Structural and Enzymatic Properties of a Gastric NADP(H)-dependent and Retinal-active Alcohol Dehydrogenase*

(Received for publication, August 21, 1998, and in revised form, April 14, 1999)

Josep Maria Peralba‡, Ella Cederlund§, Bernat Crosa‡, Alberto Moreno‡, Pere Julià‡,
Susana Eva Martínez‡, Bengt Persson§, Jaume Farrés‡, Xavier Parés‡, and Hans Jörnvall¶†

From the %Department of Biochemistry and Molecular Biology, Faculty of Sciences, Universitat Autonoma de Barcelona, 08193 Bellaterra (Barcelona), Spain and the §Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

A class IV-type, gastric alcohol dehydrogenase (ADH) has been purified from frog (Rana perezi) tissues, meaning detection of this enzyme type also in nonmammalian vertebrates. However, the protein is unique among vertebrate ADHs thus far characterized in having preference for NADP⁺ rather than NAD⁺. Similarly, it deviates structurally from other class IV ADHs and has a phylogenetic tree position outside that of the conventional class IV cluster. The NADP⁺ preference is structurally correlated with a replacement of Asp-223 of all other vertebrate ADHs with Gly-223, largely directing the coenzyme specificity. This residue replacement is expected metabolically to correlate with a change of the reaction direction catalyzed, from preferential alcohol oxidation to preferential aldehyde reduction. This is of importance in cellular growth regulation through retinoic acid formed from retinol/retinal precursors because the enzyme is highly efficient in retinal reduction (k_cat/K_m = 3.4.10⁴ M⁻¹ min⁻¹). Remaining enzymatic details are also particular but resemble those of the human class I/class IV enzymes. However, overall structural relationships are distant (58–60% residue identity), and residues at substrate binding and coenzyme binding positions are fairly deviant, reflecting the formation of the new activity. The results are concluded to represent early events in the duplicatory origin of the class IV line or of a separate, class IV-type line. In both cases, the novel enzyme illustrates enzymogenesis of classes in the ADH system. The early origin (with tetrapods), the activity (with retinoids), and the specific location of this enzyme (gastric, like the gastric and epithelial location of the human class IV enzyme) suggest important functions of the class IV ADH type in vertebrates.

In vertebrates, alcohol dehydrogenase (ADH)¹ constitutes a complex system composed of multiple molecular forms, which have been grouped into minimally six (1) and probably seven (2) or more classes according to their enzymatic and structural properties. Notably, a similar complexity is observed also for aldehyde dehydrogenase, the next enzyme in the metabolic pathway, with twelve forms presently known in humans (3). The ADH class I forms constitute the classical enzyme responsible for the liver ethanol metabolism, whereas the ADH class III form is a ubiquitous glutathione-dependent formaldehyde dehydrogenase. Both these classes are well established in structure, variability, internal architecture, origin, and function (4), with class III as a probable ancestor and class I reflecting a duplicatory emergence at early vertebrate times (5). Remaining classes are less well known in general properties or functions. All characterized forms, however, are NAD(H)-dependent.

ADH class IV exhibits a unique epithelial tissue distribution (6) and is the characteristic stomach ADH, where it has been suggested to function in the first-pass metabolism of ethanol (7). More importantly, however, class IV has been ascribed a special function, based on its high activity with retinol (8–10), in the regulation of retinoic acid formation and hence in regulation of cellular growth and differentiation in vertebrates (11, 12). Its substrate pocket has been distinguished from modeling (13) and crystallographic (14) studies as different from that of class I, supporting distinct properties. It is a problem that the class IV enzyme has hitherto not been characterized outside of mammals, although ascribed a wider role in retinoid metabolism (12). If important in that function, it would be expected to be present also in nonmammalian branches.

We now report the finding of an amphibian ADH that has both gastric distribution and substrate properties like a class IV enzyme. Surprisingly, however, the amphibian enzyme has a coenzyme preference for NADP(H) instead of NAD(H). This is in contrast to all other vertebrate ADHs thus far detected of the MDR (medium-chain dehydrogenase/reductase) type. It has implications on the origin and function of class IV-like activities and on the physiological roles of the complex vertebrate ADH system.

MATERIALS AND METHODS

Amphibian Tissue—Stomachs from Rana perezi frogs, the same species as that previously analyzed for the class I enzyme (15), were cut, cleaned, rinsed in ice-cold, distilled water, and stored at ~80 °C. Prior to analysis, the stomachs were thawed, cut into small pieces, and homogenized (1:2, w/v) in 10 mM Tris-HCl, pH 7.6, 0.5 mM dithiothreitol. The homogenates were centrifuged at 27000 × g for 1 h. Supernatants were used for ADH activity and electrophoretic analysis. Total protein was determined by the Bio-Rad protein assay method, using bovine serum albumin as standard.

Enzyme Assays and Electrophoresis—ADH activity was determined spectrophotometrically at 25 °C with a Cary 219 instrument at 340 nm. Initially, before knowledge of the NADP(H) preference, activities were measured in the usual manner with NAD(H) as coenzyme. However, once the NADP(H) preference had been established, the proper cofactor was used. Alcohol oxidation was then determined in 0.1 mM glycine/NaOH, pH 10.0, or in 0.1 mM sodium phosphate, pH 7.5, with 2.4 mM NAD⁺ or 1.2 mM NADP⁺, whereas aldehyde reduction was measured in...
Gastric NADP(H)-dependent Alcohol Dehydrogenase

0.1 mM sodium phosphate, pH 7.5, with 1.33 mM NADH or 0.17 mM NADPH. Cuvette pathlength was 0.2 cm in experiments with 1.33 mM NADH and 1 cm in all other cases. One unit of activity equals 1 µmol of cofactor transformed/min, based on an absorption coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm for NADPH. Acetaldehyde was distilled immediately before use. Kinetics with retinoids were determined as reported (10, 16). Kinetic results were processed by using the Enzfitter data analysis program (Elsevier Biosoft).

Electrophoresis on starch gel was performed as described (17). Gel slices were stained for ADH activity with 0.1 mM ethanol or 0.1 M 2-buten-1-ol as substrate. Electrophoresis in SDS-polyacrylamide gels followed the procedure of Laemmli (18) with the silver stain technique. Isoelectric focusing was performed on a MiniProtean instrument (Bio-Rad) at a pH range of 3–10, with the ADH bands detected by activity as for the starch gel electrophoresis.

**Purification and Protein Analysis of Frog Stomach ADH**—About 18 stomachs (14 g) were homogenized and centrifuged as described above. The supernatant was dialyzed against 10 mM sodium phosphate/NaOH, 2.5 mM dithiothreitol, pH 6.5, and applied to a CM-Sepharose CL-6B column (1 × 25 cm) equilibrated in the same buffer. After washing with buffer, the enzyme was eluted with 120 ml of a linear gradient of 0–150 mM NaCl. The active fractions were pooled, concentrated, and dialyzed against 10 mM sodium phosphate/NaOH, 2.5 mM dithiothreitol, pH 6.5, and applied to an AMP-Sepharose column (1 × 13 cm) equilibrated in the same buffer. The enzyme was not adsorbed under these conditions and was eluted with a further 250 ml of buffer. Proteins that bind to the column were eliminated in this step. The active fractions were concentrated, dialyzed against 100 mM HEPES, 2.5 mM dithiothreitol, pH 6.5, and rechromatographed on a second AMP-Sepharose column equilibrated in this buffer. Then, the enzyme was adsorbed and the column was washed with buffer, before elution of the enzyme with a 250-ml linear gradient of 0–100 mM NaCl. The active fractions were concentrated and stored at −80°C. The apparent molecular mass of the enzyme was determined by gel exclusion chromatography, using a column of Sephacryl S-300 superfine (2.5 × 89 cm) in 200 mM sodium phosphate/NaOH, 2.5 mM dithiothreitol, pH 6.5.

The enzyme preparation was reduced and 14C-carboxymethylated in 6 µM guanidine HCl, 0.4 mM Tris, 2 mM EDTA, pH 8.1 (15). After removal of reagents on Sephadex G-50 (4.6 × 250 mm) in 50% acetic acid, the protein was cleaved in five different 3-nmol batches with Asp-N, Glu-C, and Lys-C proteases, trypsin, and chymotrypsin, respectively, all in 0.1 M M guanidine HCl, 0.4 M Tris, 2 mM EDTA, pH 8.1 (15). After removal of reagents on Sephadex G-50 (4.6 × 250 mm) in 30% acetic acid, the protein residues 310–318 and 320–328, respectively. The resulting amino acid sequence obtained from the protein and cDNA data, phylogenetic trees relating the new ADH structure to those of other ADHs were constructed with the program Clustal W (20).

**Computer Graphics Modeling and Substrate Docking**—A three-dimensional model of the frog stomach ADH was obtained by adopting its amino acid sequence into the known conformation of the human class I ADH (21) using the program ICM (Version 2.7, 1998, Molsoft LLC, Metuchen, NJ) by a Monte Carlo procedure for energy minimization (13). Coenzyme docking calculations were performed (13) with NADP⁺ placed into the frog enzyme model to occupy a position as in the class I structure, after which the model was subjected to energy minimizations.

**RESULTS**

**Purification**—The frog stomach homogenate was found to contain two ADHs separated by starch gel electrophoresis and representing different classes. One is anodic (pI: 6.2–6.5), also detectable in the liver (Fig. 1), and corresponds to class III ADH visualized by staining for formaldehyde dehydrogenase activity in the presence of glutathione (5). The second is cathodic (pI: 8.6–8.9), as a three-band pattern absent in liver (Fig. 1) and different from the class I ADH previously characterized (15). This new ADH was purified to homogeneity by consecutive CM-Sepharose and two AMP-Sepharose chromatographies. From 14 g of gastric tissue, 22 µg of enzyme was obtained, in a 2.4% yield, with a specific activity in 0.1 M glycine/NaOH, 1 mM ethanol, and 1.2 mM NADP⁺ of 20.9 units/mg at 25°C. Electrophoresis on SDS-polyacrylamide reveals a single band with an Mr of 44,000, whereas gel exclusion chromatography on Sephacryl S-300 superfine results in a single peak with an apparent Mr of ~80,000, demonstrating a dimeric structure for the new enzyme.

**Protein Sequence**—The enzyme preparation was 14C-carboxymethylated and used for sequence analysis. After protease digestions in five different batches, high performance liquid chromatography separation of the peptides obtained, and peptide sequencer degradations, the entire amino acid sequence was obtained except for the N-terminal five-residue segment and nine other positions in total. A complicating factor was the low yield of the pure enzyme (see above) which made work with mixtures necessary. The contaminating proteins were identified as 6-phosphogluconate dehydrogenase and a homologue to protein S 47093 (Protein Identification Resource data base, corresponding to accession number Z34285 in the EBI Data Bank), which increased the number of peptides. The ADH regions were established from 86 ADH peptides of 263 peptides analyzed from all three proteins. Based on the ADH amino acid sequence, oligonucleotides were synthesized and used as primers to amplify cDNA prepared from stomach mRNA. The cDNA and protein sequences finally obtained (Fig. 2) are deposited in the EMBL database (accession number AJ002554). Three amino acid residues, at positions 73, 134, and 142, were found to differ between the sequences determined from the protein and the cDNA. The exchanges involve related residues only (Leu/Val, Lys/Arg, and Met/Val, respectively), corresponding to 1-base changes, and are without structural and functional consequence in the interpretations with the exception of position
142, corresponding to 141 in class I enzymes. These exchanges may well be related to the fact that several bands are detected on starch gel electrophoresis of the purified enzyme (Fig. 1).

Surprisingly, the new enzyme was found to have Gly at position 222 (Fig. 2), the position corresponding to 223 in the class I enzymes, which in contrast is Asp in all other vertebrate ADHs. This position is essential for coenzyme specificity (22) and made us investigate the kinetics with both NAD\(^+\) and NADP\(^+\).

Enzymology—The enzyme was found to be active with both NAD\(^+\) and NADP\(^+\) but was more efficient with NADP\(^+\) as predicted by the characteristics of the coenzyme binding site with a Gly-223 (see above). The cofactor specificity was determined (Table I).

| Coenzyme  | \(K_m\) (mM) | \(k_{cat}\) (min\(^{-1}\)) |
|-----------|-------------|-----------------|
| NAD\(^+\) | 0.2         | 1680\(^{a}\)    |
| NADP\(^+\) | 0.03        | 1660\(^{a}\)    |
| NAD\(^+\) | 0.8         | 1690\(^{a}\)    |
| NADP\(^+\) | 0.06        | 350\(^{a}\)     |
| NADH      | 0.52        | 6060\(^{a}\)    |
| NADPH     | 0.012       | 6620\(^{a}\)    |

\(^{a}\) pH 10, 1 mM ethanol.

\(^{b}\) pH 7.5, 1.5 mM octanol.

\(^{c}\) pH 7.5, 0.2 mM m-nitrobenzaldehyde.

Modelling, Coenzyme Docking, and Structural Features—The effect of the Asp/Gly exchange at position 223 (this and subsequent numbers refer to the ADH class I positions) was evaluated by modeling the present enzyme into the structure of the original Met N terminus is shown within parentheses and is not numbered to fit the situation in all other vertebrate ADHs, where it is post-translationally removed and the subsequent residue is acetylated to give the acetyl-blocked N terminus of the mature protein. Because of gaps, positional numbers differ between enzyme species and classes. At most places in the tables and in the text, positional numbers used are those of the class I human ADH.
the human class Iβ enzyme, and by subsequent docking calculations with NADP⁺. Similarly, a model with Asp-223 was used for docking evaluations. The modeling results show an active site similar to those in classes I and IV, consistent with the substrate specificity. The functionally important positions are compared in Table IV. The docking results (Fig. 3) demonstrate that NADP⁺ fits nicely into the coenzyme binding pocket when position 223 has Gly and that NADP⁺ binding is impaired with Asp-223 because of close contacts. The docking experiments are, therefore, compatible with the notion that this Asp/Gly exchange at position 223 offers a major structural explanation for the altered coenzyme specificity for NADP⁺ of the frog stomach enzyme versus that of all other vertebrate ADHs characterized. Asp-223 forms hydrogen bonds with the 2'- and 3'-hydroxyl groups of the adenosine ribose of the coenzyme in the class I enzymes (22), conferring their specificity for NAD⁺ over NADP⁺ (25). The carboxylate of Asp-223 sterically and electrostatically disfavors binding of NAD⁺ because of the cofactor 2'-phosphate, whereas Gly-223 provides the space necessary for NADP⁺. The presence of hydrophilic nearby residues (Thr-224 and His-225), also unique to the present enzyme, further favors the binding of the extra phosphate. The highly different ADHs from Thermoanaerobium brockii (26, 27), Thermoanaerobacter ethanolicus, Clostridium beijerinckii (27, 28), and Entamoeba histolytica (29) are also NADP⁺-dependent and have a Gly at the position corresponding to 223 in the class I enzyme and hydrophilic residues at those corresponding to positions 224 and 225.

Residue exchanges affecting substrate interactions concern several positions (Table IV). One is position 294, where a large residue, Leu-294, is consistent with a Kᵅᵅ for ethanol (600 mM) lower than that of the rat class IV enzyme (Kᵅᵅ; 2.4 mM; Ala-294) (23) on the basis of a tight active site pocket resulting in a strong ethanol binding. However, the human class IV enzyme with Val-294 exhibits an even lower Kᵅᵅ (37 mM), suggesting that other exchanges also, such as that at residue 116 (Table IV), are important to explain differences in Kᵅᵅ values for ethanol. Position 48 constitutes another position important for the space. The present enzyme has Ser-48 versus Thr in mammalian class Iy and mammalian class IV. Despite the extra space in the active site provided by the smaller volume of Ser, secondary alcohols are poor substrates for the frog enzyme, kᵅᵅ(Kᵅᵅ for n-butanol is 1500-fold the value for 2-butanol (Table II). The present enzyme has Gly-117, like class I, but in contrast to the deletion at position 117 in the mammalian class IV enzymes, where the deletion has been interpreted to open the active site entrance and explain the improved kinetic constants toward retinol in class IV versus class I (14). Nevertheless, retinoids are excellent substrates for the frog enzyme (Table III), better than for class I (8, 9), suggesting the existence of additional structural features in the specificity toward retinoids.

Relationships—The amino acid sequence of the frog stomach enzyme is more closely related to class I (60% identities) than to class IV (58% identities) of the human enzymes. This is surprising because both localization (stomach) and kinetic properties (although not coenzyme specificity) suggest that the new enzyme should be a class IV type of ADH. Although actual values are not differing much between classes I and IV, they are consistent and seem to suggest a class I rather than a class IV relationship. This is also noticeable in the functional positions (Table III) and in the 3'-noncoding 100 nucleotides (the frog NADP(H)-dependent ADH cDNA then exhibits 47% identities with the human class Iy cDNA, but only 22% identities with the human class IV cDNA).

**DISCUSSION**

All previously described medium-chain ADHs from animals are NAD⁺-dependent, and the cofactor specificity is an essential property to define the enzyme and to classify it in the *Enzyme Nomenclature* system (EC 1.1.1.1). The NAD⁺-dependence also indicates that the ADH family in general functions in the oxidative direction of reaction pathways. However, we report here characterization of an amphibian ADH that exhibits unique properties among ADHs expressed in animals because it is the only such ADH with preference for NADP⁺. The distinct cofactor preference must correlate with structural and functional differences. As shown by the modeling and coenzyme docking, the topology of the coenzyme binding site is compatible with the extra phosphate group of NADP⁺. The key change is the existence of Gly-223 instead of Asp-223 found in the NAD⁺-dependent ADHs. In addition, other neighboring residues are more polar (Thr-224, His-225) in the frog enzyme, resulting in a favorable environment for phosphate binding. Additional space and a less hydrophobic environment is created by the substitution 1269A. Deletions at positions 169 and 327 could also be related to the structural adaptations to the new specificity. Consequently, the NAD⁺ preference need not be the consequence of just one or a few changes but appears to be consistent with successive adaptations from the NAD⁺-specific line.

Amphibians, as well as all vertebrates from fish and upwards, have class I and class III ADHs, with properties similar to those of the corresponding homologous enzymes of mammals (4, 15). Two other classes, classes II (30) and IV (31), fairly well characterized in several species, have been deduced also to have an origin in the vertebrate line, with duplicatory emergence of class IV from the class I line (31). However, the frog expresses a seemingly different class IV ADH characteristic of the gastric mucosa, the NAD⁺-dependent enzyme now described.

Several physiological roles have been suggested for the mammalian class IV ADH: a detoxification role, performing a first-pass metabolism of ethanol and other alcohols (6, 7), elimination of aldehydes produced by lipid peroxidation, and metabolism of retinoids (8–10, 12). The presence of an NAD⁺-dependent ADH in amphibians suggests a function in these animals in which an NAD⁺-enzyme is more suitable than an NAD⁺-dependent one. In mammalian tissues (32), including gastric cells (33), NAD⁺ and NADPH are the major nicotinamide cofactor forms, making the NAD⁺/NADPH concentration ratio high and the NADP⁺/NADPH concentration ratio low.

### Table II

| Substrate                  | Kᵅ (mM) | kᵅ (min⁻¹) | kᵅ(Kᵅ/Kᵅ) (mM · min⁻¹) |
|----------------------------|---------|------------|-------------------------|
| Ethanol (pH 10)            | 70      | 1810       | 26                      |
| 2-Butanol (pH 10)          | 170     | 200        | 1.2                     |
| Butanol (pH 10)            | 1.7     | 3000       | 1800                    |
| Pentanol (pH 10)           | 0.22    | 2100       | 9500                    |
| Octanol (pH 10)            | 0.09    | 980        | 11000                   |
| Ethanol                    | 600     | 1000       | 1.7                     |
| Butanol                    | 8.2     | 640        | 70                      |
| Pentanol                   | 1.1     | 510        | 460                     |
| Hexanol                    | 0.35    | 480        | 1400                    |
| t-2-Hexanol                | 0.22    | 530        | 2300                    |
| Octanol                    | 0.15    | 340        | 2000                    |
| Acetaldehyde               | 7       | 1260       | 1800                    |
| Butanol                    | 0.11    | 15600      | 1.410⁶                  |
| Pentanol                   | 0.07    | 14400      | 2.010⁶                  |
| Hexanol                    | 0.04    | 14000      | 3.510⁶                  |
| t-2-Hexanol                | 0.06    | 2840       | 4.710⁵                  |
| Octanol                    | 0.01    | 10700      | 1.110⁶                  |
| m-Nitrobenzaldehyde        | 0.002   | 5870       | 3.010⁶                  |
Results from frog gastric mucosa are also consistent with this notion (34). Thus, the physiological concentrations of the cofactors and the $K_m$ values (Table I) support a role of the frog gastric ADH in aldehyde reduction rather than in alcohol oxidation. The enzyme would be efficient in the reduction of aldehydes generated by lipid peroxidation (Table II). Moreover, as suggested by the high catalytic efficiency with retinoids (Table III), the frog enzyme may also participate in retinoid metabolism. Several pieces of evidence suggest that mammalian class IV is involved in the physiological conversion of retinol to retinal (8–10, 12), which will be finally oxidized to retinoic acid, a molecule of powerful biological regulatory activities (cf. Ref. 12). The cofactor specificity of the frog enzyme and its high activity with all retinal isomers predict that the enzyme would

### Table III

| Substrate     | Frog NADPH-dependent ADH | Human class IV$^a$ |
|---------------|---------------------------|--------------------|
|               | $K_m$ [mM] | $k_{cat}$ [min$^{-1}$] | $k_{cat}/K_m$ [min$^{-1}$] | $k_{cat}/K_m$ [min$^{-1}$] |
| all-trans-Retinol | 0.02       | 30                  | 1500               | 4500               |
| all-trans-Retinal | 0.008      | 270                 | 33750              | 3300               |
| 9-cis-Retinol   | 0.019       | 5.7                 | 300                | 13300              |
| 9-cis-Retal     | 0.009       | 70                  | 7800               | 8980               |
| 13-cis-Retinal  | 0.015       | 63                  | 4200               | NA$^b$             |

$^a$ From Ref. 10.
$^b$ No activity.

### Table IV

| Class  | Source | Substrate-binding positions | Coenzyme-binding positions |
|--------|--------|-----------------------------|---------------------------|
|        |        | Inner | Middle | | 48 | 140 | 141 | 57 | 116 | 117 | 294 | 318 | 47 | 48 | 223 | 224 | 225 | 230 | 259 | 260 | 261 | 269 | 271 | 363 |
| IV-like frog | S L V I M G L V | G S G T H P N G G A R S |
| I frog | S G V L L G V | R S H D L N T D D G I N R |
| Iy human | S F I L I G L V | R S H D L I N A D G G I R R |
| IV human | T F M M I | R T H D L N E G N N I H N |
| IV rat | T F M M I | R T H D L N Q G N T I R Y |

Fig. 3. Coenzyme binding in the amphibian class IV-like model with NADP$^+$ (A) and in the human class I structure with NAD$^+$ (B). Binding of NADP$^+$ to the class IV-like form with Gly-223 (arrow in panel A) fits equally well into the same binding pattern as NAD$^+$ with the class I Asp-223 (arrow in panel B).
function in retinal reduction rather than in retinol oxidation, in contrast to its mammalian class IV counterpart (Table III). The NADPH-dependent ADH, present in the frog stomach but also in the intestines, may have a role in the reduction of retinol produced by the β-carotene cleavage, a main source of retinoids. All-trans-retinal is the storage form of Vitamin A in amphibian eggs (35). Thus, retinal is the most prevalent retinoid in the early Xenopus embryo, in contrast to the situation in birds and mammals where retinol is the most abundant retinoid (36). Retinal levels decrease during amphibian development (37), and retinol is the most important retinoid in adult tissues (35). The distinct metabolism of the regulatory retinoids in amphibians could be provided by the presence of the NADPH-dependent, retinal-specific ADH.

The frog NADP-dependent ADH exhibits a tissue localization similar to that of mammalian class IV. Also, the substrate specificity is similar for both enzymes. However, its structure resembles more the human ADH enzyme of class I than that of class IV. Direct sequence comparisons give no great distinctions, but many structural characteristics of mammalian class IV are not present in the frog enzyme. Thus, positions 57, 94, 116, 117, 260, 261, 309, and 317, that represent the basis of the class IV structural and kinetic features (14, 23, 31, 38), are different in the frog enzyme, suggesting that its biological functions have been reached with a different structural approach. Also, the noncoding sequences differ as mentioned above. Another essential distinction is, of course, the cofactor specificity, which results from additional differences in the structure of the coenzyme binding region. Hence, the present class IV-like frog ADH has "hybrid" or class-mixed properties, and the frog class I enzyme, is also reflected in conventional phylogenetic trees (Fig. 4). It is concluded that the frog NADPH-dependent ADH and class I ADH are derived from a gene duplication. One possibility is that this duplication is independent from the one leading to the class IV line emerging at about the same time and that therefore the present enzyme constitutes a new class ("class VIII") of the vertebrate ADH family. In that case, it may be present but not yet detected also in other vertebrate lines, including perhaps even mammals. Another possibility is that this duplication is identical to that constituting the origin of the class IV line itself and that only rapid evolutionary changes during early post-duplication events have distorted the phylogenetic tree pattern which is based on the assumption of fairly constant evolutionary rates. Independent of which of the two explanations that apply, the ADH type-I/II duplication now detected in amphibians reflects the emergence of a new enzyme, i.e. enzymogenesis, like that previously detected for the ADH I/II (in fish (39)) and ADH II/III (in ratite birds (30)) duplications. In those two cases, however, the new enzymes exhibited changes only in substrate specificity. In contrast, the ADH type-I/II duplication found in amphibians also affects coenzyme specificity.

REFERENCES

1. Jorrvall, H., and Hoig, J.-O. (1995) Alcohol Alcohol 30, 153–161
2. Farre´s, J., Moreno, A., Crosas, B., Peralba, J. M., Allali-Hassam, A., Bowman, K. D., Popov, K. M., Bosron, W. F., and Li, T.-K. (1997) J. Biol. Chem. 272, 7494–7500
3. Yonekawa, A., Abretskey, A., Hsa, L. C., and Chang, C. (1998) Eur. J. Biochem. 251, 549–557
4. Danielsson, O., Atian, S., Luque, T., Hjelmqvist, L., Gonzalez-Duarte, R., and Jorrvall, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 91, 4890–4894
5. Danielsson, O., and Jorrvall, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9247–9251
6. Boleda, M. D., Juliá, P., Moreno, A., and Parés, X. (1989) Arch. Biochem. Biophys. 274, 74–81
7. Haber, P. S., Gentry, T., Mak, K. M., Mirmiran-Yazdy, A. A., Greenstein, R. J., and Lieber, C. S. (1996) Gastroenterology 114, 863–870
8. Boleda, M. D., Sáub, N., Parés, J., and Parés, X. (1993) Arch. Biochem. Biophys. 307, 85–90
9. Yang, Z.-N., Davis, G. J., Hurley, T. D., Stone, C. L., Li, T.-K., and Bosron, W. F. (1994) Alcohol Clin. Exp. Res. 18, 587–591
10. Allali-Hassam, A., Peralba, J. M., Martínez, S., Parés, J., and Parés, X. (1998) FEBS Lett. 436, 362–366
11. Ang, H. L., Delour, L., Hayamizu, T. F., Zgombic-Knight, M., and Duester, G. (1996) J. Biol. Chem. 271, 8526–8534
12. Duester, G. (1996) Biochemistry 35, 12221–12227
13. Moreno, A., Parés, J., Parés, X., Jorrvall, H., and Persson, B. (1996) FEBS Lett. 385, 99–102
14. Xie, P., Parson, S. H., Speckhard, D. C., Bosron, W. F., and Hurley, T. D. (1997) J. Biol. Chem. 272, 18558–18563
15. Cederlund, E., Peralba, J. M., Parés, X., and Jorrvall, H. (1991) Biochemistry 30, 2811–2816
16. Parés, X., and Juliá, P. (1990) Methods Enzymol. 189, 436–441
17. Moreno, A., and Parés, X. (1991) J. Biol. Chem. 266, 1128–1133
18. Lasemilli, U. K. (1970) Nature 227, 680–685
19. Frohman, M. A. (1993) Methods Enzymol. 218, 340–356
20. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
21. Hurley, T. D., Bosron, W. F., Hamilton, J. A., and Amzel, L. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8149–8153
22. Eklund, H., Samama, J.-P., and Jones, T. A. (1987) Biochemistry 26, 5583–5596
23. Farre´s, J., Moreno, A., Crossas, B., Peralba, J. M., Allali-Hassam, A., Hjelmqvist, L., Jorrvall, H., and Parés, X. (1994) Eur. J. Biochem. 224, 549–557
24. Julia, P., Parés, J., and Parés, X. (1987) Eur. J. Biochem. 162, 179–189
25. Fan, L., Lorenzo, J. A., and Flapp, B. V. (1991) Biochemistry 30, 6397–6401
26. Peretz, M., and Burstyn, Y. (1989) Biochemistry 28, 6549–6555
27. Kirkhin, Y., Kalb-Libeck, A. M., Peretz, M., Bogan, O., Burstyn, Y., and Frow, F. (1998) J. Mol. Biol. 278, 987–981
28. Burdette, D. S, Vieille, C., and Zeitkis, J. G. (1996) Biochemistry 35, 115–122
29. Kumar, A., Shen, P.-S., Desouzaes, S., Fohl, J. Bailey, G., and Samuelson, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10186–10192
30. Hjelmqvist, L., Estonius, M., and Jorrvall, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10904–10908
31. Parés, X., Cederlund, E., Moreno, A., Hjelmqvist, L., Parés, J., and Jorrvall, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1893–1897
32. Williamson, D. H., and Bronsan, J. T. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) Vol. 4, pp. 2266–2302, Verlag Chemie/Academic Press, Weinheim, Germany
33. Sarau, H. M., Foley, J., Moonsammy, G., Wiebelhaus, V. D., and Sachs, G. (1975) J. Biol. Chem. 250, 8321–8328
34. Bannister, W. H. (1967) Experientia (Basel) 23, 715–716
35. Scadding, S. R., and Maden, M. (1994) Dev. Biol. 162, 668–671
36. Costaridis, P., Horton, C., Zeitlinger, J., Holder, N., and Maden, M. (1996) Dev. Dyn. 205, 41–53
37. Czech Kraf, J., Schuh, T., Juchau, M. R., and Kineman, D. (1994) Biochem. J. 301, 111–119
38. Kedivsli, N. Y., Bosron, W. F., Stone, C. L., Hurley, T. D., Peggs, C. F., Thomasen, H., Popov, K. M., Carr, L. G., Edenberg, H. J., and Li, T.-K. (1995) J. Biol. Chem. 270, 3625–3630
39. Danielsson, O., Eklund, H., and Jorrvall, H. (1992) Biochemistry 31, 3751–3759