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Mammalian class IV alcohol dehydrogenase (stomach alcohol dehydrogenase): Structure, origin, and correlation with enzymology

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ABSTRACT **The structure of a mammalian class IV alcohol dehydrogenase has been determined by peptide analysis of the protein isolated from rat stomach. The structure indicates that the enzyme constitutes a separate alcohol dehydrogenase class, in agreement with the distinct enzymatic properties; the class IV enzyme is somewhat closer to class I (the "classical" liver alcohol dehydrogenase; ≈68% residue identities) than to the other classes (II, III, and V; ≈60% residue identities), suggesting that class IV might have originated through duplication of an early vertebrate class I gene. The activity of the class IV protein toward ethanol is even higher than that of the classical liver enzyme. Both K_m and k_{cat} values are high, the latter being the highest of any class characterized so far. Structurally, these properties are correlated with replacements at the active site, affecting both substrate and coenzyme binding. In particular, Ala-294 (instead of valine) results in increased space in the middle section of the substrate cleft, Gly-47 (instead of a basic residue) results in decreased charge interactions with the coenzyme pyrophosphate, and Tyr-363 (instead of a basic residue) may also affect coenzyme binding. In combination, these exchanges are compatible with a promotion of the off dissociation and an increased turnover rate. In contrast, residues at the inner part of the substrate cleft are bulky, accounting for low activity toward secondary alcohols and cyclohexanol. Exchanges at positions 259–261 involve minor shifts in glycine residues at a reverse turn in the coenzyme-binding fold. Clearly, class IV is distinct in structure, ethanol turnover, stomach expression, and possible emergence from class I.**

Three classes of human alcohol dehydrogenase, I–III, were distinguished early (1) and have been characterized structurally as separate forms related by gene duplications that are intermediate between those giving rise to isozymes and those giving rise to entirely different enzymes (2). Additional classes, IV and V (3–8), of these medium-chain alcohol dehydrogenases and further isozymes in some species (9, 10) have since been added. Still more enzymes (11, 12) and another protein family, short-chain dehydrogenases (11), also belong to this system in vertebrates, and additional families and forms occur in prokaryotes, plants, yeasts, insects, and other organisms (13–16). The medium-chain alcohol dehydrogenase family is now a well-established entity with functionally, structurally, and evolutionarily distinctive features (17). The class I/III duplication has been traced to early vertebrate stages (9, 18), and the ancestral origin of the class III form, equal to glutathione-dependent formaldehyde dehydrogenase (19), is still much more distant, functionally identifying the alcohol dehydrogenase family as part of the cellular defense system (17).

In spite of this general knowledge, mammalian alcohol dehydrogenase classes IV and V are relatively poorly characterized. The class V protein has not been isolated from natural sources but has been expressed from a human liver-derived cDNA (5, 6). The complete structure of a class IV protein has not been reported as yet, making the assignment of this enzyme tentative and its details unknown. This form appears to be expressed specifically in epithelial tissues, characteristically in the stomach mucosa (3, 4, 7, 8, 20); it has been ascribed a special role in human alcohol metabolism, perhaps also correlated with sex-related differences (21) and apparently explaining the ethanol dehydrogenase activity (22, 23) of some class I-deficient animal strains (24). Among the alcohol dehydrogenases, the class IV protein has unique enzymatic properties, with exceptionally high K_m and k_{cat} values for ethanol and with activity toward alcohols and aldehydes of physiological interest (25, 26). This report gives the structure of this enzyme and correlates the structure with the enzymatic properties, to complete the characterization of a class IV enzyme and establish it as a natural part of the whole enzyme system.

MATERIALS AND METHODS

Protein. Rat (Sprague–Dawley) stomach alcohol dehydrogenase class IV was isolated by a previous procedure (25), modified to use two consecutive chromatographic steps on Blue-Sepharose instead of on AMP-Sepharose and by addition of a final Sephacryl S-300 chromatography in 100 mM Tris·HCl (pH 8.5). This yielded about 1.1 mg of protein with a high specific activity (81 units/mg at pH 10.0) in a 630-fold purification from 100 g of gastric tissue. The protein was reduced with dithiothreitol and carboxymethylated with ^{14}C-labeled iodoacetate (3). Separate batches were digested with Lys-specific *Achromobacter* protease, Glu-specific *Staphylococcus* protease, Gly-specific *Astacus* protease, Asp-specific *Pseudomonas* protease, and chymotrypsin, as described (18). Peptide mixtures obtained were fractionated by reverse-phase HPLC on C_4 and C_8 columns with gradients of aqueous trifluoroacetic acid in acetonitrile (9, 18).

Analysis. Sequence analyses were performed by degradation in a gas-phase (Applied Biosystems 477A with on-line 120A analyzer) or a solid-phase (Milligen 6600) sequencer (9, 18). Total compositions were determined with an LKB Pharmacia Alpha Plus analyzer after hydrolysis for 22 h at 110°C in 6 M HCl/0.5% phenol. Enzymatic characterization was performed at pH 10.0 and 7.5 with different primary alcohols and formaldehyde/glutathione, in the presence and absence of 4-methylpyrazole (4, 20, 25–29).

Comparisons. The structure obtained was correlated with the known conformations of classes I–III of human alcohol

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dehydrogenase (29), in turn deduced from a crystallographically analyzed class I enzyme (30, 31), and positioned in the system of other medium-chain dehydrogenases. Phylogenetic relationships were evaluated by the progressive alignment method of Feng and Doolittle (32) utilizing a set of programs provided by the authors. The conformational representation in Fig. 2 was prepared from the Protein Data Bank coordinates (33, 34) of the β_1 form of human alcohol dehydrogenase (35) by using a program supplied by the journal *Protein Science* (36).

RESULTS

Primary Structure. The amino acid sequence of the protein was determined by peptide analysis of five digests of the carboxymethylated protein. The digests utilized Lys-, Asp-, Gly-, and Glu-specific proteases, as well as chymotrypsin. Ninety-six peptides were analyzed after purification by reverse-phase HPLC. The results gave overlapping fragments for all regions and established a 374-residue polypeptide chain (Fig. 1). The N-terminal residue is blocked by an acyl group as in all other mammalian alcohol dehydrogenases analyzed so far (10). It was deblocked by treatment with nonaqueous methanolic trifluoroacetic acid, 1:1 (vol/vol) (for 16 h at room temperature) to remove the acetyl group, after which sequence degradation showed the start position and the N-terminal sequence. Because of the indirect method employing hydrolysis, the four N-terminal residues are considered tentative (Fig. 1, lowercase type), as are two residues (at positions 27 and 267 in Fig. 1) where results permit different interpretations in separate digests, indicating contaminants or possible microheterogeneities. In addition, position 230 (Gln) was difficult to interpret because of extensive susceptibility to deamidation (giving recovery of much Glu). Contaminants were directly identified in one preparation, initially questioning the nature of a few other residues. However, most of the functionally important residues rely on multiple peptide analyses as shown in Fig. 1.

Class Relationships. *Structure.* The structure now determined differs from those of the other classes of mammalian alcohol dehydrogenase (Table 1). Although the present structure is that of a rat enzyme and all the other classes have not been fully analyzed in that species (some only in the human), class IV alcohol dehydrogenase has a unique structure. It differs from classes I, II, III, and V (55–68% residue identities), much as they differ from each other (59–64% identities). The rat/human differences vary between the classes (38), but in all cases the present structure differs much more than do isozymes or mammalian species variants (Table 1). Hence, overall values for the protein are typical of interclass variations, confirming the assignment as a separate class, class IV alcohol dehydrogenase (3, 4, 7). In particular, it is not a variant of class V (5, 6), demonstrating that the two most recently discovered alcohol dehydrogenase classes are distinct and constitute separate entities.

Enzymology. The class IV protein is also enzymatically distinct. The extremely high k_{cat} value toward ethanol reported initially (25) has been found to be even higher using improved procedures for enzyme preparation, with values of 2400 min^{-1} at pH 7.5 for the rat and 1000 min^{-1} for the human enzymes (26), also confirmed by another report (8). These values are close to 100-fold those of class I (Table 2).

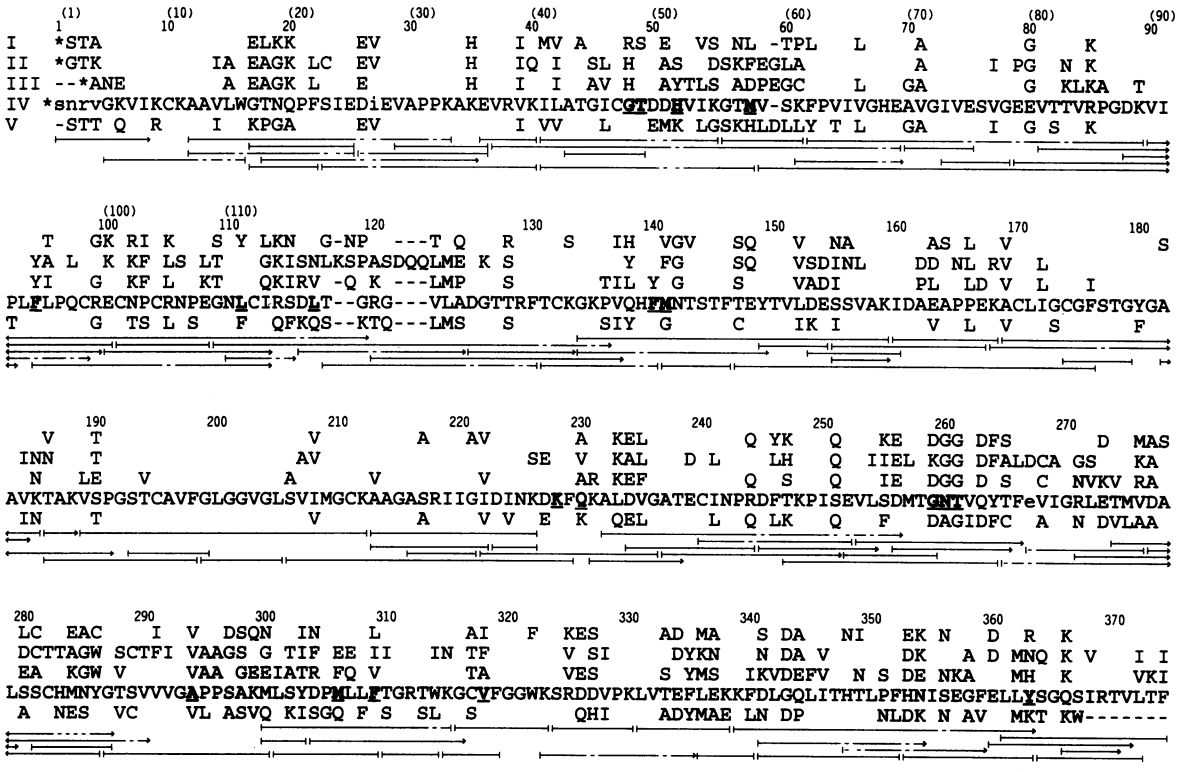

FIG. 1. Primary structure of class IV alcohol dehydrogenase and its relationship to the structures of the other classes. The class IV structure determined is from rat and is represented by the continuous type (disregarding dashes, which represent gaps toward the other classes). Differences in classes I (γ subunit), II, III, and V [all represented by the human forms (17)] are given in each line, respectively, where empty spaces indicate identities with class IV. Residues discussed in the text in relation to substrate and coenzyme binding are in boldface type and underlined. Lower lines show all peptides analyzed (solid for parts passed by sequencer degradations and dashed for remaining parts), upper numbers show the residue positions in the class IV protein and (within parentheses, where different) the classical class I enzyme for easy reference to the crystallographically analyzed forms (30, 31, 35) (numbers are identical subsequent to the gap after position 117). Heterogeneities and contaminants complicated analyses and lowercase type indicates tentative identifications. Start asterisks denote N-terminal acetylations (not yet established in class V, which is shown here without its initiator methionine since it is likely to be absent in the mature protein).

Table 1. Class relationships at the structural level

Class (human)	% identity toward structure of class IV (rat)	% intraclass identities	
		Species (human/rat)	Isozymes (human)
I (α, β, γ)	67–68	81–82	93–94
II	55	(77)	—
III	60	94	—
IV		(85)	—
V	58		—

Empty spaces indicate that values are not yet known, parentheses indicate tentative values from incompletely determined variants, and dashes in the isozyme column indicate that isozymes have not been established in mammals for those classes. Structure of class IV is from Fig. 1; remaining structures are from refs. 2–7 and 37.

However, the K_m for ethanol is also extremely high for the rat class IV enzyme, 3600-fold that for class I. The enzyme is not active toward formaldehyde in the presence of glutathione and, hence, does not have activity of the class III type. Furthermore, the activity with secondary alcohols and cyclohexanol is low (25). Although there is a substantial human/rat species variability within class IV, the general properties versus class I are present in both species, even if less pronounced in the human than the rat (Table 2). Pyrazole derivatives also inhibit class IV. K_i values are large and higher at pH 7.5 (10 mM for 4-methylpyrazole) than at pH 10 (0.2 mM) (20). With regard to coenzymes, differences are smaller toward class I, but K_m values are fairly high (25), suggesting weak binding of the coenzyme.

DISCUSSION

Mammalian class IV alcohol dehydrogenase from stomach mucosa constitutes a separate entity with a distinct structure (Fig. 1) and high turnover rate toward ethanol (Table 2). The analysis makes correlations with other structures possible to explain the enzymatic properties and to trace relationships of the vertebrate alcohol dehydrogenase system at large. In the subsequent discussion of positional numbers, the human/horse class I numbering system is followed. The alignment in Fig. 1 gives the numbering system of both the present class IV structure and the class I models, allowing for translation of numbers.

Conformational Properties. Overall, the class IV protein is a typical alcohol dehydrogenase with clear relationships to the alcohol dehydrogenases of classes I–III and V; in fact, the class IV protein is structurally closer to class I than to the other classes (Table 1). Since the tertiary structures of classes I–III have already been shown to correlate closely (29), the class IV protein should also have a similar subunit conformation, including the positions of the active site, substrate-binding cleft, coenzyme binding region, and subunit interacting segments. The characteristic features of conserved glycine residues (17), a conserved proline corresponding to a *cis*-proline at position 62 of the horse enzyme (30, 31), and two highly conserved segments (C1 and C2 in ref. 39) interspersed with three variable segments (V1, V2, and V3 in ref. 39), all constitute characteristic features also of the structure of class IV. These overall conformational conservations suggest that it may be possible to correlate specific amino acid replacements of class IV with changes in enzymatic properties.

Correlation with Enzymatic Properties. *Substrate binding.* Residues at an inner segment, a middle segment, and an outer segment of the substrate-binding cleft (31) that have been compared in class I–III alcohol dehydrogenases (29) and in many different lines of the class I enzyme (39) are underlined in Fig. 1. The spatial positions of residues considered most critical of those exchanged are shown in Fig. 2.

The inner part of the active site has large residues at all positions, Thr-48, Phe-93, Phe-140 (all as in the β-type of human class I enzyme), and Met-141 (large in all other forms except class V and identical to methionine at that position in the human class III type). In particular, Thr-48 influences the enzyme activity (40). This pattern with large residues is compatible with the high activity toward primary alcohols and low activity toward secondary alcohols and cyclohexanol because of space restrictions.

In contrast, the middle segment of the substrate cleft shows an exchange for a smaller residue, alanine at position 294 instead of valine in all other classes. This replacement, yielding additional space at the active site, appears compatible with the high activity toward aromatic substrates [*m*-nitrobenzaldehyde (25)] and with the large K_m for ethanol (Table 2). Additional water in the space would not be expected to promote binding of small molecules, such as ethanol, in agreement with similar observations on the class I/III replacements (37). In this regard, it may be noticed that the class IV protein exhibits considerable species variation in K_m for ethanol at pH 7.5 (5 M for the rat enzyme, 41 mM for the human enzyme; both, however, significantly higher than the K_m value for class I). Significantly, the human class IV protein does not have the Ala-294 replacement now found for the rat class IV structure (unpublished observations).

The outer segment of the substrate-binding cleft has large residues, Leu-110, Met-306, and Phe-309, not unique to class IV and compatible with similar size relationships as in class I.

Coenzyme binding and catalytic efficiency. Coenzyme binding is critical to the overall reaction rate in the forward direction of the enzymatic reaction. In this regard, positions 47, 363, and possibly 230 of those exchanged (Fig. 1) are of special interest. A basic residue at position 47 correlates with strong coenzyme binding (charge interactions with the pyrophosphate of the coenzyme), and enzymes with a weak or no

Table 2. Kinetic constants for class IV versus class I alcohol dehydrogenase with ethanol as substrate

Enzyme	pH 7.5			pH 10.0		
	K_m, mM	k_{cat}, min^{-1}	k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$	K_m, mM	k_{cat}, min^{-1}	k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$
Class IV						
Rat	5000	2400	0.48	340	9000	26
Human	41	1000	24	11	2100	190
Class I						
Rat	1.4	39	28	1.4	60	43
Human, γ_1	1.1	140	130	2.0	230	115
Human, α	4.2	54	13	1.5	300	200

Data for the rat class I and IV forms were from refs. 25 and 26, for the human class IV form were modified from ref. 4, and for the human class I forms were from refs. 27–29. k_{cat} values listed refer to the dimeric enzyme.

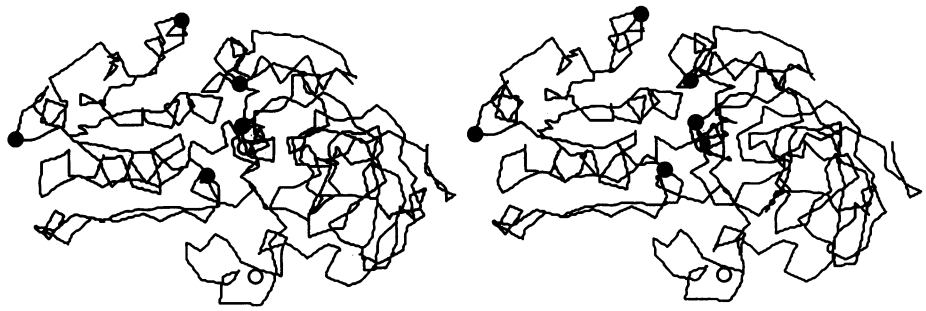

FIG. 2. Functionally important residue exchanges in the class IV alcohol dehydrogenase subunit positioned into the conformation of the human class I subunit. The conformation is that prepared from the protein data bank coordinates (33, 34) of the crystallographically analyzed class I β_1 subunit of alcohol dehydrogenase (35) using a program supplied by the journal *Protein Science* (36). The main-chain locations of the amino acid exchanged at positions 47 (center), 294 (center bottom), and 363 (center top) close to the active site and the two more distantly positioned exchanges at positions 230 (top) and 260 (far left) are shown as solid circles and their functional effects are discussed in the text. Open circles indicate the active site (center) and structural (bottom) zinc atoms.

base (such as Gly-47 of class IV and class I α) would have one factor decreasing coenzyme binding. However, the coenzyme-binding Lys-228 (31) may be especially important when a base at position 47 is lacking, as in class I α, in agreement with suggestions from mutagenesis experiments (41). Class IV is unique in having a Gln replacement at a fairly adjacent position, position 230 (instead of alanine, valine, and lysine in the other classes). In addition, class IV has Tyr-363 (instead of a basic residue in the class I enzymes) at an adjacent surface, perhaps preventing the type of compensation that may occur in class I α for the loss of the base at position 47 (42). In another segment, Val-294 in all other forms is a large residue involved in the conformational change associated with the apoenzyme/holoenzyme transition and interacting with the nicotinamide moiety of the coenzyme (31). This residue is alanine in class IV, again weakening the interaction and probably contributing to an increase in the k_{cat} value. The conserved Gly-261, characteristic of all other classes of the enzyme, is absent in class IV (Fig. 1), as is the adjacent Gly-260, which is replaced by asparagine, corresponding to a reverse turn in class I in the segment of adenine binding (30, 31, 35). This may further contribute to the weak coenzyme binding. However, part of the change in glycine pattern may perhaps be compensated for by the presence of an adjacent exchange to Gly-259, suggesting a minor shift in the reverse turn position with otherwise largely unaltered relationships.

His-51 has been ascribed a critical role in accounting for a comparatively high enzyme activity at pH 7.5 versus the activity at pH 10 and in correlating with a sensitivity to inhibition by 4-methylpyrazole (37). A relatively high activity at pH 7.5 is shared also by the class IV enzyme (Table 2), compatible with the presence of His-51. However, the rat class IV protein is weakly inhibited by 4-methylpyrazole (20, 25), suggesting that additional characteristics than just the presence of His-51 are important for that interaction.

In summary, class IV proteins lack basic residues at positions 47 and 363, where at least one basic residue occurs in other forms of the protein. Therefore, the class IV protein is unique among the alcohol dehydrogenases in lacking or having altered base relationships at two positions important for charge interactions in coenzyme binding. This effect, complemented by the changes at positions 294 and possibly 230 and 260, appears to constitute the structural explanation for decreased coenzyme binding and hence the extreme k_{cat} value of the class IV protein.

Class Relationships. As is obvious from the overall comparisons (Table 1) and the structure–function correlations (above), unique features of class IV are established. Nevertheless, residue identities are significantly higher with respect to class I than to all the other classes, and the enzyme activity is high toward ethanol (Table 2). Thus, the class IV protein, although distinct, is most closely linked both structurally and enzymatically to the class I forms, suggesting that the duplication giving rise to class IV occurred in the ancestral line to class I. Construction of a phylogenetic tree, based on presently available structures also relate class IV to class I rather than to class III (Fig. 3). Dating of the class I–IV separation is still premature, since the rate of evolutionary change may vary and is known to be different between different classes of the protein (38). Nevertheless, the origin of class IV from class I likely took place fairly early in vertebrate evolution [in agreement with a recent finding of the class IV protein also in amphibian lines (unpublished data)] but later than the origin of class I itself from class III, which has been ascribed

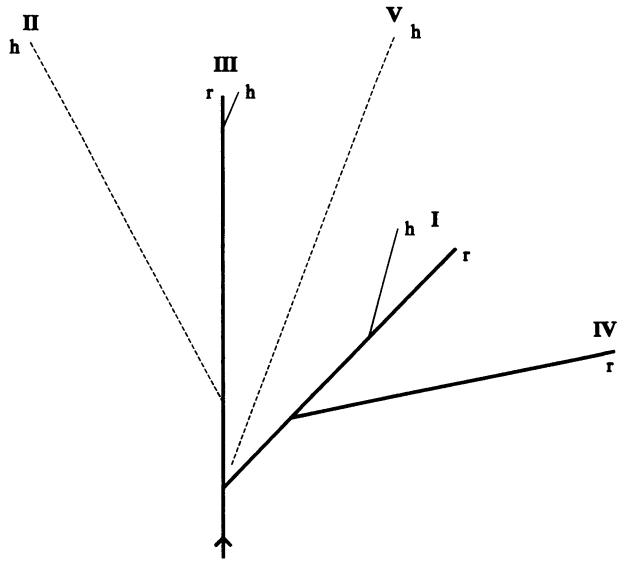

FIG. 3. Schematic representation of class relationships. The phylogenetic representation was calculated by a progressive alignment method (32) and all branch lengths are drawn to scale as obtained by the alignments. Solid lines indicate the relationship of the class IV structure toward the class I and III structures; bold lines connect all presently known rat forms. As shown, the data suggest that class IV originated from the class I line after separation of the latter from the original (9) class III line. Dashed lines indicate tentative positionings of the little studied class II and V forms, of which class II appears to be derived from class III, while the origin of class V is not yet settled, giving different possibilities depending on which other enzyme (sorbitol dehydrogenase, ζ-crystallin, yeast alcohol dehydrogenase) is used as an out-group for the rooting. Letters r and h denote the rat and human forms, respectively; both forms are characterized for classes I and III, with a larger species variation within class I than III, as represented by the correspondingly different speciation branch lengths.

tentatively to the very early part of vertebrate evolution (9, 18). The later but still early evolution of class IV suggests that this enzyme should be present in most or all mammalian animals.

Finally, it may be noticed that the class IV structure and its extensive species variation (7) are consistent with observations on a correlation between high rates of evolutionary change and late time of origin (43). Thus, class IV, like class I but not class III, exhibits considerable species variation (7) within the mammalian line.

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