The Major Piscine Liver Alcohol Dehydrogenase Has Class-Mixed Properties in Relation to Mammalian Alcohol Dehydrogenases of Classes I and III†

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ABSTRACT: The major alcohol dehydrogenase of cod liver has been purified, enzymatically characterized, and structurally analyzed in order to establish original functions and relationships among the deviating classes of the enzyme in mammalian tissues. Interestingly, the cod enzyme exhibits mixed properties—many positional identities with a class III protein, but functionally a class I enzyme—blurring the distinction among the classes of alcohol dehydrogenase. The two domain interfaces, affected by movements upon coenzyme binding, both exhibit substitutions in a manner thus far unique to the cod enzyme. In contrast, coenzyme-binding residues are highly conserved. At the active site, inner and outer parts of the substrate pocket show different extents of amino acid replacement. In total, no less than 7–10 residues of 11 in the substrate binding pocket differ from those of all the mammalian classes, explaining the substrate specificities. However, the inner part of the substrate pocket is very similar to that of the class I enzymes, which is compatible with the observed characteristics of the cod enzyme: ethanol is an excellent substrate (K_m = 1.2 mM) and 4-methylpyrazole is a strong inhibitor (K_i = 0.1 μM). These values are about as low as those typical for the ethanol-active class I mammalian enzyme and do not at all resemble those for class III, for which ethanol is hardly a substrate and pyrazole is hardly an inhibitor. Further out in the substrate pocket, several residues differ from the mammalian classes, affecting large substrates. Exchanges at positions 57 (His in the cod enzyme, Asp in human class III), 294 (Trp versus Val, but partly compensated by a gap versus Ala at 295), and 318 (Met versus Ala) narrow the middle part of the substrate-binding pocket, giving it a restricted shape, like that of the class I enzyme, and unlike that of the distally wide pocket of the class III enzyme. Overall, the piscine form exhibits both divergence within the enzyme family, with large structural changes at the active site, and apparent conservation of overall interactions, with hybrid properties illustrating class evolution and functional interactions.

Alcohol dehydrogenase constitutes a complex enzyme system. Although the major mammalian liver enzyme was early purified (Bonnichsen & Wassén, 1948) and has been much studied, novel findings have been constantly reported. Multiple forms are derived from a series of gene duplications at separate levels (Jörnvall, 1985). Three classes of the liver enzyme originally defined in the human (Vallee & Bazzone, 1983) are of widespread vertebrate occurrence, representing separate enzymes (Kaiser et al., 1989; Koivusalo et al., 1989) with widely different substrate pockets (Eklund et al., 1990). Intraclass isozymes also occur and have been structurally analyzed (Jörnvall et al., 1984; Höög et al., 1986; Eklund et al., 1987; Burnell et al., 1987). The proteins, cDNAs, and to a large extent genes have been isolated from human and other species, outlining the family, while parallel families of other types contain further alcohol dehydrogenases and additional enzymes (review in Jörnvall et al., 1991). An estimate of duplications explaining present-day human liver alcohol dehydrogenases dates a class I/III separation at early vertebrate evolution (Cederlund et al., 1991). Similarly, a mammalian stomach enzyme and yet another mammalian enzyme have been structurally defined as additional classes (Parés et al., 1990; Moreno & Parés, 1991; Yasunami et al., 1991; Yoshida et al., 1991), and corresponding complexities also apply to the metabolically linked aldehyde dehydrogenase (Jörnvall et al., 1991; Yin et al., 1991).

This insight emphasizes two questions motivating studies of the submammalian alcohol dehydrogenases. One concerns the functional consequences of all these enzymes. We now have not only one enzyme without a strictly defined substrate (classical alcohol dehydrogenase) but a whole system of families, classes, and isozymes. For elucidation of metabolic roles, knowledge of original functions would be helpful. The other question concerns modes of action. Thus, all these enzymes are structurally related, where a few amino acid replacements against a background of many amino acid differences give separate properties. It is important to know which exchanges are critical and how they exert their influences, causing enzyme divergence after the duplications. These and other questions are highlighted by our characterization of a piscine alcohol dehydrogenase.

Unexpectedly, the results show the existence of an alcohol dehydrogenase with "mixed" properties, being a functional class I enzyme (a good ethanol dehydrogenase, well inhibited by pyrazole) but a structural class III representative (regarding overall residue relationships). This phenomenon has impli-

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cations for proteins in general, illustrating transfer of one functional type into another.

Materials and Methods

Materials and Basic Methods. Liver from cod, *Gadus morhua*, Baltic origin (i.e., *Gadus callarius*), was obtained from a local fishery and stored at −70 °C until used. DEAE-Sepharose and Mono Q were from Kabi Pharmacia (Uppsala, Sweden). CapGapp-Sepharose was a gift from M. Zeppezauer (University of Saarbrücken, FRG). Enzyme activity was monitored by NADH formation in glycine/NaOH, pH 10.0, with ethanol as substrate (Dalziel, 1957), and protein amounts during purification were quantitated by the Bradford (1976) method. Purity was evaluated by SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) and isoelectric focusing on a Phast-system (Kabi Pharmacia, Uppsala, Sweden) at pH 3–9, utilizing Coomassie Brilliant Blue for protein staining and Nitro Blue tetrazolium/phenazine methosulfate for activity staining. It was also shown by FPLC on Mono Q in 10 mM Tris/HCl, pH 8.3, with a gradient of 0–0.5 M NaCl. Enzymatic parameters were determined as described (Bosron et al., 1983; Wagner et al., 1983) in 0.1 M glycine/NaOH, pH 10.0.

Purification of Cod Liver Alcohol Dehydrogenase. The major cod liver alcohol dehydrogenase was purified by methods similar to those for other alcohol dehydrogenases (Lange & Vallee, 1976; Crabb et al., 1983; Estonius et al., 1990), utilizing initial extraction and centrifugation to remove debris and lipids, a column step of ion-exchange chromatography on DEAE-Sepharose, and a step of affinity chromatography on CapGapp-Sepharose. Preparations started with 200 g of pooled cod liver, homogenized (MSE ATO mix) at 4 °C in 10 mM Tris/HCl, pH 8.4, and 0.1 mM dithioerythritol. The lipid of cod liver necessitated special filtration and careful centrifugation. Much debris and lipids were removed by filtration through filter paper, centrifugation of the solution at 27000*g* for 1 h, dialysis of the supernatant against 3 × 5 L of 10 mM Tris/HCl and 0.1 mM dithioerythritol, pH 8.4, and application to a column (2 × 30 cm) of DEAE-Sepharose, fast flow, equilibrated with the same buffer. After the column was washed with equilibration buffer, the enzyme was eluted with a linear gradient of NaCl (0–300 mM) in the buffer. Fractions with the highest specific activity were pooled and concentrated to 20 mL in an Amicon cell with a PM 10 filter. This solution was dialyzed against 4 × 1 L of 50 mM sodium phosphate and 0.1 mM dithioerythritol, pH 7.5, after which NAD was added to a final concentration of 2 mM and the enzyme was applied to a column (1 × 7 cm) of 4-[3-[*N*-(6-aminocaproyl)-amino]propyl]pyrazole–Sepharose (CapGapp-Sepharose), equilibrated with 50 mM sodium phosphate, 0.1 mM dithioerythritol, and 2 mM NAD^+, pH 7.5. After extensive washing, the enzyme was eluted with 0.5 M ethanol, 50 mM sodium phosphate, and 0.1 mM dithioerythritol, pH 7.5.

Protein Analysis. The pure protein was reduced and ^{14}C-carboxymethylated in 8 M urea, 0.4 M Tris/HCl, pH 8.1, and 2 mM EDTA, as described for other submammalian alcohol dehydrogenases (Estonius et al., 1990; Cederlund et al., 1991). Different batches of the carboxymethylated protein were cleaved with trypsin, *Lysobacter* Lys-C protease, staphylococcal Glu-C protease, and *Pseudomonas* Asp-N protease (all from Boehringer-Mannheim) at 37 °C in 0.1 M ammonium bicarbonate and 0.9 M urea, in most cases for 4 h at protease:substrate ratios of 1:50, but with the Asp-N protease for 24 h at 1:150. CNBr cleavage was carried out in 70% formic acid for 24 h at room temperature. Peptides from the enzymatic digests were purified by direct reverse-phase HPLC on C18 (Estonius et al., 1990; Cederlund et al., 1991) and C4 (Cederlund & Zimmerman, 1990). CNBr fragments were prefractionated on Sephadex G-50 in 30% acetic acid (Jeffery et al., 1984) before C18 reverse-phase HPLC.

Table I: Purification of the Major Cod Liver Alcohol Dehydrogenase[a]

step	total protein (mg)	enzyme activity (units)	specific activity (units/mg)
crude extract	3500	72	0.02
DEAE-Sepharose	120	56	0.47
CapGapp-Sepharose	11	38	3.4

[a] Activities refer to ethanol as substrate, measured at pH 10 (Dalziel, 1957).

Amino acid compositions were determined with Beckman 121M and Pharmacia alpha Plus analyzers after hydrolysis with 6 M HCl/0.5% phenol in evacuated tubes for 24 h at 110 °C. For sequence analysis, peptides were degraded with ABI 470A sequencers, utilizing an on-line 120A analyzer or a separate HPLC system as described (Kaiser et al., 1988), or degraded in a MilliGen Prosequencer 6600 with on-line detection.

Structural Comparisons and Computer Graphics Modeling. The primary structure of the cod liver enzyme was compared with those of two avian alcohol dehydrogenases (Kaiser et al., 1990; Estonius et al., 1990), the frog liver enzyme (Cederlund et al., 1991), the class III enzymes (Kaiser et al., 1988, 1989; Julià et al., 1988), and alcohol dehydrogenases earlier summarized (Jörnvall et al., 1987). Three-dimensional relationships of the cod liver enzyme were evaluated by fitting the piscine structure to the crystallographically determined holoenzyme structure of the horse class I E-type liver enzyme (Eklund et al., 1984) using the FRODO program (Jones, 1985; Jones & Thirup, 1986) as described before for relationships within (Eklund et al., 1987) and between (Eklund et al., 1990) the human enzyme classes. Molecular surface areas of the substrate clefts were obtained using the MS program (Connolly et al., 1983).

Results

Enzyme Purification. The major liver alcohol dehydrogenase from *G. callarius* was purified by extraction in aqueous medium, centrifugation to remove particulate material and lipids, DEAE-Sepharose ion-exchange chromatography, and affinity chromatography on CapGapp-Sepharose, as described in the Materials and Methods section. All steps were carried out at 4 °C and, critically, with all buffers containing 0.1 mM dithioerythritol to prevent inactivation of the enzyme.

The first purification step, utilizing DEAE-Sepharose, was performed at a relatively high pH (8.4) in order to bind the enzyme to the ion-exchange resin for separation from unbound lipids of the crude extract. The enzyme was eluted with a linear NaCl gradient and purified to homogeneity in a single step of CapGapp-Sepharose chromatography (Lange & Vallee, 1976). The preparation obtained has a specific activity of 3.4 units/mg and is recovered in a 170-fold purification with a yield of 53% (Table I), establishing alcohol dehydrogenase as a common enzyme also in piscine liver. The freshly purified enzyme gave a single band on SDS/polyacrylamide gel electrophoresis and protein staining and one band after isoelectric focusing and activity staining with Nitro Blue tetrazolium/phenazine methosulfate. It also gave a single, homogeneous peak upon FPLC on Mono Q (supplementary Figure 1). With time, additional anodic bands were noticed upon electrophoresis under nondenaturing conditions in the same manner as

Table II: Enzymatic Parameters for the Major Cod Liver Alcohol Dehydrogenase[a]

property	cod	human I	human II	human III
ethanol				
K_m (mM)	1.2	1.1 (1.2)	120	NS
k_{cat} (min^{-1})	290	140 (35)	470	
k_{cat}/K_m (mM^{-1} min^{-1})	240	130 (29)	4	
pentanol				
K_m (mM)	0.17	0.16 (0.15)	0.09	22
k_{cat} (min^{-1})	400	370 (38)	480	240
k_{cat}/K_m (mM^{-1} min^{-1})	2400	2300 (250)	5300	11
cyclohexanol				
K_m (mM)	4.3	0.042 (14.5)	210	NA
k_{cat} (min^{-1})	8	130 (14)	35	
k_{cat}/K_m (mM^{-1} min^{-1})	2	3100 (1)		
NAD^+				
K_m for (mM)	0.04	0.040 (0.037)		0.070
K_i for 4-methylpyrazole (μM)	0.1	0.3	400	≫50000

[a] Data for the cod liver enzyme are from measurements now performed in 0.1 M glycine/NaOH, pH 10.0, at 25 °C; those for remaining enzymes are at identical conditions from Eklund et al. (1990). NS, nonsaturable; NA, no activity. Human I refers to the $\gamma_1\gamma_1$ isozyme, within parentheses to the $\beta_1\beta_1$ isozyme, and regarding the K_i value to the $\beta_1\gamma_1$ isozyme. Clearly, the cod enzyme is of the functional type class I, not class III.

for other class I alcohol dehydrogenases ("primed" fractions; cf. Theorell, 1970).

Enzymatic Properties. K_m and K_{cat} values with ethanol, pentanol, cyclohexanol, and NAD^+ and inhibition with 4-methylpyrazole were determined in 0.1 M glycine/NaOH, pH 10.0 (Table II). The K_m values obtained for ethanol and pentanol and the K_i value for 4-methylpyrazole are in the same low range as those reported for mammalian class I alcohol dehydrogenases, including the human $\gamma\gamma$ enzyme and the horse EE enzyme, both typically class I with considerable ethanol activity and pyrazole sensitivity. Consequently, affinity chromatography on CapGapp-Sepharose, specifically developed (Lange & Vallee, 1976) for the pyrazole-sensitive mammalian class I enzymes, is excellent for the purification of the major cod liver enzyme (Table I).

Primary Structure. The amino acid sequence of cod liver alcohol dehydrogenase was determined by peptide analysis, utilizing five separate cleavages of the carboxymethylated enzyme, one with CNBr and four with different proteases as outlined in Figure 1. Essentially, the structure obtained (Figure 2) is based on analysis of 24 fragments from the digest with Lys-specific protease, supplemented with analysis of 7 CNBr fragments, 14 peptides from cleavages with the Glu-specific protease, and 12 from that with the Asp-specific protease. Large hydrophobic segments and low-yield cleavages at two Met–Ser and Met–Thr bonds complicated the analyses of the CNBr fragments. Direct sequence analysis of the largest CNBr fragment did not yield any results, indicating a blocked N-terminus as in most other alcohol dehydrogenases (Egestad et al., 1990). Only a few of the tryptic peptides were analyzed since at that stage much of the structure of the cod liver enzyme was already known from other digests. The total composition from acid hydrolysis of the whole protein (supplementary Table I) and relevant peptides (supplementary Table II) supports the data of the sequence analysis. A few segments presented special problems and are further detailed below.

The N-terminal blocking group was identified as acetyl by fast atom bombardment mass spectrometry of a peptide from Lys-specific cleavage (Figure 2), as separately reported (Egestad et al., 1990). Peptide E1 was treated with 10 M HCl for 3 h at room temperature to give deacylation and partial fragmentation. Direct sequence analysis of the mixture showed that two major fragments had been produced, desacetyl-E1 and des-AcAla-E1, the sequences of which could be clearly

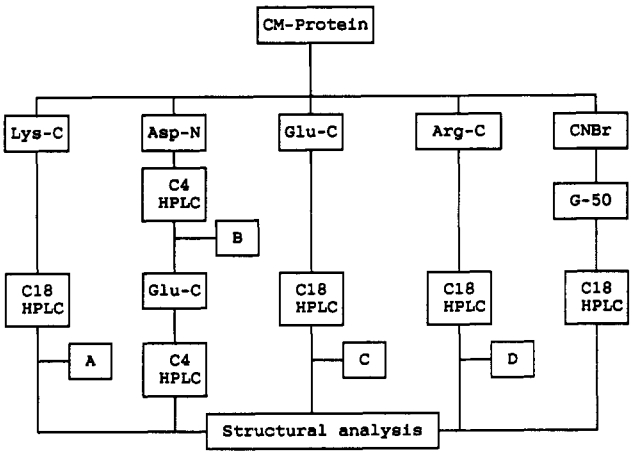

FIGURE 1: Flow scheme in the analysis of the cod liver alcohol dehydrogenase. Panels A, B, C, and D refer to the chromatograms shown in supplementary Figure 2.

identified as shown in Figure 2.

The C-terminal fragment was proven to end with Glu (Figure 2) by the results of sequence degradations and total compositions of both K24 and M7 and by the molecular weight of K24 determined by fast atom bombardment mass spectrometry (Egestad et al., 1990).

The region at positions 135–160 was a third segment requiring special analyses to establish. This is an internal, strongly hydrophobic segment frequently causing analytical problems with alcohol dehydrogenases (Kaiser et al., 1990; Cederlund et al., 1991). CNBr fragment M1 was recovered at a very low yield because of poor solubility and nonstoichiometric cleavage of the Met-124–Ser-125 bond and therefore only yielded 14 N-terminal residues when submitted to direct sequencer degradation. Similarly, the yield of peptide T3 was very low. Material sufficient for analysis and further redigestion was finally obtained from the Asp-protease cleavages and subsequent peptide purification on reverse-phase HPLC utilizing a C4 column. As shown in supplementary Figure 2B, peptide D4 was contaminated with D3 and was therefore redigested with Glu-specific protease and rechromatographed under identical conditions. The pure fragment obtained (D4E1, Figure 2) was successfully degraded with a solid-phase sequencer (MilliGen Prosequencer 6600), which allowed unambiguous identification of all residues in the sequence.

The protein chain has one more residue than several other major liver alcohol dehydrogenases, by an extra residue before position 58, one less after position 294, and one extra at the C-terminal end. The total of 375 residues are numbered in Figures 2 and 3, but in all functional discussions below positions refer to the class I human and horse enzymes to keep familiar residue numbers unchanged from the mammalian model enzymes.

The cod liver enzyme structure was compared with those of other vertebrate alcohol dehydrogenases, in particular, the human enzymes for which three classes[1] of liver protein have been shown to exhibit considerable differences tracing distant duplications (Cederlund et al., 1991). The cod enzyme is highly different (supplementary Table III) as expected from

[1] In addition, two further classes have been recently defined (Parés et al., 1990; Yasunami et al., 1991); they were included in the present overall comparisons (supplementary table III) but do not change previous class I/II/III emphasis and are therefore not mentioned in the detailed comparisons.

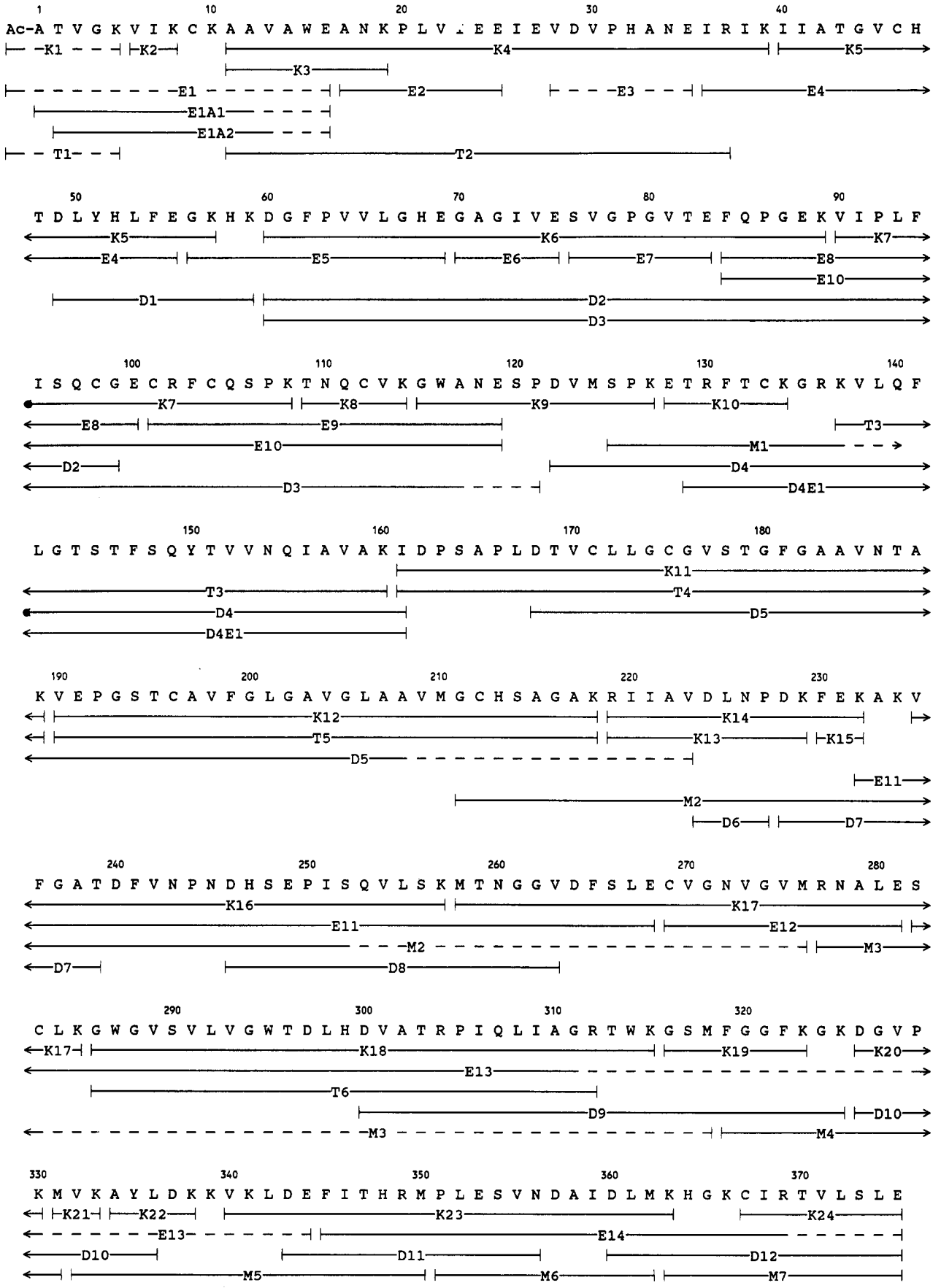

FIGURE 2: Primary structue of the major cod liver alcohol dehydrogenase and positions of all peptides analyzed. K peptides identify those obtained by Lys-C cleavages, T those by tryptic cleavages, E those by cleavages (E1A1 and -A2 subderived by partial acid hydrolysis of E1), cleavages, D those by Asp-N cleavages, and M those by Met cleavages with CNBr. Corresponding purifications are shown in supplementary Figure 2. Solid lines indicate parts of peptides analyzed by Edman degradation; dashed lines indicate remaining parts analyzed by total composition only.

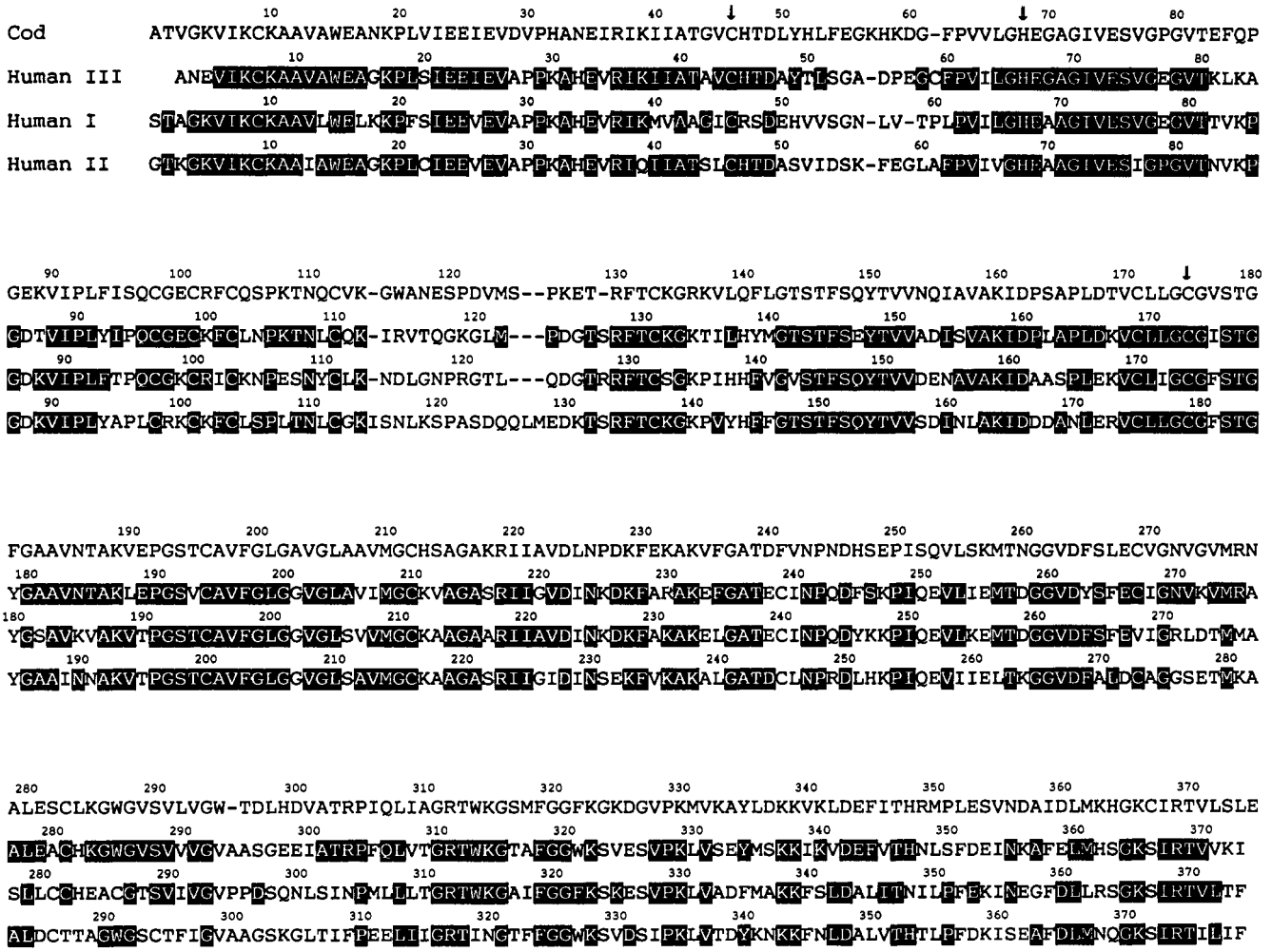

FIGURE 3: Alignment of the major cod liver alcohol dehydrogenase with those of the class I–III human enzymes. The cod line refers to the major liver enzyme, human I to the γ_1 subunit of the human enzyme, human II to the π subunit, and human III to the χ subunit. Exact alignments of gaps around positions 60 and 120 are difficult to judge but are given to represent minimal consequences for the substrate pocket around position 60 (cf. text) and around position 120 as in Eklund et al. (1990). A black background in lines 2–4 outlines identities with the top line. Positional numbers refer to each enzyme as shown, but throughout in the text, positions refer to the class I enzyme, which is the crystallographically determined model enzyme (cf. Eklund et al., 1987, 1990). The arrows indicate the residues liganding the active-site zinc atom.

the large evolutionary distance between the species, but alignments are fairly clear and contain only a few gaps (Figure 3) in spite of exchanges at almost half of all positions.

Molecular Graphics. (A) Substrate Binding. The alignment (Figure 3) allows modeling by computer graphics of the three-dimensional structure of the cod liver enzyme into that crystallographically determined for the horse class I enzyme. For the main chains, the models suggest that only minor differences exist, and all substitutions are compatible with the same fold of the molecules. This is not surprising, since previous modeling of isozymes (Eklund et al., 1987) and classes (Eklund et al., 1990) has been possible, as well as assignments of even the far more distantly related yeast alcohol dehydrogenase (Jörnvall et al., 1978), liver sorbitol dehydrogenase (Eklund et al., 1985), and ζ-crystallin (Borrás et al., 1989).

The residues binding the catalytic zinc atom (arrow-marked in Figure 3) are conserved between the cod and mammalian enzymes, as are the overall properties of coenzyme binding, while no less than 9 of 11 residues involved in substrate binding differ between the cod and the human class I enzyme (Table III). In the segment around position 60, the cod enzyme exhibits an extra residue versus the class I enzymes. This residue is not unambiguously defined from the modeling and could essentially be positioned at most places from Tyr-51 to Phe-62 of the cod enzyme, with different effects on the substrate pocket. However, from the modeling the least effects

Table III: Comparisons of Residues at the Substrate Binding Pocket[a]

position		enzyme: cod	human I	human II	human III
inner	48	Thr	Ser	Thr	Thr
	93	Phe	Phe	Tyr	Tyr
	140	Phe	Phe	Phe	Tyr
	141	Leu	Val	Phe	Met
middle	57	His	Leu	Phe	Asp
	116	Ala	Leu	Leu	Val
	294	Trp	Val	Val	Val
	318	Met	Ile	Phe	Ala
outer	110	Gln	Tyr	Leu	Leu
	B306	Ile	Met	Glu	Phe
	B309	Ile	Leu	Ile	Val
identities with cod:		(11)	2	3	1

[a] Substrate pocket residues are as defined from Figure 4 and from the human classes versus the horse enzyme (Eklund et al., 1990). For human class I, the γ_1 subunit is chosen because of its close relationships with the subunit of the horse enzyme. Differences listed are the minimal ones regarding the segment 51–61 of class I, assuming the extra residue in the cod enzyme to correspond to its Lys-57 (cf. text) as shown in Figure 3. Inner, middle, and outer refer to the corresponding parts of the pocket shown in Figure 4, while B positions refer to the opposite subunit.

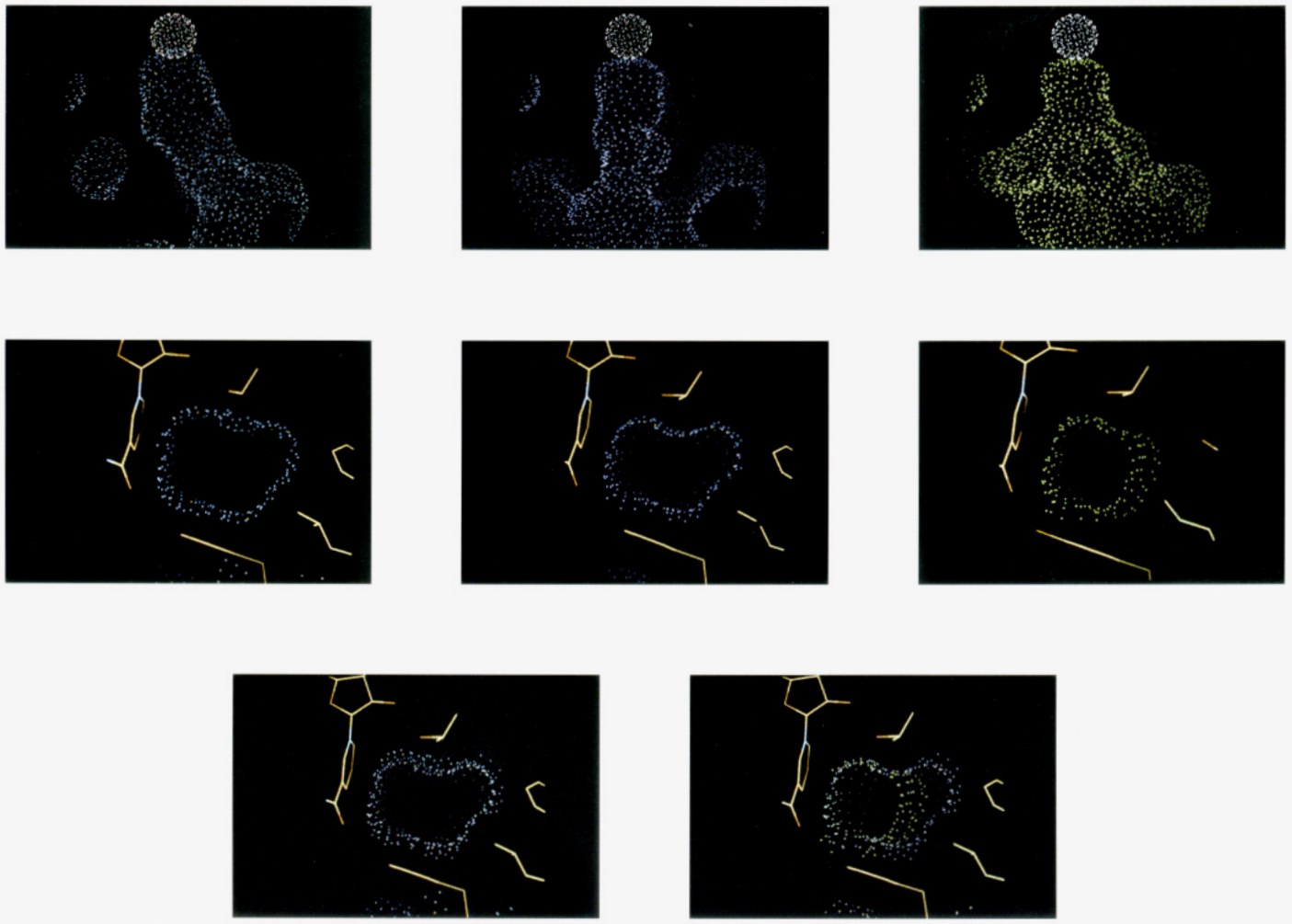

FIGURE 4: Surface outlines of the substrate pockets for the cod liver enzyme in relation to those for class I and III enzymes. (View A, top row) Side view of pockets with class I represented by the E subunit of the horse enzyme (dotted blue) and class III by the χ subunit of the human enzyme (dotted green). The cod enzyme is dotted purple, and in all cases the active-site zinc atom is represented by a small sphere at the top. (View B, bottom two rows) Inner parts of pockets from entrance side (from bottom in view A) for class I (E subunit, horse enzyme, blue), cod (purple), and class III (χ subunit, human enzyme, green). Bottom two panels are overlays of I and cod and of cod and III (using the same colors), respectively. Residues shown are 48 (top; Ser in I, Thr in cod and III), 140 (right; Phe in I and cod, Tyr in III), 141 (lower right; Leu in I and cod, Met in III), 93 (bottom; Phe in I and cod, Tyr in III) and the nicotinamide moiety of the coenzyme. Outlines were obtained by computer graphics modeling of the new structure into the crystallographically analyzed class I horse enzyme as detailed earlier for the human enzymes (Eklund et al., 1990). Both views A and B emphasize size relationships for the substrate pocket of the cod enzyme like those for class I rather than III, explaining the class I substrate specificity of the cod enzyme in spite of overall residue relationships with class III.

on the substrate pocket appear to be with the extra residue before position 58 in the class I enzyme, i.e., meaning that Lys-57 of the cod enzyme corresponds to the extra residue as shown in Figure 3. With this alignment giving minimal changes, the most important replacements are described below.

Residue exchanges affecting the substrate pocket topology are Thr-48 of the cod enzyme, which narrows the inner part of the substrate pocket (in relation to Ser-48 of most class I enzymes), and three exchanges in the middle part of the pocket. Two of these are for larger residues in the cod enzyme, Trp at position 294 (never seen in other alcohol dehydrogenases) instead of Val in class I and Met-318 (instead of Ile in class I γ subunits). The extra space consumption may be partly compensated for by an adjacent deletion at position 295 (Figure 3), allowing extra movement for the indole ring of Trp-294, and the substitution at position 318 may be partly compensated for by an exchange at position 306 (Ile in cod; Met in class I), but the overall changes affect the substrate pocket and its surface outline (Figure 4). The substrate cleft in the cod enzyme has a similar shape as in the class I enzyme in spite of the local differences. Importantly, the inner part is practically the same as for class I, explaining the ethanol activity and pyrazole inhibition. Furthermore, the exchange at position 48, affecting class I human isozyme differences in substrate specificity (Eklund et al., 1987), now gives a similar effect in the cod enzyme, for which the cyclohexanol parameters resemble those for the class I β (with Thr-48) rather than γ (with Ser-48) type of human isozymes (Table II). The inner part of the substrate cleft in the class III enzyme is significantly more narrow, while the outer part of that cleft is wider than for class I and the cod enzymes. In the middle part of the substrate pocket of the cod enzyme His-57 replaces Leu in class I. This basic/neutral residue change makes the substrate cleft more polar than that typical of class I enzymes (Table III). However, this should have little effect at basic pH and is compatible with the class I values for the cod enzyme at pH 10 (Table II), whereas an Asp replacement at the same position in class III (Table III) correlates with altered substrate specificity of class III (Juliá et al., 1988; Eklund et al., 1990). In summary, shapes (Figure 4) and residue replacements (Table III) explain the functional class I properties of the cod liver enzyme.

Table IV: Comparisons of Two Segments Susceptible to Movements at the Domain Interface upon Coenzyme Binding[a]

	residues 51–61 in the catalytic domain	residues 292–298 in the coenzyme-binding domain
Horse	H V V S G T - L V - T P L	V G V P P D S
Human Iα	H V V S G T - M V - T P L	V G V P P D S
Human Iβ	H V V S G N - L V - T P L	V G V P P A S
Human Iγ	H V V S G N - L V - T P L	V G V P P D S
Rat I	H A V S G S - L F - T P L	V G V P P N A
Mouse I	H V V S G T - L V - T P L	V G V P P S A
Human III	Y T L S G A - D P E G C F	V G V A A S G
Cod	Y H L F E G K H K D G - F	V G W - T D L

[a] Segment definitions are from Eklund et al. (1981) in relation to the horse liver class I enzyme. Residue data are from Jörnvall et al. (1987), Crabb et al. (1983), and Kaiser et al. (1988) and, for the cod enzyme, from Figure 2. Alignment is as in Figure 3 (cf. text and legend to Table III). Classes I and III and the cod enzyme are clearly different.

In contrast, coenzyme binding in the cod enzyme and the mammalian enzymes is similar. Five of 11 residues involved in coenzyme binding are completely conserved between all enzymes, and only two others differ between cod and class III (supplementary Table IV). Crucial residues at positions 47, 48, and 51 (Eklund et al., 1987, 1990) are identical in the cod enzyme. His-47 (as here) has previously been correlated with weaker coenzyme binding in a class I isozyme (Jörnvall et al., 1984), explaining a high overall rate in alcohol oxidation. However, consistent with the conclusion that other positions also are important for the rate (Eklund et al., 1987), the cod enzyme has a high specific activity (3.4 units/mg, cf. Table I), like class I β_2 type isozymes (Lange et al., 1976; Crabb et al., 1983), but a low K_m for NAD, like β_1 (and γ) type isozymes (Table II).

(B) Differences in Interface Segments at a Movable Domain Border. The comparisons reveal another unique feature of the cod liver enzyme. Thus, upon coenzyme binding, a large conformational change is triggered in alcohol dehydrogenase (Eklund et al., 1981). This involves movements at the domain interface with a shift of the segment 292–297 of the coenzyme-binding domain away from the domain interface to allow room for segment 51–59 of the catalytic domain (Eklund et al., 1981). Val-294, conserved in all mammalian alcohol dehydrogenases, is strongly affected with an oppositely pointing side chain before and after coenzyme binding. As shown in Table IV, features of these two movable segments are conserved within both class I and class III but are typically different between the two classes and exhibit compensated residue exchanges. Thus, for the segment from the catalytic domain, class III has an insertion before position 59 (Table IV), while class I instead has bulky residues in the opposite segment (Pro-Pro at 295-296, instead of Ala-Ala in class III). Significantly, the cod enzyme also has the inserted residue before position 59 (resembling class III) and a bulky residue in the opposite segment (Trp-294) that is partly compensated for by an adjacent deletion (Table IV), allowing an altered space relationship. These structures constitute unique features of the cod enzyme.

Discussion

Piscine Major Liver Alcohol Dehydrogenase—Functionally a Class I Enzyme but in Overall Primary Structure Most Similar to Class III Enzymes. The results clearly establish that piscine liver is rich in alcohol dehydrogenase. As defined from the enzymatic properties (Table II), the major cod liver enzyme is a class I enzyme with high sensitivity to pyrazole and with ethanol being a good substrate. Thus, the functional properties resemble a class I mammalian enzyme, which is the typical activity of the classical liver alcohol dehydrogenase. This extends the presence of large amounts of alcohol dehydrogenase to the livers of all major vertebrate lines, suggesting widespread, important functions of this enzyme in common.

However, in contrast to the mammalian class I enzymes, which are usually basic, the cod enzyme has a p*I* of about 7.0. The primary structure is also highly different, and as shown by the overall comparisons (supplementary Table III), similarities in primary structure are greatest with the class III type of mammalian enzyme. Thus, the cod enzyme exhibits "class-mixed" properties derived from the class separation early in vertebrate evolution (Cederlund et al., 1991), which again suggests important liver functions for the class I enzyme activity. This is the activity that includes ethanol and many other alcohols, coupled with sensitivity toward pyrazole and its derivatives. Combined, these relationships, when correlated with corresponding insight into the properties of the other classes, help to define original functions and hence metabolic roles of this divergent enzyme system in humans.

Three aspects of the overall comparisons are important. One concerns the actual identity values (supplementary Table III). This is the first time that a species difference for a vertebrate alcohol dehydrogenase from class I of the human enzyme has been found to be greater than the class differences among the human enzymes. Thus, while the human enzyme classes exhibit residue identities at the level of roughly 60%, and the previously most distant species divergence (human–frog) a residue identity of 68% (Cederlund et al., 1991), the cod enzyme reveals a clearly smaller identity, 55% (supplementary Table III). This fact supports the conclusion that the class I/III duplication occurred early in vertebrate evolution and that therefore present-day class divergence in humans is of the same order of magnitude as the distant vertebrate divergence of piscine and mammalian lines. An initial timing attempt suggested the duplication to have occurred about 450 million years ago (Cederlund et al., 1991), which seems reasonable also with the properties of the cod enzyme. A more detailed recalculation is not yet motivated since known structures still constitute just single cases. It should be noted, though, that not only do the overall residue identities suggest maximal fits of the cod enzyme to the class III structural type (supplementary Table III) but also several of the specific residue similarities do so. For example, the cod enzyme has Tyr-51 (and residues at 55 other positions) like class III proteins and in contrast to class I forms (Figure 3). Several of these other residues are also present in plant alcohol dehydrogenases, and Tyr-51 has previously been suggested to be typical for non-class I functions (Julià et al., 1988; Eklund et al., 1990). Obviously, this conclusion must now be modified, but it establishes that overall residue identities give patterns similar to those from identities at individual positions.

The second aspect of the overall comparisons is more important and shows, as noted above, that the cod enzyme is more related to another class (class III, 64% residue identities, supplementary Table III) than its functional assignments

suggest (class I, above). This deviation between structural and functional assignments is a property now also met for the first time in animal alcohol dehydrogenases and appears to be compatible with the fact that we study enzymes from ancestors separating at about the time of class separation. The third aspect concerns analysis of conformational (Figure 4) and residue (Table III) differences at the active sites, yielding direct correlations between residue replacements and new functions (below) and making it possible to trace molecular features critical for the class distinctions.

Functional Correlations. The present comparisons correlate functional assignment of class I properties with specific residue exchanges at the substrate pocket, altering substrate specificities and enzyme features (Table III, Figure 4). At the same time as class distinctions are blurred by the mixed properties of the cod enzyme, the residue replacements show how new functions can be obtained by differently sized substrate pockets and hence how class transfer can originate.

Exchanges at positions ascribed crucial roles in previous correlations of alcohol dehydrogenase isozymes and classes (Eklund et al., 1987, 1990) are of interest also in the cod structure. In addition, the cod enzyme shows several new and unique features, including a tryptophan (position 294) in the substrate pocket and replacements in movable segments at the domain interface (Table IV) also involving this Trp residue.

The cod enzyme structure questions the exact role of some of the residues previously discerned as important. For example, it has been pointed out that the sensitivity to inhibition with methylpyrazole appears to relate to conditions at position 51 (Juliã et al., 1988; Eklund et al., 1990). Thus, all the class I enzymes, with His-51, are strongly inhibited (K_i = 0.3 μM at pH 10); class II enzymes with Ser-51 or Thr-51 (Höög et al., 1988) are much less inhibited (K_i = 0.4 mM at pH 10), while class III enzymes with Tyr-51 are almost insensitive ($K_i \gg 50$ mM). In contrast, the cod liver enzyme (Table II) with Tyr-51 is still strongly inhibited by 4-methylpyrazole (K_i = 0.1 μM) and maize alcohol dehydrogenase, also with Tyr-51, has a considerable but lower 4-methylpyrazole inhibition (K_i = 17 μM; Pryor & Huppatz, 1982). This demonstrates that residues other than the one at position 51 are also important, like the exchanges at position 93, as shown by class comparisons (Eklund et al., 1990). Clearly, one important factor for the ethanol activity and hence class I function is restricted space in the substrate pocket, allowing additional interactions. It excludes water molecules interfering with the hydride transfer (Tapia et al., 1982) and hence with the catalytic efficiency in the case of small substrates.

Concerning residues strictly conserved in all alcohol dehydrogenases, the cod enzyme lacks one of the Asp residues previously found to be conserved (Jörnvall et al., 1987). Thus, Asp-87 of mammalian class I alcohol dehydrogenase is replaced by Glu in the cod enzyme. However, this exchange need not have conformational consequences, as Asp-87 in the horse enzyme is in a turn where the side chain is hydrogen bonded to the peptide nitrogen of residue 84, and this bond appears to be possible to form also with Glu at position 87. Nevertheless, although still an acidic residue, this position is no longer strictly conserved of those originally discerned [neither is Gly-260 in other enzymes (Kaiser et al., 1990; Estonius et al., 1990)], meaning that strictly conserved residues in all alcohol dehydrogenases now are limited to 20 residues. In summary, the cod liver enzyme structure, its relationships, and its enzymatic properties reveal a protein with class-mixed features explained at the molecular level. The correlations can be tested in further natural and site-directed variants. When correlated with similar data from all classes, they will establish original functions of these enzymes to define metabolic roles.

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Supplementary Material Available

Four tables, showing the total composition of the protein and peptides, residue identities in enzyme comparisons, and residues at the coenzyme-binding site, and two figures, showing FPLC of the enzyme analyzed and HPLC peptide purifications (7 pages). Ordering information is given on any current masthead page.

Registry No. NAD^+, 53-84-9; alcohol dehydrogeanse, 9031-72-5; ethanol, 64-17-5; pentanol, 71-41-0; cyclohexanol, 108-93-0; 4-methylpyrazole, 7554-65-6; alcohol dehydrogenase (cod liver protein moiety reduced), 138721-89-8.

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Quantitation of Lateral Stress in Lipid Layer Containing Nonbilayer Phase Preferring Lipids by Frequency-Domain Fluorescence Spectroscopy†

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ABSTRACT: Frequency-resolved fluorescence measurements have been performed to quantitate the lateral stress of the lipid layer containing nonbilayer phase preferring dioleoylphosphatidylethanolamine (DOPE). On the basis of a new rotational diffusion model, the wobbling diffusion constant (D_w), the curvature-related hopping diffusion constant (D_H), and the two local orientational order parameters ($\langle P_2 \rangle$ and $\langle P_4 \rangle$) of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPH-PC) in fully hydrated DOPE and DOPE/dioleoylphosphatidylcholine (DOPC) mixtures were calculated from the frequency-domain anisotropy data. The values of $\langle P_2 \rangle$, $\langle P_4 \rangle$, and D_H for DOPE were found to increase significantly at ~12 °C, the known lamellar liquid crystalline (L_α) to inverted hexagonal (H_{II}) phase transition temperature of DOPE. Similar features as well as a decline of D_w were detected in the DOPE/DOPC mixtures as the DOPE content was increased from 85% to 90% at 23 °C, corresponding to the known lyotropic phase transition of the DOPE/DOPC. In contrast, for DOPC (0-40 °C) and DOPE/DOPC (0-100% DOPE at 3 °C), which remained in the L_α phase, ese changes were not detected. The most probable local orientation of DPH-PC in the DOPE/DOPC mixtures shifted progressively toward the normal of the lipid/water interface as the content of DOPE increased. We concluded that the curvature-related lateral stress in the lipid layer increases with the content of the nonbilayer phase preferring lipids.

The structural morphologies of the lipids in the lamellar liquid crystalline (L_α) and inverted hexagonal (H_{II}) phases have been recognized and established (Luzzati & Husson, 1962; Cheng & Hui, 1986; Tate & Gruner, 1989; Rand et al., 1990). In the L_α phase, the molecules are arranged in stacked lipid bilayers with sheets of water between the polar surfaces of the lipids. However, in the H_{II} phase, the molecules rearrange themselves to form long water-cored cylindrical tubes in which the lipid polar head groups are facing the long symmetric axes of the cylinders. The L_α to H_{II} phase transition involves a change in the lipid/water surface curvature and a

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