Molecular Basis for Differential Substrate Specificity in Class IV Alcohol Dehydrogenases

A CONSERVED FUNCTION IN RETINOID METABOLISM BUT NOT IN ETHANOL OXIDATION*

Received for publication, December 18, 1999, and in revised form, May 3, 2000
Published, JBC Papers in Press, May 26, 2000, DOI 10.1074/jbc.M910040199

Bernat Crosas‡‡, Abdellah Allali-Hassani‡‡, Susana Eva Martínez‡‡, Silvia Martras‡‡, Bengt Persson‡, Hans Jörnvall‡, Xavier Pares‡, and Jaume Farrés‡†

From the ‡Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Barcelona, Spain and the †Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden

Mammalian class IV alcohol dehydrogenase enzymes are characteristic of epithelial tissues, exhibit moderate to high \( K_m \) values for ethanol, and are very active in retinol oxidation. The human enzyme shows a \( K_m \) value for ethanol which is 2 orders of magnitude lower than that of rat class IV. The uniquely significant difference in the substrate-binding pocket between the two enzymes appears to be at position 294, Val in the human enzyme and Ala in the rat enzyme. Moreover, a deletion at position 117 (Gly in class I) has been pointed out as probably responsible for class IV specificity toward retinoids. With the aim of establishing the role of these residues, we have studied the kinetics of the recombinant human and rat wild-type enzymes, the human G117ins and V294A mutants, and the rat A294V mutant toward aliphatic alcohols and retinoids. 9-cis-Retinol was the best retinoid substrate for both human and rat class IV, strongly supporting a role of class IV in the generation of 9-cis-retinoic acid. In contrast, 13-cis retinoids were not substrates. The G117ins mutant showed a decreased catalytic efficiency toward retinoids and toward three-carbon and longer primary aliphatic alcohols, a behavior that resembles that of the human class I enzyme, which has Gly\(^{117}\). The \( K_m \) values for ethanol dramatically changed in the 294 mutants, where the human V294A mutant showed a 280-fold increase, and the rat A294V mutant a 50-fold decrease, compared with those of the respective wild-type enzymes. This demonstrates that the Val/Ala exchange at position 294 is mostly responsible for the kinetic differences with ethanol between the human and rat class IV. In contrast, the kinetics toward retinoids was only slightly affected by the mutations at position 294, compatible with a more conserved function of mammalian class IV alcohol dehydrogenase in retinoid metabolism.

Many alcohol dehydrogenase (ADH,\(^1\) EC 1.1.1.1) forms exist in mammals. These forms have been grouped into at least six classes according to their structural and enzymatic properties (1). The physiological function of each class can be estimated from its kinetic characteristics and organ distribution. Thus, classes I and II, with low or moderate \( K_m \) for ethanol, are those mostly responsible for hepatic ethanol metabolism in humans. Class III exhibits activity with long chain primary alcohols, but its major function is the elimination of formaldehyde, acting as a glutathione-dependent formaldehyde dehydrogenase (2). Classes V and VI are little defined and only detected at the mRNA level. Recently, much effort has been devoted to the functional and molecular characterization of class IV. Its localization in the stomach and a moderate \( K_m \) for ethanol of the human enzyme make it suitable for a contribution to the first-pass metabolism of ethanol (3, 4). Moreover, class IV is characteristic of epithelial tissues, like the mucosa of the upper gastrointestinal tract, the cornea, and the blood vessel endothelium (5–7). It is very active in retinol oxidation (8–11), strongly suggesting a role of the class IV enzyme in the formation of retinoic acid. The latter compound is known to regulate morphogenesis and epithelial cell differentiation. A function of class IV in development was supported by the findings that retinoic acid synthesis in embryonic tissues correlates spatiotemporally with the expression of the class IV ADH gene (12). Moreover, class IV knock-out mice have a decreased production of retinoic acid from retinol (13) and have an increased risk of embryonic lethality during vitamin A starvation (14).

Although class I ADH is also active with retinoids, class IV, or \( \sigma \), is the most active ADH form in humans (9). Structural features responsible for class IV specificity have been investigated using molecular models, in which docking simulations with retinoid isomers indicate a better binding of these molecules to the class IV active site in comparison to that of class I, suggesting that deletion at position 117 facilitates their binding (15–17). Recently, the x-ray structure of human class IV ADH has revealed an active site more suitable for oxidation of long chain aliphatic alcohols, such as retinols, than for short chain molecules, e.g. ethanol, in contrast with the opposite behavior of the class I \( \beta \) subunit (18, 19).

An intriguing feature of class IV enzymes is the strong variation of kinetic properties between enzymes of different mammalian species. Thus, the human enzyme exhibits a \( K_m \) for

\* This work was supported by Grant BTO4-CT97-2123 from the Commission of the European Union, Grants PN96-0069 and PB98-0855 from the Spanish Dirección General de Enseñanza Superior e Investigación Científica, Projects 13X-3532 and 03P-11312 from the Swedish Medical Research Council, and the Magnus Bergvall Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^TM\) / EBI Data Bank with accession number(s) X98746.
‡ These authors made equal contributions to this study.
\(^{1}\) The abbreviations used are: ADH, alcohol dehydrogenase; PCR, polymerase chain reaction; DTT, dithiothreitol; \( K_m \), dissociation constant for NAD\(^+\); \( K_

25180 This paper is available on line at http://www.jbc.org
ethanol—100-fold lower than that of the rat form, at pH 7.5 (20). However, the two enzymes show 88% identity in amino acid sequence, and exchanges at the substrate-binding site are scarce, the most interesting being at position 294. This position is a Val in most animal ADHs, but an Ala in the rat and mouse class IV enzymes, both with high Km values for ethanol (21, 22). The exchange to a smaller and less hydrophobic residue in this area is compatible with low affinity for small substrate molecules, and we hypothesized that this substitution was responsible for the dramatic kinetic differences between the rat and human class IV enzymes (20, 23).

In the present work, the influence of the Val/Ala exchange on the class IV kinetics with retinoids and ethanol has been assessed from the inversely complementary properties of the human class IV V294A and the rat A294V mutants. Moreover, we have investigated the role in substrate specificity of the deletion at position 117, a specific feature of class IV, by constructing a human class IV mutant with Glu117 (G117ins), as found in class I. The differences in the substrate-binding pocket between the x-ray structure of human class IV (20), including recognition sites (underlined) for the G117ins mutant but using HUM5 (5′-CATGGAGGAGGATCATGGATCAGGAC-3′) and the antisense HUM7 (5′-CTTGGGTTGCATTCTCTACACTGTGG-3′) primers, containing recognition sequence (underlined) for restriction enzyme NdeI and BamHI, respectively. The PCR products were cloned into pBluescript II SK (+) and sequenced. After NdeI/EcoRI or BamHI excision, the cDNAs were subcloned into the expression vector pET5a (Promega). The E. coli strain BL21(DE3)/pLysS was transformed with the final constructs pET/HUM12 and pET/RAT78, and grown in 5 liters of liquid Terrific Broth medium. When an OD at 595 nm of 0.65 was reached, a 2-h induction of 1 m M isopropyl-1-thio-b-D-galactopyranoside to the culture.

The Human V294A, G117ins, and Rat A294V Mutants—To obtain the V294A mutant, four primers were used in three PCR reactions. The primers HUM1 and HUM3 (5′-CATCGTGCTGATGAGGAGGCTCTACAGACAT-3′) and HUM5 (5′-GGTACCTCC-3′) were used in the PCR2 construct. The products from PCR1 and PCR2 were mixed with HUM1 and HUM2 primers in a third PCR reaction, obtaining a 3′-ADH coding region, including the V294A mutation. The same procedure was followed for the G117ins mutant but using HUM5 (5′-CATCGTGCTGATGAGGAGGCTCTACAGACAT-3′) and the mutagenic primer HUM4 (5′-GGTACCTCC-3′), containing the G117ins mutation (underlined), and HUM2 were used in the PCR2 construct. The products from PCR1 and PCR2 were mixed with HUM1 and HUM2 primers in a third PCR reaction, obtaining the 5′-ADH coding region, including the G117ins mutation. The corresponding wild-type DNA fragment. All mutated DNA fragments were checked by sequencing prior to expression.

Recombinant Wild-type Human Class IV ADH and V294A and G117ins Mutants—Isopropyl-1-thio-b-D-galactopyranoside-induced cells from a 5-liter culture (18 g wet weight) were harvested by centrifugation at 5000 × g for 20 min. The pellets were frozen at −80 °C to facilitate cell lysis and resuspended in 50 mM Tris-HCl, 1 mM EDTA, pH 7.0, 10% sucrose, 0.05% sodium azide, and 2 mM b-mercaptoethanol. Glass beads (diameter 0.5 mm) were added, and the mixture was stirred at 4 °C for 30 min and incubated with DNase (18 μg/ml) at room temperature for 30 min to reduce sample viscosity. The homogenate was centrifuged at 11,000 × g for 20 min, the supernatant was treated with 0.6% proteamine sulfate and incubated on ice for 30 min, and it was centrifuged at 20,000 × g for 15 min. The supernatant resulting from the centrifuga-tion was dialyzed against 20 mM Tris-HCl, 0.5 mM DTT, pH 8.0, and applied to a DEAE-Sepharose (Amersham Pharmacia Biotech) column (15 cm × 1 cm) equilibrated with the same buffer (3). The enzyme was eluted with a 0–150 mM NaCl linear gradient (1000 ml), and the active fractions were pooled, concentrated, dialyzed against the initial buffer, and applied to a AMP-Sepharose (Amersham Pharmacia Biotech) column (1 × 15 cm). The enzyme was eluted with a linear gradient (500 ml) of 20–100 mM Tris-HCl, 0.5 mM DTT, pH 8.2, and the active fractions were pooled, concentrated, and stored at −80 °C. Starch gel electrophoresis, followed by activity staining with ethanol or 2-buten-1-ol as a substrate (28), and electrophoresis in SDS-polyacrylamide gel with subsequent silver staining were used to assess the degree of purity after each step of purification. For the V294A mutant enzyme the same purification procedure was used. For the G117ins mutant, an additional gradient from 100 to 400 mM Tris-HCl, 0.5 mM DTT, pH 8.2 (500 ml) was necessary to elute the enzyme from the AMP-Sepharose column.

Recombinant Wild-type Rat Class IV ADH and the A294V Mutant—Only the differences with the purification procedure for the human enzyme are detailed. The complete cell lysate was obtained by shaking the cell resuspension with glass beads (diameter 0.5 μm) in a bead beater (Bioprec Products). The buffer used during the purification was 20 mM Tris-HCl, 0.5 mM DTT, pH 7.9. The dialyzed supernatant was applied to a DEAE-Sepharose (Amersham Pharmacia Biotech) column (2.5 × 40 cm) equilibrated with the same buffer, and the enzyme was eluted with a 0–150 mM NaCl linear gradient (500 ml). The active fractions were pooled, concentrated, and applied to a Blue-Sepharose (Amersham Pharmacia Biotech) column (1 × 15 cm). The enzyme was eluted with a linear gradient (400 ml) of 20–300 mM Tris-HCl, 0.5 mM DTT, pH 7.9. The active fractions were pooled, concentrated, dialyzed against the initial buffer, and applied to a Q-column (Waters). Elution
of the enzyme was performed with a 0.2–250 mM sodium acetate linear gradient.

Enzyme Assays—Alcohol dehydrogenase activity with alcohols was determined by monitoring the formation of NADH at 25 °C in a Varian SpectraMax 190 spectrophotometer by measuring absorbances at 340 nm. Alcohol oxidation was measured in 0.1 M sodium phosphate, pH 7.5, or in 0.1 M glycine-NaOH, pH 10. Kinetic parameters were obtained from activity measurements, with substrate concentrations that ranged from 0.1× \( K_m \) to 10× \( K_m \), except for ethanol with the V294A mutant and rat wild-type enzyme, where saturation with this substrate could not be reached. Each individual rate measurement was run in duplicate. Three determinations were performed for each kinetic constant. The dissociation constant for NAD(H) was determined by monitoring the formation of NADH at 25 °C in a Varian SpectraMax 190 spectrophotometer by measuring absorbances at 340 nm.

RESULTS

Cloning and Sequence Analysis of the cDNA Coding for Rat Class IV ADH—A composite full-length sequence for rat class IV ADH, comprising 2052 bp (Fig. 1) and was obtained via screening of a rat lung cDNA library and using reverse transcription-PCR of rat liver RNA. The sequences isolated from the lung cDNA library included nucleotides 1–1685, whereas an overlapping PCR fragment from liver RNA extended the sequence up to position 2052. A part of the 5'-noncoding regions showed a notable sequence identity with those corresponding to the human (2.3 kilobases) (34) and mouse (2.1 kilobases) (35) class IV ADH nucleotide sequences, further indicating the correctness of the rat sequence. The 3'-untranslated region containing nucleotides 1182–2017 was also amplified from rat genomic DNA and sequenced, matching the sequence isolated from liver RNA and showing that this was not a cloning artifact. Both the 5'- and 3'-noncoding regions showed a notable sequence identity with those corresponding to the human (20) and mouse (33) class IV ADH nucleotide sequences, further indicating the correctness of the rat sequence. The 3'-untranslated region contained a putative polyadenylation signal at position 1885 and a poly(A) tail starting at position 2042. Northern blot analysis showed a 2.1-kilobase class IV mRNA band at position 1885 and a poly(A) tail starting at position 2042.

Therefore the reported cDNA sequence likely represents the complete rat class IV ADH transcript.
molecular basis for substrate specificity in class IV ADH

Table I
Bisubstrate kinetics of the human and rat wild type and mutant class IV ADH enzymes, with ethanol and NAD⁺, at pH 7.5

| Substrate | Kᵦ (μM) | Kᵦ (μM) | kₗcat (min⁻¹) | Kᵦ (μM) | kₗcat/Kᵦ (mM⁻¹·min⁻¹) | Kᵦ (μM) | kₗcat/Kᵦ (mM⁻¹·min⁻¹) |
|-----------|---------|---------|---------------|---------|-------------------------|---------|-------------------------|
| Ethanol   | 42 ± 3  | 2570 ± 60 | 61 ± 5        | 12 ± 2  | 1000 ± 100               | 83 ± 16 | 2500 ± 400               |
| Propanol  | 11 ± 1  | 1750 ± 60 | 160 ± 15      | 8.7 ± 1.1 | 950 ± 55               | 110 ± 17 | 480 ± 55                |
| Butanol   | 1.0 ± 0.1 | 2390 ± 80 | 2390 ± 250    | 4.9 ± 0.8 | 1040 ± 55             | 210 ± 36 | 47 ± 3                  |
| Pentanol  | 0.31 ± 0.03 | 2100 ± 65 | 6780 ± 890   | 1.15 ± 0.17 | 950 ± 46           | 830 ± 130 | 63.3 ± 0.5               |
| Hexanol   | 0.14 ± 0.012 | 1370 ± 35 | 9790 ± 875   | 0.46 ± 0.03 | 750 ± 17           | 1630 ± 110 | 1.9 ± 0.1               |
| Octanol   | 0.050 ± 0.008 | 740 ± 40 | 14800 ± 1500 | 0.18 ± 0.04 | 550 ± 55           | 3060 ± 750 | 0.67 ± 0.01            |

Table II
Kineti constants of the human class IV enzyme with primary aliphatic alcohols at pH 7.5

| Substrate | Kᵦ | kₗcat | hₗcat/Kᵦ | Kᵦ | kₗcat/Kᵦ | Kᵦ | kₗcat/Kᵦ |
|-----------|----|-------|----------|----|----------|----|----------|
| Ethanol   | 42 | 2570  | 61       | 12 | 1000     | 83 | 2500     |
| Propanol  | 11 | 1750  | 160      | 8.7| 950      | 110| 480      |
| Butanol   | 1.0| 2390  | 2390     | 4.9| 1040     | 210| 47       |
| Pentanol  | 0.31| 2100 | 6780     | 1.15| 950      | 830| 63.3     |
| Hexanol   | 0.14| 1370 | 9790     | 0.46| 750      | 1630| 1.9      |
| Octanol   | 0.050| 740 | 14800    | 0.18| 550      | 3060| 0.67     |

The amino acid sequence deduced is identical to that obtained from sequence analysis of the protein (23), except for six residue differences. Notably, the first four N-terminal residues (Met-Asp-Thr-Ala) are different from those previously reported (Ser-Asn-Arg-Val), which had been considered tentative because of the fact that the N-terminal residue was blocked by an acetyl group, as in all other mammalian ADHs. Indeed, the present rat N-terminal sequence resembles more closely the human (20) and mouse (33) class IV ADH sequences except for the presence of an Asp (instead of Gly) following the initiator Met. Asp is quite an unusual residue at this position (mostly Ser, Ala, or Gly) in other ADHs. Human and mouse class IV have a Gly, which implies loosing Met and the subsequent acetylation of the Gly residue. Interestingly, when Met is retained and acetylated, Asp is the predominant penultimate residue found in eukaryotic proteins (36). Because the rat protein analysis had revealed that the N terminus contained one additional residue compared with the human class IV enzyme (20, 23), it is tempting to speculate that in rat class IV the initiator Met may be retained and acetylated. The other two observed differences, a Glu for Gly at position 109 and a Val for initiator Met may be retained and acetylated.

The kinetic constants for the G117ins mutant were similar to those of the human wild-type enzyme. No significant change was observed between the Kᵦ values, although the Kᵦ and kₗcat values were lower for the mutant enzyme, and there was a 2–3-fold decrease in the Kᵦ value for ethanol. Consistent with this finding, the Kᵦ for the competitive inhibitor 4-methylpyrazole was also decreased.

The kinetic constants with ethanol of the human V294A and rat A294V mutants compared with those of the corresponding wild-type enzymes were quite altered, although less so for the rat enzymes (Table I). The V294A mutant exhibited a Kᵦ value for ethanol, which was 280-fold that of the wild-type human class IV enzyme. Accordingly, the inhibition constant for 4-methylpyrazole was also decreased.

PCR-based site-directed mutagenesis was used to construct several mutant class IV enzymes: human V294A, rat A294V, and human G117ins. The mutants were purified to homogeneity and their kinetic properties were compared with those of the wild-type enzymes (Tables I-III).

Kinetic Properties of the Mutant and Wild-type Enzymes with Aliphatic Alcohols—Bisubstrate kinetics were performed by covariation of NAD⁺ and ethanol concentrations. The experimental data could be fitted to the equation for the sequential ordered Bi Bi mechanism (29), supporting the conclusion that the wild-type and mutant enzymes follow this mechanism. The kinetic constants Kᵦ (Kᵦ for NAD⁺), Kᵦ (dissociation constant for NAD⁺), Kᵦ (Kᵦ for ethanol), and kₗcat could be determined (Table I). In addition, Kᵦ (dissociation constant for NADH) and Kᵦ for 4-methylpyrazole were calculated by fitting the data to the equation for competitive inhibition with NAD⁺ and ethanol, respectively.

The kinetic constants for the G117ins mutant were similar to those of the human wild-type enzyme. No significant change was observed between the Kᵦ values, although the Kᵦ and kₗcat values were lower for the mutant enzyme, and there was a 2–3-fold decrease in the Kᵦ value for ethanol. Consistent with this finding, the Kᵦ for the competitive inhibitor 4-methylpyrazole was also decreased.

The kinetic constants with ethanol of the human V294A and rat A294V mutants compared with those of the corresponding wild-type enzymes were quite altered, although less so for the rat enzymes (Table I). The V294A mutant exhibited a Kᵦ value for ethanol, which was 280-fold that of the wild-type human class IV enzyme. Accordingly, the inhibition constant for 4-methylpyrazole increased significantly in this mutant. Although the Kᵦ value for NAD⁺ increased notably in the V294A mutant, the Kᵦ and Kᵦ values did not vary substantially. Thus, the marked increase in the kₗcat value with respect to the wild-type human class IV enzyme cannot be correlated with dissociation of the coenzyme. Because dissociation of coenzyme is likely to be the limiting step (kₗcat remains constant for different substrates, Table II), variation of kₗcat cannot be excluded. A reciprocal tendency was observed in the A294V mu-
Molecular Basis for Substrate Specificity in Class IV ADH

Table III

Kinetic constants of human and rat class IV with retinoids at pH 7.5

| Substrate | Wild type | G117ins | V294A | Rat | A294V |
|-----------|-----------|---------|-------|-----|-------|
| All-trans-retinol | | | | | |
| $K_m$ (µM) | 15 ± 4 | 15 ± 3 | 10 ± 3 | 7.6 ± 0.6 | 7.7 ± 1 |
| $k_{cat}$ (min⁻¹) | 67 ± 10 | 24 ± 1.2* | 30 ± 2* | 4.4 ± 0.1 | 25 ± 1* |
| $k_{cat}/K_m$ (µM⁻¹·min⁻¹) | 4500 ± 1370 | 1600 ± 330 | 3000 ± 900 | 550 ± 50 | 3250 ± 440* |
| 9-cis-retinol | | | | | |
| $K_m$ (µM) | 30 ± 4 | 39 ± 10 | 58 ± 7* | 25 ± 2 | 18 ± 0.4* |
| $k_{cat}$ (min⁻¹) | 475 ± 44 | 170 ± 15* | 430 ± 21 | 77 ± 8 | 300 ± 50* |
| $k_{cat}/K_m$ (µM⁻¹·min⁻¹) | 13300 ± 2020 | 4360 ± 1100* | 7400 ± 960 | 3080 ± 400 | 16670 ± 2800* |
| 13-cis-retinol | N.A. | N.A. | N.A. | N.A. | N.A. |
| All-trans-retinal | | | | | |
| $K_m$ (µM) | 34 ± 6 | 5.4 ± 1.3* | 9.4 ± 0.6* | 8 ± 1 | 6.8 ± 0.6 |
| $k_{cat}$ (min⁻¹) | 110 ± 25 | 13 ± 1* | 84 ± 2 | 14 ± 0.7 | 71 ± 2* |
| $k_{cat}/K_m$ (µM⁻¹·min⁻¹) | 3300 ± 960 | 2410 ± 660 | 8940 ± 880* | 1750 ± 230 | 10440 ± 970* |
| 9-cis-retinal | | | | | |
| $K_m$ (µM) | 21 ± 5 | 34 ± 7 | 9.3 ± 0.5 | 12 ± 2 | 5 ± 0.75* |
| $k_{cat}$ (min⁻¹) | 190 ± 24 | 80 ± 4* | 360 ± 2 | 31 ± 1.5 | 68 ± 3* |
| $k_{cat}/K_m$ (µM⁻¹·min⁻¹) | 8980 ± 2350 | 2350 ± 500* | 38710 ± 2400* | 2580 ± 450 | 13600 ± 2100* |
| 13-cis-retinal | N.A. | N.A. | N.A. | N.A. | N.A. |

* Data taken from Ref. 10.

Significant differences ($p < 0.01$) of mutant enzyme values with respect to those of wild-type enzymes are indicated with an asterisk. N.A., no activity was detected using up to 150 µM 13-cis-retinol or 100 µM 13-cis-retinal.
distances between the oppositely positioned residues 116 and 294 in the substrate-binding pocket could be measured. Whereas in the human structure, Ile116 and Val294 were separated by only 4.8 Å, in the rat enzyme the atomic distance between Leu116 and Ala294 was 6.8 Å (Fig. 2). In the human enzyme, this distance is close to the threshold value for water accessibility (2.8 Å), when the contribution of van der Waals radii is considered. Consistent with the kinetic results (Table I), each of the 294 mutations reverts the narrowness of the bottleneck; in the A294V mutant the atomic distance was 5.5 Å.

Docking of all-trans-, 9-cis-, and 13-cis-retinol isomers to the x-ray structure of human class IV and to the model of the rat class IV ADH revealed that the atomic distance between the
catalytic zinc and the oxygen of the hydroxyl group of the all-trans-retinol and 9-cis-retinol isomers was 2.4 Å in both structures. However, the distance was 3.1–3.3 Å for 13-cis-retinol, a value excessively large for catalysis to be productive. Moreover, 13-cis-retinol bound in an orientation different from that of the other two isomers, with its β-ionone ring much closer to the 114–120 loop. The docking energy values for the three isomers studied ranged from −14 to −27 kcal/mol (Fig. 3).

**DISCUSSION**

Distinct class IV structural features are observed in the middle region of the substrate-binding pocket, where all members of the class exhibit a deletion at position 117 (Gly in class I), and the rodent enzyme has Ala in place of Val. Both the deletion at 117 (15, 16, 18, 19) and the exchange at 294 (20, 23) have been suggested to explain the distinct kinetic properties of the class IV enzymes. Interestingly, these residues are located within the variable segments V2 and V3 of ADH structures (37).

Deletion of residue 117 in class IV ADH shortens the loop including residues 114–120, and thus it has been reported to widen the entrance to the substrate-binding pocket permitting the efficient binding of retinol (15, 16, 18). Here we have shown that by reverting the deletion at position 117 (G117I mutation), kinetics with primary aliphatic alcohols (Table II) and with retinoids (Table III) are affected. In terms of the kinetic properties toward primary aliphatic alcohols, the results obtained with wild-type class IV versus those with the G117I mutants (Table II) are compatible with previous studies on class I mutations: human β2/β1, L116A (38) and horse EE D115del (39), which likewise diminish the size of loop 114–120 (either by substitution by a smaller side chain or by deletion). In all cases, when the size of the loop decreased, the Km values for short-chain aliphatic alcohols (ethanol and propanol) increased, whereas at the same time the enzymes became more specific for alcohols with four or more carbons.

Residues 116 and 294 define a narrow bottleneck in the middle region of the substrate-binding pocket that restricts the access of bulky substrates to the bottom of the active site (Fig. 2; 18, 40). The presence of Met57, Met41, and Phe389 also contributes to narrowing the middle region of the substrate pocket compared with the relationships in class I ADH (18, 19). It is tempting to speculate that the deletion at position 117 in class IV and the V294A mutation could create additional space for water molecules to enter the active site. This effect increases the number of nonproductive conformations and it would impair the correct binding of short-chain alcohols, such as ethanol, resulting in increased Km values. In fact, it is possible to establish a good correlation between the exchange Val/Ala in the middle region of the substrate-binding pocket, the atomic distance between residues 116 and 294, and the Km values for ethanol (Table 1). The V294A exchange also increased the Km values for longer aliphatic alcohols, although to a lesser extent, probably because of their ability to establish interactions with several residues in the active site pocket.

This work provides an in depth kinetic characterization of rat class IV ADH regarding retinoid metabolism. No data have ever been reported for 9-cis- and 13-cis-retinol oxidation with this enzyme. The present kinetic values toward the all-trans compounds do not differ significantly from those previously reported (8), with the exception of a higher kcat value for all-trans-retinol, which may be due in part to a somewhat higher specific activity obtained in the preparation of purified recombinant rat class IV.

Among the isomers assayed, 9-cis-retinol was the best substrate in terms of catalytic efficiency. The pathway that leads to the bioactive compound, 9-cis-retinoic acid, has not been clearly established yet. As suggested in previous works (10, 41), isomerization at the level of 9-cis-retinol and subsequent oxidation to 9-cis-retinol is one possibility, supported now by favorable kinetics of both human and rat class IV ADHs. No activity was found with 13-cis-retinol, which is a competitive inhibitor of all-trans-retinol and 9-cis-retinol oxidation in human class IV (10). In good agreement with its inhibitory behavior, our docking simulations show that 13-cis-retinol could bind to human and rat class IV. However, the rigidity provided by the double bond in cis conformation at C13 appears to prevent the hydroxyl group from reaching a catalytically productive distance or proper orientation with respect to the zinc atom. In contrast, all-trans- and 9-cis-retinol docking simulations showed a binding effective for catalysis, in good agreement with other docking studies (15–18).

The G117I mutant showed kcat values for retinoids that were below those of the wild-type human class IV enzyme but far above those of the class I enzymes (9, 11). This implies that deletion of residue 117 contributes to the high specificity of class IV toward retinoids but also that this is not the only amino acid exchange involved in the creation of such a specificity. It is conceivable that other substitutions, such as the smaller Leu110 (in class IV) could contribute to differences in substrate specificity. Interestingly, the two 294 mutations altered minimally the kinetic constants toward retinoids, despite the fact that they profoundly altered ethanol oxidation.

From the present results, it can be concluded that human and rat class IV ADH are enzymes similarly efficient toward retinoids, although they differ dramatically with respect to ethanol oxidation. This difference is extremely relevant when drawing conclusions from alcohol toxicity studies using rodents as model animals. Ala389 in rodent class IV, in substitution of a Val394 in human class IV (a residue highly conserved in vertebrate ADH), is clearly the change responsible for the physiological inefficiency of the rat enzyme toward ethanol (Km = 2.4 mM) in contrast to the moderate activity of the human class IV enzyme (Km = 42 mM). Evolution has allowed the presence of a residue in the rodent class IV that makes the enzyme unsuitable for ethanol elimination but still permitting retinoid metabolism. Together with the enzyme localization in epithelial tissues but not in liver (5), this is a strong evidence of a role of class IV in a specific metabolic step such as the conversion of retinol to retinal in the crucial pathway of retinoid acid synthesis, rather than in an unspecified alcohol detoxification.
Molecular Basis for Substrate Specificity in Class IV ADH

25187