **6:** 1541–1551

40 Crosas B., Cederlund E., Torres D., Jörnvall H., Farrés J. and Parés X. (2001). A vertebrate aldo-keto reductase active with retinoids and ethanol. J. Biol. Chem. **276:** 19132–19140