Genetics of alcohol dehydrogenase and aldehyde dehydrogenase from *Monodelphis domestica* cornea: further evidence for identity of corneal aldehyde dehydrogenase with a major soluble protein

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Summary

A didelphid marsupial, the gray short-tailed opossum (*Monodelphis domestica*), was used as a model species to study the biochemical genetics of alcohol dehydrogenases (ADHs) and aldehyde dehydrogenase (ALDH) in corneal tissue. Isoelectric point variants of corneal ALDH (designated ALDH3) and a major soluble protein in corneal extracts were observed among eight families of animals used in studying the genetics of these proteins. Both phenotypes exhibited identical patterns following PAGE-IEF and were inherited in a normal Mendelian fashion, with two alleles at a single locus (ALDH3) showing codominant expression. The data provided evidence for genetic identity of corneal ALDH with this major soluble protein, and supported biochemical evidence, recently reported for purified bovine corneal ALDH, that this enzyme constitutes a major portion of soluble corneal protein (Abedinia et al. 1990). Isoelectric point variants for corneal ADH were also observed, with patterns for the two major forms (ADH3 and ADH4) and one minor form (ADH5) being consistent with the presence of two ADH subunits (designated γ and δ), and variant phenotypes existing for the γ subunit. The genetics of this enzyme was studied in the eight families, and the results were consistent with codominant expression of two alleles at a single locus (designated ADH3). It is relevant that a major detoxification function has been proposed for corneal ADH and ALDH, in the oxidoruduction of peroxidic aldehydes induced by available oxygen and UV-B light (Holmes & VandeBerg, 1986a). In addition, a direct role for corneal ALDH as a UV-B photoreceptor in this anterior eye tissue has also been proposed (Abedinia et al. 1990).

1. Introduction

It is fitting that this research paper on the biochemical genetics of mammalian alcohol and aldehyde dehydrogenase should appear in association with Dr Mary Lyon, since the early gene mapping studies on both of these enzymes in mouse were carried out by R.S.H at Harwell, during a period of study leave from Australia (Holmes, 1978, 1979). Dr Lyon's support and advice associated with this work were invaluable, particularly in the development and making available of linkage testing stocks of mice for biochemical genetic analyses of enzymes. These studies provided the foundation for a wide range of biochemical and genetic studies using the mouse animal model in examining complex families of enzymes involved in alcohol and aldehyde metabolism (reviewed in Holmes, 1987).

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retrieved 65–70% amino acid and cDNA sequence homology between the three human liver ADH classes (see Jörnvall et al., 1987), and the responsible genes have all been mapped to a small region on the long arm of chromosome 4 (see Smith, 1986). Biochemical genetic studies on mouse liver Class I and stomach Class II ADHs have also revealed close linkage for the genes encoding these enzymes on chromosome 3 (see Holmes et al., 1983).

Mammalian ALDHs have also been divided into at least three classes, based on comparative biochemical properties, tissue, and subcellular distributions and sequences. Class 1 ALDHs comprise the major liver cytosolic isozyme, with intermediate \( K_m \) values with acetaldehyde as substrate (designated as \( E_2, \) ALDH II, or ALDH1, in humans, depending on individual nomenclature), whereas Class 2 ALDHs are predominantly localized in liver mitochondria, and show sub-\( \mu \)M \( K_m \) values with acetaldehyde (designated as \( E_2, \) ALDH I, or ALDH2 in humans) (see Greenfield & Pietruszko, 1977; Li & Bosron, 1986; Holmes, 1988 a). Class 3 ALDHs (designated ALDH3 in humans) are characterized by their "high-\( K_m \)" (mm) values with acetaldehyde as substrate, dual coenzyme specificity and exhibit highest activity in stomach and corneal extracts (Mather & Holmes, 1984; Holmes & VandeBerg, 1986 a; Evces & Lindahl, 1989), and in liver tumors (see Jones et al., 1988). Molecular genetic studies have supported the concept of three distinct but related classes for this enzyme (Hsu et al., 1985; Jones et al., 1988), with the responsible genes (ALDH1, ALDH2, and ALDH3) being localized on separate chromosomes of the human genome; on chromosomes 9q, 12q, and 17 respectively (Human Gene Mapping 10, 1989).

Recent comparative studies have reported very high activity levels for corneal ADH in a number of mammals, including rat (Messiha & Price, 1983; Evces & Lindahl, 1989), baboon (Holmes & VandeBerg, 1986 a), pig, sheep, and cattle (Holmes et al., 1989), and human (Holmes, 1988), and a role has been proposed in detoxification of UV light induced peroxidic aldehydes (Holmes & VandeBerg, 1986 a). Moreover, bovine corneal ADH has been purified and identified as the major soluble protein in this anterior eye tissue, and a further role proposed for this enzyme in serving as photoreceptor of UV-B light, absorbed by the cornea (Abedinia et al. 1990). Comparative studies on mammalian corneal ADH have also been undertaken, and very high specific activities reported in baboon (Holmes & VandeBerg, 1986 a), rat (Julia et al., 1987), and pig (Holmes, 1988 b).

Biochemical genetic studies on mouse stomach and corneal ADH (ADH3) and ALDH (AHD4) provided evidence for the genetic identity of these enzymes, in each case, with the responsible loci being mapped to chromosomes 3 and 11, respectively (Holmes et al., 1983, 1988). In this present study of corneal ADH and ALDH from a nocturnal South American marsupial species (Monodelphis domestica), evidence is presented for the genetic identity of corneal ALDH with a major soluble protein of corneal extracts. These data lend further support to the proposal that corneal ALDH is the major soluble protein in the cornea, and may therefore play a role as a UV-B photoreceptor. The genetics of corneal ADH was also studied, and evidence is reported for two distinct ADH subunits, with one exhibiting electrophoretic variant phenotypes in the population.

2. Materials and methods
(i) Animals and crosses

The gray short-tailed opossums (Monodelphis domestica) used in this study were produced at the Southwest Foundation for Biomedical Research. This South American marsupial species (Fig. 1) has been recently established as a laboratory animal capable of being produced in large numbers and is highly suitable for genetic research (see VandeBerg, 1989; van Oorschot & VandeBerg, 1989). Twenty-two families consisting of a sire, dam, and at least four mature offspring were killed and dissected, and tissues were stored at –80 °C to form a family panel. Ear tissue of all sires and dams within the family panel were types for ADH (ADH3 isozyme) and ALDH (ALDH3 isozyme). Eight families (comprising 44 offspring) were selected for further analysis of corneal tissue, according to observed variant phenotypes for these enzymes. They were chosen in such a way that products of several different ADH3 and ALDH3 phenotype combinations between parents were analysed. Details of the genetics and biochemical properties of ear and stomach ADH and ALDH will be published elsewhere (Holmes et al., 1990 a, b). These studies had demonstrated coincidence of variant ADH3 and ALDH3 phenotypes within the population for the stomach, ear, and corneal enzymes.

Fig. 1. An adult male Monodelphis domestica (gray short-tailed opossum).
Genetics of opossum corneal ALDH and ADH

(ii) Chemicals

Acrylamide, N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylethylene diamine were obtained from Biorad Laboratories (Richmond, CA); polyacrylamide gel support medium from FMC Corporation (Rockland, ME); ampholytes (Servalyte 6–8 and 3–10 ranges) from Serva Fine Chemicals (Garden City, NY); and Titan III cellulose acetate plates from Helena Laboratories (Beaumont, TX). Nicotinamide adenine dinucleotide (NAD), phenazine methosulfate (PMS), methyl thiazolyl blue (MTT), sodium pyruvate, ammonium persulfate and tricine [N-tris (hydroxymethyl) methylglycine] were obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were of analytical grade purity.

(iii) Corneal extracts

Eyes were excised from freshly killed gray short-tailed opossums and stored at −80 °C until required. Corneas were dissected from the anterior region of the eye, to obtain tissue samples free of lens and pigmented iris, and homogenized in 10 volumes of 50 mM-Tricine-sodium hydroxide, pH 8.0, buffer, using a Model PT 10-35 Polytron homogenizer (Westburg, NY). The homogenates were then centrifuged (45000 g, 30 min, 4 °C) prior to isoelectric focusing or electrophoresis. Corneal extracts used for PAGE–IEF and cellulose acetate electrophoretic analyses of soluble protein were subjected to a concentration procedure using Centricon 30 microconcentrators (Amicon, Danvers, MA), resulting in a five- to tenfold increase in concentration of corneal protein.

(iv) Isoelectric focusing and staining

Homogenate supernatants were subjected to polyacrylamide gel isoelectric focusing (PAGE–IEF) according to the method of Radola (1980). A 50:50 mixture of pH ranges, 6–8 and 3–10 ampholytes was used to resolve and compare corneal ALDH, ADH, and soluble protein, whereas a 50:50 mixture of 4–6 and 3–10 pH ranges of ampholytes was used routinely for resolution of corneal ADH isoforms. The gels were prefocused at 9 °C for 30 min by setting the constant-wattage power supply (E-C Apparatus Corporation, St Petersburg, FL) at 5 W. Aliquots (10 μl of dilute or concentrated extracts) were then applied to the gel, and IEF commenced for 30 min at 5 W. After removal of the sample applicator, PAGE–IEF proceeded for a further 30 min at a constant 8 W.

Following PAGE–IEF, ADH or ALDH activity was stained using the following mixture: 100 mM-tricine-sodium hydroxide (pH 8.0), 0.25 mM-NAD, 2.5 mM-pyruvate (to inhibit lactate dehydrogenase activity), 0.5 mM-PMS, 3 mM-MTT, and alcohol or aldehyde substrate, respectively. Ten mM-trans-2-hexen-1-ol was used to stain ADH activity, and 1 mM-heptaldehyde as substrate for ALDH staining. Appropriate control stains (in the absence of substrate and/or coenzyme) were also used to distinguish oxidase activity on the gels (none was observed for corneal extracts under the conditions used). The gels were stained at 37 °C for periods up to 15 min and destained in cold 5% acetic acid for 30 min. Protein stains were performed using 0.2% Coomassie Blue G in methanol:acetic acid:water (5:5:1), and destained in the same solvent mixture. Following washing, the gels were air dried at 37 °C overnight and photographed.

(v) Cellulose acetate electrophoresis and staining

Corneal extracts were also subjected to zone electrophoresis in Titan III cellulose acetate plates (60 × 75 mm) with Tris-glycine buffer (25 mM-Tris; 192 mM-glycine), pH 8.2, at 250 V for 30 min at 4 °C. Corneal ALDH was stained by an agar-overlay procedure, previously described (Holmes et al. 1988), using 1 mM-heptaldehyde as substrate. The gels were incubated at 37 °C for periods of up to 15 min, the overlays were washed from the surface of the plates with distilled water, and the plates were destained in distilled water for 60 min, air dried, and photographed. Corneal protein was stained using the procedure described earlier for PAGE–IEF.

3. Results

(i) Isoelectric focusing of corneal ALDH, soluble protein, and ADH

The PAGE–IEF patterns for corneal soluble protein, aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) from two animals exhibiting homozygous and heterozygous patterns for ALDH3 are described later. For animal 1, a single major ALDH3 activity zone (pI = 8.3) was observed, consistent with the B phenotype shown following cellulose acetate electrophoresis (Fig. 3), whereas for animal 2, three major ALDH3 activity zones were apparent, with pI values of 8.3, 8.05, and 7.8, for the dimeric isoforms, ALDH3C2, C1C2, and C12, respectively (see (iii)). Corneal soluble protein PAGE–IEF phenotypes showed two major protein zones, with pI values of 7.0 and 8.3 for animal 1 (Fig. 2). For sample 2, however, the higher pI protein region showed three protein zones, identical with those observed for ALDH3 variant forms of activity. Animals exhibiting the A phenotype (designated ALDH3 Cl), also showed correspondence in pI values between ALDH3 and the 7.8 pI soluble protein zone (data now shown).

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Fig. 2. Polyacrylamide gel-isoelectric focusing patterns of *M. domestica* corneal alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and soluble protein, showing variation of ALDH and protein phenotype. Animal 1, B phenotype for ALDH3 and soluble protein (also designated as ALDH-C\textsubscript{2}); animal 2, AB phenotype for ALDH3 and soluble protein (designated as ALDH \textsubscript{C2}, ALDH \textsubscript{C'C2} and ALDH \textsubscript{C4} for the three dimeric isozymes. Note identity of pl values and pattern for ALDH and the high pl soluble protein zone. These individuals were homozygous for corneal ADH variants (see Fig. 4).

Fig. 3. Cellulose acetate zymogram of *M. domestica* corneal aldehyde dehydrogenase (ALDH3), showing variation of enzyme phenotype observed among members of 24 gray short-tailed opossums analysed; 1 = animal 1, B phenotype or ALDH-C\textsubscript{2}; 2 = animal 2, AB phenotype, comprising three allelic isozymes, ALDHs \textsubscript{C2}, \textsubscript{C'C2}, and \textsubscript{C4}; and 3 = animal 3, A phenotype or ALDH-C\textsubscript{2} (note that a subband was observed for this phenotype); 4 = a mixture of corneal extracts showing A and B phenotypes for ALDH3.

(iii) Genetics of corneal ALDH3 and soluble protein zone

In all 60 animals tested, the corneal ALDH3 banding patterns (phenotypes A, B, and AB) corresponded with the banding patterns of the same pis in the corneal homogenate supernatants, stained for soluble protein. Furthermore, the family data on the inheritance of corneal ALDH3 patterns and the corneal soluble protein pattern showed that they cosegregated in every instance, further supporting the hypothesis that ALDH3 is a major protein in the cornea.

The data presented in Fig. 2, Fig. 3, and Table 1 are in accordance with the hypothesis that ALDH3 is controlled by two codominant alleles, \textit{ALDH3}\textsuperscript{A} and \textit{ALDH3}\textsuperscript{B}, at a single autosomal locus: \textit{ALDH3}.

(iv) Isoelectric focusing of corneal ADHs

The PAGE–IEF patterns (using a 50:50 mixture of pI range ampholytes 4–6 and 3–10) for *M. domestica* corneal ADH isozymes, from two animals with distinct phenotypes, are shown in Fig. 4. Sample 1 showed three zones of ADH activity staining with 10 mM-trans-2-hexen-1-ol as substrate, with wide differences in pI values (ADH3 [7-8]; ADH4 [6-8]; and ADH5 [5-7]). Sample 2 exhibited a number of additional forms, including three zones in the ADH3 region of the gel, and two major zones in the ADH4 region. A higher pI subband for ADH4 was also observed in both samples. Based upon the known subunit structures for mammalian ADHs, and the pI values for variant isozymes, the following subunit compositions are proposed as a basis of multiplicity for corneal ADH isozymes: ADH3 (\textit{y} isozyme, with two electro-
### Table 1. Family data on the inheritance of corneal ALDH3 and ADH3

<table>
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<th>Protein</th>
<th>Parental phenotypes</th>
<th>Number of families</th>
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<th>Phenotype AB</th>
<th>Phenotype B</th>
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<td>7</td>
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<td>1</td>
<td>3</td>
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</tbody>
</table>

**:5-7-** ADH 5

**6-8-** •

**7-8-** •

Fig. 4. Polyacrylamide gel-isoelectric focusing patterns of *M. domestica* corneal alcohol dehydrogenase (ADH), showing variation of phenotype. pi values indicated at left of zymogram for the three major isozymes resolved: ADH3 (7-8); ADH4 (6-8), and ADH5 (5-7). 1 = Animal 1, A phenotype for ADH3 (designated γ2 isozyme) and ADH4 (γ1δ isozyme), involving a model of allelic variation at the proposed ADH3 locus (γ1 and γ2 designate variant subunits). 2 = Animal 2, AB phenotype for ADH3 (γ1, γ1γ2, and γ2 isozymes); and ADH4 (γ1δ and γ2δ isozymes). • designates major subbands for variant ADH4 isozymes. Note that ADH5 is electrophoretically invariant in these animals, and has a δ2 subunit structure, according to this model.

4. Discussion

This biochemical genetic study of *Monodelphis domestica* alcohol dehydrogenase (ADH3) and aldehyde dehydrogenase (ALDH3), and previous analyses of mouse (Holmes et al. 1988), rat (Messiha & Price, 1983; Julia et al. 1987; Evces & Lindahl, 1989), baboon (Holmes & VandeBerg, 1986 a, b), pig, sheep, and cattle (Holmes et al. 1989), and human (Holmes, 1988b) anterior eye tissues, provide evidence for cornea serving as a major site of ADH and ALDH activity in mammalian organisms. In addition, in the case of mouse corneal ADH and ALDH isozymes (designated as ADH3 and AHD4, respectively), these enzymes are encoded by genes which are distinct to those encoding the major liver isozymes, in each case (Holmes et al. 1988). Mouse corneal (and stomach) ADH is encoded by the *Adh3* locus, which is closely linked with *Adh-1* (encoding liver ADH) on chromosome 3 (Holmes et al. 1983, 1988); whereas mouse corneal ALDH is encoded by the *Ahd-4* locus on chromosome 11, and is distinct from liver cytosolic and mitochondrial ALDHs which are encoded by genes on chromosomes 19 and 4, respectively (Holmes, 1978; Timms & Holmes, 1981). The genetic analyses of *M. domestica* corneal ALDH3 support the existence of a locus (designated *ALDH3*) encoding allelic variants for this enzyme, forming three dimeric allelic isozymes in heterozygous individuals – γ1, γ1γ2 and γ2; ADH4 (γδ isozyme – with two forms in heterozygous animals; γ1δ and γ2δ); and ADH5 (δ2).

(v) Genetics of corneal ADH isozymes

The data on the inheritance of corneal ADH3 are presented in Table 1. The phenotypes of all progeny were consistent with autosomal codominant inheritance. However, the cross of A2 × AB3 produced a significant excess of progeny exhibiting ADH3 A (x2 = 5.4, P < 0.05). The smaller data set derived from the reciprocal cross (AB3 × A2) was consistent with Mendelian expectations, although the two data sets did not differ significantly from each other in a 2 × 2 contingency x2 test (x2 = 1.28, P = 0.258).
PAGE–IEF analyses of *M. domestica* corneal ADHs revealed two major forms (ADH3 and ADH4) and one minor form (ADH5) of activity in homozygous individuals (Fig. 4). These forms have been characterized by substrate and inhibitor specificity studies as Class II ADHs (Holmes et al. 1990a). Based on the electrophoretic variant phenotypes (Fig. 4), as well as the limited family data of these corneal ADHs (Table 1 and Holmes et al. 1990a), the most parsimonious interpretation is codominant allelic inheritance at one (ADH3) of two loci (ADH3 and ADH4) which encode ADH subunits, designated γ and δ respectively. According to this model, corneal ADH forms in homozygous individuals comprise three isozymes, involving the dimeric association of two ADH subunits: γ2 (ADH3); γδ (ADH4) and δ2 (ADH5); with heterozygous individuals exhibiting six major forms of activity: γ2, γ'γ2, and γ2; (ADH3); γδ and γδ (ADH4) and δ2 (ADH5). The formation of homodimeric and heterodimeric ADH isozymes, involving multiple ADH subunits, has been previously reported for the human liver Class I ADHs, for which three genetically distinct ADH subunits (α, β, and γ) have been described (see Smith, 1986). The present report, however, provides the first evidence for this phenomenon involving multiple Class II ADH subunits.

The distorted segregation ratio of the putative ADH3 alleles in the cross of A9 x AB(J, if confirmed by more extensive data, will be of considerable interest. Some testable hypotheses that would arise from such confirmation are: (1) that heterozygous embryos (or fetuses) are at a selective disadvantage when carried by homozygous mothers, (2) that ADH3 is linked to a locus with a recessive lethal or semi-lethal allele, and (3) that ADH3 is linked to a locus that causes segregation distortion, such as the t-complex in mice.

Recent biochemical studies using purified preparations of corneal ALDHs (Evces & Lindahl, 1989; Abedinia et al. 1990), as well as other reports involving analyses of purified stomach ALDH (Algar & Holmes, 1989) and ADHs (Algar et al. 1983; Julia et al. 1987) (on the basis that genetic studies have supported genetic identity between stomach and corneal ADH and ALDH, in each case), have proposed a distinct biochemical role for these enzymes in peroxidic aldehyde detoxification. These aldehydes, including a range of C6-C9 aliphatic aldehydes, are generated in vivo by lipid peroxidation processes, following exposure to oxygen and/or ultraviolet light in the environment (Schauenstein et al. 1977; Bhuyan et al. 1984). Thus, high specific activity corneal ADH and ALDH, which share a preference for medium-chain length aliphatic aldehydes such as those generated by lipid peroxidation, may function in a reductive and oxidative detoxification process, respectively, thereby removing cytotoxic aldehydes from this tissue environment.

A recent study of the biochemical properties of purified bovine corneal ALDH has proposed an additional function for this enzyme, in serving as a UV-B photoreceptor in this anterior eye tissue (Abedinia et al. 1990). The mammalian cornea is predominantly responsible for the absorption of available ultraviolet (UV) light in the 290–320 nm wavelength range (UV-B light), and serves to protect sensitive retinal cells against UV-induced tissue damage (Boettner & Wolters, 1962; Zigman, 1983). The photokeratitis action spectrum for abiotic UV-light strongly suggests that the photochemical reaction is associated with absorption by a soluble protein or enzyme contained within corneal cells (Cogan & Kinsey, 1946; reviewed by Ringvold, 1980). Abedinia et al. (1990) reported that bovine corneal ALDH is the major soluble protein in this tissue, and constitutes around 0.5% wet weight of tissue. The UV absorption spectrum for this corneal enzyme revealed significant overlap with that previously reported for the photokeratitis action spectrum of the cornea, and a possible role for corneal ALDH as UV-B photoreceptor (or one of a range of photoreceptors) was proposed. This present study of *M. domestica* corneal ALDH lends further support to this proposal, since genetic analyses have indicated identity between ALDH3 and a major soluble protein in the cornea.

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References


