Amphibian Alcohol Dehydrogenase, the Major Frog Liver Enzyme. Relationships to Other Forms and Assessment of an Early Gene Duplication Separating Vertebrate Class I and Class III Alcohol Dehydrogenases†

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ABSTRACT: Submammalian alcohol dehydrogenase structures can be used to evaluate the origins and functions of the different types of the mammalian enzyme. Two avian forms were recently reported, and we now define the major amphibian alcohol dehydrogenase. The enzyme from the liver of the Green frog *Rana perezi* was purified, carboxymethylated, and submitted to amino acid sequence determination by peptide analysis of six different digests. The protein has a 375-residue subunit and is a class I alcohol dehydrogenase, bridging the gap toward the original separation of the classes that are observable in the human alcohol dehydrogenase system. In relation to the human class I enzyme, the amphibian protein has residue identities exactly halfway (68%) between those for the corresponding avian enzyme (74%) and the human class III enzyme (62%), suggesting an origin of the alcohol dehydrogenase classes very early in or close to the evolution of the vertebrate line. This conclusion suggests that these enzyme classes are more universal among animals than previously realized and constitutes the first real assessment of the origin of the duplications leading to the alcohol dehydrogenase classes. Functionally, the amphibian enzyme exhibits properties typical for class I but has an unusually low K_m for ethanol (0.09 mM) and K_i for pyrazole (0.15 μM) at pH 10.0. This correlates with a strictly hydrophobic substrate pocket and one amino acid difference toward the human class I enzyme at the inner part of the pocket. Coenzyme binding is highly similar, while subunit-interacting residues, as in other alcohol dehydrogenases, exhibit several differences. The frog enzyme has a lower p*I* than mammalian class I alcohol dehydrogenases, showing that electrophoretic migration is not a reliable indicator of the class distinction. The p*I* difference is explained by amino acid substitutions resulting in three more negative charges in the frog than in the human class I γ_1 subunit. In conclusion, the amphibian enzyme allows a rough positioning of the divergence of the alcohol dehydrogenase classes, shows that the class I type is widespread in vertebrates, and functionally conforms with greater variations at the substrate-binding than the coenzyme-binding site.

At least six different genes code for mammalian alcohol dehydrogenases and reflect a number of duplications at different levels (Jörnvall et al., 1989; Parés et al., 1990; Yoshida et al., 1991; Moreno & Parés, 1991). In an attempt to ascertain present-day functions of the human enzymes, which are unclear, the class (Vallee & Bazzone, 1983) origins and the original functions are of particular interest. Structural data for the submammalian animal enzymes first characterized (two avian proteins) recently suggested that the segregation of the present-day human enzyme classes I, II, and III occurred in steps at separate time periods, giving the class III enzyme structure partly mixed properties of those of classes I and II (Kaiser et al., 1990; Estonius et al., 1990), but those analyses did not allow further conclusions regarding the origins and separate functions of the human enzymes.

We decided to analyze a still more distantly related submammalian alcohol dehydrogenase and therefore purified and characterized an amphibian alcohol dehydrogenase. The results give further insight into the enzyme relatinships and position for the first time one of the duplications to roughly the time of the vertebrate evolution. In addition, the data add strength to conclusions on coenzyme-binding, substrate-binding, and subunit-interacting residues (Eklund et al., 1990).

Materials and Methods

Enzyme Protein. The major liver alcohol dehydrogenase was purified from the Western Mediterranian Green frog *Rana perezi* by use of DEAE-Sepharose ion-exchange chromatography and AMP–Sepharose affinity chromatography (J. M. Peralba and X. Parés, unpublished results). The final preparation obtained was recovered in a 110-fold purification with a yield of 23%, establishing that alcohol dehydrogenase is one of the common enzymes also in amphibian liver. The pure protein was reduced and ^{14}C-carboxymethylated in the same manner as for analysis of other alcohol dehydrogenases (Kaiser et al., 1990; Estonius et al., 1990).

Enzymology. During purification, enzyme activity was monitored with ethanol as substrate. K_m values for the pure protein and inhibition with pyrazole were measured as described (Julià et al., 1987).

Structural Analysis. Different batches of the carboxymethylated protein were cleaved with proteolytic enzymes and CNBr. Resulting peptides were purified by HPLC exclusion chromatography on Ultropac TSK G2000 SW in 30% acetic acid and by reverse-phase HPLC on C18 (Kaiser et al., 1990;

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Table I: Total Composition of the Major Frog Liver Alcohol Dehydrogenase[a]

residue	acid hydrolysis (molar ratio)	sum of sequence (residues)
Cys	13.6	16
Asp	33.5	21
Asn		12
Thr	24.5	25
Ser	24.7	25
Glu	24.1	20
Gln		2
Pro	17.7	18
Gly	39.0	38
Ala	25.5	24
Val	31.3	35
Met	4.4	5
Ile	25.4	29
Leu	26.5	26
Tyr	7.7	8
Phe	18.1	18
Trp		2
Lys	33.6	33
His	8.0	8
Arg	10.8	10
sum		375

[a] Values given are molar ratios after acid hydrolysis (110 °C, 24 h, 6 M HCl/0.5% phenol) and, for comparison, the sum of the sequence analysis as given in Figure 2.

Estonius et al., 1990) and C4 (Cederlund & Zimmerman, 1990) columns. Compositions were determined with Beckman 121M and Pharmacia Alpha Plus analyzers after acid hydrolysis for 24 h at 110 °C with 6 M HCl/0.5% phenol. Sequencer degradations were performed with an Applied Biosystems 477A sequencer and an on-line phenylthiohydantoin analyzer 120, an Applied Biosystems 470A sequencer with separate HPLC identification as described (Kaiser et al., 1988), or a Milligen Prosequencer solid-phase instrument with an on-line HPLC, allowing utilization of different methods for special problems. Enzyme digestions were carried out in 0.1 M ammonium bicarbonate with protease to substrate ratios of 1:10–1:100 and digestion times of 4–20 h at 37 °C.

For interpretation of differences found, known relationships within (Eklund et al., 1987) and between (Eklund et al., 1990) the human enzyme classes were utilized.

Results

Determination of the Primary Structure. The major liver alcohol dehydrogenase was purified from the frog *Rana perezi* by a two-step chromatographic procedure. The protein was ^{14}C-carboxymethylated (Estonius et al., 1990) and submitted to sequence analysis. Direct sequencer degradation failed to yield any results indicating that the protein chains are blocked as in most other alcohol dehydrogenases (Egestad et al., 1990).

The entire structure was determined by separate analysis of six different digests, one with CNBr and five with different enzymes, as summarized in Figure 1. Because of large hydrophobic fragments and nonstoichiometric cleavages at Met–Thr bonds, only some of the CNBr fragments were recovered. Similarly, it was necessary to analyze only a few of the peptides from the complex peptic and chymotryptic digests. Peptides were purified by reverse-phase HPLC on C18 columns or, for fractions with large peptides, C4 columns, directly or after initial separation by exclusion HPLC on Ultropac TSK G2000 SW. In this manner, peptides covering all regions of the protein were recovered and analyzed.

The steps of all peptide analyses are summarized in Figure 1 and the results given in Figure 2, allowing an unambiguous

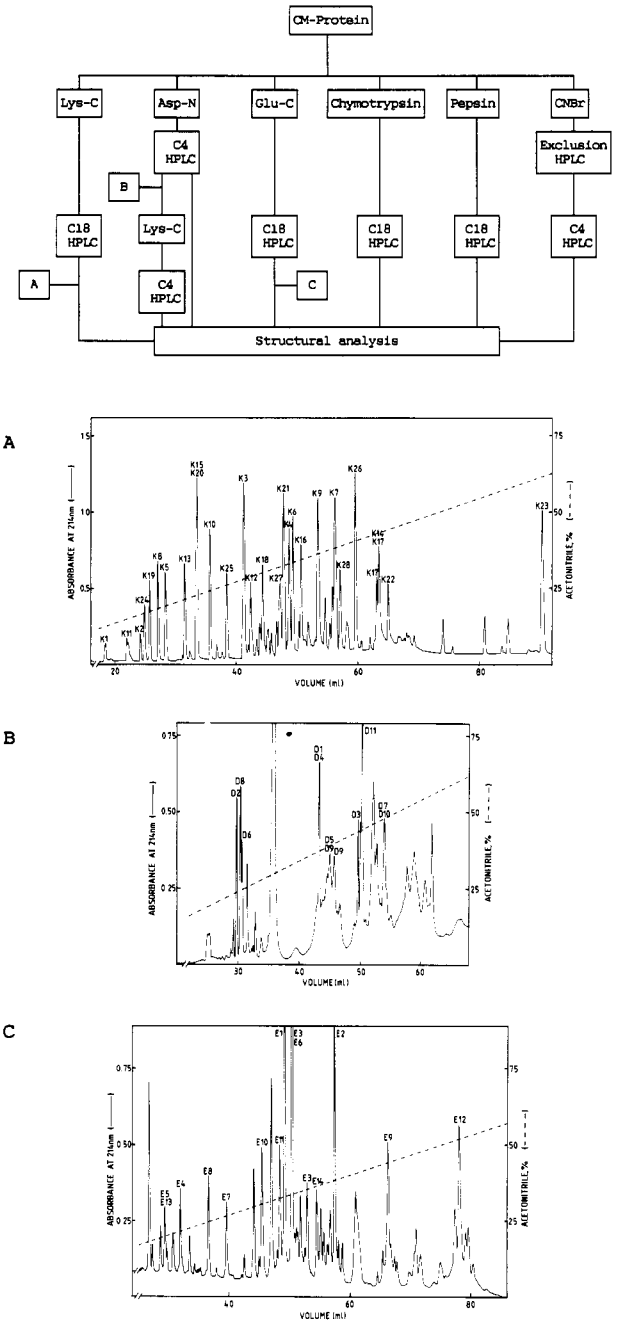

FIGURE 1: Flow schemes (I) summarizing peptide generations, purifications (II), and analyses. Peptide designations in II are as given in Figure 2. Chromatograms show elution profiles upon reverse-phase HPLC with a gradient of acetonitrile and on columns of C4 or C18, as indicated.

determination of the entire structure. Total compositions of pure peptides (supplemental table; see paragraph at end of paper regarding supplementary material), fully support the sequence data, as does the total composition of the whole protein (Table I).

Regions frequently causing large analytical problems with alcohol dehydrogenases are parts of the internal segments exhibiting substantial hydrophobicity (Kaiser et al., 1990; Estonius et al., 1990). In the present case, special advantage was taken of peptic and Asp-protease cleavages and of the fact that a solid-phase sequencer (Milligen Prosequencer) allowed reliable identification of unstable residues at late cycles. Thus, although the initial yield was lower than in other sequencers (sometimes down to 10%), the part of the peptide that was degraded was observed without extensive background; once

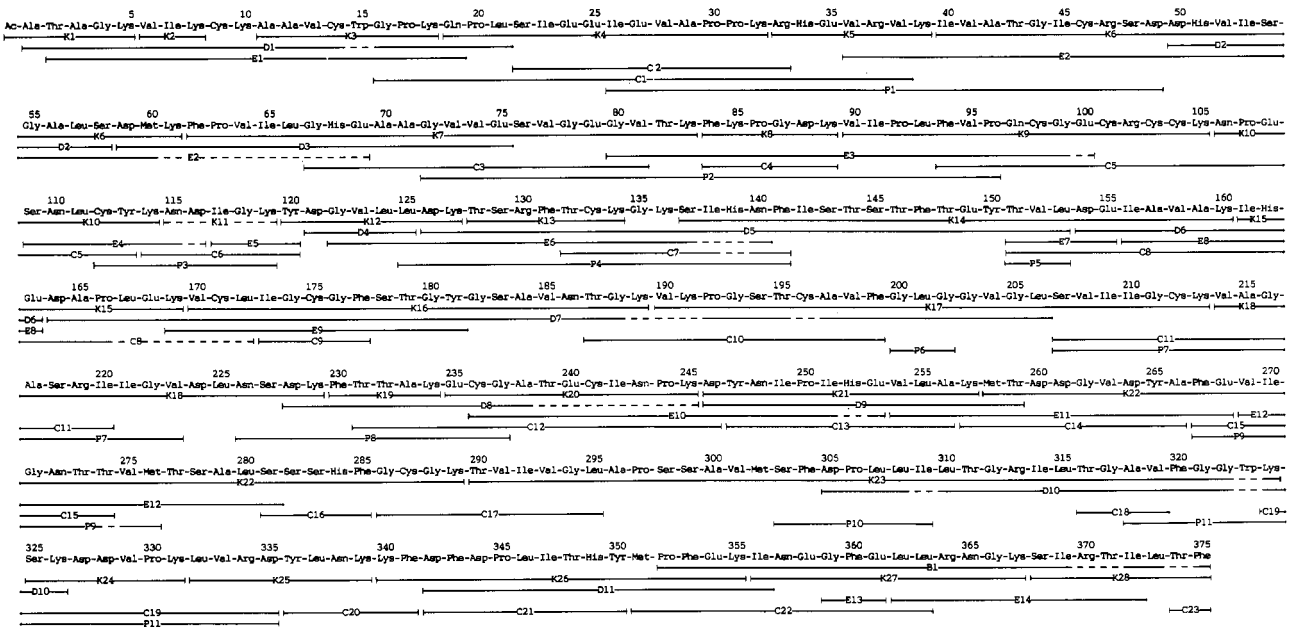

FIGURE 2: Primary structure of the major frog liver alcohol dehydrogenase and positions of all peptides analyzed. Solid lines indicate peptide parts proven by sequencer degradations and dashed lines remaining parts proven by total compositions only. Peptides derived from cleavages with the Lys-specific protease are given by K, the Glu-specific protease by E, the Asp-specific protease by D, chymotrypsin by C, pepsin by P, and CNBr by M, in all cases followed by a number to indicate the relative order of fragments. Positions of these peptides in the elution profiles during purification are given by the same nomenclature in Figure 1.

coupled, repetitive yields approached 98%, allowing distinction of Ser, Arg, Trp, Thr, and His from background values at very late positions (40–60 or more) in the low picomole range. All Lys-protease cleavage products were recovered except for two nonimportant dipeptides (positions 9-10 and 135-136). The peptides were degraded successfully and gave clear results also for repetitive hydroxy residues and extended degradations (for example, peptides K14, K22, and K23; Figure 2).

The blocked N-terminus was recovered in peptides from Lys-specific and Asp-specific cleavages. The blocking group was determined by fast atom bombardment mass spectrometry as reported separately (Egestad et al., 1990). Peptide K1 was deblocked by prior treatment with acid (10 M HCl, 6 h, room temperature) and in one case also by prolonged precycling in the sequencer, resulting in roughly 10% deblocking, presumably because of the repeated TFA treatments [cf. Wellner et al. (1990)]. The C-terminal peptides were recovered in three digests and analyzed as shown in Figure 2. The complete structure consists of 375 residues, without detectable microheterogeneity or analytical ambiguities.

Comparison with Other Dehydrogenases. The structure obtained is homologous to those of other liver alcohol dehydrogenases but related fairly distantly, as expected from the wide separation between amphibians and other species from which the enzyme has been characterized. Toward the mammalian enzymes, the similarities are greatest with class I (Table II), establishing that the major frog liver enzyme also belongs to the class I type. This is supported further by the functional properties (below).

The amphibian enzyme gives new data on the separation of the mammalian enzyme classes. Thus, as shown by the alignment in Figure 3 and summarized by the values in Figure 4, the frog enzyme exhibits sequence differences almost exactly in between those for the human enzyme classes and the latter versus characterized submammalian enzymes of avian origin. This stands in sharp contrast to all other animal alcohol dehydrogenases studied previously, where intermammalian enzyme variations are considerably fewer than the class differences (Jörnvall et al., 1989) and where the avian enzymes just

Table II: Overall Relationships of the Frog Enzyme Now Analyzed, the Three Classes of Mammalian Alcohol Dehydrogenases Characterized, and the Only Other Nonmammalian Animal Alcohol Dehydrogenase Characterized (Avian Form)[a]

major frog enzyme subunit	residue identity
major quail	262 (70%)
human class I	255 (68%)
human class II	212 (56%)
human class III	230 (61%)

[a] Regarding the three classes, the human forms are those utilized for the comparison. Other species variants affect values only marginally. Regarding class I, the β_1 form is the one listed, although again differences are marginal (the human class I γ_1 subunit exhibits 258 identities with the frog enzyme, β_1 exhibits 256, and α exhibits 255). Regarding the avian form, two species representatives are known, chicken (Estonius et al., 1990) and quail (Kaiser et al., 1990). The one now listed is the quail enzyme, although here, too, species influences are marginal (quail enzyme exhibits 262 identities with the frog enzyme versus 258 for the chicken enzyme).

initiated the distinction of sublevels in the duplications behind the mammalian classes (Kaiser et al., 1990; Estonius et al., 1990) but failed to approach variations similar to those between the classes. Still, in the present case, the frog/human differences are smaller than the human interclass differences, but not more than to allow reasonable estimates for original class separations.

Thus, as shown in Table III, the present results suggest a separation of the lines leading to the class I and III enzymes at a time about 430 million years ago, which approaches early events in vertebrate evolution (Minkoff, 1983; Dayhoff, 1972). Of course, the time estimate is still highly approximate. Thus, mutational distances have been corrected [for multiple hits; footnote *a* of Table III; cf Dayhoff (1972)], samples are small, species origins are not well-defined [footnote *c* of Table III; cf. Minkoff (1983) and Fernholm et al. (1989)], and the evolutionary rates for each protein need not be constant. Nevertheless, minimal and maximal estimates for the different branches give similar end values for the duplications, and evolutionary rates have been fairly distinct for each alcohol dehydrogenase class in more recent periods (Kaiser et al.,

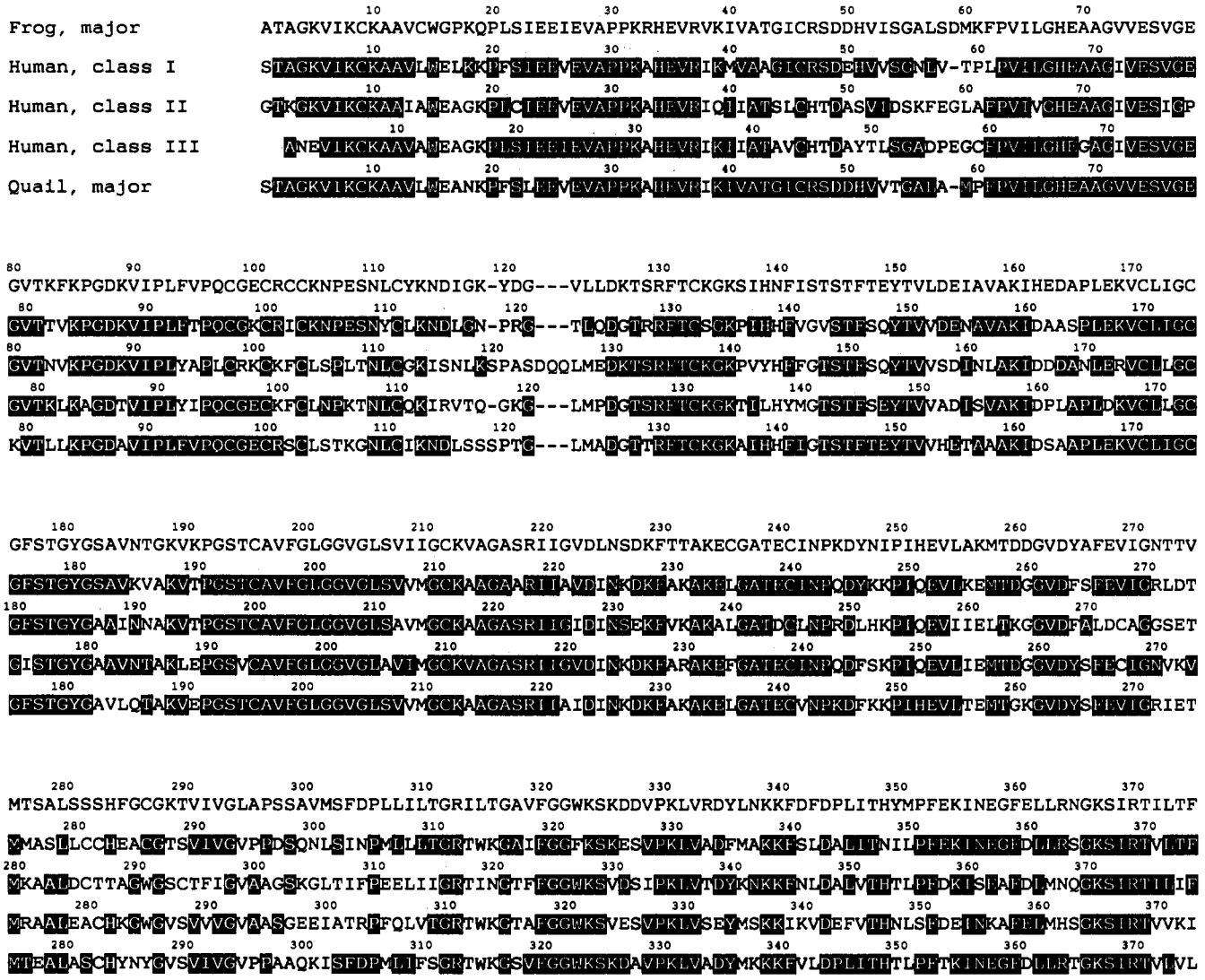

FIGURE 3: Alignment of the major frog liver enzyme with the three classes of mammalian enzyme characterized and with the other submammalian enzyme type (avian). In the cases of species variants, the human forms and the most frog-like of the avian ones have been chosen although as pointed out in the legend of Table II differences are marginal. For the human class I, the γ_1 subunit is listed; for the avian enzyme, the major quail form is listed.

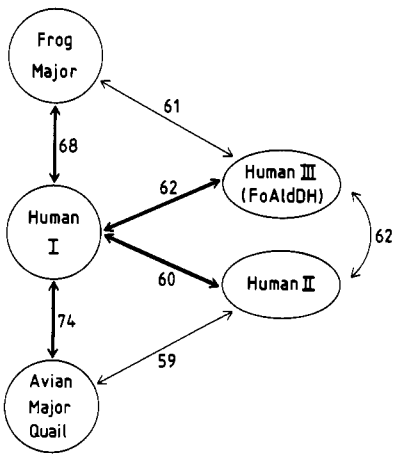

FIGURE 4: Overall relationships between the mammalian enzyme classes and the two known types of submammalian animal alcohol dehydrogenase, an avian form (below) and an amphibian form (top, present work). Data for the human classes form Jörnvall et al. (1990), the quail enzyme from Kaiser et al. (1990), and the frog enzyme from Figure 2. Values show extent of residue identities in percent. FoAldDH, glutathione-dependent formaldehyde dehydrogenase, which is identical with class III alcohol dehydrogenase (Koivusalo et al., 1989).

1989). The present calculation may therefore provide a realistic estimate that the duplication separating the lines leading to present-day mammalian classes I and III may have occurred close to the origin of vertebrates or early in their radiation. This places the alcohol dehydrogenase gene duplications more distantly back than commonly anticipated and suggests that enzymes corresponding to the classes will be found to be widespread in most or all vertebrates. This conclusion is in agreement with the conclusions from comparisons of known alcohol dehydrogenase structures (Jörnvall et al., 1987) and their genes (Yokoyama et al., 1990). It also adds further strength to the notion derived from the speed of evolutionary changes (Kaiser et al., 1989) and direct measurement of substrate specificities (Koivusalo et al., 1989) that the classes represent separate enzymes and do not just constitute isozyme forms.

Functional Properties. Enzymatic properties of the major frog liver enzyme resemble those of the class I mammalian enzymes. Thus, there is considerable activity with ethanol as substrate, and the K_m values are low, 0.09 mM at pH 10 and 0.1 mM at pH 7.5. Similarly, the enzyme is strongly inhibited by pyrazole, with a K_i value of 0.15 μM at pH 10.0. The major frog liver enzyme is therefore a class I enzyme, defined from

Table III: Estimates of Relationships of Two Major Vertebrate Alcohol Dehydrogenases of Class I and the Human Class III Enzyme versus the Human Class I Enzyme

enzyme compared to the human class I form	residue identities (Figure 4) (%)	observed differences (%)	estimated mutational differences (%)[a]	emergence (MY ago)[b]
avian	74	26	33	140, 250[c]
amphibian	68	32	43	300,[c] 350[c]
human class III	62	38	54	X[d]

[a] Calculated from observed differences by correction for multiple mutational hits to reflect actual mutations and hence evolutionary distances (Dayhoff, 1972). [b] First time gives approximate emergence (MY, million years) from paleontological records (birds and frogs, respectively) and second time the estimated time of separation of their ancestors from those leading to the mammalian line; the latter time is more distant than the former because of coevolution with other lines (reptiles in the case of birds) before bird and frog emergence (Dayhoff, 1972; Minkoff, 1983; Fernholm et al., 1989). X is the corresponding calculated time for the class I/class III separation, assuming linear relationships between time and the corrected extent of mutations. [c] Times are approximate and difficult to assess from different estimates. ±20–50 MY appears possible, especially for the avian separation and the frog emergence; even larger deviations are possible for the extrapolated value X. [d] Calculation for class I/class III separation, X: ~430 MY ago.

both enzymatic properties and structural properties. However, the electrophoretic behavior of the major frog liver enzyme differs from that of class I mammalian enzymes. Thus, the p*I* of the frog enzyme is about 7.0, whereas that of the mammalian class I enzymes is usually basic. This difference corresponds to three additional negative charges in the frog enzyme compared to the class I human γ_1 form.

The functional properties are explained directly by the structures of the coenzyme-binding site and the substrate-binding pocket, as further discussed below.

Discussion

Structure Deduced. The primary structure was determined by analysis of 89 different peptides from six proteolytic digests. All overlapping regions were recovered, no ambiguities were detected, and the end results agree well with the total composition of the whole protein (Table I). In particular, recoveries of the peptides from the cleavages with the Lys-specific protease and extended degradations in a solid-phase sequencer were successful. Consequently, the complete structure deduced is likely to represent a reliable structure of the major frog liver alcohol dehydrogenase.

Methodologically, a deacetylation of N-terminally blocked peptides was noticed upon prolonged sequencer precycling. It resembles the effects of the acid treatments recommended in separate peptide handling (Wellner et al., 1990) and may prove generally useful by allowing direct analytical access to blocked peptides.

Relationships. The enzyme clearly is a class I type of alcohol dehydrogenase, with functional class-distinguishing residues (below) of the class I type. The overall similarities are greatest with class I of the mammalian system (Table II and Figure 4) with segments strictly homologous to the class I enzyme (Figure 3).

As concluded above, the present analysis traces one of the ancestral duplications in the alcohol dehydrogenase system to roughly the vertebrate divergence (Table III). In fact, this poses the possibility that the class I function may have emerged with the evolution of the liver. The class III function (glutathione-dependent formaldehyde dehydrogenase; Koivusalo et al., 1989) is present in still more primitive species (Uotila & Koivusalo, 1989) and has a slower evolutionary rate (Kaiser et al., 1989), while the class II structure also appears more distantly related (Kaiser et al., 1990). In any event, apart from giving a first, approximate timing of one class duplication, the present results establish that the class I function has been of continuous importance, since the class I enzyme is a major liver alcohol dehydrogenase in both mammals and amphibians.

Table IV: Residues Corresponding to Those Lining the Substrate-Binding and Coenzyme-Binding Sites of the Human Enzyme Classes

position	enzyme: frog major	quail major	human Iγ_1	human II	human III
(A) Substrate-Binding Site (All Exchanged)					
48i	Ser	Ser	Ser	Thr	Thr
93i	Phe	Phe	Phe	Tyr	Tyr
140i	Phe	Phe	Phe	Phe	Tyr
141i	Ile	Ile	Val	Phe	Met
57m	Leu	Leu	Leu	Phe	Asp
116m	Ile	Leu	Leu	Asn	Val
294m	Leu	Val	Val	Val	Val
318m	Val	Val	Ile	Phe	Ala
110o	Leu	Leu	Tyr	Leu	Leu
306o	Leu	Met	Met	Glu	Phe
309o	Leu	Phe	Leu	Ile	Val
identities with frog	(11)	7	5	2	1
(B) Coenzyme-Binding Site (178, 203, 223, 228, and 369 Identical in All and Not Listed)					
47	Arg	Arg	Arg	His	His
48	Ser	Ser	Ser	Thr	Thr
51	His	His	His	Ser	Tyr
224	Leu	Ile	Ile	Ile	Ile
269	Ile	Ile	Ile	Ala	Ile
271	Asn	Arg	Arg	Gly	Asn
identities with frog (including five unchanged)	(11)	9	9	5	7

[a] Data for the frog enzyme from Figure 2, for the major quail enzyme from Kaiser et al. (1990), and for the human enzyme classes and for the conformational interpretations from Eklund et al. (1990). All positional numbers refer to the mammalian class I type. i indicates internal part of the substrate pocket; m, middle part; o, outer part.

Functional Correlations. Residues at the substrate-binding and coenzyme-binding sites are of particular interest. A comparison with the three mammalian classes and the other submammalian animal alcohol dehydrogenase characterized (avian) reveals the following properties (Table IV):

First, overall differences in substrate binding are much greater than in coenzyme binding. About half of all coenzyme-interacting residues are conserved in all these enzymes (Table IVB) independent of species and class, whereas variation is much larger at the substrate-binding site (Table IVA). This pattern was noticed already in the mammalian enzymes (Eklund et al., 1990) but is now discernible also in the submammalian forms, defining a general property of the enzyme. In fact, the coenzyme-interacting residues in the amphibian enzyme are all typical for those of alcohol dehydrogenases, and the crucial residues at positions 47, 48, and 51 are of clear class I type (Table IV).

Second, the variability at the substrate-binding site gives the frog enzyme a substrate pocket highly typical for class I (Table IVA). Almost no residue is the same as those in the class II and III enzymes, clearly showing that the functional properties of the major amphibian enzyme correlate with the assignment drawn from the alignment at large (Figure 4). The class I structural type explains the considerable activity toward ethanol and the high sensitivity toward pyrazole actually determined by direct analysis.

Finally, the residue changes that do occur correlate well with the enzymatic properties observed. Thus, the substrate-binding pocket of the amphibian enzyme is extremely hydrophobic, even more so than the classical class I γ_1 type of human enzyme, because the amphibian enzyme has Leu instead of Tyr at position 110 and two larger residues (Ile instead of Val at position 141 and Leu instead of Val at position 294). Although to some extent this may be compensated by Val instead of Ile at position 318, the overall effect of the changes is an extremely hydrophobic and somewhat space-restricted substrate-binding site. Since large and polar substrate pockets with a resultant presence of water molecules have been suggested to constitute the structural explanation for the largely complete lack of ethanol dehydrogenase activity of the class III enzyme (Juliâ et al., 1988), the properties of the amphibian substrate-binding pocket now observed correlate well with the comparatively tight binding of ethanol and pyrazole revealed by the low K_m and K_i values (above).

In summary, the analysis of the major amphibian enzyme establishes the common occurrence of class I alcohol dehydrogenases in the vertebrate system, clearly distinguishes the different classes as highly separate entities, suggests an estimate for the time of divergence of the class I type, and gives further functional correlations between residue changes and enzymatic properties, illustrating the extensive variability at the substrate-binding site.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Table of the total compositions of relevant peptides for the subunit structure deduced (1 page). Ordering information is given on any current masthead page.

Registry No. Alcohol dehydrogenase, 9031-72-5; ethanol, 64-17-5; pyrazole, 288-13-1; alcohol dehydrogenase I (*Rana perezi* liver reduced), 131635-25-1.

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