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Fundamental molecular differences between alcohol dehydrogenase classes

(*Drosophila* octanol dehydrogenase/class III alcohol dehydrogenase/molecular patterns/zinc enzyme family)

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ABSTRACT **Two types of alcohol dehydrogenase in separate protein families are the "medium-chain" zinc enzymes (including the classical liver and yeast forms) and the "short-chain" enzymes (including the insect form). Although the medium-chain family has been characterized in prokaryotes and many eukaryotes (fungi, plants, cephalopods, and vertebrates), insects have seemed to possess only the short-chain enzyme. We have now also characterized a medium-chain alcohol dehydrogenase in *Drosophila*. The enzyme is identical to insect octanol dehydrogenase. It is a typical class III alcohol dehydrogenase, similar to the corresponding human form (70% residue identity), with mostly the same residues involved in substrate and coenzyme interactions. Changes that do occur are conservative, but Phe-51 is of functional interest in relation to decreased coenzyme binding and increased overall activity. Extra residues versus the human enzyme near position 250 affect the coenzyme-binding domain. Enzymatic properties are similar—i.e., very low activity toward ethanol (K_m beyond measurement) and high selectivity for formaldehyde/glutathione (*S*-hydroxymethylglutathione; k_{cat}/K_m = 160,000 $min^{-1} \cdot mM^{-1}$). Between the present class III and the ethanol-active class I enzymes, however, patterns of variability differ greatly, highlighting fundamentally separate molecular properties of these two alcohol dehydrogenases, with class III resembling enzymes in general and class I showing high variation. The gene coding for the *Drosophila* class III enzyme produces an mRNA of about 1.36 kb that is present at all developmental stages of the fly, compatible with the constitutive nature of the vertebrate enzyme. Taken together, the results bridge a previously apparent gap in the distribution of medium-chain alcohol dehydrogenases and establish a strictly conserved class III enzyme, consistent with an important role for this enzyme in cellular metabolism.**

The "classical" alcohol dehydrogenase is part of a widespread system of zinc-containing enzymes (1). In mammalian tissues, at least six classes of this enzyme occur. They differ considerably and represent stages between separate enzymes and ordinary isozymes. Class I is the well-known liver enzyme with ethanol dehydrogenase activity (2), class III is identical with glutathione-dependent formaldehyde dehydrogenase (3), class IV is a form preferentially expressed in stomach (4, 5), while classes II, V, and VI, although little studied, are known also to exhibit distinct properties (6, 7, 44). The class origins have been traced to gene duplications early in vertebrate evolution [the I/III duplication (8)] or during that evolution [the IV/I duplication (5)], with emerging activities toward ethanol (9); class III corresponds to an ancestral form. These properties and the different evolutionary patterns, with class III being "constant" and class I "variable" (10), result in a consistent picture of the enzyme system and place the classes of medium-chain alcohol dehydrogenases as separate enzymes in the cellular metabolism.

Similarly, another protein family, short-chain dehydrogenases, has also evolved into a family comprising many different enzyme activities, including an alcohol dehydrogenase (11). This form operates by means of a completely different catalytic mechanism and is related to mammalian prostaglandin dehydrogenases/carbonyl reductase (12). Thus far, this alcohol dehydrogenase has been found in insects, the *Drosophila* enzyme being recognized early to differ from the zinc-containing alcohol dehydrogenases (13, 14). Its properties in various *Drosophila* species are well established (15).

These two alcohol dehydrogenase types demonstrate that ethanol dehydrogenase activity has evolved in different manners, with many organisms now employing a medium-chain enzyme, while others depend on a short-chain enzyme. The medium-chain family has not been identified in insects, although it is of ancient origin and has been characterized in other eukaryotes and in prokaryotes. We now show that the family is indeed present also in insects and that its major representative is the typical class III type. In fact, the enzyme turns out to be identical to *Drosophila* octanol dehydrogenase, long known (16–20) but little studied. We have characterized the enzyme from *Drosophila melanogaster* enzymatically and structurally to prove its consistency with other class III forms. We also have identified and sequenced the corresponding gene§ and detected its transcription product at all developmental stages. Thus, the family is now known to be present essentially in all life forms, supporting the view that medium-chain alcohol dehydrogenases are universal factors in cellular defense mechanisms from prokaryotes to humans. In addition, we find fundamental differences between the class I and III enzymes, defining separate properties of these related proteins.

MATERIALS AND METHODS

Protein. *D. melanogaster* whole flies were bred and harvested as described (21). After homogenization, centrifugation, isoelectric focusing, and activity staining with 1 mM octanol or 33 mM ethanol at pH 10 and 1 mM glutathione/1 mM formaldehyde at pH 8, alcohol dehydrogenase activity of the medium-chain type was detected and purified by utilizing ion-exchange chromatography on DEAE-Sepharose, affinity chromatography on AMP-Sepharose, and a fast protein liquid chromatography (FPLC) step on Mono Q as described for the class III enzyme from other sources (9). Short-chain alcohol dehydrogenase activity was also monitored (isopropanol at

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U07641).

pH 8.6), pooled after the ion-exchange chromatography step, and purified to homogeneity on Blue-Sepharose (22). The strain used contained the adh^F allele yielding the rapidly migrating form of the short-chain enzyme.

Structural Analysis. The pure protein was carboxymethylated by treatment with ^{14}C-labeled iodoacetate and digested in separate batches with proteolytic enzymes (8). Peptide digests were fractionated by reverse-phase HPLC, and all fragments obtained were submitted to structural analysis. Amino acid compositions were determined with a Pharmacia LKB Alpha plus analyzer after acid hydrolysis for 24 hr at 110°C with 6 M HCl/0.5% phenol, and sequence degradations were carried out with MilliGen Prosequencers 6600 and 6625 utilizing arylamine coupling for membrane attachments or with an Applied Biosystems 470 sequencer with an on-line 120A analyzer.

PCR Amplification and Northern Analysis. Peptide structures determined were utilized for construction of two 23-mer degenerate oligonucleotide probes (corresponding to amino acid residues 92–99 and 261–268), which served as primers for PCR amplification with genomic DNA of several *Drosophila* species. Total cellular RNA was isolated by the guanidinium isothiocyanate method (23) from larval, pupal, and adult *D. melanogaster*; separated by 1.2% agarose/formaldehyde gel electrophoresis; and transferred to a nylon membrane (Amersham) for hybridization at high stringency (42°C in 50% formamide) (24). A control rehybridization was performed with a *D. melanogaster* actin gene probe. Autoradiographs were measured with an UltroScan XL (Pharmacia LKB) enhanced laser densitometer.

Enzymatic Characterization. Substrate specificities were screened by activity staining with ethanol, isopropanol, octanol, and *S*-hydroxymethylglutathione (formed by spontaneous reaction of formaldehyde and glutathione) (9). K_m and k_{cat} values were determined with alcohols at pH 10.0 and with *S*-hydroxymethylglutathione at pH 8.0 (5).

Structural Comparisons. The structure obtained was correlated with the three-dimensional model deduced for human class III alcohol dehydrogenase (25) to evaluate all replacements. The conformational representation in Fig. 4 was prepared by using a program supplied by Protein Science (26) and the coordinates (27) in the Protein Data Bank (28, 29) of the related (25) class I human alcohol dehydrogenase. Alignments required only few insertions and utilized the class III alcohol dehydrogenases from a prokaryote (30), yeasts (31, 32), a cephalopod (33), and vertebrates (34).

RESULTS

Presence of Class III Alcohol Dehydrogenase (Octanol Dehydrogenase). Homogenates of *D. melanogaster* were submitted to isoelectric focusing and subsequent activity staining with ethanol, isopropanol, octanol, and formaldehyde/glutathione. Results (Fig. 1) clearly show the presence of two enzyme types with partly overlapping substrate specificities. One, active with ethanol, isopropanol, and octanol, represents the well-known *Drosophila* short-chain alcohol dehydrogenase (13–15), which is known in multiple electrophoretic forms (35), and confirms that this is the only ethanol-active alcohol dehydrogenase in *Drosophila*. The other is active with octanol and formaldehyde/glutathione in a manner typical of mammalian medium-chain class III alcohol dehydrogenase. This suggests that the zinc-containing class III enzyme is present in *Drosophila* and that this glutathione-dependent formaldehyde dehydrogenase may represent the little-studied *Drosophila* octanol dehydrogenase previously reported (16–20) but not characterized structurally.

The glutathione-dependent formaldehyde dehydrogenase was purified 1900-fold in a 52% yield, resulting in a homogeneous preparation (Fig. 1, lanes 5 and 8) after chromatography on DEAE-Sepharose, AMP-Sepharose, and Mono Q FPLC column in a protocol similar to that for other class III alcohol dehydrogenases (9). All octanol dehydrogenase activity was monitored and coincided either with that of the glutathione-dependent formaldehyde dehydrogenase through all purification steps or with the short-chain alcohol dehydrogenase, which was also purified. The pure glutathione-dependent formaldehyde dehydrogenase exhibited both activities, with values (Table 1) typical of class III alcohol dehydrogenase and patterns similar to those of the corresponding human enzyme (36–38). The specific activity obtained was 12 units/mg with glutathione/formaldehyde, while the activity toward 1 mM octanol was 11 units/mg. Chromatography on Superose-12 indicated a molecular mass of about 80 kDa, compatible with a dimer of 40-kDa subunits and in agreement with the estimate from SDS/polyacrylamide gel electrophoresis (Fig. 1*C*). Combined with the structural analysis (below), all results now show that *Drosophila* octanol dehydrogenase is a class III medium-chain alcohol dehydrogenase and establish the conserved catalytic activity of this enzyme.

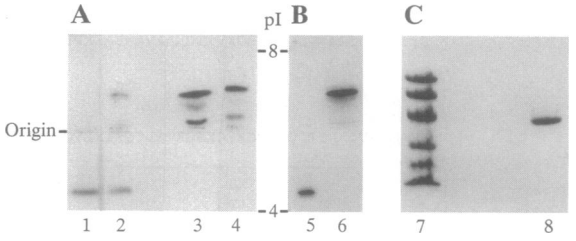

FIG. 1. Gel electrophoretic patterns of homogenates (*A*) and purified alcohol dehydrogenases (*B* and *C*) of *D. melanogaster*. (*A*) Isoelectric focusing under native conditions and activity staining with formaldehyde/glutathione (lane 1), octanol (lane 2), isopropanol (lane 3), and ethanol (lane 4). (*B*) Isoelectric focusing as in *A*, but with protein staining of the purified proteins, octanol dehydrogenase (lane 5), and short-chain dehydrogenase (lane 6). (*C*) Isoelectric focusing after SDS/polyacrylamide gel electrophoresis and protein staining of standard proteins (lane 7; molecular masses 94, 67, 43, 30, 20.1, and 14.4 kDa from top to bottom) and pure octanol dehydrogenase (lane 8). In lanes 3, 4, and 6, a minor more-acidic form is visible, representing one of interconvertible forms in flies homozygous at the *ADH* locus (35).

Table 1. Enzymatic properties of *D. melanogaster* medium-chain class III alcohol dehydrogenase compared to those of human class III alcohol dehydrogenase

Substrate	K_m, mM	k_{cat}, min^{-1}	k_{cat}/K_m, $min^{-1} \cdot mM^{-1}$
Ethanol	NS		
	(NS)		
Pentanol	5.4	840	160
	(22)	(240)	(11)
Octanol	0.51	1,300	2,500
	(1.2)	(220)	(180)
12-Hydroxydodecanoate	0.04	840	21,000
	(0.060)	(170)	(2,800)
NAD^+(alc)	0.13		
	(0.070)		
S-Hydroxymethyl-GSH	0.006	960	160,000
	(0.004)	(200)	(50,000)

Values for the *Drosophila* enzyme experimentally determined at pH 10 in 0.1 M glycine/NaOH for the alcohol substrates and at pH 8 in 0.1 M sodium pyrophosphate with 2.4 mM NAD^+ for *S*-hydroxymethylglutathione. Values for the human enzyme from refs. 36–38 are given below, within parentheses for comparison. NS, not saturable. NAD^+(alc) indicates values in the alcohol dehydrogenase reaction measured with 0.5 mM octanol (pH 10).

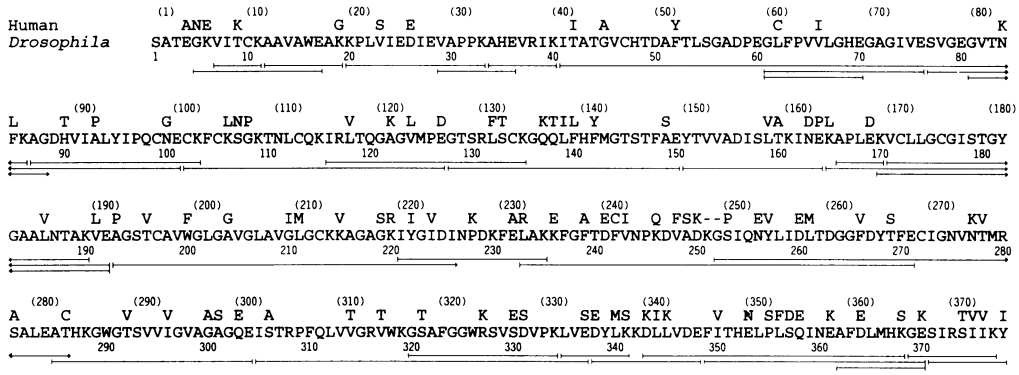

FIG. 2. Primary structure of *D. melanogaster* class III alcohol dehydrogenase (lower, continuous sequence) and its relationship to the human class III structure (upper sequence; residues cited only where different; in addition, the human enzyme lacks residues corresponding to the three first residues of the *Drosophila* form). Peptides passed by Edman degradations are denoted by lines beneath the sequences and were obtained by cleavages with a lysine-specific protease (upper lines) and with a Glu-specific protease (lower lines). Positional numbers (within parenthesis) above the human sequence line refer to the human class I enzyme and are given to allow correlation with the functional residues, which are generally known under their numbers in the class I enzymes, while numbers below the *Drosophila* sequence refer to the *Drosophila* class III enzyme now determined. Initiator methionine is not included since the protein is N-terminally blocked and is concluded to be acetylated like the human form.

Structure, Presence in Different Species, and Expression at All Developmental Stages. The enzyme was carboxymethylated and digested in different batches with glutamic acid- and lysine-specific proteases, respectively. Each digest was fractionated by reverse-phase HPLC, and peptides were submitted to sequence analysis. These results, combined with DNA data (below), gave the primary structure of the protein chain (Fig. 2). Only the N-terminally blocked peptides were not degraded, and positions 1–3 rely on the DNA data.

Oligonucleotides (corresponding to positions 92–99 and 261–268) were synthesized and used for PCR amplifications with DNA from five *Drosophila* species belonging to three different subgenera. Data obtained (Fig. 3*A*) show that the gene coding for the class III enzyme is present in all of the species tested. However, slightly different sizes of the amplified fragments suggest that there is some variability in the genomic region flanked by the two PCR primers.

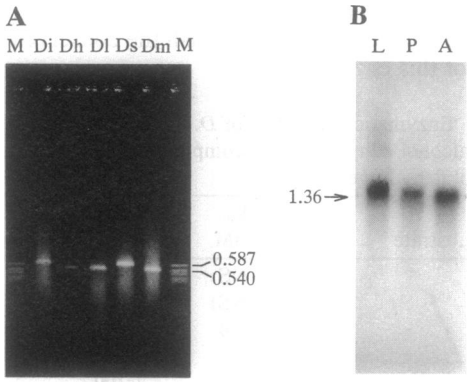

FIG. 3. Presence of a class III alcohol dehydrogenase/octanol dehydrogenase gene in several *Drosophila* species (*A*) and expression in different developmental stages (*B*). (*A*) PCR amplification with DNA of five *Drosophila* species: *D. immigrans* (lane Di); *D. hydei* (lane Dh); *D. lebanonensis* (lane Dl); *D. subobscura* (lane Ds); and *D. melanogaster* (lane Dm). Lane M shows *Hae* III-digested pBR322 as molecular weight markers. Amplification was performed in 100 μl containing 1 μg of genomic DNA, 40 pmols of each degenerate primer, 1.5 units of *Taq* polymerase (Promega) in 2.5 mM $MgCl_2$, and 200 μM each dNTP. After 2 min at 94°C and 45 cycles of 94°C for 60 s, 50°C for 90 s, and 72°C for 90 s, 15 μl was resolved on 1.2% agarose gels in Tris borate/EDTA buffer and stained with ethidium bromide. Units are in kb. (*B*) Northern analysis with larval (lane L), pupal (lane P), and adult (lane A) total cellular RNA from *D. melanogaster*. Units are in kb.

Using the PCR-amplified products, we cloned and sequenced the *D. melanogaster* gene, confirming the protein structure. Total RNA from larval, pupal, and adult *D. melanogaster* was also used for Northern analysis with the PCR-amplified product (Fig. 3*B*; an actin gene probe was also used). A single band of comparable intensity (as measured by laser densitrometry with the actin control) and ≈1.36 kb was detected in each sample. This indicates that expression of the class III gene is not confined to a particular developmental stage but appears to be abundant during the life span of the organism. Together, all data support a wide distribution of the enzyme in the *Drosophila* genus. The constant presence of the transcript at all developmental stages is compatible with a constitutive pattern of expression.

DISCUSSION

Identification of Medium-Chain Alcohol Dehydrogenase as Octanol Dehydrogenase in *Drosophila*. The demonstration of a medium-chain alcohol dehydrogenase in *Drosophila* establishes that this protein family occurs throughout the living world. It has previously been characterized structurally in prokaryotes (30) and in many highly different eukaryotic lines (31–34) but thus far not in *Drosophila*, where the short-chain alcohol dehydrogenase appeared to constitute a peculiar feature of alcohol oxidation. Nevertheless, the short-chain enzyme is the only one with appreciable ethanol dehydrogenase activity in *Drosophila* (Fig. 1), even though the medium-chain family is present, as is now evident. Its class III form coexists with the short-chain enzyme and has both unaltered enzymatic properties and a structure conserved in relation to the human enzyme. The absence of a class I medium-chain alcohol dehydrogenase in *Drosophila* apparently reflects the later enzymogenesis of that enzyme type (9) but does not imply that the protein family as such or the class III ancestral form is absent.

The extremely wide occurrence of this protein highlights its general importance and suggests that it has a role in basic cellular metabolism. Moreover, the present results identify octanol dehydrogenase, which has been discussed previously (16–20) but with somewhat contradictory estimates regarding molecular weight and quaternary structure, as being identical to the class III medium-chain alcohol dehydrogenase. Thus, the octanol dehydrogenase is involved also in glutathione-dependent elimination of formaldehyde. However, negative mutants are viable (32, 39), suggesting further multiplicity and the existence of a back-up system.

Correlation with Functional Properties. The identification and analysis of *Drosophila* class III alcohol dehydrogenase means that five divergent forms (conserved residues, 46%) of class III alcohol dehydrogenase have now been characterized structurally—mammalian forms including the human enzyme (34), a cephalopod enzyme (33), a *Drosophila* enzyme (this work), and two yeast enzymes (31, 32). Their properties can be compared with those of the classical liver alcohol dehydrogenase of class I, whose structure, within the same family, has been analyzed recently to a level showing approximately the same residue divergence (conserved residues, 42%) by analysis of five major vertebrate lines (40).

The conservation of functionally important residues in class III is extensive. Of 35 positions participating in coenzyme or substrate interactions in the class I enzyme (41) and considered to do so also in the class III enzyme (25, 42), no less than 28 are strictly conserved between the human and *Drosophila* class III enzymes, and all but 1 of the 7 exchanged constitute just minor variations encountered also in other species. Position 51 is the single exception. There, the class III *Drosophila* enzyme has a phenylalanine residue, as does the *Escherichia coli* enzyme (30), while human and the other class III forms have a tyrosine residue [and the whole family has a histidine, tyrosine, or serine residue (5, 33, 40)]. This residue usually participates in hydrogen bonding with the ribose moiety of the NMN part of the coenzyme (25). Although the hydrogen bond may have little effect (43), Phe-51 cannot donate such a bond to the coenzyme. Therefore, this exchange may contribute an explanation to the weakened coenzyme binding of the *Drosophila* enzyme and, hence, to its increased activity relative to the human enzyme (Table 1).

Disregarding the functionally important residues, the *Drosophila* enzyme has two extra residues in relation to the human class III form. These extra residues, close to position 250 (Lys-251 and Gly-252 in Fig. 2, or adjacent residues, depending on the alignment chosen), are also present in the yeast enzyme (31, 32) but not in the octopus enzyme (33). This region corresponds to a surface helix in the coenzyme binding domain (Fig. 4 *Upper Left*), and it appears possible to extend the helix without adverse consequences.

The conservation of class III stands in marked contrast to the spread within the class I enzyme. Just among vertebrates, class I enzyme residue conservation [55% for the human/fish pair (42)] is lower than that for class III across separate eukaryotes (63% for the human/yeast pair). Among the 35 functionally important positions, no fewer than 20 vary, many to a great extent (40, 42). Glycolytic enzymes—e.g., glyceraldehyde-3-phosphate dehydrogenase (human/yeast, 63–65% residue identity, depending on the types compared) and enolase (human/yeast, 61–64% identity)—demonstrate that the class III alcohol dehydrogenase variation (human/yeast, 63%) is "normal" for an enzyme of this type in a fixed metabolic pathway. It is the faster evolving class I that has an "atypical" variation. In fact, the human/yeast values for the two glycolytic enzymes and the class III alcohol dehydrogenase are surprisingly close, just within ±2%, suggesting similar functional restrictions for these three metabolically

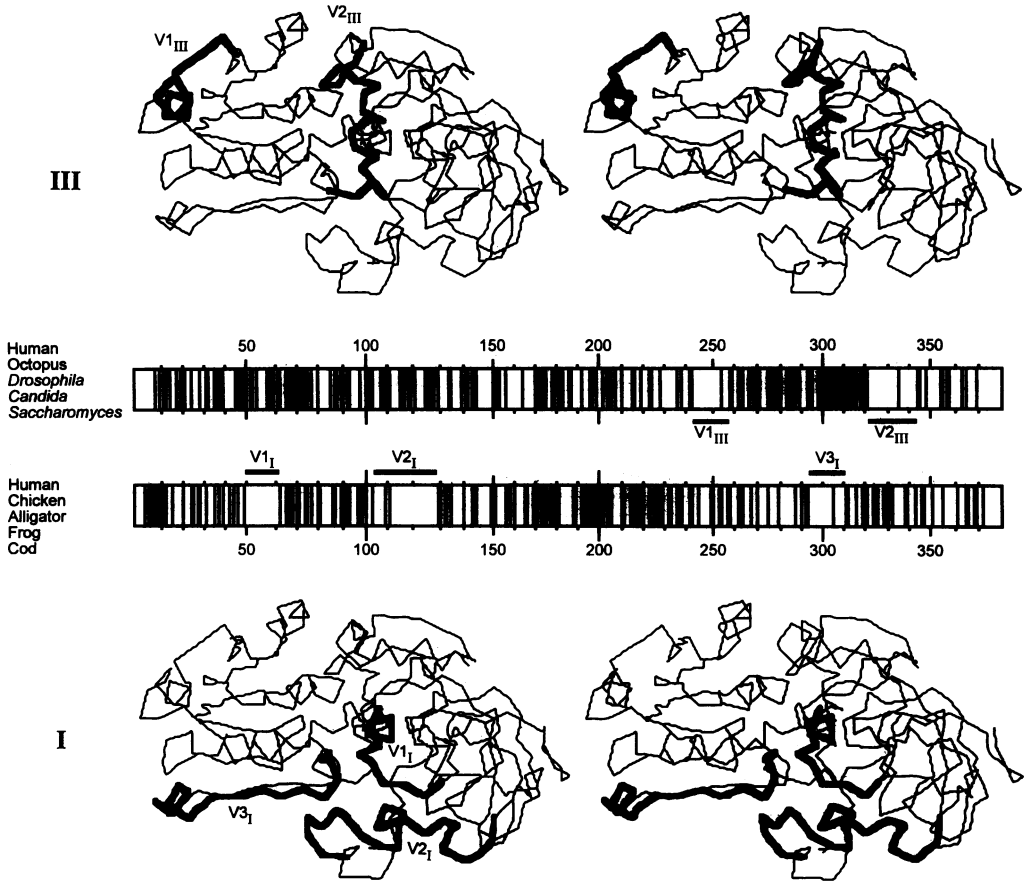

FIG. 4. Differences in patterns of variability for classes I (bar and conformation) (*Lower*) and III (bar and conformation) (*Upper*). The bars give a schematic representation of the residue conservations along the polypeptide chains (positions numbered), with black vertical lines denoting positions with strictly conserved residues among the species shown. Unfilled bar areas correspond to variable segments, $V1_I$–$V3_I$ in class I and $V1_{III}$–$V2_{III}$ in class III. Their spatial positions are shown with thick lines inside the models of the class I and III enzymes (25, 27). The conformation is that from the Protein Data Bank coordinates (28, 29) of the human class I enzyme (27) when using a program supplied by Protein Science (26).

fixed enzymes. This agreement adds strength to the conclusion of significantly different natures of the class I and III alcohol dehydrogenases.

Fundamental Differences in Molecular Architecture of Related Proteins. Apart from the differences in overall conservation, the actual distributions of the constant and variable segments of the class I and III alcohol dehydrogenases are fundamentally different. In class I, three segments ($V1_I$–$V3_I$) stand out as variable (empty spaces in the bottom bar in Fig. 4) and have been noted to make class I peculiar among proteins in general by suggesting hypervariability in important segments (40). As shown in the conformation (Fig. 4 *Lower*), they correspond to a segment adjacent to the active site ($V1_I$), a part of the loop around the second zinc atom ($V2_I$), and a part of the subunit–subunit interacting segment ($V3_I$). These three segments all are concentrated on one side of the molecule (toward the observer in Fig. 4). Therefore, apart from affecting the functional areas, their covariability may have even further implications, suggesting variability of a particular side of the class I molecule.

In contrast, these segments are not at all variable in class III but are exactly those much conserved (bar in Fig. 4 *Upper*), supporting the view that class III represents a "normal" enzyme, with maximal conservation at the active site and other important segments. Nevertheless, class III also has segments of variability, but its two such segments ($V1_{III}$ and $V2_{III}$) affect nonfunctional, superficial regions, completely differently positioned regarding both functional representation and molecular surfaces (Fig. 4 *Upper*). Interestingly, though, $V2_{III}$ corresponds to the helix constituting one of the two domain-interconnections of the subunit (Fig. 4). Since domain movements are known to be associated with coenzyme binding in the class I enzyme (41), this segment may indicate further functional differences in catalytically active states between the classes.

The remaining classes are not yet known in similar detail. Nevertheless, their inclusion in bar comparisons of the type in Fig. 4 (as given in ref. 40) suggests that the class I pattern is typical of variable classes of medium-chain alcohol dehydrogenases.

In conclusion, we notice that fundamentally different internal variability patterns affect two related proteins of a protein family. These patterns add to the overall differences between the classes and show that differences apply to building elements in the molecular architecture of the proteins. Class III behaves as enzymes in general, constant in function, enzymology, overall structure, and important segments, while class I is emerging in function, exhibiting enzymatic differences, rapid evolutionary changes, and variability at important regions. The two variability patterns (Fig. 4) illustrate fundamental differences between an evolving and a constant protein within a single family, and correlate the differences with the molecular architecture and enzymatic properties.

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