The vertebrate alcohol dehydrogenase system: Variable class II type form elucidates separate stages of enzymogenesis

Lars Hjelmqvist, Mats Estonius, and Hans Jörnvall

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

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ABSTRACT A mixed-class alcohol dehydrogenase has been characterized from avian liver. Its functional properties resemble the classical class I type enzyme in livers of humans and animals by exhibiting low $K_m$ and $k_{cat}$ values with alcohols ($K_m = 0.7$ mM with ethanol) and low $K_i$ values with 4-methylpyrazole (4 $\mu$M). These values are markedly different from corresponding parameters of class II and III enzymes. In contrast, the primary structure of this avian liver alcohol dehydrogenase reveals an overall relationship closer to class II and to some extent class III (69 and 65% residue identities, respectively) than to class I or the other classes of the human alcohol dehydrogenases (52–61%), the presence of an insertion (four positions in a segment close to position 120) as in class II but in no other class of the human enzymes, and the presence of several active site residues considered typical of the class II enzyme. Hence, the avian enzyme has mixed-class properties, being functionally similar to class I, yet structurally similar to class II, with which it also clusters in phylogenetic trees of characterized vertebrate alcohol dehydrogenases. Comparisons reveal that the class II enzyme is ~25% more variable than the “variable” class I enzyme, which itself is more variable than the “constant” class III enzyme. The overall extreme variability, the activity unexpected for a class II enzyme, and the unusual chromatographic behavior may explain why the class II enzyme has previously not been found outside mammals. The properties define a consistent pattern with apparently repeated generation of novel enzyme activities after separate gene duplications.

Known alcohol dehydrogenase complexity has increased rapidly during the last few years. The zinc-containing liver enzyme first characterized is now recognized to form part of a system of separate forms or classes (1), with different activities and corresponding gene multiplicity in humans and most vertebrates (2). Presently, at least six classes have been described, of which five have been structurally characterized from human origin (cf. ref. 3). Properties and relationships have been best established for three classes (4, 5), the classical liver enzyme with considerable ethanol activity (class I), the ubiquitous class III enzyme with a major glutathione-dependent formaldehyde dehydrogenase activity (6), and the stomach-expressed class IV enzyme with the highest ethanol activity (7). In molecular terms, these classes have ~60% residue identity. Between species, class I structures are fairly variable, class III is more constant, and class IV is intermediate (4, 5). Class III appears to be the ancestral type, present in prokaroytes (8) and invertebrates (4). An evolutionary tree based on a series of gene duplications starts to be discernible (9). Tentatively, the class III/I duplication, with the subsequent emergence of class I activity, has been traced to early vertebrate times. This dating has been deduced from estimates of molecular changes (10), the apparent absence of class I in lower life forms (4), and the presence of an enzyme in bony fish that still exhibits mixed properties, structurally closest to class III but functionally of class I type, presumably reflecting the original enzymogenesis (11).

This emerging knowledge reveals many details that need to be established. One of particular interest is the late origin of the vertebrate enzyme multiplicity, which is noteworthy since enzyme multiplicity is present also in forms of much earlier origin, such as yeasts and plants. This suggests the possibility of functional convergence in separate lines, with repeated emergence of ethanol activity (2). Unresolved questions also occur for the other human and mammalian enzyme classes, classes II, V, and VI, of which little is known. They have not been defined or even detected in submammalian forms, although considerable structural divergence between the forms is seen in humans, and evolutionary trees suggest distant origins (9).

During recent screening of nonmammalian sources, we detected another alcohol dehydrogenase form* in ostrich liver. The present analysis reveals it to be structurally of class II derivation vs. the human enzymes, while functionally it is of class I activity type. It evidently represents an ancestral duplication other than the class III/I duplication previously traced in bony fish (11). Furthermore, it suggests a distinct pattern with which also the class II enzyme is consistent in a system that eventually will define the original functions of the alcohol dehydrogenase classes.

MATERIALS AND METHODS

Protein Purification. Alcohol dehydrogenase from ostrich liver, obtained from Kolmården Zoological Park, Sweden, was purified by ion-exchange chromatography on DEAE-Sepharose, affinity chromatography on AMP-Sepharose, and exclusion chromatography on Sephadex G-100, essentially as described for the class I enzyme from this source (12). The ethanol class I alcohol dehydrogenase of this liver binds to DEAE-Sepharose (12), but, unexpectedly, we found that much ethanol dehydrogenase activity did not bind. We therefore applied the flow-through fraction to AMP-Sepharose for purification. After elution of the class III activity with a gradient of 0–0.5 mM NAD+ in Tris-HCl (pH 8.0), we continued with a second gradient to 10 mM NAD+. To increase yields, the column was then eluted with a gradient of 0–2 M KCl in the same buffer. The fraction obtained was desalted by exclusion chromatography and submitted to enzymatic and structural characterization.

Enzymatic Characterization. Activities were tested (6, 13) with ethanol, pentanol, octanol, cyclohexanol, and formaldehyde/glutathione by monitoring NAD+ reduction spectrophotometrically at 340 nm. $K_m$ and $k_{cat}$ values and the $K_i$ for 4-methylpyrazole were determined at pH 10.0 in 0.1 M glycine-NaOH buffer (13). Constants were calculated with

*The sequence reported in this paper has been deposited in the Swiss-Prot data base (accession no. P80468).
the program ENZYME (14), and $k_{cat}$ values are given per subunit.

Structural Analysis. The protein was carboxymethylated by reduction with dithiothreitol and treatment with iodo-
$[^{14}C]$acetate (12). It was then cleaved in separate batches with Lys-specific, Glu-specific, or Asp-specific proteases, with chymotrypsin, or with CNBr, and the resulting peptide digests were fractionated by reverse-phase HPLC (12). Amino acid compositions were determined with a Pharmacia LKB Alpha Plus analyzer after acid hydrolysis with 6 M HCl/0.5% phenol for 24 h at 110°C in evacuated tubes. Amino acid sequences of peptides were determined in Applied Biosystems model 477A and MilliGen Prosequencers 6600 and 6625 instruments with on-line analyzers. The N-terminal amino acid sequence and blocking group were determined by mass spectrometry (15).

The primary structure obtained was correlated with the three-dimensional model deduced for human class II alcohol dehydrogenase (16). Sequence relationships were expressed quantitatively in terms of a phylogenetic tree constructed with the program CLUSTAL W (17).

RESULTS

Protein Purification and Enzymatic Properties. Alcohol dehydrogenase purification from liver usually involves a DEAE ion-exchange chromatography step, an affinity step, and an exclusion chromatography step (4, 10, 12, 18). By using this protocol to purify alcohol dehydrogenases from ostrich liver, we noticed that recovery of total alcohol dehydrogenase activity was low at the intermediate AMP-Sepharose step (37%) after completion of the gradient of NAD$^+$ from 0–10 mM [in 50 mM Tris-HCl (pH 8.0)]. We therefore continued elution with a salt gradient, 0–2 M KCl, in the same buffer. This produced a second fraction of alcohol dehydrogenase that was eluted at about 1 M KCl and increased the total activity recovered to acceptable levels (68%). This second fraction was desalted by the usual exclusion chromatography step and characterized.

In enzymatic screenings, this alcohol dehydrogenase behaved as a class I enzyme, demonstrating considerable activity to ordinary alcohols, including ethanol. The presence of two other alcohol dehydrogenases during purification was also confirmed. The class III alcohol dehydrogenase previously not reported from this species but expected to be present from its occurrence in other vertebrates (2, 6, 11) was detectable by its glutathione-dependent formaldelyde dehydrogenase activity. The class III enzyme was present in the same fraction as the present enzyme during the first purification step but was separated from it by earlier elution from AMP-Sepharose. Similarly, the class I enzyme, known by structural and enzymatic properties (12), had separated during the first purification step by absorbing to DEAE. $K_{m}$ and $k_{cat}$ values for the present enzyme with most alcohols were low. The ones with ethanol and the $K_{i}$ value with the class-distinguishing inhibitor 4-methylpyrazole were in the range of those of the human class I enzymes (Table 1). Values with ethanol and 4-methylpyrazole, the substrate and inhibitor often used to distinguish the properties of the classes (7, 13, 16, 19), are considerably higher for the characterized class II form (from humans) and very much higher for all class III forms known (Table 1).

In conclusion, the purification and enzymatic characterization suggest the presence of a third type of alcohol dehydrogenase in ostrich liver, in addition to the established class I and III types of mammals and birds. Its unexpected behavior during purification, requiring high salt for elution in the affinity step, may explain why it has not been previously observed.

Structural Characterization and Relationships. The present alcohol dehydrogenase was homogeneous after the final exclusion chromatography step, as judged by SDS/PAGE and subsequent protein staining, showing an apparent 40-kDa band, similar to that of other vertebrate alcohol dehydrogenases. The protein was $[^{14}C]$carboxymethylated and subjected to five types of cleavage in separate batches to generate peptides for structural analysis. The fractions obtained produced overlapping peptides covering all regions of the molecule, yielding a continuous amino acid sequence of 379 residues (Fig. 1). The N terminus is blocked by an acetyl group as in all other characterized vertebrate alcohol dehydrogenases and was established by mass spectrometry of an N-terminal peptide (15). The C terminus was established as Phe-379 by identical ends of peptides from three digests (Fig. 1) with different cleavage specificity (Lys-specific protease, Asp-specific protease, and CNBr).

Table 1. Enzymatic properties of the present enzyme form, compared with those of the characterized human enzymes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ostrich I</th>
<th>Ostrich II</th>
<th>Human II</th>
<th>Human I</th>
<th>Human III</th>
<th>Human IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.71</td>
<td>1.2</td>
<td>1.1</td>
<td>120</td>
<td>NS</td>
<td>11</td>
</tr>
<tr>
<td>Pentanol</td>
<td>0.025</td>
<td>0.15</td>
<td>0.16</td>
<td>0.09</td>
<td>22</td>
<td>0.08</td>
</tr>
<tr>
<td>Octanol</td>
<td>0.003</td>
<td>0.013</td>
<td>0.010</td>
<td>0.007</td>
<td>1.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Cyclohexanol</td>
<td>3.4</td>
<td>14.5</td>
<td>0.042</td>
<td>210</td>
<td>NS</td>
<td>140</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>4-Methylpyrazole</td>
<td>4.0</td>
<td>0.3</td>
<td>(β1γ)</td>
<td>400</td>
<td>&gt;=50,000</td>
</tr>
</tbody>
</table>

$K_{m}$ values (mM) are the upper value in each pair and $k_{cat}$ values (min$^{-1}$) are the lower value for aliphatic alcohols and cyclohexanol measured at pH 10. NS, nonsaturable. $K_{i}$ values (μM) for the class I inhibitor 4-methylpyrazole in the presence of ethanol as substrate are shown. Values for the ostrich class II were determined herein; remaining values are from the literature (7, 13, 16, 19).

The structure immediately revealed features distinct from those of class I, in contrast to its class I enzymatic properties. Thus, the enzyme is 5 residues longer than the class I form, and alignment of sequences (also shown in Fig. 1) reveals that these residues correspond to insertions in the class I enzyme sequence, one close to position 60 and four in a segment close to position 120. All these positions of internal differences coincide with those known for the human class II enzyme. Overall residue identities also relate the form to the structure of class II, with an identity toward the human enzyme of 69%. Lower values were obtained toward all other alcohol dehydrogenase classes [61% for human class I $y$, 65% for human class III, 58% for human class IV, 58% for human class V, and 52% for deer-mouse class VI enzymes (20)]. In addition, several residue positions characteristic of class distinctions are related to those of the human class II type enzyme. Residues at class I positions 57 and 93 [of importance in class III distinctions (19)], residues at class I positions 110, 309, and 318, and the residues at class II positions 115 and 128 are as in the human class II enzyme and distinguish it from the class I enzyme (Fig. 1). Furthermore, the structure clusters with the human and rat class II structures in a phylogenetic tree constructed with the program CLUSTAL W (17) (Fig. 2) and values from bootstrap analysis (22) are statistically significant. In separate phylogenetic trees constructed for the N-terminal and C-terminal halves of the structures, the structure also clusters with the class II enzymes.

The structure determined and the alignments with known forms of the enzyme establish the present enzyme as an alcohol dehydrogenase that is related to the class II form, reported in humans (26) and also in rats (21). Overall size, insertions, residue identities, and actual residue types show class II relationships, although the residue types also show some...
**Fig. 1.** Primary structure of the class II type alcohol dehydrogenase from avian liver. The 379-residue protein chain is determined by the continuous sequence designated Ostrich II. For comparison, residue deviations at equivalent positions in the human class I–III enzymes are indicated. Empty positions, residue identities with the ostrich II structure; dashes, gaps. Residues in boldface type and underlined are functional residues further outlined in Table 2. Asterisks show acetylated N termini. Thin lines below the sequences show all peptides now analyzed (dashed parts indicating tentative identification in individual sequencer deviations). Thick lines indicate three segments variable within class I (4) and herein shown to be variable also within class II. Numbers above the sequences give residue positions in the enzyme, and numbers in parentheses give those of the class I enzymes (human and horse) for reference. The two numbering systems deviate from position 60 and still more from position 115, because of the extra residues in class II (16) vs. class I.

class I characteristics compatible with the enzymatic activity. It may be concluded that the present enzyme constitutes a form with mixed properties, enzymatically related to class I and structurally related to class II.

**DISCUSSION**

**Active-Site Structure—Function Relationships.** Computer graphics model building has established (16) that the class II human enzyme is compatible with an overall fold closely related to that of the crystallographically determined class I conformations (23, 24). Subsequently, an even more distantly related enzyme (the major cod liver alcohol dehydrogenase with 45% residue differences) has also been established by modeling (18) and actual x-ray crystallography (S. Ramaswamy, M. El-Ahmad, O. Danielsson, H.J., and H. Ekland, unpublished data) to be compatible with the same overall fold. Hence, the identity level and class II relationship in the present case make it meaningful to correlate functional residues of the enzyme with those of other alcohol dehydrogenases based on the positional assignments. Alignment of 11 substrate-interacting and 5 coenzyme-interacting critical positions (Table 2) reveals both conservation and divergence, in agreement with the enzymatically and structurally mixed properties. In particular, coenzyme-interacting residues are largely conserved in relation to those of class I, as they are in alcohol dehydrogenases in general, but some differences exist, all seen before in other alcohol dehydrogenases. Differences are larger with the substrate-binding site; relationships are closest to those of the class II enzyme (Table 2), corroborating the overall structural assignment. However, positions 116, 141, and 306 have residues differing from those of the human class II form. Unexpectedly, however, two of these residues, although different, are not of class I type, suggesting that enzymatic properties in the present enzyme are defined not only by known interactions in the substrate pockets but also by additional interactions that probably include those at the coenzyme-binding site, which is similar to that of the class I enzyme (Table 2).

**Enzyme Evolution and Emergence of Novel Forms.** The present structure can be incorporated into a phylogenetic tree...
relating the characterized six classes of the enzyme (Fig. 2). Importantly, the present form clusters with class II, reflecting the separate duplicatory origins of class I (11) and II (this work). Exact branching is still premature for final judgement since few structures are known, but separate groupings of the classes are apparent, distinguishing the class concept. The duplication giving rise to class II was previously enigmatic, since no class II structure had been established outside mammals. We now see that such structures occur, compatible with early duplication and with the overall pattern of the evolution of the enzyme system.

The analysis reveals that the structural divergence within class II is extensive, even more extensive than that for the class I protein, previously concluded to be the “variable” type of the classes then characterized (4). Thus, all the classes apparently have quite distinct evolutionary patterns. Class III is “constant,” differing very little between separate mammalian lines; class I is “variable,” differing ≈3-fold more; and class IV is intermediate [the human/rat differences varying by 6%, 18% (25), and 13% (5), respectively]. We see that class II is particularly variable, being 25% more variable than class I for the human/ostrich forms. In total, therefore, the speed of evolutionary change is now seen to vary up to 4-fold between the relatively constant class III enzyme of ancient origin (4) and the class II enzyme of later emergence. Apart from distinguishing the separate classes, this shows a system of highly different rates of evolutionary change although all folds and enzyme activities are related.

Within the subunits, separate patterns of class-distinguishing segment variability have been observed (4). In particular, three functional segments of variability distinguish class I from class III in a manner atypical of species differences in general. Checking all regions in class II, we find that the atypical pattern of class I also applies to class II. The three regions outlined in class I (4) are variable also in class II (V1–V3, Fig. 1). In particular, V1 and V3 are highly variable within class II, with only a few residues conserved between the species variants (Fig. 1). The variable segments set up a pattern of extensive exchanges at the center of the dimer (Fig. 3), involving both subunit interactions and the entrance to the active site. Hence, it may be concluded that the patterns constitute general phenomena, distinguishing evolving enzymes from constant ones by an atypical vs. a typical pattern, affecting functional and nonfunctional segments, respectively. It appears significant that the overall most variable class II enzyme also is the alcohol dehydrogenase with the largest variation at the active site (V1) and the dimer interaction area (V3) (Fig. 3).

The finding of an alcohol dehydrogenase with mixed-class properties is of interest. Previously, this has been encountered in the major cod liver enzyme, where it was interpreted to reflect enzymogenesis of class I properties from the class III ancestral form (11). We now see that mixed-class properties also relate to class II. The present enzyme has functional properties of class I, overall structural properties of class II, and root relationships with class III. Although branchings are thus far based on few known structures, and additional classes

Table 2. Important residues at substrate and coenzyme-binding pockets as determined for the class I alcohol dehydrogenases crystallographically analyzed (23, 24), the human class II and III enzymes modeled (16), and the present enzyme

<table>
<thead>
<tr>
<th>Source</th>
<th>Inner</th>
<th>Middle</th>
<th>Outer</th>
<th>Coenzyme-binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrich II</td>
<td>48</td>
<td>93</td>
<td>140</td>
<td>141</td>
</tr>
<tr>
<td>Human Iβ</td>
<td>Thr</td>
<td>Tyr</td>
<td>Phe</td>
<td>Met</td>
</tr>
<tr>
<td>Human Iγ</td>
<td>Ser</td>
<td>Phe</td>
<td>Phe</td>
<td>Val</td>
</tr>
<tr>
<td>Human II</td>
<td>Thr</td>
<td>Tyr</td>
<td>Phe</td>
<td>Val</td>
</tr>
<tr>
<td>Human III</td>
<td>Thr</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Met</td>
</tr>
</tbody>
</table>

Replacements at the various amino acid positions indicated relative to the present enzyme are underlined, and positional numbers refer to class I. Additional positions in coenzyme interactions but conserved between the human classes (16) are not listed. Inner, middle, and outer refer to the relative positions in the substrate-binding cleft.

FIG. 3. Structural assignment of residue replacements in variable segments of the alcohol dehydrogenase dimer. The dimer symmetry axis is perpendicular to the paper and passes through the cross at the center. Solid circles, Cx positions of replacements within class II between the human and ostrich enzymes in three variable segments, V1–V3; bars between Cx positions, replacements corresponding to gaps in class I; open circles, positions of the zinc atoms. These segments, outlined in Fig. 1 and defined in ref. 4, show the dense representation of replacements along subunit-interacting segments in the dimer and part of the entrance to the active site. The conformation shown is that of the crystallographically determined human class I alcohol dehydrogenase (24), obtained from the Brookhaven Protein Data Bank, and actual replacements are as given in Fig. 1.
or mixed forms may still be encountered altering details regarding gene numbers, duplicatory orders, or class assignments, the delineation of the alcohol dehydrogenase system illustrates successive duplications and subsequent evolution of novel enzyme activities. The enzyme activities emerge with changes affecting the active site, even when the general relationships still reflect the ancestral origin from another class.

The mixed-class forms constitute a nomenclature problem. Previously the enzyme with mixed-class properties in cod liver was called class I from its functional assignment, because no other class I enzyme was detected in that species. The present mixed-class enzyme is intuitively regarded as class II from its structural properties because another class I enzyme is already known in this species (12). Although clarifying the distinctions, this alternative naming of enzymes after function or structure is inconsistent, but reconsideration may best be postponed until further forms, origins, and properties have been established.

Finally, the assignment of each class into separate groups of distinct evolutionary patterns is concluded to reflect correspondingly distinct metabolic roles. Class III is the most constant form of distant ancestral origin and is involved in cell defense and detoxication (see refs. 2 and 6). The form characterized herein reveals extensive differences in class variability yet shows a constant pattern in each class, once established. These class patterns and functional differences will be useful in defining the metabolic role(s) of all forms, including the human alcohol dehydrogenase complex with its still further isozyme gene multiplicity.

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