Characteristics of Mammalian Class III Alcohol Dehydrogenases, an Enzyme Less Variable than the Traditional Liver Enzyme of Class I†

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ABSTRACT: Class III alcohol dehydrogenase, whose activity toward ethanol is negligible, has defined, specific properties and is not just a "variant" of the class I protein, the traditional liver enzyme. The primary structure of the horse class III protein has now been determined, and this allows the comparison of alcohol dehydrogenases from human, horse, and rat for both classes III and I, providing identical triads for both these enzyme types. Many consistent differences between the classes separate the two forms as distinct enzymes with characteristic properties. The mammalian class III enzymes are much less variable in structure than the corresponding typical liver enzymes of class I: there are 35 versus 84 positional differences in these identical three-species sets. The class III and class I subunits contain four versus two tryptophan residues, respectively. This makes the differences in absorbance at 280 nm a characteristic property. There are also 4–6 fewer positive charges in the class III enzymes accounting for their electrophoretic differences. The substrate binding site of class III differs from that of class I by replacements at positions that form the hydrophobic barrel typical for this site. In class III, two to four of these positions contain residues with polar or even charged side chains (positions 57 and 93 in all species, plus positions 116 in the horse and 140 in the human and the horse), while corresponding intraclass variation is small. All these structural features correlate with functional characteristics and suggest that the enzyme classes serve different roles. In addition, the replacements between these triad sets illustrate further general properties of the two mammalian alcohol dehydrogenase classes. The variation between them is marginally less in the coenzyme binding than in the catalytic domain. Both enzyme classes demonstrate that evolutionary changes appear to be slowest in the human and fastest in the rat (as reflected indirectly by 31 positions with unique residues in the human class I and class III enzymes combined versus 43 positions in the two horse forms and 67 positions in the two rat forms). Provided these values do not reflect unknown differences in the capacity for DNA repair, they could, in accord with data obtained for a few other proteins, reflect the importance of short generation times to acquire rapid evolutionary changes.

Mammalian alcohol dehydrogenases constitute a complex protein subfamily composed of at least three enzyme classes (Vallee & Bazzone, 1983), with additional isozyme subforms (Smith et al., 1971) especially within class I. Class I represents the "classical" liver enzyme that is active toward ethanol and has been studied extensively in several species. The major human class I isozymes have been characterized at the protein, cDNA, and genomic levels [cf. Jörnvall et al. (1989)] and can be induced by steroid hormones of both the androgen and glucocorticoid type (Ceci et al., 1986; Dong et al., 1988; Felder et al., 1988).

Class III alcohol dehydrogenase, in contrast, is barely active toward ethanol. Its tissue distribution is different, and it is virtually the only alcohol dehydrogenase in some organs such as placenta, brain, and testis (Parês & Vallee, 1981; Beisswenger et al., 1985; Dafeldecker & Vallee, 1986). Its primary structure has been determined only recently (Kaiser et al., 1988; Julià et al., 1988), and the class III enzyme is apparently expressed constitutively (Smith, 1988). These marked differences between the class I and class III enzymes suggest that they, in spite of the common appellation "alcohol dehydrogenase", may now represent distinct and separate enzyme forms rather than merely isozymes (Jörnvall et al., 1987a). There is at least one more class, i.e., class II (Vallee & Bazzone, 1983), the primary structure of which is known also (Höög et al., 1987).

In view of previous extensive work on the class I enzyme, the differences among the classes, and the superimposed species variations, it was important to ascertain the primary structure of the horse class III enzyme to identify the characteristic and unique properties in each case. The determination allows comparison of classes I and III in identical sets of three species, i.e., human, horse, and rat. Previous comparisons of alcohol dehydrogenase structures then available have revealed other properties concerning relationships at large (Jörnvall et al., 1987b), conformational aspects (Eklund et al., in preparation), or the initial class III structure characterized (Kaiser et al., 1988), but have not differentiated variations within classes from those between species. This has now become possible, and the results for the triad comparison establish the general structural properties of the class III enzyme, define its characteristics, and show that the enzymes of classes I and III are separate and distinct forms. Moreover, the results establish that the class III enzyme is the one which is most conserved, implying that it likely serves important functional roles. In both classes the variations differ from species to species, being

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largest in the rat and smallest in the human. Finally, the data reveal the existence of overall domain properties common to the two classes.

Materials and Methods

Horse liver class III alcohol dehydrogenase (the χχ enzyme) was purified as described (Dafeldecker & Vallee, 1982) by using affinity chromatography on CapGapp–Sepharose and AMP–hexane–agarose (Lange & Vallee, 1976; Wagner et al., 1984). The pure enzyme was ^{14}C-carboxymethylated after solubilization in 8 M urea, 0.4 M Tris, and 2 mM EDTA, pH 8.15, reduction with dithiothreitol, and alkylation with ^{14}C-labeled neutralized iodoacetic acid as described (Kaiser et al., 1988). Reagents were removed by extensive dialysis against distilled water.

The carboxymethylated protein was treated with trypsin in 0.1 M ammonium bicarbonate and 0.9 M urea (initially added as 9 M urea to solubilize the protein before dilution with bicarbonate) and with CNBr in 70% formic acid (initially added as concentrated formic acid for solubilization) as described (Kaiser et al., 1988). The resultant peptides were prefractionated on Sephadex G-50 in 30% acetic acid and purified further by reverse-phase high-performance liquid chromatography, using acetonitrile gradients in aqueous trifluoroacetic acid (Jeffery et al., 1984).

Pure peptides were submitted to sequence analysis in a Beckman 890D liquid-phase sequencer, an Applied Biosystems 470A gas-phase sequencer, or an Applied Biosystems 470A gas-phase sequencer equipped with a 120A PTH analyzer. Phenylthiohydantoin derivatives from the former two instruments were analyzed by high-performance liquid chromatography (Hewlett Packard 1090) on Nucleosil C18 with an acetonitrile gradient in sodium acetate (Kaiser et al., 1988). Total compositions were determined with a Beckman 121M amino acid analyzer after hydrolysis with 6 M HCl/0.5% phenol at 110 °C for 22 h in evacuated tubes. Large fragments were redigested with staphylococcal Glu-specific extracellular protease (Miles) and *Lysobacter* Lys-specific protease (Boehringer). The products were purified by high-performance liquid chromatography and analyzed as above.

Results

Horse liver class III alcohol dehydrogenase was ^{14}C-carboxymethylated and cleaved with trypsin and CNBr in separate batches to generate two sets of overlapping peptides. Resulting fragments were purified in two steps by exclusion chromatography followed by high-performance liquid chromatography (Figure 1) in a manner similar to that used for the corresponding human and rat enzymes (Kaiser et al., 1988; Juliá et al., 1988). All pure peptides were analyzed for composition and amino acid sequence. Large fragments were redigested with staphylococcal Glu-specific protease and *Lysobacter* Lys-specific protease, followed by purification and structural analysis.

Peptides covering all parts of the protein subunit were identified. The structure deduced and the peptides analyzed are shown in Figure 2. Owing to a high degree of homology with the corresponding human and rat enzymes (positional identities of 95% and 92%, respectively, cf. below), it was not necessary to identify every peptide overlap by Edman degradation. They were unequivocally proven by total compositions, specificities of the proteolytic cleavages, and chromatographic properties of peptides in relation to the corresponding fragments from the human and rat enzymes. Compositions of relevant peptides isolated from the horse enzyme that differ from their human and rat counterparts have been determined (supplementary material). The amino acid sequence deduced is in excellent agreement with the total composition of the original preparation (Table I). The same agreement was obtained for each of the pure peptides analyzed. Results are apparent from Figure 2 and do not require further comment, save for two segments where special problems were encountered.

Table I: Total Composition of the χ Subunit of Class III Horse Liver Alcohol Dehydrogenase[a]

residue	acid hydrolysis	sum of sequence
Cys	13.2	14
Asp, Asn	22.3	21 {14, 7}
Thr	24.2	24
Ser	25.2	24
Glu, Gln	34.9	34 {26, 8}
Pro	16.4	16
Gly	40.2	40
Ala	34.7	35
Val	34.7	39
Met	7.3	7
Ile	23.8	27
Leu	21.2	21
Tyr	6.9	7
Phe	13.9	14
Trp	+	4
Lys	30.9	31
His	7.3	7
Arg	8.2	8
sum		373

[a] Values show molar ratios as determined by acid hydrolysis and the sum of the sequence determined. Cys analyzed as Cys(Cm). Trp present (+) but not quantitatively analyzed. The low hydrolytic values for Val and Ile are due to slow release of Val-Val, Ile-Val, Val-Ile, and Ile-Ile sequences (cf. Figure 1).

One is the long, internal segment comprising residues 141–208 (B3), which is very hydrophobic. It was obtained in low yield because of solubility problems and could not be redigested readily. However, solubilization in 9 M urea, dilution, and subsequent redigestions provided the fragments that were analyzed. It should be noted that analogous analytical problems were encountered with precisely the same region of the human protein (Kaiser et al., 1988).

The other segment presenting special problems was the N-terminal region that was inaccessible to direct analysis, since the protein is blocked, as is the case for all other mammalian alcohol dehydrogenases characterized so far. However, the blocked peptides were identified in the proteolytic digests, and mass spectrometry (Fairwell et al., 1987) of peptide T1 provided the N-terminal structure.

The equine class III structure (Figure 2) is compared in Table II with the two class III alcohol dehydrogenase structures from the human and the rat. Among the three species, differences occur at a total of 35 positions. However, also at these positions the three structures are interrelated: whenever a residue is unique to one of the three species, the same position in the other two have an alternative that is the same in both of them (Table II). Such a pattern is compatible with a close relationship among the sequences and suggests, at each position, a limited number of mutations from a common alternative. Where the two types of residue at each position are compatible with a one-base change (30 of the 35 positions), the alternatives observed may actually reflect the mutation itself. The original residue will then correspond to the two-species alternative more often than to the one-species alternative. This correlation will not be exact because of the possibility of multiple mutations at a position, but the number of unique residues in each species will in a relative manner

reflect differences in the accumulation of mutations among the species.

The positions of the residue exchanges are of interest both because of their relation to the two domains of the protein and because of the particular species involved. Thus, residue exchanges are somewhat more common in the catalytic than in the coenzyme-binding domain, but this difference is only marginal considering the different sizes of the domains (23 versus 12 differences, corresponding to 10% versus 8%). A similar degree of change is present in the class I enzymes from the same species (Table II). With regard to species variations in class III, the relative estimates of the mutational differences in the human enzyme appear to be only about one-third of the number of those found in the other two species (Table III). In class I, this spread is smaller, and for the two classes combined there is a gradual increase from human, to horse, to rat in the number of unique residues and, hence, in the relative estimates of the mutations observed.

Finally, the majority of variations within class III of the three species affect residues to which little functional importance has been ascribed. Among 10 residues at the substrate-binding site (Eklund et al., unpublished data) only one position, 116, differs between the horse and the human class III enzymes (Table IV). Even in that instance, the difference, although affecting polarity, is minor (Val in human and rat, Thr in horse). Similarly, at this site the only difference between the rat and the human/horse enzymes is a conservative replacement, Phe for Tyr at position 140. These differences all increase the polarity. Thus, the substrate-binding pocket of class III is considerably less hydrophobic than that of class I (Kaiser et al., 1988; Julià et al., 1988).

Discussion

Class III versus Class I Enzyme Characteristics. The results establish the structure of the class III enzyme of the horse (Figure 1). As a consequence, the primary objective, knowledge of an identical triad set of class III and class I enzymes has been obtained, and the structure and evolutionary properties characteristic of these two classes from three mammalian species can now be compared (Figure 3).

(*a*) *Structure Variations.* The structure of the class III enzyme is considerably more conserved than that of the class I. Species variations among the three class III forms affect only 35 positions compared with 84 substitutions in class I forms from the same species. Thus, class III is more than twice as well conserved (Figure 3). Interclass differences are largely species independent ranging from 19 to 30 for class III versus 44–70 for class I. This shows that the greater conservation of class III is a genuine property, suggesting a stricter requirement on the class III than on the class I enzyme or a generally more conserved metabolic role of the class III enzyme than that of the class I.

(*b*) *Physicochemical Properties.* All class I enzymes contain two tryptophan residues, but all class III enzymes contain four, accounting for their higher molar absorptivity at 280 nm. This parameter, easily measured, can therefore be added to those that have proven useful in differentiating between the forms of the mammalian alcohol dehydrogenases. Similarly, the charges of class III and class I enzymes differ, regardless of species: there are 4–6 fewer positive charges in class III (Table II). Consequently, electrophoretic properties also reflect their characteristic differences. This is particularly important since

Table II: Comparison of the Residues at All Positions with Exchanges in the Identical Three-Species Set of Figure 2[a]

Class III

Position	Human	Horse	Rat	Position	Human	Horse	Rat	Position	Human	Horse	Rat
3	Ala	**Ser**	Ala	163	Leu	Leu	**Ser**	302	Ala	Ala	**Ser**
4	Asn	**Ala**	Asn	176	Ile	**Val**	Ile	328	Val	**Ile**	Val
5	Glu	Glu	**Gln**	189	**Leu**	Val	Val	348	His	His	**Gly**
8	Lys	Lys	**Arg**	194	**Val**	Thr	Thr	349	Asn	**Ser**	Asn
21	Leu	**Val**	Leu	197	Val	**Ile**	Val	354	**Glu**	Gln	Gln
26	Ile	**Val**	Ile	222	Val	Val	**Ile**	357	Lys	**Glu**	Lys
32	Lys	Lys	**Gln**	231	**Arg**	Lys	Lys	360	Glu	Glu	**Asp**
61	Cys	**Ser**	Cys	238	Thr	**Ser**	Thr	364	Ser	**Ala**	Ser
107	Lys	**Gln**	Lys	249	Pro	Pro	**Ser**	366	Lys	Lys	**Asn**
116	Val	**Thr**	Val	264	Tyr	Tyr	**Phe**	372	Val	Val	**Leu**
136	Thr	Thr	**Pro**	277	Ala	Ala	**Ser**	374	**Ile**	Leu	Leu
140	Tyr	Tyr	**Phe**	282	Cys	Cys	**Ala**				
							Sum	35	5	14	16

Positions with exchanges

Total enzymes	35 (9% of all positions)
Catalytic domains (residues 1-175 + 318-374)	23 of 232 (10%)
Coenzyme-binding domain (residues 176-317)	12 of 142 (8%)

Table II (Continued)

Class I

Position	Human	Horse	Rat	Position	Human	Horse	Rat	Position	Human	Horse	Rat
17	Leu	Glu	Pro	118	Asn	Met	Gln	258	Thr	Ser	Thr
18	Lys	Lys	His	120	Arg	Arg	Lys	259	Asp	Asn	Asp
22	Ser	Ser	Thr	122	Thr	Thr	Ala	276	Met	Val	Thr
25	Glu	Glu	Asp	123	Leu	Met	Leu	277	Ala	Thr	Ser
26	Val	Val	Ile	124	Gln	Gln	Leu	278	Ser	Ala	Ala
43	Ala	Thr	Thr	128	Arg	Ser	Ser	280	Leu	Ser	Leu
45	Ile	Ile	Val	131	Thr	Thr	Ser	281	Cys	Cys	Ser
50	Glu	Asp	Asp	133	Ser	Arg	Arg	283	His	Gln	His
52	Val	Val	Ala	141	Val	Leu	Leu	284	Glu	Glu	Ser
56	Asn	Thr	Ser	142	Gly	Gly	Ser	286	Cys	Tyr	Cys
58	Val	Val	Phe	143	Val	Thr	Thr	288	Thr	Val	Val
63	Val	Val	Ala	154	Glu	Glu	Asp	297	Asp	Asp	Ser
64	Ile	Ile	Val	155	Asn	Ile	Ile	298	Ser	Ser	Ala
65	Leu	Ala	Leu	156	Ala	Ser	Ala	300	Asn	Asn	Ser
69	Ala	Ala	Gly	164	Ser	Ser	Ala	303	Ile	Met	Val
76	Val	Ile	Ile	167	Glu	Glu	Asp	307	Leu	Leu	Ser
82	Thr	Thr	Cys	185	Lys	Lys	Gln	310	Thr	Ser	Leu
84	Lys	Arg	Lys	191	Pro	Gln	Pro	326	Glu	Asp	Asp
94	Thr	Thr	Ser	208	Val	Ile	Val	327	Ser	Ser	Ala
102	Ile	Val	Ile	209	Met	Met	Ile	341	Ser	Ala	Pro
105	Asn	His	His	213	Ala	Ala	Thr	343	Asp	Asp	Glu
108	Ser	Gly	Ser	218	Arg	Arg	Lys	344	Ala	Pro	Pro
110	Tyr	Phe	Leu	221	Ala	Gly	Ala	348	Asn	His	His
112	Leu	Leu	Gln	235	Leu	Val	Leu	349	Ile	Val	Val
113	Lys	Lys	Thr	239	Glu	Glu	Asp	358	Gly	Gly	Ala
114	Asn	Asn	Lys	241	Ile	Val	Ile	364	Ser	Ser	Ala
115	Asp	Asp	Asn	247	Lys	Lys	Thr	366	Lys	Glu	Lys
117	Gly	Ser	Thr	255	Lys	Thr	Gln	371	Val	Ile	Val
							Sum	84	26	29	51

Positions with exchanges

Total enzymes	84 (22% of all positions)
Catalytic domains (residues 1-175 + 318-374)	55 of 232 (24%)
Coenzyme-binding domain (residues 176-317)	29 of 142 (20%)

[a] Structures for class III are from Figure 2, this work, Kaiser et al. (1988), and Julià et al. (1988). Class I structures are from a previous summary (Jörnvall et al., 1987b). Where the intraclass isozymes differ, the γ_1 residues are those listed for the human enzyme and the E residues those for the horse enzyme. For ease of comparison, all positional numbers are given in the 374-residue nomenclature of the class I enzymes. Hence, identical numbers in the two classes refer to equivalent positions, although single deletions/insertions in the class III structures occur and the total number of residues in class III is only 373. At all positions with species differences in class III and most such positions in class I (73 of 84), only one species deviates. Deviating residues that constitute unique alternatives are boxed. (At 11 of the 84 positions all three species differ). The sum in the position column gives the total number of positions affected by a exchange; the sums in remaining columns give the number of unique residues in each species.

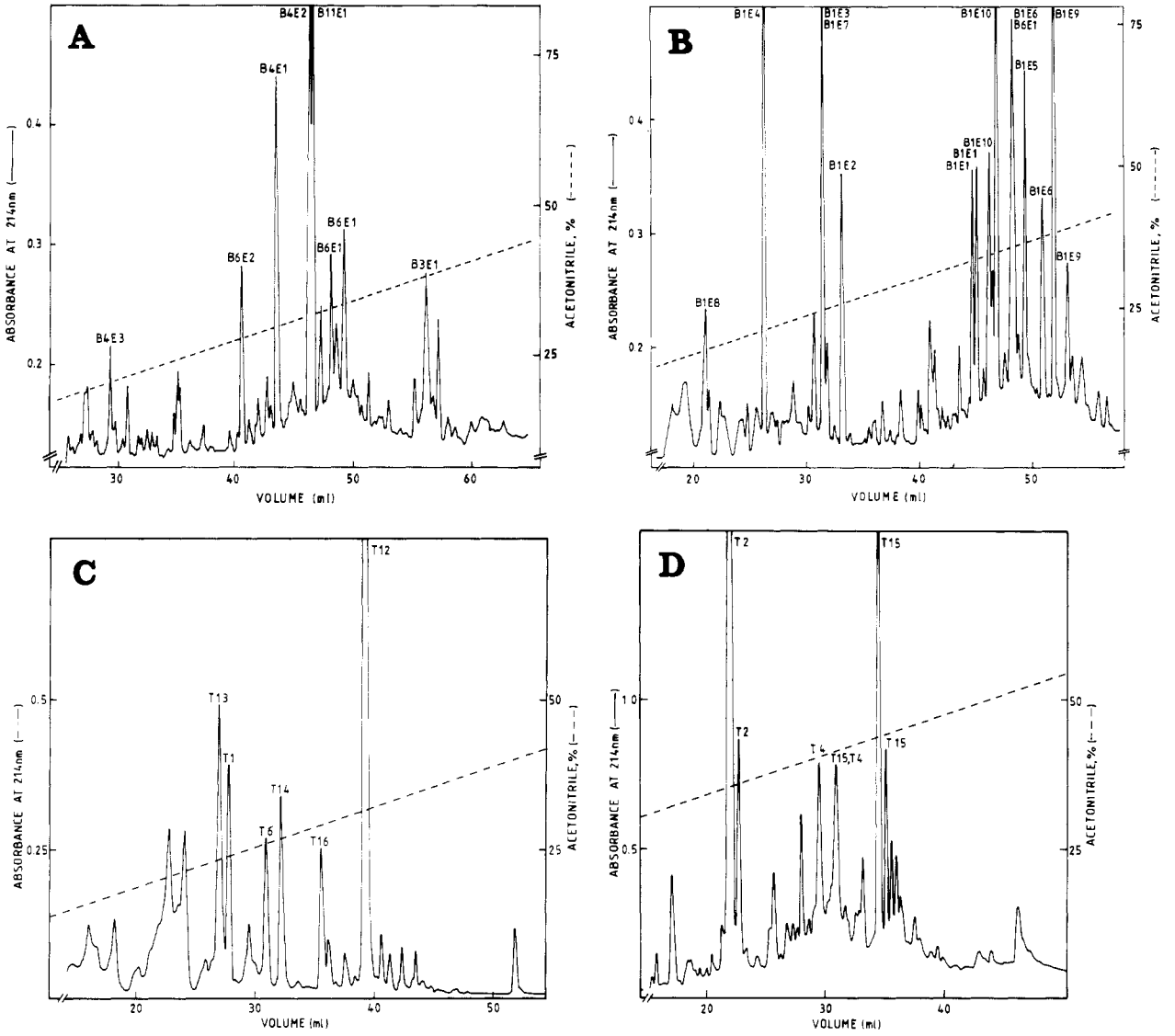

FIGURE 1: Final HPLC purification of relevant peptides containing amino acid replacements in relation to the human enzyme. In each case, peptides were prefractionated by Sephadex G-50 chromatography followed by reverse-phase HPLC. (A) Purification of the material in the fifth peak from the Sephadex fractionation of the CNBr fragments; (B) chromatogram of the second peak of the CNBr fractionation after redigestion with Glu-specific protease; (C and D) HPLC patterns from Sephadex fractions 5 and 6, respectively, in the fractionation of the tryptic digest. Both the N-terminal and C-terminal tryptic peptides are purified in (C).

Table III: Sum of Unique Residues in Each of the Enzymes and Species from the Three-Member Sets of the Class I and Class III Enzymes[a]

species	class III	class I	sum, class I + class III
human	5	26	31
horse	14	29	43
rat	16	51	67

[a] Residues included are those boxed in Table II, corresponding to the sums listed in Table II for each species. Patterns are similar in class I and class III but vary in detail, as expected for biological properties. The sum shows a pattern consistent with the different generation times. As stated in the text, the unique positions indirectly or inexactly reflect mutational events; the correlation is not absolute, and additional mutations could have occurred. Nevertheless, the sum values estimate relative mutational differences among the species.

Table IV: Residues Contributing to the Substrate-Binding Cleft of Alcohol Dehydrogenase[a]

	III			I		
	human	horse	rat	human	horse	rat
48	Thr	Thr	Thr	Ser	Ser	Ser
57	Asp	Asp	Asp	Leu	Leu	Leu
67	His	His	His	His	His	His
93	Tyr	Tyr	Tyr	Phe	Phe	Phe
116	Val	Thr	Val	Leu	Leu	Leu
140	Tyr	Tyr	Phe	Phe	Phe	Phe
141	Met	Met	Met	Val	Leu	Leu
294	Val	Val	Val	Val	Val	Val
306	Phe	Phe	Phe	Met	Met	Met
318	Ala	Ala	Ala	Ile	Ile	Ile

[a] Residues at 10 positions defined for the class I enzyme (Eklund et al., 1987) are shown for both the three-enzyme sets. Residue positions are from Figure 2 and previous papers as in Table II. The only three positions that differ are underlined.

considerable differences in the relative patterns for all human and rat enzymes, especially for class II, have been reported (Julià et al., 1987). Nevertheless, the charges of class I and class III enzymes appear distinctive: typically, class I is basic and class III considerably more acidic.

(*c*) *Substrate-Binding Pocket.* The overall conformations of the class I and class III enzymes have been thought to be related, but their substrate-binding pockets differ significantly (Eklund et al., unpublished data) and are compatible with the marked, known differences of substrate specificity (Vallee & Bazzone, 1983). It is now clear that this class difference is characteristic of mammalian alcohol dehydrogenases in gen-

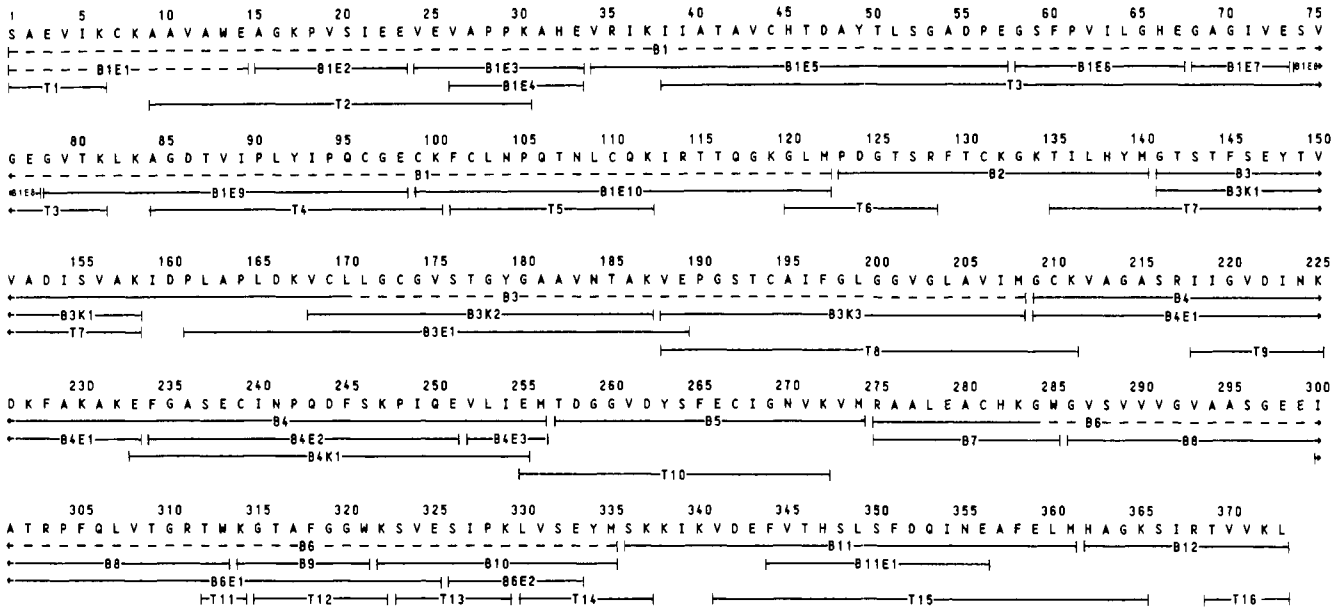

FIGURE 2: Summary of the amino acid sequence analysis of class III horse alcohol dehydrogenase. The amino acid sequence determined and all peptides analyzed are shown. Solid lines indicate regions of a peptide identified by Edman degradations, and dashed lines indicate remaining regions analyzed by total composition. Peptide nomenclature: T, trypsin; B, cyanogen bromide; E, Glu-specific protease; K, Lys-specific protease; letters give consecutive numbering of all peptides purified from a digest. Multiple letter-number designations indicate redigestions. The residue positions are given in absolute numbers corresponding to the class III structure and therefore do not directly transfer to the numbers in Table II, where, for ease of comparison with previous data, the numbering system is according to the traditional class I enzymes, which deviate by one to three residues because of insertions/deletions. For direct comparisons of the numbering systems, alignments have previously been given (Kaiser et al., 1988).

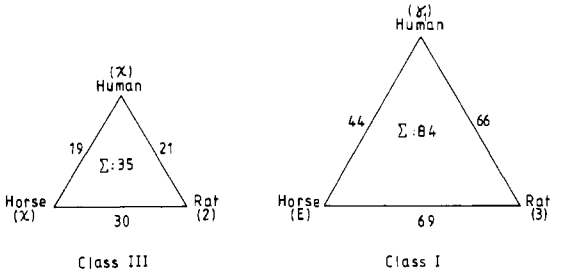

FIGURE 3: Differences within each identical three-species set of human class III and class I enzymes. Values along the sides of the triangles give number of amino acid replacements between each species pair of either enzyme class, while values inside the triangles give the total number of positions affected by any species variation for each class. The values clearly quantitate the differences between the classes, as also reflected by the relative sizes of the triangles. Subunit or enzyme names are shown within parentheses. Structural data are from Figure 2 and as listed in Table II. For class I, where different isozymes have been characterized, the subunits chosen are γ_1 for the human and E for the horse enzyme since they are the most related subunits of this class between these species. However, the use of other isozyme chains alters the numbers only marginally (class I human/horse gets a value of 48 instead of 44 when β_1 instead of γ_1 is used for the calculations).

eral, independent of species. Thus, nearly all residues of the substrate-binding pocket of class III differ from those of class I, resulting in a more hydrophilic binding site, including a charged residue (Table IV). In fact, only 2 of 10 positions in the substrate-binding pockets are conserved between classes I and III (positions 67 and 294), making this part one of the more variable areas of the structure. Several of the class III replacements result in polar or charged residues in the hydrophobic substrate-binding barrels of class I. Thus, in class III of all species, positions 57 and 93 are Asp and Tyr, but in class I, they are Leu and Phe. In the horse class III enzyme, position 116 is polar (Thr) as is position 140 (Tyr) in both the human and horse class III enzymes (Table IV). In contrast to the extensive interclass variation of the substrate-binding segments, affecting 8 of 10 residues altogether, species variations are confined to three positions, and only two of these involve nonhydrophobic residues, position 116 and 140, respectively, in class III only. Again, these properties, like the differences in evolutionary rate (above), suggest separate functional roles of the two enzyme classes in metabolism. They also imply specific functions for each class, independent of species.

Mammalian Alcohol Dehydrogenases. In spite of the characteristic differences between classes I and III and the ensuing inference of separate and distinct functional properties, they share important structural characteristics with alcohol dehydrogenases in general. Thus, the replacements in the coenzyme-binding domain are marginally fewer than in the catalytic domain; the relative differences between the two domains are virtually identical in the enzyme of both classes (20% versus 24% of all positions in class I, for the coenzyme-binding and the catalytic domains, respectively, and 8% versus 10% in class III). The conservation of overall structural properties is particularly apparent from the conservation of Gly residues, which constitute half of all invariant residues in the enzyme (Jörnvall et al., 1987b) and are associated with the conformational pattern characteristic of coenzyme binding.

Beyond this, the three hypervariable regions noted in classes I and II (Jörnvall et al., 1987a) are also apparent in horse, human, and rat class III. The three regions include (1) the loop that binds the noncatalytic zinc atom, (2) part of the active-site segment, consistent with the different substrate specificities, and (3) the major area of subunit interactions that accounts for the lack of cross-hybridization between subunits of different classes (Jörnvall et al., 1987a; Briganti et al., 1989). Since the active sites of proteins are usually conserved, the present class variability in this region of alcohol dehydrogenase should be noted. However, variability at functional sites has also been found in some other proteins, such as ovomucoids (Laskowski et al., 1987). Although the variability there involves species differences, rather than mere class variations as in alcohol dehydrogenase, the parallel might

not be too remote. Thus, ovomucoids also exhibit multiplicity (domain repeats), and alcohol dehydrogenases exhibit not only class but also species variations at the active site. In any event, the alcohol dehydrogenase differences now defined appear to relate to the evolution of classes with separate and distinct properties.

Species Differences in Evolution. The rate of evolutionary change in a protein relates to structural and functional requirements, resulting in great variations of evolutionary speed (Dickerson, 1971) and allowing differentiation of separate functional properties (cf. above). However, deviations in the rate of evolutionary change of a single protein have been observed. These variations appear to relate to differences in generation time or other properties, such as possibly the capacity for DNA repair, as noted for globins (Goodman, 1985; Li & Tanimura, 1987), aldehyde dehydrogenases (Johansson et al., 1988), and now the class I and class III alcohol dehydrogenases.

In all class III and most class I forms, one residue of the species variants differs from that in the other two, as indicated by boxes in Table II, a pattern that is compatible with a highly limited number of mutations at each position resulting in a unique residue. Most of the unique residues, which inexactly reflect relative numbers of mutations, occur in the rat, with fewer ones in the horse, and the smallest number in the human enzymes (Table III). Significantly, this trend applies to both classes I and III, although there is variation in the actual numbers (Table III) as would be expected for any biological property.

In summary, the structures explain physicochemical and enzymatic properties. The class differences can be monitored by electrophoresis, absorbance, and substrate specificity. In each case, the trends in the structural differences reflect general properties of the two enzymes, independent of species variations which are considerably smaller. Class III is 2–3-fold more conserved than the class I enzyme, again independent of species, suggesting distinct metabolic roles for the two classes, with stricter requirements or a more unaltered function for class III than for class I. The present analyses define the enzymes of the different mammalian classes as consistently unique proteins, albeit with characteristic properties common to alcohol dehydrogenases in general and with further evolutionary differences in particular species.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Table of the total compositions of peptides of the horse class III alcohol dehydrogenase (1 page). Ordering information is given on any current masthead page.

Registry No. Alcohol dehydrogenase, 9031-72-5; alcohol dehydrogenase (horse liver class III isoenzyme reduced), 122487-66-5.

REFERENCES

Beisswenger, T. B., Holmquist, B., & Vallee, B. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8369–8373.

Briganti, F., Fong, W. P., Auld, D. S., & Vallee, B. L. (1989) *Biochemistry 28*, 5374–5379.

Ceci, J. D., Lawther, R., Duester, G., Hatfield, G. W., Smith, M., O'Malley, M. P., & Felder, M. R. (1986) *Gene 41*, 217–224.

Dafeldecker, W. P., & Vallee, B. L. (1982) *J. Protein Chem. 1*, 59–69.

Dafeldecker, W. P., & Vallee, B. L. (1986) *Biochem. Biophys. Res. Commun. 134*, 1056–1063.

Dickerson, R. E. (1971) *J. Mol. Evol. 1*, 26–45.

Dong, Y., Poellinger, L., Okret, S., Höög, J.-O., von Bahr-Lindström, H., Jörnvall, H., & Gustafsson, J.-Å. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 767–771.

Eklund, H., & Brändén, C.-I. (1988) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 3, pp 74–142, Wiley, New York.

Eklund, H., Horjales, E., Vallee, B. L., & Jörnvall, H. (1987) *Eur. J. Biochem. 167*, 185–193.

Fairwell, T., Juliâ, P., Kaiser, R., Holmquist, B., Parês, X., Vallee, B. L., & Jörnvall, H. (1987) *FEBS Lett. 222*, 99–103.

Felder, M. R., Watson, G., O'Malley Huff, M., & Ceci, J. D. (1988) *J. Biol. Chem. 263*, 14531–14537.

Goodman, M. (1985) *BioEssays 3*, 9–14.

Höög, J.-O., von Bahr-Lindström, H., Hedén, L.-O., Holmquist, B., Larsson, K., Hempel, J., Vallee, B. L., & Jörnvall, H. (1987) *Biochemistry 26*, 1926–1932.

Jeffery, J., Cederlund, E., & Jörnvall, H. (1984) *Eur. J. Biochem. 140*, 7–16.

Johansson, J., von Bahr-Lindström, H., Jeck, R., Woenckhaus, C., & Jörnvall, H. (1988) *Eur. J. Biochem. 172*, 527–533.

Jörnvall, H., Höög, J.-O., von Bahr-Lindström, H., & Vallee, B. L. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2580–2584.

Jörnvall, H., Persson, B., & Jeffery, J. (1987b) *Eur. J. Biochem. 167*, 195–201.

Jörnvall, H., von Bahr-Lindström, H., & Höög, J.-O. (1989) in *Human Metabolism of Alcohol* (Crow, K. E., & Batt, R. D., Eds.) Vol. II, pp 43–64, CRC Press, Boca Raton, FL.

Juliâ, P., Farrês, J., & Parês, X. (1987) *Eur. J. Biochem. 162*, 179–189.

Juliâ, P., Parês, X., & Jörnvall, H. (1988) *Eur. J. Biochem. 172*, 73–83.

Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. L., & Jörnvall, H. (1988) *Biochemistry 27*, 1132–1140.

Lange, L. G., & Vallee, B. L. (1976) *Biochemistry 15*, 4681–4686.

Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202–221.

Li, W.-H., & Tanimura, M. (1987) *Nature 326*, 93–96.

Parês, X., & Vallee, B. L. (1981) *Biochem. Biophys. Res. Commun. 98*, 122–130.

Smith, M. (1988) *Biochem. Soc. Trans. 16*, 227–230.

Smith, M., Hopkinson, D. A., & Harris, H. (1971) *Ann. Hum. Genet. 34*, 251–271.

Vallee, B. L., & Bazzone, T. J. (1983) in *Isozymes: Current Topics in Biological & Medical Research* (Rattazzi, M. C., Scandalios, J. G., & Witt, G. S., Eds.) pp 219–244, Liss, New York.

Wagner, F. W., Parês, X., Holmquist, B., & Vallee, B. L. (1984) *Biochemistry 23*, 2193–2199.