Volume 298, number 2,3, 297–300 FEBS 10763 February 1992

Reptilian alcohol dehydrogenase

Heterogeneity relevant to class multiplicity of the mammalian enzyme

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Received 18 December 1991

Liver alcohol dehydrogenase of the ethanol-active type ('class I enzyme') from the lizard, *Uromastix hardwickii*, was purified and screened for relationships with other vertebrate forms of the enzyme. Two different acetylated N-termini (acetyl-Gly and acetyl-Ser) and further positional differences already in the N-terminal segments establish the presence of two types of protein chain. The multiplicity is different from that hitherto detected within vertebrate class I alcohol dehydrogenase isozymes but typical of that which would be expected for subunits of different classes. In particular, relationships to class II or to class II-related forms appear likely. This may indicate yet further vertebrate alcohol dehydrogenase multiplicity or discovery of a class II non-mammalian enzyme. The results give prospects of defining gene duplications corresponding to more than one alcohol dehydrogenase class split to at an early vertebrate stage.

Enzyme family; Alcohol dehydrogenase; Isozymes; Heterogeneity; Amino acid exchanges

1. INTRODUCTION

The known complexity of the vertebrate alcohol dehydrogenase system is rapidly increasing. Gene duplications at several levels have created a protein family composed of highly different enzymes, intermediately different classes and more similar isozymes [1]. The classes have distant origins, and a first, rough estimate for the timing of one gene duplication, the class I/III separation, has been obtained from analysis of an amphibian enzyme [2]. Thus far, the N-terminal structures appear to be typical for each class, and differ in position and type of the residue acetylated [3]. In order to study the class origins and the isozyme developments further, we decided to analyse reptilian forms of the protein. They have hitherto not been characterized at all. From an *Uromastix* lizard liver, we find evidence both for alcohol dehydrogenase multiplicity and for variation of a type relevant to the one for distinction of mammalian enzyme classes. The results suggest the presence of enzymes with hybrid properties of a novel type and may trace yet one further class origin, possibly the class I/II separation.

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2. MATERIALS AND METHODS

2.1. *Proteins*

Liver alcohol dehydrogenase of the ethanol-active type [4] from *Uromastix hardwickii* was purified by chromatography on DEAE-Sepharose, AMP-Sepharose utilizing elution with NAD^+, and Sephadex G-100, essentially as in recent purifications of the enzyme from other sub-mammalian vertebrate lines [5]. The major ethanol dehydrogenase obtained eluted from the penultimate purification step, AMP-Sepharose, midway in a 0–10 mM gradient of NAD^+ and was recovered at 5 mM NAD^+ in batch-wise elutions.

The protein was pure as judged by SDS/polyacrylamide gel electrophoresis, revealing approximately 40 kDa subunits, and was carboxymethylated with iodo-[^{14}C]acetate as described [2].

2.2. *Structural analysis*

The carboxymethylated protein was cleaved in separate batches for 4 h at 37°C with Achromobacter Lys-specific protease (Wako) and Staphylococcal Glu-specific protease (Boehringer), at protease:substrate ratios of 1:6–1:20 in 0.1 M ammonium bicarbonate, with up to 2.2 M urea for solubilization. Resulting peptides were separated by reverse-phase HPLC on TSK ODS-120T columns (Pharmacia LKB Biotechnology) as described [2]. Peptides were submitted to amino acid analysis after acid hydrolysis and to sequencer analysis [2]. When N-terminally blocked, they were analysed by plasma desorption mass spectrometry in a ^{252}Cf time of flight instrument [6].

3. RESULTS

3.1. *Functional properties*

Electrophoresis under native conditions and differential activity staining with ethanol, pentanol and formaldehyde/glutathione revealed that *Uromastix* liver contains two separate activities of alcohol dehydrogenase,

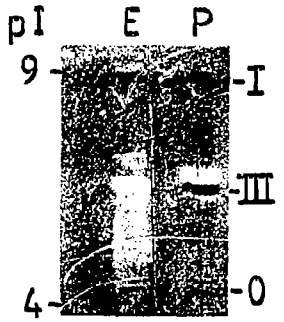

Fig. 1. Differential activity staining with ethanol (E) and pentanol (P) of *Uromastix* liver alcohol dehydrogenase crude extract after isoelectric focusing. I and III denote activity bands of the type suggesting the presence of class I and III enzymes, respectively. O, origin. Electrophoresis in a Pharmacia Phast system as described [5].

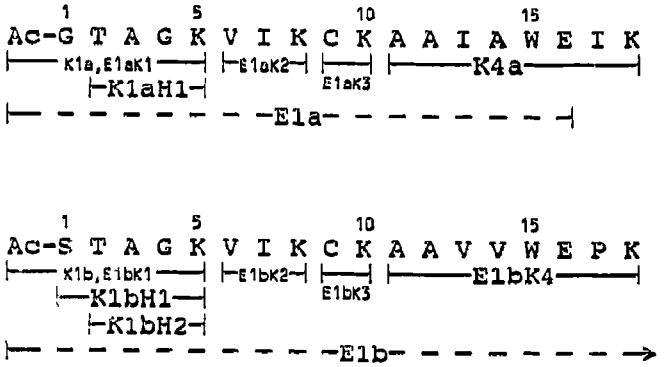

Fig. 3. Amino acid sequences and positions of the peptides analysed to establish the presence of two different types of protein chain. Corresponding peptides from the two different chains are denoted with postscripts a and b, respectively. K1 and K4, Lys-cleaved fragments; E1, Glu-cleaved fragments; E1K1-E1K4, re-cleavage products after Lys-protease treatment of E1; K1H1, the K1 fragments obtained after deblocking with HCl. Overlaps follow from the E1 total compositions and from the homology with other class I alcohol dehydrogenases [1].

one behaving like a class III type, visible with the formaldehyde/glutathione or pentanol staining, and one like a classical class I type, visible most clearly with ethanol (Fig. 1). The latter form is the major component and was purified by ion exchange and affinity chromatography (see section 2.1.). The product obtained exhibited a symmetrical peak upon the final purification step, a single band upon SDS/polyacrylamide gel electrophoresis, and an overall purification of 50-fold after the initial homogenization and centrifugation steps. All these properties are compatible with an apparent homogeneity of a class I vertebrate alcohol dehydrogenase.

3.2 *Blocked N-terminal structure*

The *Uromastix* alcohol dehydrogenase preparation did not yield any result upon attempts at direct sequence analysis, suggesting that the reptilian enzyme, like other medium-chain alcohol dehydrogenases, is N-terminally blocked. The carboxymethylated protein was therefore cleaved in separate batches with Lys-specific and Glu-specific proteases. The digests were submitted to reverse-phase HPLC to purify N-terminally blocked peptides. This is possible in a specific manner since class I liver alcohol dehydrogenase has a basic residue at position 5 and a Trp–Glu sequence at positions 15–16, meaning that the blocked N-terminal peptide after Lys-specific cleavage typically corresponds to the first A_{214} peak from the HPLC recording, and to the first big A_{280} peak after Glu-specific cleavage (class I alcohol dehydrogenase frequently has only 2 Trp).

The *Uromastix* alcohol dehydrogenase digests showed doublets of both these peaks. Thus, from the Lys-C protease digest, the first A_{214}-absorting fraction was a doublet (Fig. 2), and in the Glu-C protease digest,

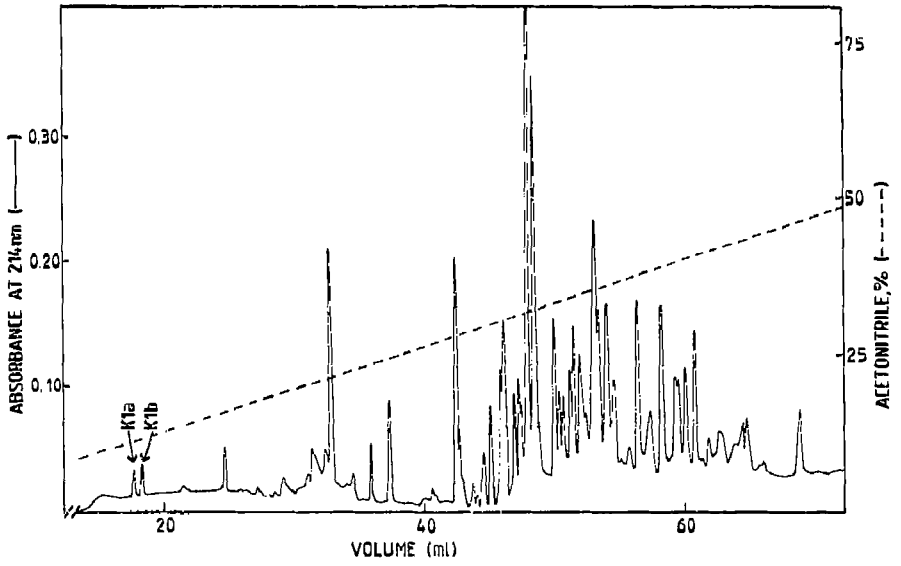

Fig. 2. Reverse-phase HPLC purification of the blocked N-terminal peptide in digests with the Lys-specific protease. The early eluting doublet of N-terminal peptides, K1a and K1b, (see Fig. 3) is indicated by arrows.

two fairly early eluting A_{280}-absorbing fractions were obtained. Analysis of the two peptide pairs in each case showed them to correspond to the N-terminal segments and to represent two separate structures (Fig. 3). Deblocking of the acyl-blocked pentapeptides with 8 M HCl at room temperature for 4 h [2] produced a tetrapeptide lacking the blocked residue. In total, the peptide analyses established four positional differences in the N-terminal regions (Fig. 3).

3.3. *Blocking group*

Plasma desorption mass spectrometry of the N-terminal pentapeptides showed masses corresponding to the presence of N-terminal acetyl groups in both cases (Table I). Consequently, the structures have been determined to be as given in Fig. 3.

4. DISCUSSION

The present results establish three facts of importance for the understanding of vertebrate alcohol dehydrogenase forms and functions.

The first is that reptiles have a major, ethanol-active liver alcohol dehydrogenase of a type typical for a class I enzyme. This finding is consistent with conclusions from structures for the amphibian and piscine alcohol dehydrogenases [2,5] and suggests related metabolic roles for the protein in all major vertebrate lines.

Secondly, the results show that *Uromastix* liver ethanol dehydrogenase has at least two different types of subunit. They differ in both the nature of the residue acetylated and in a large proportion of all positions (4 of 18 analysed, Fig. 3). These two types of difference are atypical for vertebrate intra-class isozyme variation but are typical for class variability [1]. Consequently, the results indicate either a new type of vertebrate isozyme variability, corresponding to one further level of gene duplication than the ones known (e.g. see [1]), or trace the emergence of a divergence that eventually led to the class distinction presently observed in the mammalian alcohol dehydrogenase system.

Table I

Data for the two N-terminal peptides analysed by mass spectrometry

	K1a	K1b
Thr	0.9 (1)	0.8 (1)
Ser	– –	1.0 (1)
Gly	2.0 (2)	1.0 (1)
Ala	0.9 (1)	0.8 (1)
Lys	0.8 (1)	0.9 (1)
Sum	5	5
Molecular weight		
Calculated	475.4	504.5
Mass spectrometry	474.9	504.3

Top values show compositions supporting the sequence identification (Fig. 3), while bottom mass values show the molecular weight agreements establishing the acetyl group.

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B   S T A G K V I K C K A A V V W E P K
A   G T A G K V I K C K A A I A W E I K

I   S T A G K V I K C K A A V L W E L K
II  G T K G K V I K C K A A I A W E A G
III   A N E V I K C K A A V A W E A G
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Identities	A	B
I	14	(16)
II	(15)	12
III	11	11

Fig. 4. Class comparisons in relation to the two new structures detected. (A and B) The two N-terminal structures now determined for *Uromastix* alcohol dehydrogenase. (I–III) Constitute three of the five known classes of the human enzyme (class I–III in [1]; class IV [9] and V [10] also considered but now not further detailed since they constitute divergent structures still incompletely known), with the γ form shown for the isozyme variation within class I [1]. As shown by the encircled values, overall relationships for form B are closest to class I, and for form A to class II. Boxed positions indicate A/B differences.

Thirdly, the two forms isolated exhibit properties that appear class-mixed or hybrid in relation to the mammalian alcohol dehydrogenase classes. In particular, they do so in relation to the class I/II enzymes (Fig. 4). Thus, the two forms were purified functionally as ethanol dehydrogenases, but structurally one (form A) exhibits larger similarities with class II mammalian alcohol dehydrogenase, regarding both positional identities (Fig. 4) and nature of the blocked N-terminus (Gly), the latter thus far largely typical for each class [3]. Consequently, it appears possible that the two forms now detected in a preparation of liver ethanol dehydrogenase represent descendants of a class separation, class I from class II, or from a class II-related form. The structures have diverged in a manner towards that typical for the separation of the mammalian alcohol dehydrogenase classes. Functionally, the two *Uromastix* forms are purified as class I enzymes, co-migrating in the chromatographic steps and exhibiting ethanol dehydrogenase activity in a preparation suggesting only minor differences, like between isozymes, but structurally the differences are large and of a class-distinguishing type (Fig. 4). All the *Uromastix* heterogeneity positions now found, except position 17, affect residues and positions that are different from those known for isozyme variations in other vertebrate alcohol dehydrogenases [1,7,8].

If the heterogeneity represents a descendant from the emergence of class II, this would constitute the first time a class II enzyme has been found in a sub-mammalian species. In the same manner as a major piscine alcohol dehydrogenase represents a class I/III hybrid molecule, structurally related to class III but functionally related to class I [5], the A form of the two *Uromastix* forms could constitute a class I/II hybrid, structurally resembling class II, but functionally purified as a class I enzyme. This would mean that two gene duplications have now been traced, and that the class I/II separation, although not previously detected, would also be of early vertebrate origin, like the class I/III separation. If the present form A presents a class II structure, it would further mean that class II alcohol dehydrogenase exhibits considerable variation, suggesting it to be of the 'variable' type, like class I, and not of the 'constant' type, like the class III enzyme [11] found in many living lines [12]. Independent of this possibility regarding variability, the present structure shows a sub-mammalian enzyme form that appears to illustrate early relationships for the classes of mammalian alcohol dehydrogenase.

Acknowledgements: We are grateful to Carina Palmberg for assistance and for drawing the figure. This work was supported by grants from The Swedish Medical Research Council (project 03X-3532), The Swedish Alcohol Research Fund, the Swedish Institute (fellowships to J.S. and A.R.S.), and the Endowment for Research in Human Biology, Boston, MA (fellowships to L.H. and J.S.).

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