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Alcohol dehydrogenase of class III: consistent patterns of structural and functional conservation in relation to class I and other proteins

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Abstract Class III alcohol dehydrogenase from the lizard *Uromastix hardwickii* has been characterized. This non-mammalian, gnathostomatous vertebrate class III form allows correlations of structures and functions of this class, the traditional class I alcohol dehydrogenase, and other well-studied proteins. Catalytically, results show similar recoveries and activities of all vertebrate class III forms independent of source, similar activities also in invertebrates but in lower amounts, and considerably higher specific activities in microorganisms. Structurally, variability patterns are consistent throughout the vertebrate system with a ratio in accepted point mutations versus class I of 0.4. This ratio between different classes of a zinc enzyme is comparable to that between different heme proteins (cytochrome *c* and myoglobin), suggesting defined but non-identical functions also for the alcohol dehydrogenase classes.

Key words: Alcohol dehydrogenase; Reptilian protein; *Uromastix hardwickii*; Amino acid sequence; Molecular evolution

1. Introduction

Vertebrate liver alcohol dehydrogenase constitutes a complex system composed of minimally six classes of related enzymes [1], further subdivided into isozymes, and forming parts of a larger protein family, MDR (medium-chain dehydrogenases/reductases), with additional enzymes [2]. The classes have related three-dimensional folds [3,4], and at least some alcohol dehydrogenase activity in common [5], but differ widely in primary structure and enzyme activity. Classes I and III are those best defined [6], class I being alcohol dehydrogenases with low K_m values for ethanol, class III primarily a glutathione-dependent formaldehyde dehydrogenase [7], and remaining classes little studied regarding properties throughout vertebrates.

The classes also differ extensively in molecular properties [6]. In particular, class I enzymes exhibit more differences among each other than the class III enzymes. Class I is of more recent origin, and has most probably originated through a gene duplication at early vertebrate times. This dating is based on three lines of evidence, estimates of molecular changes [8], apparent absence of class I in species diverging before the supposed class III duplication [6,9], and presence of enzymes with class-mixed properties in species originating at that time (fish [10]).

Although the alcohol dehydrogenase classes now constitute a model system with an apparent class III parent form and with repeated descendents from separate gene duplications, class III has not been characterized in any sub-mammalian traditional vertebrate (Superclass Gnathostomata). Many of the conclusions therefore depend on the recently characterized insect enzyme [6] and cannot be directly correlated with variants of class I which have been studied in sub-mammalian vertebrate lines. We have therefore analyzed the *Uromastix* class III alcohol dehydrogenase, bridging the gap between invertebrates and mammals. The results allow firm assignment of constant class III properties throughout vertebrates. They also allow correlations with other well-studied protein families, such as those of the heme proteins cytochrome *c* and myoglobin, relating, in extent of variability, to alcohol dehydrogenase of classes III and I, respectively.

2. Materials and methods

2.1. Protein purification

Livers from *Uromastix hardwickii* (collected in Pakistan) were used for purification of class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase, utilizing DEAE-Sepharose and Mono Q for anion-exchange chromatography, AMP-Sepharose for affinity chromatography, and Sephadex G-100 for gel filtration (all from Pharmacia), in 10 mM Tris-Cl, pH 8.0, essentially as in purifications of other alcohol dehydrogenases [6,8]. Protein was monitored during purification with the Bradford method [11] and in pure state by amino acid analysis after hydrolysis. Purities were evaluated by SDS/polyacrylamide gel electrophoresis (Phast system, Pharmacia) with Coomassie brilliant blue for protein staining. Activity staining after isoelectric focusing was performed with Nitro blue tetrazolium/phenazine methosulphate.

2.2. Protein characterization

Enzyme activities at 25°C were measured in 0.1 M sodium pyrophosphate, pH 8.0, with glutathione-conjugated formaldehyde [7], and in 0.1 M glycine/NaOH, pH 10.0, with ethanol and other alcohols [12].

For structural characterization, the protein was ^{14}C-carboxymethylated and cleaved in separate batches with Lys-, Glu-, and Asp-specific proteases, chymotrypsin, and CNBr. Peptides were fractionated by reverse phase HPLC [8]. Total compositions were determined by amino acid analysis (Pharmacia Alpha Plus analyzer) after hydrolysis with 6 M HCl/0.5% phenol. Peptide structures were determined with an Applied Biosystems 477A sequencer or with MilliGen prosequencers 6600/6625, all with on-line HPLC detection. The N-terminally acetyl-blocked peptides were structurally analyzed by tandem mass spectrometry [13] and molecular masses were monitored with a Lasermat 2000 laser desorption mass spectrometer. For calculation of relationships among all structures, the program CLUSTAL W [14] was used with distances corrected for multiple substitutions.

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The sequence reported in this paper has been deposited in the SWISS-PROT data base (accession no. P80467).

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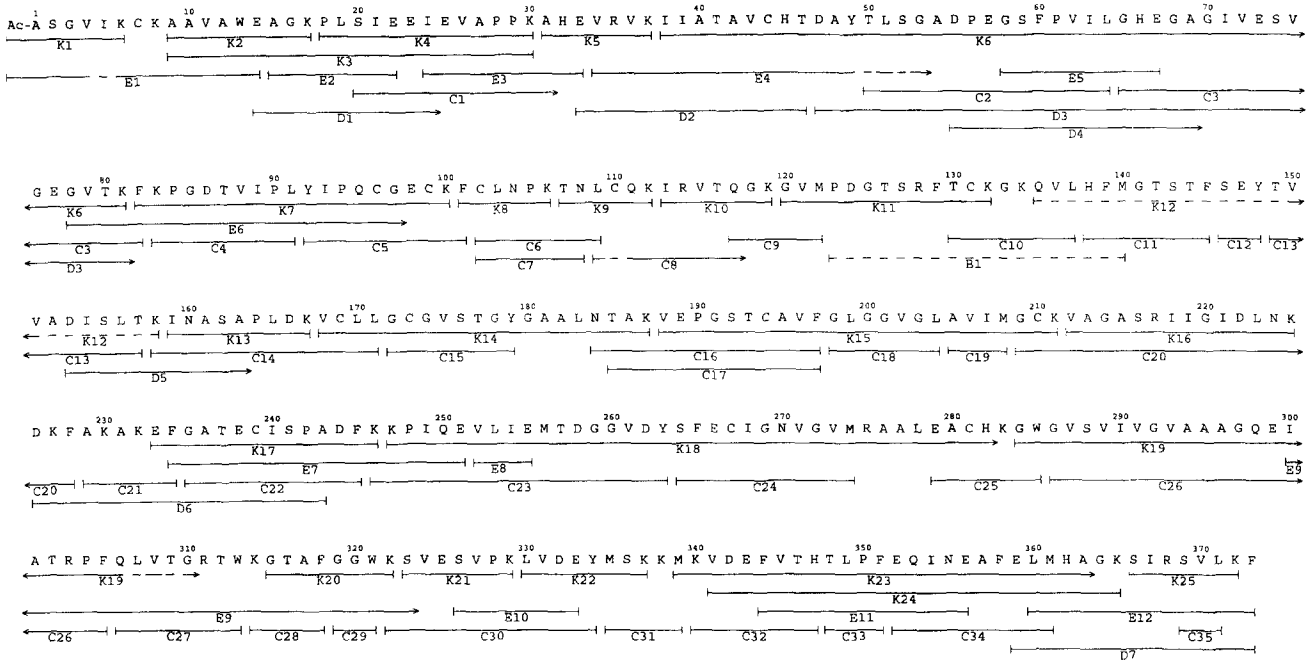

Fig. 1. Primary structure of *Uromastix* liver class III alcohol dehydrogenase. Solid lines indicate parts of peptides analyzed with Edman degradation or tandem mass spectrometry for K1 and E1), dashed lines remaining parts. Peptide names are given by K for origins by cleavages with Lys-specific protease, E for those with the Glu-specific protease, D for those with the Asp-specific protease, C for those with chymotrypsin, and B for those with CNBr. Dashed peptides B1 and K12 were analyzed by laser desorption mass spectrometry, while peptides K1 and E1 were determined from collision activation dissociation mass spectra as previously reported [13]. Position 5 was established as Ile (not Leu) by amino acid analysis of K1.

3. Results

3.1. *Class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase*

The class III enzyme from *Uromastix hardwickii* livers was purified by a 4-step chromatographic method. Separation from the ethanol-active class I alcohol dehydrogenase, also present [13], was achieved in the first anion exchange step, in which the ethanol dehydrogenase does not bind and is eluted with the wash-through. In the subsequent affinity step on AMP-Sepharose, class III alcohol dehydrogenase was eluted mid-gradient using 0–0.5 mM NAD^+ in the buffer. The enzyme was then submitted to FPLC on Mono Q, and for final purification to gel filtration on Sephadex G-100. The protein was pure according to SDS/polyacrylamide gel electrophoresis, and isoelectric focusing revealed it to have a pI around pH 6.5.

The enzyme was active with hydroxymethylglutathione (with a specific activity of 5.2 U/mg) and long-chain alcohols, like human class III alcohol dehydrogenase (which has a specific activity of 3.2 U/mg for the glutathione-dependent formaldehyde dehydrogenase activity [15]).

3.2 *Protein structure*

Peptides for determination of the primary structure were generated by proteolytic cleavages in separate batches of the carboxymethylated protein. Analysis of the overlapping peptides yielded an amino acid sequence of 373 residues (Fig. 1). Most peptides were analyzed by Edman degradations, while the acetyl-blocked N-terminally derived peptides [13] and a few peptides used to establish overlaps were analyzed by mass spectrometry.

The 373-residue size of the protein chain is exactly the same as that for the mammalian forms of the enzyme. In particular, the N-terminus is acetylated, and although the start position is different in different class III enzymes, this reptilian form had a start position like in the mammalian class III alcohol dehydrogenases (corresponding to position 3 of the class I forms)

Table 1
Estimates of amounts and specific activities of class III alcohol dehydrogenase in tissues from vertebrates and invertebrates, fungi and prokaryotes

Species		Estimated amount (mg/100 g tissue)	Specific activity (U/mg)
A Liver	Human	9	3.2
	Rat	6	2.5
	Uromastix hardwickii	5	5.2
	Hagfish	4	11
B Other	Octopus	1	3.7
	Drosophila melanogaster	2	12
	Candida boidinii	40*	48
	Saccharomyces cerevisiae	3	47
	Escherichia coli	1	57

Uromastix values from this work, remaining values calculated from literature (human [15], rat [16], hagfish [9], octopus [17], *Drosophila* [6], *Candida* [18], *Saccharomyces* [19], *E. coli* [20]). Although values are from separate preparations and dependent on purifications, possible inhibitors and other factors, fairly similar estimates are noticed for all liver enzymes, considerably lower amounts of most non-liver forms, and significantly higher activities of the forms from microorganisms.
*Enzyme measured after induction due to growth on methanol, explaining the high value.

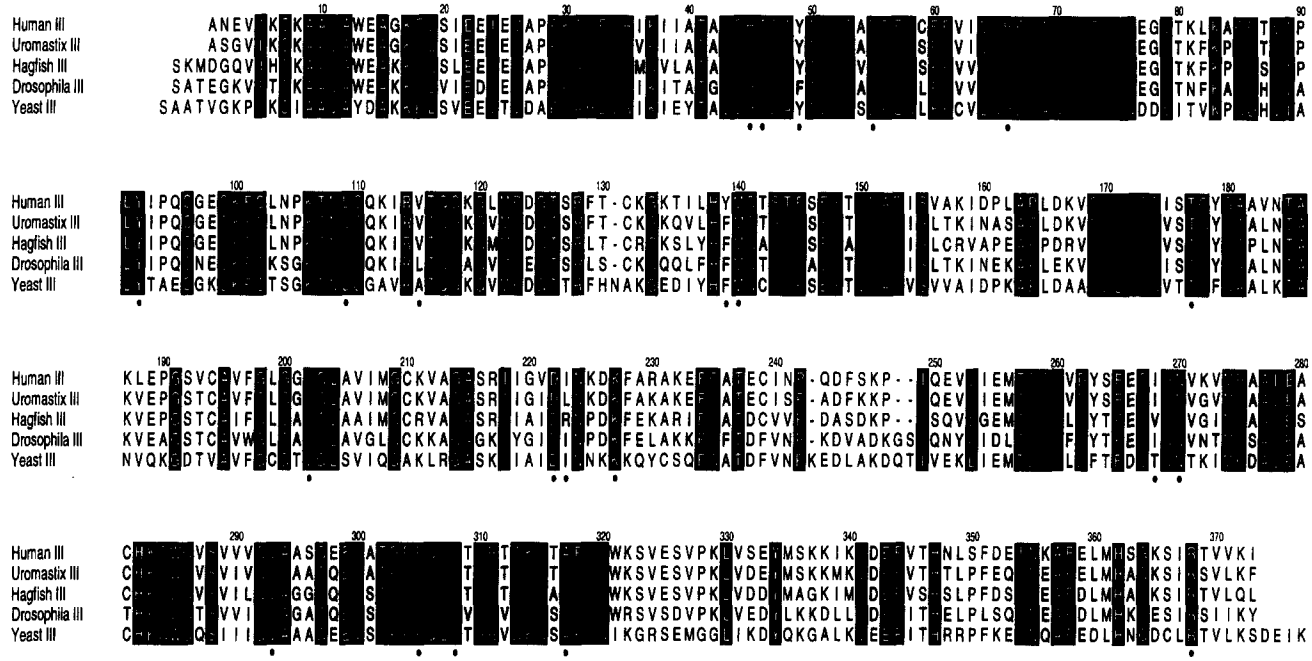

Fig. 2. Alignment of the class III alcohol dehydrogenase from *Uromastix hardwickii* with those from human, hagfish, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*. Residues conserved in all five species are given against a black background. Dots denote residues in substrate and coenzyme binding interactions [3,21]. Positional numbers refer to the *Uromastix* enzyme and are identical in the human enzyme. Sequences from data banks except for the present structure (from Fig. 1).

rather than like in the longer [13] class III enzymes of cartilaginous fish, cyclostomes and invertebrates.

3.3. *Substrates, specific activity and yields. Comparative aspects within class III*

Class III alcohol dehydrogenase has now been purified and characterized from a non-mammalian gnathostomatous liver, allowing rough comparisons of amounts and specific activities in different types of life forms, ranging from mammals [15,16], via the present form, to cyclostomes [9], and to invertebrates [6,17], fungi [18,19] and prokaryotes [20]. Of course, such estimates from total activities and yields in routine preparations are rough and may deviate because of different enzyme stabilities, possible presence of enzyme inhibitors or activators in the preparations, and other factors. Nevertheless, the values, as obtained (Table 1), suggest that all class III enzymes thus far reported from vertebrates have similar activities and occur in similar amounts, while those from invertebrates, fungi and prokaryotes differ to a large extent probably reflecting separate environments [19]. However, the different enzymes have a strict specificity for glutathione-conjugated formaldehyde, similar activity with long-chain alcohols, and similar low activity with ethanol (enzyme unsaturable with ethanol because of very high K_m). These properties suggest similar functional roles in formaldehyde elimination of all class III alcohol dehydrogenases.

4. Discussion

4.1. *Structures and evolutionary relationships*

The amino acid sequence now determined is homologous with that of other class III forms (Fig. 2), reflecting its intermediary position from a branch between the mammalian and lower vertebrate or invertebrate forms known before. The residue identity with the human enzyme is 89%, and that with the hagfish form is 81%. Comparisons of 22 residues in substrate and coenzyme binding interactions [3,21] reveal largely conserved structures throughout the vertebrate system. The present enzyme only differs at two of those positions from the human enzyme, at position 140 in the inner part of the substrate binding pocket, now Phe but Tyr in the human form (this variation is seen also in most of the other forms), and at position 224 (coenzyme-binding), now Leu but Ile in the human form (numbering according to the crystallographically investigated class I enzymes [cf. 3]). This conservation in interacting

Table 2
Differences between species sets for two different alcohol dehydrogenases (class III and class I enzymes) and two different heme-binding proteins (cytochrome *c* and myoglobin)

Human versus	Differences (PAM units)				Ratios of differences	
	ADH III	Cyt *c*	ADH I	Mb	ADH III / ADH I	Cyt *c* / Mb
Horse	5	13	13	14	0.39	0.93
Rat	6	10	20	–	0.28	–
Mouse	8	10	17	19	0.45	0.53
Reptile	12	15	29	36	0.40	0.42

Right-hand values are calculated from the ratio of differences between the two alcohol dehydrogenases and between the two heme-binding proteins, respectively. Similar relationships are shown for the alcohol dehydrogenase pair and the heme protein pair, respectively. The reptilian sequences used are from *Uromastix* lizard (class III alcohol dehydrogenase; this work), alligator (class I alcohol dehydrogenase), and *Varanus varius* lizard (cytochrome *c* and myoglobin). Sequence data from data banks except for the present structure (Fig. 1). Dash for rat myoglobin indicates lack of sequence data. ADH, alcohol dehydrogenase; Cyt *c*, cytochrome *c*; Mb, myoglobin; PAM, accepted point mutation.

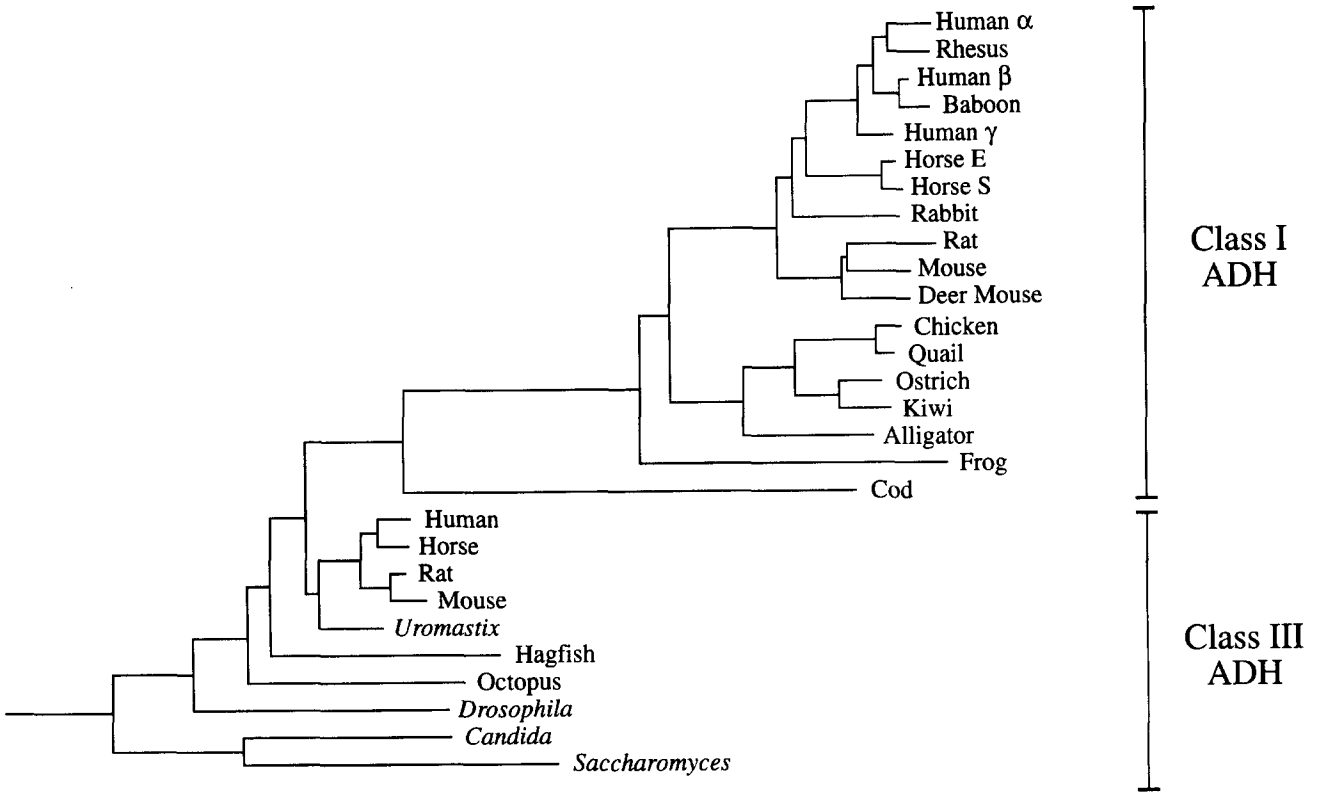

Fig. 3. Evolutionary tree, relating the present enzyme to other known class III and class I alcohol dehydrogenases. Sequence data from data banks except present structure (Fig. 1). Relationships shown are those obtained with the program CLUSTAL W [14], with distances corrected for multiple substitutions. Alcohol dehydrogenase from *Paracoccus denitrificans* was used as outgroup structure.

residues also in the present form (Fig. 2) again suggests strict functional conservation of the class III enzyme.

The variability can now be compared between identically separated life forms in both the class III and class I lines. The phylogenetic tree obtained (Fig. 3) is significant in showing two aspects.

One is apparent origin of class I from class III, with the class I connection between the present *Uromastix* form and the Hagfish enzyme (Fig. 3). This is compatible with a duplicatory class I origin at early vertebrate times, and with the cod class I form occupying a class-intermediate position (Fig. 3) in agreement with its mixed properties [10]. These facts confirm conclusions on the duplicatory origin from class III of the traditional class I liver alcohol dehydrogenase.

The other and more important aspect is the consistent pattern of longer branch lengths (meaning faster variation) of class I lines than of the corresponding class III lines in those four mammals (human, horse, rat, mouse) and one reptile (alligator/*Uromastix*) where both class I and III structures are known Fig. 3). The values show that class III evolves at a speed of about 0.4 of that for class I (Table 2). The pattern is largely independent of species lines compared, reflecting true differences between the classes maintained in the separate branches which evolve independently. The similarly sized ratios (Table 2) suggest that both classes now have fixed, though different physiological functions. Regarding class III a fixed function was already established from the substrate specificity and constant properties (3.3, above), but regarding class I, with its more variable structure, a fixed function has not been clear.

4.2. *Parallel patterns with heme proteins*

The present pair of zinc enzyme classes allows comparisons with other proteins well-studied in many species, such as the heme proteins of myoglobin and cytochrome *c*, for both of which human, mouse and reptilian forms are established. The heme proteins also differ in rate of evolutionary changes, with cytochrome *c* more constant than globins [22,23]. Comparisons now reveal (Table 2) that within a reasonable spread, the two alcohol dehydrogenase classes I and III differ by the same order of magnitude in evolutionary change as the two heme proteins myoglobin and cytochrome *c*. Thus, the ratios between corresponding species differences (reflecting the evolutionary rates), as well as the actual mutational difference values in PAM units, position the alcohol dehydrogenase pair in the same category as the heme protein pair. Class III with its constant properties exhibits mutational acceptance values like cytochrome *c*, while class I more closely parallels the changes in myoglobins. It may be concluded that patterns of variability are consistent over wide periods, reflecting functions, and including both the constant class III and the variable class I alcohol dehydrogenases, relating them to similar patterns for heme proteins, and presumably for proteins in general.

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