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"Enzymogenesis": Classical liver alcohol dehydrogenase origin from the glutathione-dependent formaldehyde dehydrogenase line

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ABSTRACT **Analysis of the activity and structure of lower vertebrate alcohol dehydrogenases reveals that relationships between the classical liver and yeast enzymes need not be continuous. Both the ethanol activity of class I-type alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) and the glutathione-dependent formaldehyde activity of the class III-type enzyme [formaldehyde:NAD$^+$ oxidoreductase (glutathione-formylating), EC 1.2.1.1] are present in liver down to at least the stage of bony fishes (cod liver: ethanol activity, 3.4 units/mg of protein in one enzyme; formaldehyde activity, 4.5 units/mg in the major form of another enzyme). Structural analysis of the latter protein reveals it to be a typical class III enzyme, with limited variation from the mammalian form and therefore with stable activity and structure throughout much of the vertebrate lineage. In contrast, the classical alcohol dehydrogenase (the class I enzyme) appears to be the emerging form, first in activity and later also in structure. The class I activity is present already in the piscine line, whereas the overall structural-type enzyme is not observed until amphibians and still more recent vertebrates. Consequently, the class I/III duplicatory origin appears to have arisen from a functional class III form, not a class I form. Therefore, ethanol dehydrogenases from organisms existing before this duplication have origins separate from those leading to the "classical" liver alcohol dehydrogenases. The latter now often occur in isozyme forms from further gene duplications and have a high rate of evolutionary change. The pattern is, however, not simple and we presently find in cod the first evidence for isozymes also within a class III alcohol dehydrogenase. Overall, the results indicate that both of these classes of vertebrate alcohol dehydrogenase are important and suggest a protective metabolic function for the whole enzyme system.**

Alcohol dehydrogenases are common in nature. Two such enzymes widely separated, the yeast and horse liver forms, were among the first dehydrogenases studied (see ref. 1) and shown to have catalytic zinc (2–5), important cysteine residues (6, 7), and homologous structures (8). After the determination of the horse enzyme tertiary structure (9), the conformation was interpreted also for the yeast enzyme (10). Consequently, these distantly related enzymes [25% residue identity (11)] have for a long time been considered species variants, with standard albeit large variability.

After these basic concepts were established, alcohol dehydrogenase became the subject of much continued research. Three matters have been successively clarified, revealing a complex enzyme system. First, the *Drosophila* enzyme was found to have a completely different primary structure (12, 13) even though the mammalian, yeast (8, 10), and prokaryotic (14) forms were clearly related to each other. This fact was explained when it was established that two evolutionary lines exist and that convergence in function has given rise to separate families of alcohol dehydrogenases from different ancestral lineages (15), apparently utilized to different extents in different organisms. The *Drosophila* line is now known to be part of another large protein family, short-chain dehydrogenases, encompassing also human prostaglandin and steroid dehydrogenases (16–18).

Second, separate classes of human alcohol dehydrogenase were discovered (19). These classes have been structurally characterized and shown to typify mammalian alcohol dehydrogenases, with separate evolutionary properties (20). The explanation is a series of gene duplications at different stages (21), generating a system of isozymes and enzymes, which have been studied for structure–function relationships (22, 23).

Third, analysis of amphibian alcohol dehydrogenase recently established a first estimate of the timing of one of the duplications that explain the classes. This timing placed the class I/III separation early in vertebrate development (24), and analysis of the ethanol-active cod enzyme showed this to be a protein with mixed-class properties (25). These results seemed puzzling, since both glutathione-dependent formaldehyde-active class III [formaldehyde:NAD$^+$ oxidoreductase (glutathione formylating), EC 1.2.1.1] and ethanol-active class I (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) forms are wide-spread in living organisms (1, 26, 27) that originated much earlier than at the time estimated for the duplication. This puzzle is now resolved by the characterization of a second alcohol dehydrogenase in cod liver, which establishes the relationships and clarifies the origin of the class I enzyme. These results illustrate successive creation of activities, highlight the metabolic importance of alcohol dehydrogenase, and establish further isozyme complexity.

MATERIALS AND METHODS

Liver from cod of Baltic origin (*Gadus morhua*) was used for alcohol dehydrogenase preparation. The ethanol-active form was prepared as described (25) except that AMP-conjugated Sepharose was utilized in the affinity chromatography step instead of Cap-Gapp-Sepharose {4-[3-(*N*-6-aminocaproyl)-aminopropyl]-pyrazole-Sepharose}. The enzyme was obtained in 50% yield with a specific activity of 3.4 units/mg after a 170-fold purification. A second form was purified by monitoring the glutathione-dependent formaldehyde dehydrogenase activity ("class III alcohol dehydrogenase"). The two enzyme forms were separated by chromatography (*i*) on DEAE-Sepharose (2.5 × 30 cm) in 10 mM Tris·HCl/0.1 mM dithioerythritol, pH 7.6, or in the same buffer adjusted to pH 8.4 (elution was with a linear gradient of NaCl (0 → 300 mM) in the buffers]; and (*ii*) on AMP-Sepharose (2.2 × 13 cm) in 50 mM sodium phosphate/0.1 mM dithioerythritol, pH 7.5 [elution was with a linear gradient of NAD$^+$ (0 → 2 mM) in the same buffer]. The enzymes were then applied to a fast protein liquid chromatography (FPLC) Mono Q (HR 5/5) column equilibrated in 10 mM Tris·HCl (pH 8.3) (class I enzyme) or in the same buffer adjusted to pH 7.8 (class III enzyme) and eluted with a 20-ml NaCl gradient (0 → 0.5 M).

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Enzyme activities with ethanol, pentanol, and glutathione/formaldehyde and inhibitions with 4-methylpyrazole were determined as described (1, 23, 25, 26). Purity was evaluated by SDS/polyacrylamide gel electrophoresis (28) and electrophoresis under nondenaturing conditions in a Phast-system (Pharmacia LKB) utilizing Coomassie brilliant blue for protein staining and nitro blue tetrazolium/phenazine methosulfate for activity staining. Reduction, ^{14}C-carboxymethylation, and cleavage with aspartic acid-specific protease in 0.1 M ammonium bicarbonate were performed as described (25). Peptides were separated by reverse-phase HPLC (Ultropac TSK ODS-120T; 5 μm; 4.6 × 250 mm) with a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. Sequence analysis utilized an Applied Biosystems 470 instrument with separate phenylthiohydantoin detection, and a MilliGen ProSequencer 6600 with peptide attachment to acrylamine membranes. For sequence comparisons, the alignment previously determined for isozymes (22), different classes (23), and the major cod liver enzyme (25) was utilized with the peptide structures of the second cod liver enzyme.

RESULTS AND DISCUSSION

Cod Liver Has at Least Two Alcohol Dehydrogenase Activity Types. Monitoring ethanol and glutathione-dependent formaldehyde dehydrogenase activities reveals the presence of both of these enzymes from the alcohol dehydrogenase system in cod liver. One is the previously established ethanol-active type (functionally of class I, but structurally overall closer to class III) (25). The second is a typical class III enzyme, with low ethanol dehydrogenase activity and high specificity for glutathione/formaldehyde. This latter enzyme was separated from the class I form by DEAE chromatography by using a NaCl gradient at pH 7.6 (conditions under which the ethanol activity was eluted in the flow-through). A less complete separation was obtained at pH 8.4. In this case subsequent AMP-Sepharose chromatography separated the activity peaks into one of class I type and two of class III type, III H and III L (Fig. 1). The H and L designations correspond to high and low specific activity with hydroxymethylglutathione—40 and 4.5 units/mg of protein, respectively. FPLC on Mono Q showed that forms I and III H are essentially pure, while the III L fraction separated into two forms, III L and a more acidic subform, III L_a, with the same specific activity. Nondenaturing electrophoresis and activity-staining identified the I and III forms as separate enzymes with different mobilities and activities (Fig. 2). The type III forms are hardly active with ethanol at low substrate concentrations but are specifically visible with glutathione/formaldehyde. The class I form has the opposite properties, and both forms are visible with pentanol, as is typical for the corresponding mammalian enzymes of classes I and III (19, 23, 26). Activity measurements and pyrazole inhibitions further show the differences between the cod forms I and III as well as the similarities with the human class I/III enzymes (Table 1). These results establish the presence of both class I and III activities in vertebrate lines down to the stage of bony fishes.

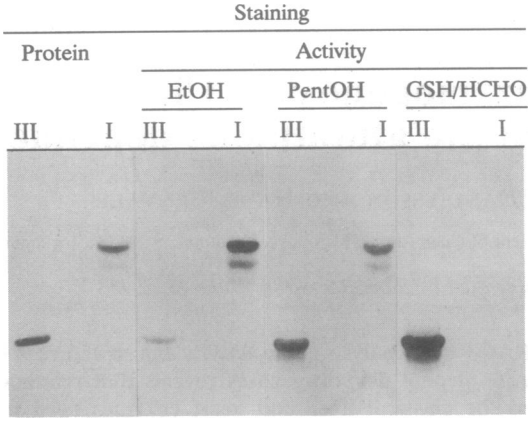

FIG. 2. Polyacrylamide gel electrophoresis under nondenaturing conditions of the cod I and III alcohol dehydrogenase forms. Protein staining was performed with Coomassie brilliant blue, and the activity staining was with ethanol (EtOH), pentanol (PentOH), and glutathione plus formaldehyde (GSH/HCHO). For the class III enzyme, the L type is shown [L and L_a do not separate on this gel; H runs just cathodic of (above) L]. For the class I form, bands of increasing anodic migration appear with time (see the visible extra band that appears with the ethanol staining) as with all of the class I enzymes from other species.

The Two Cod Enzyme Types Differ in Structure and Are Related to Mammalian Classes I and III. The class III form (III H and III L, Fig. 1) was reduced, carboxymethylated, and submitted to peptide sequence analysis after digestion with aspartic acid-specific protease and fragment separation by

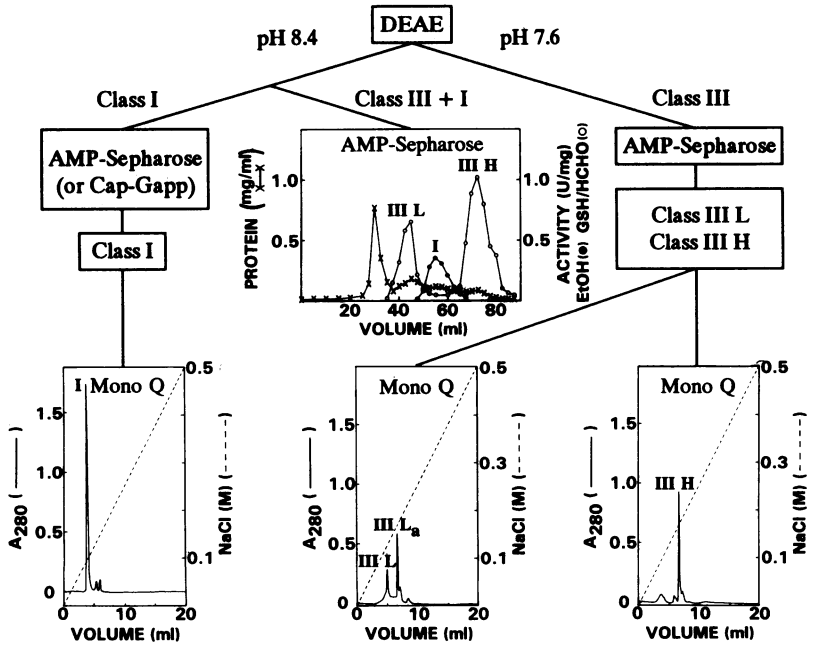

FIG. 1. Purification scheme for the different piscine alcohol dehydrogenases. Class I refers to the ethanol-active enzyme, and class III refers to the glutathione-dependent formaldehyde dehydrogenase, with L and H for low-activity and high-activity forms, respectively, and with L_a indicating a more acidic subform. The high lipid content of cod liver necessitated the use of different pH buffers for optimal DEAE chromatography of the cod enzymes. The cod class I enzyme was eluted together with lipids at pH 7.6 and required a pH of 8.4 to be retarded in the DEAE step and separated from lipids.

Table 1. Comparison of the enzymic characteristics of the classes of cod and human alcohol dehydrogenase (ADH)

ADH	Enzyme activity, units/mg of protein		Enzyme inhibition, K_i (μM)
	Ethanol	GSH/HCHO	4-Methylpyrazole
Class I			
Cod	3.4	0	0.1
Human*	1.2	0	0.3
Class III			
Cod†	(−)‡	4.5	(−)‡
Human	(−)‡	3.2	(−)‡

GSH, glutathione.

*For human class I, $\gamma_1\gamma_1$ is listed, since $\gamma\gamma$ is the isozyme closest to the mammalian type I enzymes in general. Values for specific activities of the human classes are from refs. 19 and 27.

†For cod class III, the L form is listed, since that represents the homodimer and the one closest to other class III enzymes (see text).

‡(−) indicates very high K_m with ethanol (>3 M; see ref. 23), giving no meaningful activity at low substrate concentration; and very high K_i values with 4-methylpyrazole (>50,000 μM; see ref. 23), giving no inhibition at low pyrazole concentration.

HPLC (Fig. 3). Comparison of the structures obtained with that of the cod I enzyme (25) established three features.

First, the two type-III forms have largely identical peptide patterns, with single peptides differing in elution position (3a from 3 in Fig. 3*A*) because of residue exchanges (Fig. 3). This pattern correlates with the finding that both the H and L forms have glutathione-dependent formaldehyde dehydrogenase activity but different turnover numbers and suggests that they constitute isozymes within class III. Peptide positions and heights upon HPLC (Fig. 3) suggest that the L form represents a homodimer and the H form represents a heterodimer. This is the first time structurally distinct isozymes have been established in a class III enzyme from any species investigated. The III L form is the one most closely related to the human and other class III enzymes in specific activity and in structure (Fig. 3*B*) and appears to represent the line in common.

Second, comparison of the structure of the class III peptides with those of the corresponding regions of the cod class I alcohol dehydrogenase (25) (Fig. 3) shows that the cod I and III forms differ by about 41%, a value typical of class distinctions in alcohol dehydrogenases (20, 23, 24). This distinction for the cod enzymes is also found with respect to their activity (Fig. 2) and suggests that the cod forms I and III now isolated represent the counterparts to class I and III mammalian alcohol dehydrogenases, respectively.

Third, the structural (Fig. 3) and enzymatic (Fig. 2) differences are confirmed by detailed comparisons with mammalian class I and III enzymes. Thus, specific class III residues of the human class III form, such as Tyr-49 and Asp-55 [instead of His-51 and Leu-57 in the class I enzyme (23)] are indeed related to the novel structures of the cod type III form. Residue differences between the class III human/cod enzymes are 18% (Table 2), which correspond to 21% after correction for repeated mutations in a position (29) and hence to ≈5% observable differences per 100 million years [assuming the teleost/mammal divergence at about 400 million years ago (see ref. 30)]. This is close to the value for the variation within mammalian class III alcohol dehydrogenase (≈8% observable differences per 100 million years) and differs greatly from the more variable class I enzyme [by a factor of 3 (20) or more]. Our conclusion is that the new enzyme isolated from cod is a typical class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase not only in its substrate specificity (Table 1) and its structure (Table 2) but also in its residue variability. Therefore, our results show that this enzyme maintains its enzymatic specificity and has a conserved structure throughout the vertebrate system, merely exhibiting conservative species differences from bony fishes to mammals.

Class III Appears To Be the Original Form and Class I the Emerging Form, Revealing Development of an Enzyme Activity. Previous studies of vertebrate lines more recent than the fishes established a common origin for the mammalian class

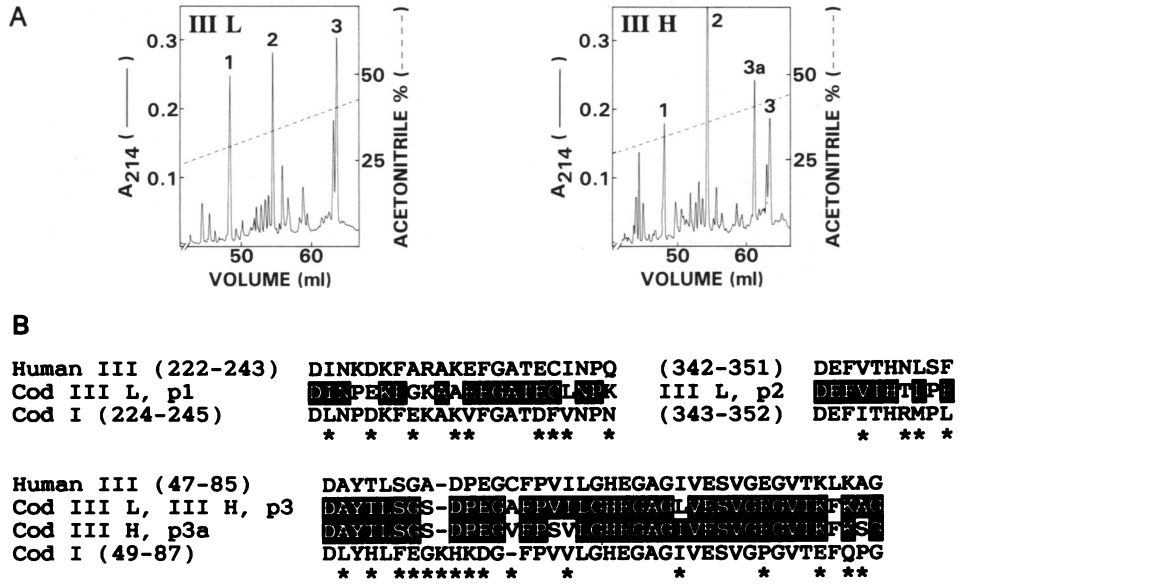

FIG. 3. Separation of cod liver alcohol dehydrogenase class III peptides (*A*) and corresponding structures (*B*). HPLC separations of class III peptides after digestion of the carboxymethylated H and L enzymes with aspartic acid-specific protease reveal similar patterns, with three prominent peaks and different relative peak heights in class III H showing its heterodimeric nature. Structural analysis of peptides 1–3 (p1, p2, p3 in B) showed complete identity between peptide 3 from the class III L and the III H enzymes. In addition, it established that peptide 3a from III H is a peptide 3 variant with five exchanges; the remaining peptides in III H were not analyzed because of the heterodimeric nature (peak 2 in III H corresponds to two components). Black background indicates residue identities with the human class III structure (20), and asterisks show residue differences between the cod I (25) and III L structures. Numbers within parentheses refer to residue positions. Gap positions were defined from alignment data (25).

Table 2. Sequence comparison of the classes of cod and human alcohol dehydrogenase

Cod enzyme*	Human enzyme, % residue identity	
	Class I†	Class III
Class I	55	64
Class III	61	82

*For the class I enzyme, values given refer to comparisons of the whole structures (see ref. 25), but those for the class III enzyme refer only to portions analyzed (data from Fig. 3). However, calculation of values for the same portions of class I as those utilized for class III give similar values (51% instead of 55%, and 58% instead of 64%). This validates the suggestion that the regions used for class III are sufficiently extensive for deriving relevant conclusions.
†See first footnote (asterisk) of Table 1.

I and III enzymes and gave an estimate that the gene duplication occurred about 450 million years ago, corresponding to the vertebrate and liver evolution (24). Since the timing is based on just a few known structures, it may well be underestimated. However, even if off by a factor of about 2 or more to include also the plant alcohol dehydrogenases as descendants from the duplication, the common origin of classes I and III was confirmed by the recent elucidation of the cod class I enzyme structure (25). That showed its hybrid nature, functionally of class I and structurally closer to class III (25), but did not reveal which of the two classes is closer to the original form and which is the one emerging after the duplication. The results now obtained show that the class III alcohol dehydrogenase is the form evolutionarily oldest and that the cod class I enzyme is the emerging form. Hence, the two cod enzyme structures define the earlier events and illustrate the "enzymogenesis," or emergence of a unique enzyme. This interpretation is compatible with the constancy of properties within the class III enzyme from mammals (20) and the presence of this form also in plants, yeast (27), and prokaryotes (31). For the class I form, on the other hand, gradual changes are observed (25) towards the new enzyme class represented by the classical alcohol dehydrogenase of mammalian liver.

Functional Conclusions. Fig. 4 summarizes relationships of the major class I/III vertebrate enzymes. It suggests that ethanol-active alcohol dehydrogenases of yeast (1) do not have a common duplicatory origin with the ethanol-active major form in vertebrates. Instead, these two classical types of ethanol dehydrogenase appear to be derived from separate duplicatory events and to have evolved towards similar substrate specificities by convergence (from a class III type of activity, at least for the vertebrate class I form, Fig. 4). Therefore, the relationships are complex, and the ethanol activity may have multiple origins. This could explain the old observation that yeast alcohol dehydrogenase deviates functionally from the mammalian ethanol dehydrogenases (1). The multiple origins are also compatible with the different quaternary structures (1) and gap patterns (10, 11) of the classical alcohol dehydrogenases (the yeast line is tetrameric; the mammalian line, dimeric). The multiple early duplications at the origins of the classes are compatible with the frequent duplications in the protein family that occur also at later stages (21)—i.e., the separate isozyme developments in the mammalian class I enzymes (21, 32) and those now found also for the piscine liver class III enzyme (III H and L; see above). In short, the system is complex and susceptible to repeated duplications and continuous variability. However, patterns are distinct and illustrate the emergence of a new enzyme activity in the form of the classical ethanol dehydrogenase in the vertebrate line (Fig. 4).

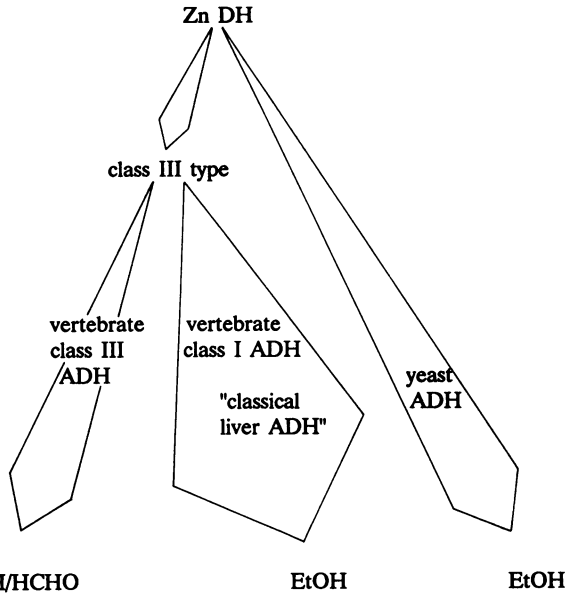

FIG. 4. Schematic outline of enzyme relationships in the zinc dehydrogenase (Zn DH) enzymes. The zinc dehydrogenases relate to still further enzyme families (15, 18), but the figure emphasizes the class III type with its glutathione-dependent formaldehyde dehydrogenase activity (GSH/HCHO) as the origin for both class I and III vertebrate alcohol dehydrogenases (ADHs), the emergence of the class I enzyme, and the convergence of classical liver ADH and yeast ADH to ethanol-active (EtOH) ADH from separate origins. For the vertebrate enzymes, widths of arrows are proportional to the evolutionary speed [class I ADH exhibiting a roughly 3-fold larger rate of residue replacements than class III ADH (20)].

Finally, the observation that the formaldehyde activity is the ancestral one suggests that one of the primary functions of the system is the elimination of low molecular weight reactive substances. Alcohol dehydrogenases exhibit several properties resembling those of glutathione transferases (33) and cytochromes P450 (34). All of these three enzyme systems have multiple forms with several classes and further isozyme developments from duplications at several stages. This parallelism suggests that an important function for the alcohol dehydrogenase system is to participate in basic cellular defense mechanisms. Furthermore, the constant nature of class III in keeping its functional properties and the apparently repeated emergence of class I-type ethanol activity (Fig. 4) suggest that both classes are important, although differing in substrates, structural properties, and evolutionary paths.

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