Isozyme Multiplicity with Anomalous Dimer Patterns in a Class III Alcohol Dehydrogenase. Effects on the Activity and Quaternary Structure of Residue Exchanges at "Nonfunctional" Sites in a Native Protein†

Olle Danielsson, Jawed Shafqat, Mats Estonius, Mustafa El-Ahmad, and Hans Jörnvall*

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

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ABSTRACT: The isozymes of class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase from cod were characterized. They exhibited three unexpected properties of general interest. First, these dimeric isozymes, derived from two types of subunit (*h* and *l*, for high- and low-activity forms), were recovered from liver preparations in only the homodimeric *ll* and heterodimeric *hl* combinations. Dissociation and reassociation of the isolated *hl* form *in vitro* also resulted in lower yields of the *hh* than the *ll* homodimer, although class III subunits are usually freely associable over wide borders of divergence (human and *Drosophila*). The *h* and *l* primary structures show that both chain types are characteristic of class III enzymes, without large amino acid replacements at positions of known subunit interactions. Hence, the *hh* dimer partial restriction indicates nontraditional alterations at *h*-subunit interfaces. The structure provides a possible explanation, in the form of *h*-chain modifications that may influence the anchoring of a loop at positions of two potentially deamidative β-aspartyl shifts at distant Asn-Gly structures. Second, the *ll* and *hl* forms differ in enzymatic properties, having 5-fold different K_m values for NAD^+ at pH 8, different K_m values for *S*-(hydroxymethyl)glutathione (10 versus 150 μM), and different specific activities (4.5 versus 41 units/mg), with *ll* resembling and *hl* deviating from human and other class III alcohol dehydrogenases. However, functional residues lining substrate and coenzyme pockets in the known conformations of homologous forms are largely identical in the two isozymes [only minor conservative exchanges of Val/Leu116, Val/Leu203, Ile/Val224, and Ile/Val269 (numbering system of the human class I enzyme)], again indicating effects from distantly positioned *h*-chain replacements. Third, the two isozymes differ a surprising amount in amino acid sequence (18%, the same as the piscine/human difference), reflecting a remarkably old isozyme duplication or, more probably, discordant accumulation of residue exchanges with greater speed of evolution for one of the subunits (*h* chain) than is typical for the slowly evolving class III alcohol dehydrogenase.

The zinc-containing medium-chain alcohol dehydrogenases exhibit a high extent of multiplicity. Six classes with distinct kinetic and structural properties have been described in vertebrates [summarized in Jörnvall and Höög (1995)], classes I−III (Vallee & Bazzone, 1983) and IV−VI (Parés et al., 1994; Yasunami, 1991; Zheng et al., 1993). Class I constitutes the traditional ethanol-oxidizing liver enzyme, class III the glutathione-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989), and class IV a special, epithelial form (Farrés et al., 1995; Žgombić-Knight et al., 1995). The particular properties of classes II, V, and VI are still little known. The classes have a common origin through a series of gene duplications (Jörnvall et al., 1987) during early vertebrate phylogeny, initially traced for the class I/III separation (Cederlund et al., 1991), presumably reflected by mixed-class properties of the piscine class I enzyme now known in tertiary structure (Ramaswamy et al., 1996) and interpreted to constitute early enzymogenesis (Danielsson & Jörnvall, 1992).

In addition to this class multiplicity, the enzyme occurs in multiple forms within a class. Three subunit types in dimeric combinations explain the isozymes of the human class I enzyme (Smith et al., 1971). Further multiplicity is derived from allelic variability [cf. Jörnvall et al. (1987)] and posttranslational modifications. Isozymes of class I have been characterized in several vertebrate species, including human, horse (Jörnvall, 1970; Park & Plapp, 1991), and lizard (Hjelmqvist et al., 1996) forms. In each of these lines, the isozyme duplications constitute independent events (Jörnvall et al., 1995). In spite of this multiplicity within class I, a similar multiplicity has not been defined for the other classes (except for the cod class III enzyme, below), suggesting that isozyme duplications are more common within the rapidly evolving class I.

Class III alcohol dehydrogenase separates electrophoretically into subforms (Parés & Vallee, 1981; Adinolfi et al., 1984; Valkonen & Goldman, 1988), which appear to be derived from posttranslational modifications, as deduced from analysis of the human (Kaiser et al., 1991) and cyclostome (Danielsson et al., 1994b) forms. Primary structures of the class III alcohol dehydrogenase have been reported from highly divergent organisms, covering vertebrates (Kaiser et al., 1988; Julià et al., 1988; Danielsson et al., 1994b; Hjelmqvist et al., 1995c), cephalopods (Kaiser et al., 1993), insects (Danielsson et al., 1994a), yeasts (Sasnaukas et al., 1992; Wehner et al., 1993), plants (Shafqat

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* Corresponding author: Hans Jörnvall, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden. Phone: +46-8-728 7702. Fax: +46-8-33 74 62.

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et al., 1996; Martínez et al., 1996), bacteria (Gutheil et al., 1992; Ras et al., 1995; Peretz & Burstein, 1989), and archaeons (Ammendola et al., 1992). However, except for the situation with functionally different forms in fish (Danielsson & Jörnvall, 1992), no structural evidence for class III isozymes has been detected. Here, we characterize these isozymes in cod and find that the functional differences have unexpected structural explanations.

MATERIALS AND METHODS

Protein. Different organs from cod of Baltic origin (*Gadus morhua*) were examined for the presence of class III alcohol dehydrogenase activity. Liver, brain, eye, heart, stomach, pyloric ceaca, large intestine, and spleen were dissected, washed in ice-cold distilled water, and stored at −70 °C until purification or electrophoresis. A purification protocol modified from Danielsson and Jörnvall (1992) was used. Pooled cod livers were homogenized at 4 °C in buffer (0.1 M Tris/HCl and 0.5 mM dithiothreitol at pH 8.5 readjusted to pH 8.5 in the homogenate by addition of 2 M Tris base) and centrifuged at 27000*g* for 60 min. The supernatant was collected after careful removal of the lipid layer and filtered through cloth to remove additional debris and lipids before dialysis against 20 mM Tris/HCl and 0.5 mM dithiothreitol at pH 8.5 with two rapid buffer changes. The dialysate was applied to a 5 × 30 cm column of DEAE-Sepharose Fast Flow (Pharmacia Biotech) in the same buffer, also used for initial washing. Additional washing was performed with 20 mM Tris/HCl at pH 7.6 before elution with an NaCl gradient (0 to 300 mM) in 20 mL at 1 mL/min. Fractions with glutathione-dependent formaldehyde dehydrogenase activity were pooled, dialyzed against 50 mM sodium phosphate at pH 7.5, and applied to an AMP-Sepharose column (2 × 12 cm, Pharmacia Biotech). After being washed, the activity eluted in two peaks with an NAD^+ gradient (200 mL; 0 to 2 mM NAD^+). The two corresponding pooled fractions were concentrated after buffer exchange (Amicon PM10 ultrafiltrator and Centricon 10 microconcentrators) to 20 mM Bis-Tris at pH 7.1, applied separately to Mono Q HR 5/5 FPLC, and eluted under identical conditions, employing a 20 mL 0 to 0.5 M NaCl gradient. The material corresponding to the early peak from the AMP-Sepharose was submitted to FPLC rechromatography on Mono Q in 20 mM Tris/HCl at pH 8.8 and was eluted with an NaCl gradient in the same buffer.

Protein concentrations during purification were estimated colorimetrically (Bradford, 1976), while amounts of pure proteins were determined by amino acid analysis after hydrolysis.

Activity Measurements. Enzyme activity was determined spectrophotometrically at 25 °C by monitoring the change in absorbance at 340 nm with a Beckman DU 68 spectrophotometer. Alcohol oxidation was measured with 2.4 mM NAD^+ in 0.1 M glycine/NaOH at pH 10.0 (Dalziel, 1957), while formaldehyde dehydrogenase activity (Koivusalo et al., 1989) was measured in 0.1 M sodium pyrophosphate at pH 8.0 with 2.4 mM NAD^+ and *S*-(hydroxymethyl)-glutathione (formed by the spontaneous reaction of formaldehyde and glutathione). One unit of activity is defined as the amount of protein necessary to catalyze the conversion of 1 μmol of NAD^+ to NADH in 1 min on the basis of a molar absorptivity of 6220 cm^{-1} for NADH at 340 nm. Alcohols were of analysis grade. NAD^+, grade III, and glutathione were from Sigma, and formaldehyde was from Ladd Research Industries (VT) as a methanol-free 20% aqueous solution. Octanol and 12-hydroxydodecanoate were dissolved in 50% aqueous acetonitrile, yielding 2.5% acetonitrile in the reaction mixture. The program ENZYME (Lutz et al., 1986) was used to fit the data points for calculation of kinetic parameters, all values of which are the mean from two separate protein preparations.

Isoelectric Focusing and Gel Electrophoresis. A Pharmacia Phast gel system was used with samples loaded onto gels of pH 3−9 and 4−6.5. The gels were stained for activity (Koivusalo et al., 1989; Danielsson et al., 1992) and protein (with Coomassie Brilliant Blue). Isoelectric focusing under denaturing conditions was performed after incubation of precast dried gels with Ampholine 5-8, 8 M urea, and 20 mM dithiothreitol, and the gels were silver stained.

Homogeneous (12.5%) precast Phast gels (Pharmacia Biotech) were used for SDS and native runs with gel staining as above. Electrophoretic titration curves were performed according to the manufacturer and stained with silver nitrate.

Immobiline gels for two pH unit gradients (pH 6−8) were prepared largely as described (Pharmacia Biotech). Immobiline stock solutions were stored at −20 °C. Gels were 245 × 110 × 0.5 mm and were placed in the gel rehydration cassette after washing with 5% glycerol, drying overnight, and rehydration with 8 M urea and 20 mM dithiothreitol. Electrophoresis was run overnight (2.5 W at 15 °C) under denaturing conditions. The edges of the gels were cut and stained with Coomassie Brilliant Blue for protein identification, after which the corresponding gel bands were excised and electroeluted (Bergman & Jörnvall, 1987).

Generation and Separation of Scrambled Isozymes. Before dissociation, enzyme preparations were made free of NAD^+ by chromatography on Affigel Blue affinity resin (Biorad). The enzymes (0.4−0.5 mg/mL) in 0.1 M sodium phosphate at pH 7.0 and 1 mM dithiothreitol were frozen in a dry ice/acetone mixture for 20 min and thawed at 25 °C, and activities were measured at specified time intervals (Briganti et al., 1989). After the freezing and thawing procedure, the samples were subjected to analytical polyacrylamide gel (12.5%) electrophoresis.

Structural Analysis. Batches of the carboxymethylated proteins were chemically cleaved with CNBr (0.2 mg/mL) in 70% formic acid for 24 h or hydroxylamine (2 M) in 0.2 M Tris at pH 9.3 and 8 M urea at 45 °C for 5 h. Other batches were digested for 4−24 h at 37 °C with Lys-specific protease (Wako, Neuss), Asp-specific protease (Boehringer Mannheim), and Glu-specific and Gly-specific proteases (Sigma) in 0.1 M ammonium bicarbonate at pH 8 and 1−2 M urea, at protease/protein ratios from 1/20 to 1/200. Resulting peptides were purified by reverse phase HPLC on Vydac C_4 (5 μm, 4.5 × 250 mm) and C_8 (5 μm, 2.1 × 250 mm) and TSK ODS-120T C_{18} (5 μm; 4.6 × 250 mm) columns with 0.1% trifluoroacetic acid/acetonitrile linear gradients. Fragments from hydroxylamine cleavage were purified by exclusion chromatography on Superose 12 (10 × 300 mm) (Pharmacia Biotech) at 0.3 mL/min. Sequence degradations were carried through with Applied Biosystems 470A and 477A and MilliGen 6600 instruments. Phenylthiohydantoin derivatives were analyzed with sodium acetate/acetonitrile gradients on a Hewlett-Packard 1090 instrument or on-line with Applied Biosystems 120A or Waters 440 HPLC instruments. Amino acid compositions were determined with an Alpha Plus instrument (Pharmacia Biotech) after acid hydrolysis at 110 °C for 24 h in 6 M HCl/0.5%

Table 1: Purification of Class III Isozymes from Cod Liver[a]

step	total protein (mg)	enzyme activity (units)	specific activity (units/mg)	yield (%)	purification (fold)
initial common steps for *ll* and *hl*					
crude extraxt	8400	160	0.019	100	1
DEAE-Sepharose	240	120	0.50	75	26
final purification steps for *ll*					
AMP-Sepharose	15	25	1.7	16	89
Mono Q (pH 7.1)	5.2	14	2.7	8.8	140
Mono Q (pH 8.8)	1.7	7.7	4.5	4.8	240
final purification steps for *hl*					
AMP-Sepharose	2.5	73	29	49	1500
Mono Q	1.2	49	41	31	2200

[a] Starting material, 400 g of liver tissue.

phenol. Molecular masses and sequences of N-terminally blocked peptides were determined separately by mass spectrometry (Hjelmqvist et al., 1995b).

Structural Comparisons. Primary structures obtained were correlated with the three-dimensional structure deduced for human class III alcohol dehydrogenase (Eklund et al., 1990) and with the one recently analyzed for a cod class I "hybrid" enzyme (Ramaswamy et al., 1996). To evaluate critical residues, the replacements were further compared to those of the other five mammalian classes, as well as to the structurally characterized class III alcohol dehydrogenases from yeasts (Sasnauskas et al., 1992; Wehner et al., 1993), a cephalopod (Kaiser et al., 1993), an insect (Danielsson et al., 1994a), and vertebrates (Kaiser et al., 1988; Julià et al., 1988; Hjelmqvist et al., 1995c).

RESULTS

Distribution of Class III Alcohol Dehydrogenase Activity. Homogenates from eight different cod tissues were examined. Polyacrylamide gel electrophoresis under native conditions and subsequent activity staining with glutathione/formaldehyde revealed two bands in all tissues analyzed (not shown), both clearly reproducible and independent of time and conditions. The results demonstrate that at least two forms of class III alcohol dehydrogenase of wide tissue distribution exist in cod, in agreement with initial studies of the liver enzyme (Danielsson & Jörnvall, 1992).

Purification of Class III Alcohol Dehydrogenase Isozymes from Cod Liver. The two electrophoretically distinguishable isozymes exhibiting glutathione-dependent formaldehyde dehydrogenase activity were purified. They coeluted in the DEAE chromatography step but were separated on AMP-Sepharose with a linear NAD^+ gradient. Each isozyme was then chromatographed on Mono Q FPLC, at pH 7.1, after buffer exchange and concentration (cf. Materials and Methods). The most basic form (denoted *hl*) eluted as an apparently pure fraction in this step, with a specific activity of 41 units/mg after a 2200-fold purification, yielding one band on electrophoresis in both SDS and native gels (Figure 1). The activity eluting earlier on AMP-Sepharose contained the acidic form (denoted *ll*) but required an additional Mono Q step at pH 8.8 for pure recovery as judged by electrophoresis in SDS and native gels (Figure 1) after a 240-fold purification. The specific activity of this form was almost 10-fold lower (4.5 units/mg). The yield of *hl* was 31% and that of *ll* 5% (Table 1).

Kinetic Properties. The kinetic parameters of the liver-derived class III isozymes (*ll* and *hl*) toward *S*-(hydroxymethyl)glutathione and primary alcohols (Table 2) were

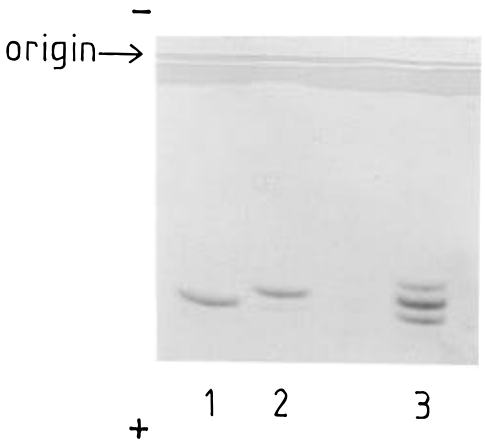

FIGURE 1: Polyacrylamide gel electrophoresis under nondenaturing conditions in the absence of SDS of purified cod class III *ll* (lane 1) and *hl* (lane 2) isozymes and of the forms produced from dissociation and reassociation of *hl* (lane 3). The latter gel pattern is consistent with formation of *ll*, *hl*, and *hh* isozymes, although *hh* homodimer is formed in lower yield than *ll* homodimer, as shown by the lower relative intensity.

Table 2: Enzymatic Properties of Cod Class III Isozymes Compared with Those of the Human Class III Enzyme[a]

property	*ll*	*hl*	human
octanol			
K_m (mM)	1.2	1.1	1.2
k_{cat} (min^{-1})	340	330	220
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	280	300	180
12-hydroxydodecanoate			
K_m (mM)	0.081	0.095	0.060
k_{cat} (min^{-1})	230	210	170
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	2800	2200	2800
S-(hydroxymethyl)glutathione			
K_m (mM)	0.010	0.150	0.004
k_{cat} (min^{-1})	390	3600	200
k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	39000	23000	50000
NAD^+			
K_m at pH 8.0 (mM)	0.015	0.080	0.009
K_m at pH 10.0 (mM)	0.070	0.14	0.070
structural differences			
substrate-interacting			
position 116	Val	Leu	Val
position 140	Phe	Phe	Tyr
coenzyme-interacting			
position 202	Ala	Ala	Gly
position 203	Val	Leu	Val
position 224	Ile	Val	Ile
position 269	Ile	Val	Ile

[a] Residue numbers refer to the horse and human enzymes of class I. The exchanges at the substrate and coenzyme pockets are indicated as deduced from structures of the classes (Eklund et al., 1990; Ramaswamy et al., 1996). As shown, exchanges are minor, indicating the possibility of distant exchanges with indirect effects (cf. text).

examined under conditions allowing comparisons with those obtained for the human enzyme. The isozymes exhibit very similar values for K_m and k_{cat} toward octanol and 12-hydroxydodecanoate. The catalytic efficiencies (k_{cat}/K_m) toward *S*-(hydroxymethyl)glutathione are also very similar, but the K_m and k_{cat} values toward this substrate differ significantly. The heterodimer exhibits a K_m of 150 μM and a k_{cat} of 3600 min^{-1}, while these values are 10 μM and 390 min^{-1}, respectively, for the *ll* isozyme. The affinities for NAD^+ are comparable with 5-fold differences at pH 8 (K_m values of 80 and 15 μM, respectively) down to a factor of 2 at pH 10 for *hl* and *ll* (Table 2).

Dissociation and Reassociation of the Heterodimer. The freeze−thaw procedure conducted on the heterodimer re-

sulted in the formation of three enzymatically active forms as judged from analytical polyacrylamide gels in the absence of SDS (Figure 1). Recoveries of activity were up to 90%. In a number of scrambling experiments, the relative intensity of the *ll*, *hl*, and *hh* bands on the analytical gels indicated an underrepresentation of the *hh* form in relation to the *ll* form. This is consistent with a pattern expected if the subunits recombine with a restriction on *hh* dimers, in agreement with the observation from the native preparations.

Preparation of Homogeneous h Subunits. Peptide mapping of *ll* and *hl* isozymes on HPLC after Asp-N-specific cleavage (Danielsson & Jörnvall, 1992) and after Lys-C cleavage (Figure 2) demonstrated the heterodimeric nature of *hl*. Pure *h* subunit for sequence analysis was obtained by separation of the subunits of the heterodimer. The *hl* form, chromatographically purified, was denatured, reduced, and applied to an Immobiline pH gradient (6 to 8) gel containing 8 M urea and 20 mM dithiothreitol (cf. Materials and Methods). Typical yields range from 15 to 40%.

Primary Structure. Attempts at direct sequence analysis of the ^{14}C-carboxymethylated *ll* and *hl* proteins failed to yield results, suggesting acetyl-blocked N termini of both the *l* and *h* chains as is typical of other alcohol dehydrogenases, and was confirmed by mass spectrometry of N-terminal peptides (Hjelmqvist et al., 1995b). The primary structures were determined by multiple cleavages as outlined in Figure 3.

The *ll* form was analyzed using five separate cleavages of the carboxymethylated enzyme, one with CNBr and four with different proteases (Asp-N, Glu-C, Gly-C, and Lys-C, cf. Figures 2 and 3). The amino acid sequence of the *h* subunit was similarly determined from peptides of the Lys-C and CNBr cleavages, with overlaps determined from peptides obtained after Glu-C and Asp-N cleavages of the carboxymethylated heterodimer, taking advantage of the different HPLC pattern versus that from the peptides from *ll* in selecting the relevant fragments for analysis (Figure 2).

An analytical problem was caused by two Asn-Gly structures less than 10 residues apart, making a region at positions 127−149 difficult to establish. This problem was circumvented by cleavage of the carboxymethylated heterodimer with hydroxylamine, producing one short and two large fragments derived from the *h* subunit. Relevant peptides could be analyzed for sequence after purification by reverse phase HPLC on a C_{18} column (NG1, Figure 3B) and by gel filtration FPLC (NG2, Figure 3B).

The N termini of both *h* and *l* subunits have been analyzed by electrospray mass spectrometry (Hjelmqvist et al., 1995b), utilizing Glu-cleaved fragments. The C termini were established through consistent end results with peptides from different cleavages of both subunits.

The *h* chain was found to contain two Asn-Gly structures, at positions 126−127 and 134−135 (Figure 3B). Such structures are known to be labile, easily susceptible to β-aspartyl formation [cf. Jörnvall (1974)]. Sequence degradations now in part passed through the structures, showing the presence of some Asn but with over 50% drops in yield at each place, indicating also cessation of degradation from the presence of chains with Aspβ−Gly structures that block Edman degradations. Hence, the structures are concluded to be Asn-Gly in the native state, but susceptible to modification.

Line and Class Relationships. The homologies of the cod isozymes are unequivocally of the class III alcohol dehydrogenase type. Identities with the human enzyme are high, 76−82%, compatible with the conserved nature of the class III protein [cf. Danielsson et al. (1994a)]. The *l* subunit is

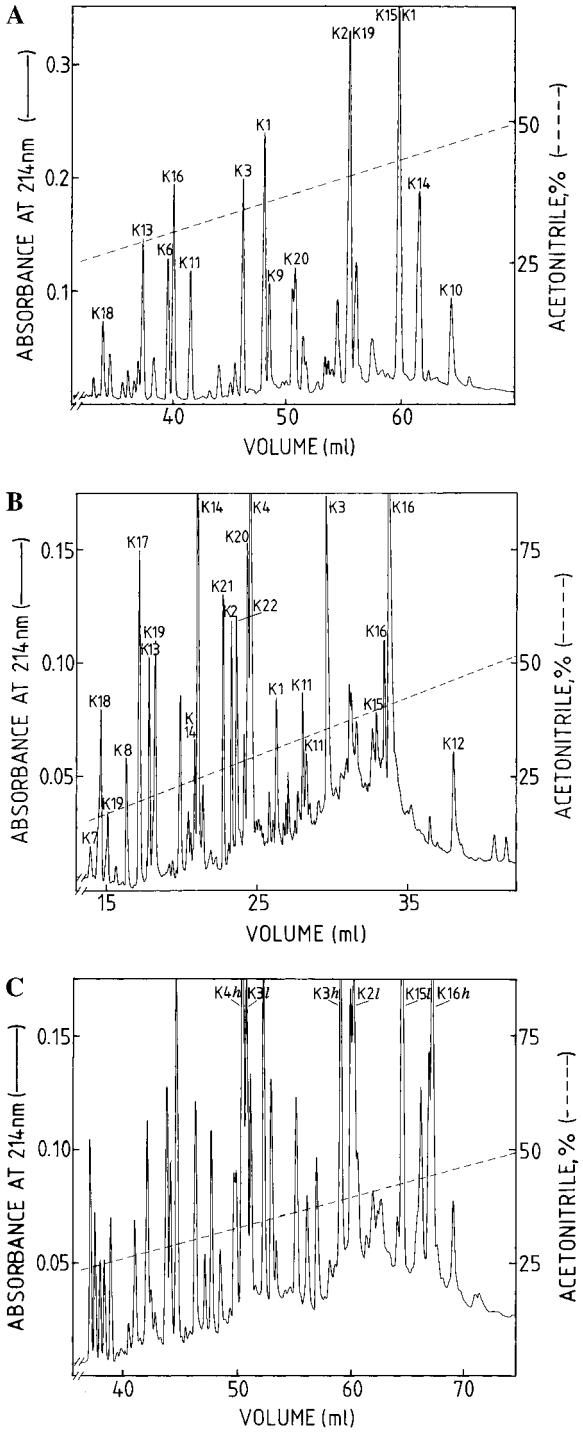

FIGURE 2: Purifications of peptides derived from digests with Lys-specific protease of carboxymethylated *ll* homodimer (A), purified *h* subunit (B), and *hl* heterodimer (C). The elution positions of all peptides identified are shown by the peptide designations corresponding to those in Figure 3A (*l* form) and Figure 3B (*h* form). The peptides from the digest of the *h* subunit (B) were purified on a Vydac C_8 apparatus (5 μm, 2.1 × 250 mm), while those from the digests shown in panels A and C were purified on a TSK ODS-120T C_{18} apparatus (5 μm, 4.6 × 250 mm). In panel C, three pairs of peptides with similar peak heights in each pair are shown. Structural analysis reveals that they correspond to three homologous regions in the two subunits with residue exchanges as given in panels A and B of Figure 3. The remaining peptides of that digest were not analyzed from this batch because of its heterodimeric nature but were determined from the digests of pure subunits (A and B).

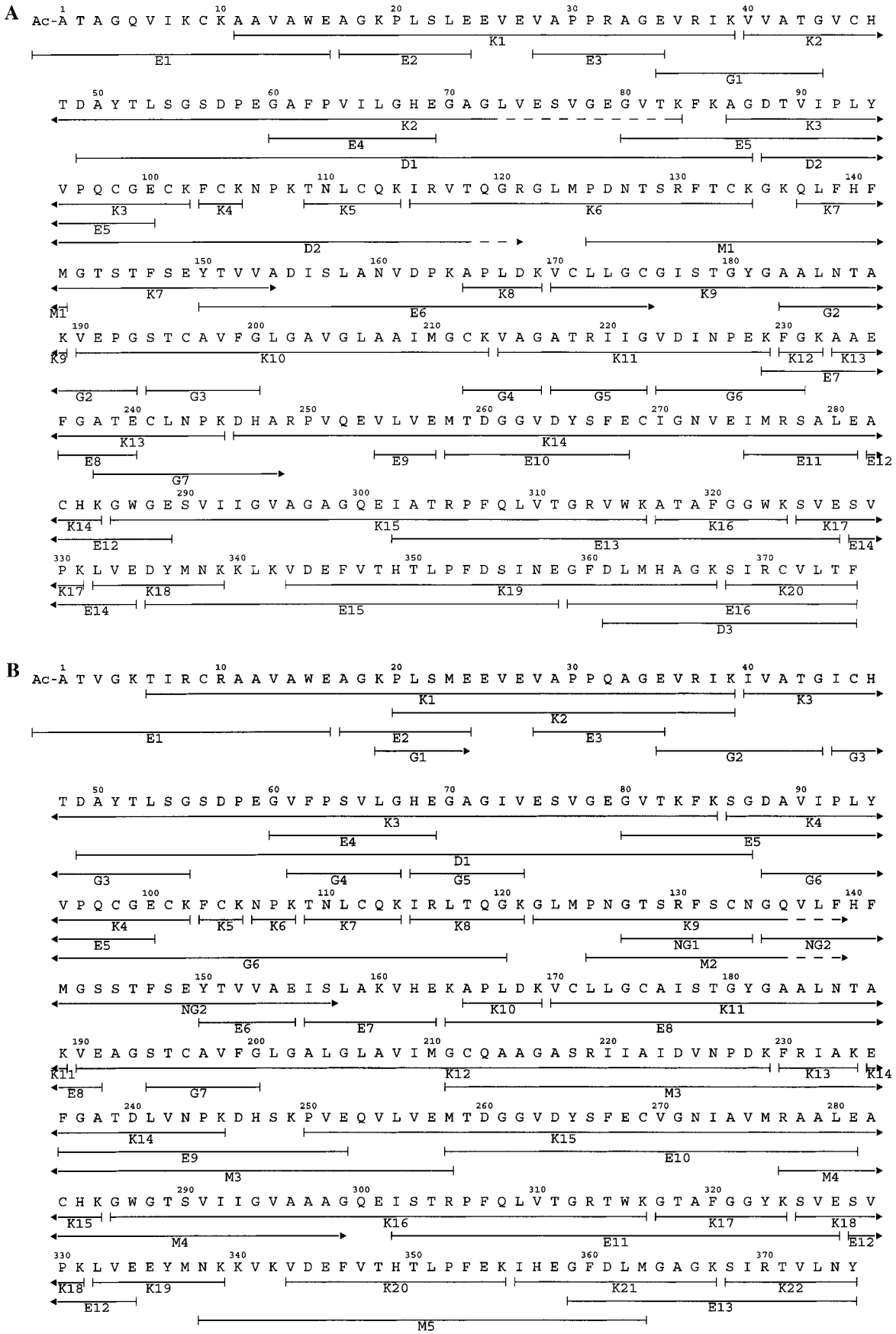

FIGURE 3: Primary structures of the *l* (A) and *h* (B) subunits of cod liver class III alcohol dehydrogenase. Solid lines indicate parts of peptides analyzed with Edman degradation or for the N-terminal peptides by mass spectrometry. K indicates peptides obtained by Lys-C cleavage, E those by Glu-C cleavage, D those by Asp-N cleavage, G those by Gly-specific cleavage, M those by CNBr cleavage, and NG those by hydroxylamine cleavage.

the one most related to other vertebrate class III forms, in agreement with its similarly conserved catalytic properties (Table 2). Differences between the *l* and *h* forms are surprisingly large, 18% (68 positions, Figure 4). This is as extensive as the human/piscine differences (Table 3, also showing the relationships with the enzymes of other lines

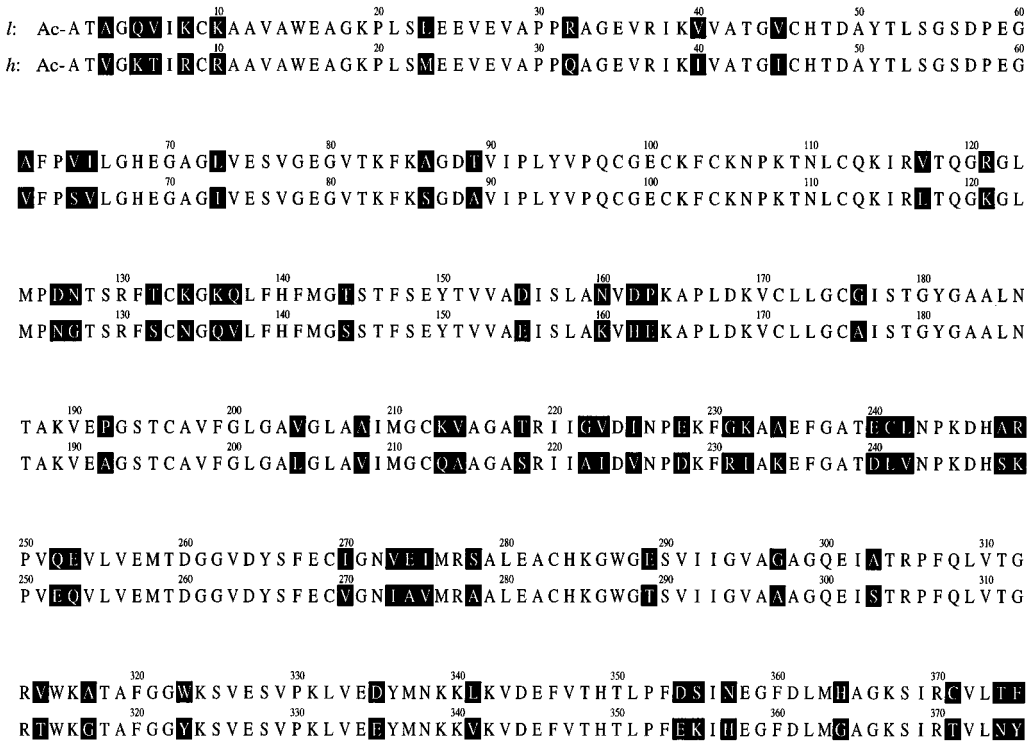

FIGURE 4: Structural comparison of the *l* and *h* subunits of cod class III alcohol dehydrogenase. Residues with a black background show the 68 positions where the *h* and *l* subunits differ.

Table 3: Overall Residue Identities between Cod Class III *l* and *h* Subunits, Other Relevant Class III Subunits (A), and Those of Other Classes (B)[a]

		cod III *l*	cod III *h*
A	human III	82	76
	hagfish III	78	75
	octopus III	75	74
	Drosophila melanogaster III	74	73
	pea III	67	65
	Saccharomyces cerevisiae III	61	61
	Candida maltosa III	64	63
B	human I	62	59
	cod I	61	61
	human II	62	58
	human IV	58	55
	human V	57	53
	deer mouse VI	51	51

[a] In B, only the human forms are included, except for class I, where a cod structure is also known (Danielsson et al., 1992), and class VI, where only the deer mouse structure is known (Zheng et al., 1993).

and classes) and is interpreted to indicate nonlinear evolutionary changes of the *h* subunit during early postduplicatory divergence (cf. Discussion).

DISCUSSION

The results establish the presence and properties of isozymes of a vertebrate class III alcohol dehydrogenase. Isozymes of the "classical" class I alcohol dehydrogenase are previously well known in vertebrates and have been much studied in human, other primates, horse, and lizard (cf. introductory section), but isozymes have previously not been fully characterized in other classes of alcohol dehydrogenase. Although it may still be early to judge the distribution of isozymes in general, most vertebrate lineages have now been studied (Jörnvall et al., 1995), and at this stage, two characteristics of the isozyme formations appear discernible in the vertebrate alcohol dehydrogenase system. Each case thus far studied is unique, representing separate gene duplications in a taxon-specific manner. This has been defined for the three class I isozyme lines structurally characterized (Hjelmqvist et al., 1996), and the present cod class III isozymes show a fourth duplicatory origin. Isozyme formation appears more common in class I than in class III.

In view of the distinct evolutionary variability of the classes [cf. Danielsson et al. (1994a)], the isozyme duplications are of interest because they correlate with further properties. Three unexpected results of general interest concern the quaternary structure, the active site, and the evolutionary divergence.

Quaternary Structure. The cod class III alcohol dehydrogenase isozyme pattern exhibits an anomaly; in spite of fairly abundant *ll* and *hl* isozymes, there is an apparent lack of *hh* forms in liver-derived preparations and a lower yield of *hh* formation from *in vitro* dissociated and reassociated assemblies. This is not explained by a large abundance of *l* chains over *h* chains. Also, there is no absolute prevention of *hh* formation, since some *hh* dimers are formed upon dissociation and reassociation (Figure 1). Consequently, it appears as if relative or variable restrictions toward *hh* formation apply, suggesting the presence of two or more types of *h* chains, one forming and one not forming *hh* homodimers. Such restrictions are not typical of class III subunits in general, which are freely associable over wide species ranges from human to *Drosophila* (Danielsson et al., 1994b). Positions regarded as subunit-interacting (Eklund et al., 1990; Danielsson et al., 1994a; Ramaswamy et al., 1996) appear to give no structural explanation for the *hh* restrictions (Table 4). Consequently, the cod isozyme patterns may reflect a case where distantly positioned substitutions may cause conformational changes, preventing facile interactions in a homodimer of the *h* chains. The sequence analysis does point to possible explanations of this type. One is that the *h* chain has two Asn-Gly structures, at positions 126−127 and 134−135 (Figure 4). Such structures

Table 4: Comparisons between the Cod Class III Isozymes and the Human Class III Enzyme for Residues Participating in Subunit Interactions[a]

position	*l*	*h*	human
272_i	Val	Ile	Val
292_i	Ile	Ile	Val
299_e	Gln	Gln	Glu
302_e	Ala	Ser	Ser
316_i	Ala	Gly	Gly

[a] Invariant residues not listed. Positions given refer to the familiar class I structure with subscript indications i and e for internal and outside positions, respectively. Interacting residues are defined as given by comparisons (Eklund et al., 1990). As shown, exchanges are fairly few and minor, indicating the possibility that distant replacements produce further differences through indirect effects (cf. text).

are known to be susceptible to deamidative β-aspartyl formation [cf. Jörnvall (1974)], which would produce altered local geometries and an extra negative charge from the carboxyl group formed. The sequencer results, although continuous over both structures (showing the asparagine), drop considerably at each of the two positions, suggesting that considerable amounts of the peptide occur in the β-aspartyl form at these positions. Hence, it appears likely that the *h* chains as isolated from the *hl* heterodimer constitute a mixture of the original Asn-Gly structures and of Asp$\beta-$Gly structures at these two positions. The positions are at reverse turns in the tertiary structures (Eklund et al., 1990; Ramaswamy et al., 1994) and contribute to subunit interactions. Consequently, an altered structure may restrict dimer formation when both monomers have unsuitable geometry, while *hl*-dimer formation might be preserved because of a stabilizing effect of an intact *l* surface. The tendency to form some correct *hh* homodimers upon dissociation and reassociation may be derived from unmodified *h* chains. Other distantly positioned replacements may also be conceivable, and even if the Asn-Gly segments constitute an example where protein lability could cause notable differences between homologous subunits in the isozymes prepared, the *hh* and *hl* forms *in vivo* may still have normal structures with Asn-Gly.

Active Site. The two isozymes isolated differ markedly in some but not all catalytic properties (Table 2). These differences would be expected to reflect substitutions in functional residues. However, the residues likely to line the substrate and coenzyme binding pockets give minimal explanations (Table 2). A few conserved active site exchanges are noted, but all are observed in other class III structures without having an influence on the overall properties. Thus, also in respect to the changes at the active site, it appears possible that distant or "nonfunctional" positions influence the activity changes. If so, it may be noted that an isozyme-variable region occurs in the coenzyme-binding domain (bar and three-dimensional representation in Figure 5) of the enzymes. This region or the Asn-Gly structures in the *h* subunit appear to be possible candidates for distant, indirect effects. Hence, also in relation to activity, *h* replacements may illustrate a case of unexpected and distant influences in a native protein where the other isozyme counterpart is a "normal" *l* chain. This possibility also means that the actual values determined for the heterodimer should be regarded as tentative. This form may be influenced by different interactions, and if so, the actual K_m, k_{cat}, and turnover numbers measured may just reflect an average of the type of *h* form present.

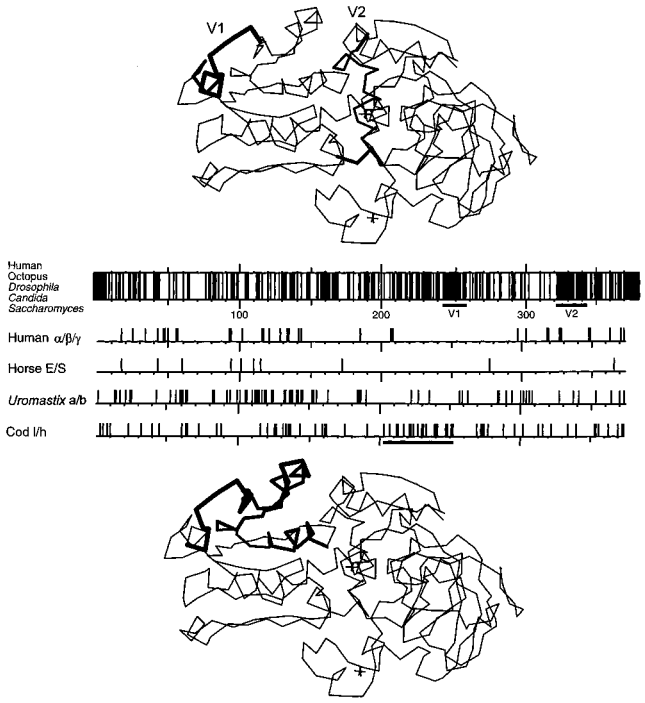

FIGURE 5: Positions of class III and isozyme variabilities in medium-chain alcohol dehydrogenases. The top bar shows those positions where the class III alcohol dehydrogenases from highly divergent species vary (indicated by black vertical lines), establishing two regions of high continuous variability (V1 and V2) as shown before (Danielsson et al., 1994a). Their positions in the conformation are shown at the top, with the conformation from Data Bank coordinates of the human class I enzyme using a program by Protein Science (Richardson & Richardson, 1992; Hurley et al., 1991; Bernstein et al., 1977; Abola et al., 1987). The lower bars show the positions of isozyme variations in the reported class I isozyme patterns and in the class III isozymes now established. One *h/l* variable region is indicated by the thick line below the bottom bar, and its position in the three-dimensional structure is given in the bottom panel, utilizing the same model and programming as that for construction of the top panel. The separate nature of the class I and III isozyme variabilities is noticeable, as well as the overlap between the class III species variability region and the class III isozyme variability region, establishing that the cod isozyme formation to some extent follows the same pattern as the class III variability at large.

Nonlinear Divergence. Finally, the great difference among the two isozyme subunits may be noticed. Although the class III enzyme is highly conserved from prokaryotes to humans (Danielsson et al., 1994a), and the distance from fish to human encompasses only an 18% divergence, the two isozyme chains now detected in cod class III alcohol dehydrogenase differ as much as fish/human species variants. If this enzyme is reflecting a linear divergence similar to those for other class III forms, this isozyme divergence would indicate a very distant gene duplication. Such an origin is unlikely since no trace of this duplication or of the same class III multiplicity has been found in the many species examined to date (Jörnvall et al., 1995). Instead, it appears possible that this great *h*/*l* subunit divergence indicates a rapid evolution of one form with fast accumulation of isozyme differences between the forms, deviating from the constant picture of class III in general. If so, it would appear that the *l* chain is the "traditional" class III function, being the one with most structural (Table 3) and functional (Table 2) similarities to other class III forms, including the human enzyme. The *h* form would then be the "novel" partner and presumably the duplicatory product undergoing rapid evolutionary changes. It may be significant that this finding of

a possibly nonlinear evolutionary divergence is found just in fish, representing a vertebrate of early origin, since the class I/III duplication has also been dated to roughly early vertebrate times, with similar nonlinear changes and partly "hybrid" properties also in the class I line. Consequently, the large *h/l* differences now found in class III may constitute an example of rapid evolutionary changes subsequent to formation by a duplicatory event, more or less like for the class I (Danielsson & Jörnvall, 1992) or for the class II enzymogenesis (Hjelmqvist et al., 1995a). In all these cases, the patterns appear to illustrate nonlinearity in evolutionary changes before functional properties have been locked in new restrictions.

In conclusion, the characterization of the cod class III alcohol dehydrogenase suggests the presence of spatially distant structural influences in a native enzyme, highlights isozyme pairs, and illustrates evolutionary changes. This proves the potentials from analysis of related enzymes in different species and gives future crystallographic analysis of the class III cod liver enzyme [cf. El-Ahmad et al. (1995)] additional interest.

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