cDNA Cloning and Characterization of a New Human Microsomal NAD\(^+\) -dependent Dehydrogenase that Oxidizes All-trans-retinol and 3α-hydroxy-steroids*  

Wendy H. Gough, Sarah VanOoteghem, Thaw Sint, and Natalia Y. Kedishvili‡  

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122  

We report the cDNA sequence and catalytic properties of a new member of the short chain dehydrogenase/reductase superfamily. The 1134-base pair cDNA isolated from the human liver cDNA library encodes a 317-amino acid protein, retinol dehydrogenase 4 (RoDH-4), which exhibits the strongest similarity with rat all-trans-retinol dehydrogenases RoDH-1, RoDH-2, and RoDH-3, and mouse cis-retinol/androgen dehydrogenase (≥73% identity). The mRNA for RoDH-4 is abundant in adult liver, where it is translated into RoDH-4 protein, which is associated with microsomal membranes, as evidenced by Western blot analysis. Significant amounts of RoDH-4 message are detected in fetal liver and lung. Recombinant RoDH-4, expressed in microsomes of SF9 insect cells using BaculoGold Baculovirus system, oxidizes all-trans-retinol and 13-cis-retinol to corresponding aldehydes and oxidizes the 3α-hydroxy-steroids androstan-3β-diol and androsterone to dihydrotestosterone and androstane-dione, respectively. NAD\(^+\) drostane-diol and androsterone to dihydrotestosterone.  

The physiological role of these NADP\(^+\)-dependent enzymes in retinoic acid biosynthesis in vivo has been questioned because of the cellular ratios of the reduced and oxidized forms of NADP\(^+\) (13). In the liver cytosol, and presumably other tissues, the NADP\(^+\)/NADPH ratio is about 0.01, whereas the NAD\(^+\)/NADH ratio is about 1000 (14), suggesting that enzymes that prefer NAD\(^+\) function in the reductive rather than oxidative direction.  

We became interested in finding an isoenzyme of RoDH that would function efficiently in the oxidative direction (i.e. prefer NAD\(^+\) as cofactor) and would recognize all-trans-retinol as substrate.

Short chain alcohol dehydrogenases/reductases are either cytosolic or membrane-bound enzymes with a subunit molecular mass of 25–35 kDa that utilize a vast variety of substrates, including steroids and progestins (1). Recently, this family of enzymes has expanded to include the retinol-oxidizing dehydrogenases (2–6). Retinol dehydrogenases are involved in the biosynthesis of all-trans-retinoic acid, the activating ligand for a family of nuclear receptors (7). All-trans-retinoic acid is produced from all-trans-retinol in two oxidative steps: all-trans-retinol is oxidized to all-trans-retinal and then further to all-trans-retinoic acid. Retinol dehydrogenases catalyze the rate-limiting step: the oxidation of retinol to retinaldehyde (8). Although the effects of retinoic acid on gene transcription and regulation have been intensively studied during the last decade, the exact enzymes that synthesize this morphogen and the mechanisms that regulate its production in tissues are not fully understood. Enzymatic activity capable of oxidizing retinol to retinaldehyde is readily detected in the cytosolic and microsomal fractions of total cell homogenates (9). The cytosolic activity has been linked to the NAD\(^+\)-dependent medium-chain alcohol dehydrogenases (ADHs),1 which, in addition to trans and cis forms of retinol, oxidize a variety of aliphatic and a number of cyclic alcohols (10). In the cells, most retinol is bound to the cellular retinol-binding protein (CRBP) (11). ADHs cannot oxidize the bound form of retinol.2

The first enzyme purified by following its ability to oxidize CRBP-bound retinol, RoDH-1, turned out to be a microsomal NADP\(^+\)-dependent short chain dehydrogenase/reductase (3). The cDNA for RoDH-1 was initially isolated from a rat liver cDNA library, and later on, its mouse homolog was cloned and found to share 98% amino acid sequence identity with the rat enzyme (6). Two more closely related enzymes were subsequently cloned, RoDH-2 (4), with 82% sequence identity to RoDH-1, and RoDH-3 (12), with 95% sequence identity, establishing a multigene family of all-trans-retinol dehydrogenases.  

The physiological role of these NADP\(^+\)-dependent enzymes in retinoic acid biosynthesis in vivo has been questioned because of the cellular ratios of the reduced and oxidized forms of NADP\(^+\) (13). In the liver cytosol, and presumably other tissues, the NADP\(^+\)/NADPH ratio is about 0.01, whereas the NAD\(^+\)/NADH ratio is about 1000 (14), suggesting that enzymes that prefer NAD\(^+\) will function in the reductive rather than oxidative direction.

W. F. Boston, unpublished observations.

1 The abbreviations and trivial names used are: ADH, alcohol dehydrogenase; CRBP, cellular retinol-binding protein; RoDH, retinol dehydrogenase; HPLC, high performance liquid chromatography; MeSO\(_2\), dimethyl sulfoxide; 3α-diol, 5α-androstane-3α,17β-diol; androsterone, 5α-androstane-3α,17β-dione; dihydrotestosterone, 5α-androstane-17β-ol-3-one; androstane-dione, 5α-androstane-3,17-dione; CRAD, cis-retinol/androgen dehydrogenase; PCR, polymerase chain reaction.

2 N. Y. Kedishvili, W. H. Gough, W. I. Davis, S. Parsous, T.-K. Li, and W. F. Boston, unpublished observations.
substrate, because it was clear that RoDH exists in multiple isoenzymic forms. Here, we report a cDNA sequence and catalytic properties of a new human short chain dehydrogenase/ reductase that shares more than 70% sequence identity with rat RoDHs, recognizes all-trans-retinol, and prefers NAD+ over NADP+.

MATERIALS AND METHODS

cDNA Cloning—A human liver agt10 cDNA library (CLONTECH Inc., Palo Alto, CA) was screened with the cag-RII/ATP-labeled coding region of rat RoDH-1 prepared by PCR amplification of the 5′ ATAIL-PCR specific primers designed according to a published sequence (3). The hybridization conditions were as follows: 25% formamide, 5× Denhardt’s solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400), 5× saline-sodium-phosphate-EDTA, 0.1 mg/ml salmon sperm DNA, and 0.1% SDS at 42 °C overnight. After hybridization, the Nick-trans filters were washed several times in 6× SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.5), 0.1% SDS at room temperature, and the final wash was performed in 0.2× SSC, 0.1% SDS. Positive recombinant phage plaques were purified, and phage DNA was isolated using a Qiagen Lambda kit (Qiagen, Chatsworth, CA). The DNA insert was obtained by digestion with EcoRI and subcloned into a M13mp18RF digested with EcoRI. Sense and antisense single-strand cDNAs were synthesized from 20,000–50,000 cDNA inserts at least 300 nucleotides in length and digested with restriction endonucleases EcoRI and HindIII, respectively (underlined). The C-terminal fragment of RoDH-4 cDNA was amplified by PCR using primers Eco5′ (sense, CGG GAA TTC CAG GTG CTG AGC CAC CTG; nucleotides at position 609–628) and HindIII (antisense, CGA AAG CTG TGG CTC TCA CGC ACT CC; nucleotides at position 430–446). The primers carried recognition sites for restriction endonucleases EcoRI and HindIII, respectively (underlined). The C-terminal fragment of RoDH-4 cDNA was amplified using primers Hind5′ (sense, CCA AAG CTG TGG TGG TAC ACG TCT CCA GT; nucleotides at position 609–628) and Xho3′ (antisense, AGA CTC GAG TGG CAT CCC ACC GAG CAG CTG; nucleotides at position 981–998), which carried recognition sites for restriction endonucleases HindIII and XhoI, respectively (underlined). Both PCR primers were used for 5 min at 94 °C and cooled to 72 °C, 2 μl of Ffu polymerase (Stratagene) were added, and 30 cycles were run as follows: denaturing at 94 °C for 45 s, annealing at 52 °C for 45 s, and extension at 72 °C for 6 min. The PCR fragments were purified by electrophoresis in 1% agarose gel and subcloned into pET32a vector (Novagen) digested with EcoRI/HindIII and HindIII/XhoI, respectively, using Rapid ligation kit (Boehringer Mannheim, Indianapolis, IN). Competent BL21 E. coli cells were transfected with ligation mixtures and spread onto TY hard agar plates containing 200 μg/ml ampicillin. The pET32a vectors that contained inserts were sequenced by verifying the sequence of the inserts and were transfected into BL21(DE3) cells. The expression of recombinant proteins in BL21(DE3) cells was induced by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at A600 of 0.8–1.0. The cultures were incubated for 24 h at 30 °C. Cell pellets were resuspended in ice-cold 1× binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9) and homogenized twice using a French press. The homogenate was centrifuged at 20,000 × g for 15 min, the supernatant was discarded, and the pellet was resuspended in 1× binding buffer, sonicated, and centrifuged to remove all soluble proteins. The pellet was resuspended in the 1× binding buffer supplemented with 6 mM urea and incubated on ice for 1 h shaking. The solubilized proteins were loaded onto ice-cold 1× binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.4) and equilibrated with 1× binding buffer plus 6 mM urea. Protein was eluted with 1× elute buffer plus 6 mM urea. From a 1-liter culture, 5.5 mg of the N-terminal fragment and 3.5 mg of the C-terminal fragment were obtained. The purified proteins were dialyzed against 10 mM Tris-HCl, pH 7.4, to remove urea. Rabbits were injected subcutaneously with 500-μg portions of each protein mixed 1:1 with adjuvant five times at 3-week intervals. A 1:2000 dilution of each anti RoDH-4 antisemur detected ~1 ng of the corresponding recombinant protein.

Expression of RoDH-4 in S99 Cells—S99 cells were purchased from Invitrogen and were grown in a monolayer at 27 °C in Grace's insect cell culture medium (Life Technologies, Inc.) supplemented with yeastolate and lactalbumin hydrolysate (each to 3330 μg/liter), fetal bovine serum (10%), gentamycin (10 μg/ml), and amphotericin (2.5 μg/ml). At confluency, the cells were sloughed off and split 1:3.

The RoDH-4 cDNA was subcloned into the XbaI/BglII restriction sites of pVL1392 vector (Pharmingen, San Diego, CA). The expression construct was sequenced to verify the sequence of RoDH-4. The cotransfection of Sf9 cells with RoDH-4-pVL1392 and BaculoGold DNA was performed according to manufacturer's protocol (Pharmingen). Briefly, 2.5 million S99 cells were cotransfected with a mixture of 2 μg of sterile RoDH-4-pVL1392 and 0.5 μg of BaculoGold DNA. The infected cells were incubated for 5 days at 27 °C, and the medium was collected and centrifuged for 10 min at 5000 × g to remove detached cells. The supernatant was amplified two more times, and the titer of the amplified supernatant was determined by plaque assay. To produce recombinant RoDH-4, attached S99 cells were infected at virus:cell ratio of 10:1. Cells were collected after 3 days of incubation at 27 °C; resuspended in 0.01 mM potassium phosphate, pH 7.4, 0.25 μM sucrose, 0.1 mM EDTA, 0.1 mM DTT; and homogenized using a Dounce homogenizer. The unbroken cells, cellular debris, and mitochondria were removed by centrifugation for 15 min. Microsomal suspension was aliquoted into small portions and stored frozen at −80 °C. Protein concentration was determined by Lowry (15) and by dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

Northern Blot Analysis—A sample of frozen human liver was homogenized in 50 mM Heps, pH 6.8, 0.5% Triton X-100, 2 mM DTT, 1 mM benzamidine, and 1 mM EDTA. The homogenate was centrifuged at 20,000 × g for 30 min, and the supernatant was recenterfuged at 105,000 × g for 2 h to isolate the membranes. Twenty-one micrograms of protein from each fraction, 105,000 × g supernatant, and 105,000 × g pellet were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin in PBS, washed several times with PBST, and incubated with a 1:2000 dilution of either anti-N-terminal or anti-C-terminal antisemur overnight. After washing with PBST, the membrane was incubated with a 1:20000 dilution of 125I-labeled protein A. The bands were visualized by overnight exposure to x-ray film (Kodak X-OMAT AR).

Preparation of CRBP-bound Retinol—The coding region of CRBP (type I) cDNA (16) was amplified from rat liver total RNA by reverse transcription-PCR using Ffu polymerase. The gene-specific nucleotide primers carried restriction sites for BamHI and EcoRI endonucleases. The PCR product was subcloned into the corresponding sites in pGEX-2T expression vector and sequenced. The recombinant protein was produced in TG-1 E. coli cells in the presence of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and 200 μg/ml ampicillin as a fusion with glutathione S-transferase. The cell pellet was resuspended in ice-cold PBS, 2 mM EDTA, 0.1% β-mercaptoethanol and lysed using a French press. The fusion protein was purified to homogeneity using affinity chromatography on a glutathione-agarose column. The purified fusion protein was cleaved with thrombin in 50 mM Tris, pH 8.0, 0.1% β-mercaptoethanol, 150 mM NaCl, 2.5 mM CaCl2. CRBP was separated from glutathione S-transferase by elution with 0–500 mM NaCl gradient in 10 mM Tris, pH 7.4, 1 mM DTT from a Q Sepharose column. The yield of CRBP was about 14 mg per liter of culture. The final preparation exhibited a single protein band of approximately 16 kDa by SDS-polyacrylamide gel electrophoresis. The amount of functional protein was determined from the fluorescence titration curve of apo-CRBP with retinol (17). Excitation was at 350 nm; emission was measured at 480 nm. The fluorescence values were corrected for contribution of free retinol.

The purified recombinant CRBP was incubated with excess of free retinol for 2–2 h in the dark. The free retinol was separated from the bound by elution of the S Superose column with 10 mM sodium phosphate buffer, pH 7.4. The final preparation of CRBP-retinol had a ratio of A350/A280 of 1.75.

Determination of Kinetic Constants—Steady-state kinetics were performed in 90 mM potassium phosphate, pH 7.3, and 40 mM KCl at 37 °C in siliconized glass tubes. The radiolabeled steroids (NEN Life Science Products) 5α-androstan-3α,17β-diol (3α-adiol) (41 Ci/mmol), 5α-androstan-3α-ol-17-one (androsterone) (45 Ci/mmol), and 5α-androstan-17β-
ol-3-one (dihydrotestosterone) (43.5 Ci/mmole) were diluted with cold
steroids (Sigma) to achieve the required specific radioactivity of each
steroid. Aqueous solutions of the substrates were prepared by adding
100× stock of the radiolabeled substrate in dimethyl sulfoxide (Me2SO),
so that the final concentration of Me2SO in the reaction mixture did not
exceed 1%. Equimolar amounts of bovine serum albumin were added to
improve the solubility of steroids. The suspensions were sonicated for
10 min, and the concentration of the radiolabeled substrate in the
aqueous phase was verified by counting an aliquot of the suspension.
The 250-μl reactions were started with the addition of cofactor and
stopped after 15 min by addition of 3.5 ml of methylene chloride. The
aqueous phase was removed and methylene chloride was evaporated
under a stream of N2. Retinoids were dissolved in 200 μl of ethanol; 10 μl
were spotted on alumina oxide thin layer chromatography plates (Sig-
ma) and resolved by development in chloroform/ethyl acetate (3:1),
according to Biawas and Russell (18). The Rf values for steroids under
these conditions were 0.26 for 3α-adiol, 0.48 for androstenedione, 0.53
for dihydrotestosterone, and 0.82 for 5α-androstane-3,17-dione (androst-
edione). The lanes were cut into pieces ~1 cm wide and counted in
scintillation liquid (Bio-Safe II). For determination of apparent Km
values, five concentrations between 0.6 and 1 μM were used for andros-
terone, 0.25–4 μM for dihydrotestosterone, and 0.1–2.5 μM for adiol.
Initial velocities (nmol of product formed/mg of protein) were obtained
by linear regression. The amount of product formed was less than 10%
within the 15-min reaction time and was linearly proportional to the
amount of microsomes added. The Km values for oxidation of all-
trans-retinol were determined at a fixed NADPH (1 mM) concentration; for reduction of
dihydrotestosterone, values were determined at a fixed NADH (0.5 mM)
concentration. Each Km determination was repeated at least three
times. A control without added cofactor was included with each exper-
iment. The apparent Km values for cofactors were determined with six
concentrations between 0.05–6.4 μM for NAD+, 0.25–8 μM for NADP+, 15–1000 μM for NADPH, and 0.15–10 μM for NADH.

Retinol inhibition of steroid oxidation was evaluated by incubating
RoDH-4-containing microsomes with 1 μM NAD+, 4 concentrations of
androstenedione, and 3 concentrations of retinol in reaction buffer. All
retinol solutions and reaction mixtures were kept in the dark. Each
data set was evaluated for fit to different types of inhibition (19). Km
estimations were evaluated by non-linear regression of inhibition equations using the method of Marquart (20).

**Retinol Assays and HPLC Analysis of Reaction Products—** Assays of
RoDH-4-catalyzed oxidation and reduction of retinoids were performed
in 90 mm potassium phosphate, pH 7.3, and 40 mM KCl at 37°C in
silanized glass tubes. Retinoid stock solutions in Me2SO were added to
the reaction buffer along with equimolar bovine serum albumin and
sonicated for 10 min. Experiments with tritiated retinol showed that
this procedure improved solubilization of retinol. The concentration
of Me2SO in the reaction mixture did not exceed 1%. The 500-μl reactions
were started with the addition of cofactor and stopped after 10 min by
addition of an equal volume of cold ethanol supplemented with 100
μg/ml butylated hydroxytoluene and an internal standard, retinol ace-
tate. Reactions were placed on ice and extracted twice with 7 volumes of
hexane. The aqueous phase was removed, and hexane was evaporated
under a stream of N2. Retinoids were dissolved in 200 μl of mobile
phase, and an aliquot was analyzed by HPLC.

All HPLC procedures were performed using an automatic injector
710WIS from Waters and a Varian 9010 pump. Elution was monitored
at 370 nm with a variable wavelength Varian 9050 detector connected
to a Hewlett Packard P39090 integrator. The stationary phase was a
Beckman ultrasphere ODS column (4.6 mm x 15 cm). The mobile
phase consisted of 0.05 M ammonium acetate, pH 7.0:acetonitrile:trihydro-
furan (70:168:12). The flow rate was 1 ml/min. Under these conditions,
Me2SO in the reaction mixture did not exceed 1%. The 500-
this procedure improved solubilization of retinol. The concentration of
sonicated for 10 min. Experiments with tritiated retinol showed that
the reaction buffer along with equimolar bovine serum albumin and

Kinetic parameters for RoDH-4 were obtained using non-linear
regression. The amount of product formed was less than 10%
within the 15-min reaction time and was linearly proportional to the
amount of microsomes added. The Km values for NAD+, 0.25–8 mM for NADP+, 15–1000 μM for NADPH, and 0.15–10 μM for NADH.

**RESULTS**

To isolate human homologs of rat RoDH isoenzymes, we
preparied a RoDH-1 cDNA probe by reverse transcription-PCR
amplification of rat liver mRNA with gene-specific primers.
The radiolabeled RoDH-1 cDNA was used to screen the human
liver λgt10 cDNA library. We applied low stringency conditions
for screening the library, because our goal was to find an
enzyme with similar (recognition of all-trans-retinol) but not
identical properties (preference for NAD+, not NADP+). Four
positives were identified during the first round of screening.
Two of these positive clones were purified and sequenced. The
longest clone contained a 1308-base pair cDNA (Fig. 1), which
exhibited 78% nucleotide sequence identity with RoDH-1 cDNA
in the coding region. Similar to RoDH-1, the human cDNA
encoded a 317-amino acid protein including a starting Met. The
deduced protein product exhibited features characteristic of the
short chain dehydrogenase/reductase family of enzymes, such as
putative consensus sequence for the cofactor binding site,
G(X)G, at Gly-36, and the active site consensus sequence,
X(Y)K, at Tyr-176. The new human short chain dehydrogen-
ase/reductase showed the highest sequence identity with the
recently reported cists-retinol/androgen dehydrogenase from
mouse (73%), rat RoDH forms 1 and 3 (72%), and rat RoDH-2
(71%). Because rat RoDHs share 98% sequence identity with
their mouse homologs, we assumed that the human cDNA
cloned in this study encoded a previously unknown form of
short chain dehydrogenase/reductase, and we called the new
isoenzyme RoDH-4.

The tissue distribution of RoDH-4 mRNA was analyzed by
Northern blot analysis (Fig. 2). A very strong signal was ob-
served in human liver, similar to the expression patterns of rat
all-trans-retinol dehydrogenases (3, 4). In addition, rela-
atively high levels of hybridizing message were detected in fetal
liver and lung (Fig. 2). The size of the message in fetal lung was
somewhat smaller than in fetal liver. This could be due to
cross-hybridization with a closely related gene product or to a
different processing of the mRNA. The tissue distribution of
RoDH-4 was distinctively different from that of the mouse
cis-retinol/androgen dehydrogenase (6) and the two human
cis-retinol dehydrogenases that are not active toward all-trans-
retinol (2, 5).

Next, we tested whether the mRNA for RoDH-4 is translated
into RoDH-4 protein in the human liver by Western blot anal-
ysis. Antibodies were raised against the N-terminal and the
C-terminal fragments of RoDH-4 expressed in E. coli as de-
scribed under “Materials and Methods.” A sample of frozen
human liver was homogenized and fractionated by centrifuga-
tion into cytosol and microsomes. RoDH-4 protein was detected
by incubation with either anti-N-terminal or anti-C-terminal
antiserum (Fig. 3). A single band of 35 kDa appeared in the
105,000 × g membrane fraction using either antiserum (Fig. 3,
lane P), indicating that RoDH-4 message is translated into a
protein in human liver, and the protein is associated with
membranes. This is similar to the subcellular localization of
other RoDH isoenzymes.

To obtain a catalytically active enzyme, the cDNA for
RoDH-4 was expressed in insect cells using the BaculoGold
Baculovirus system. The full-length cDNA was subcloned into
the pVL1392 transfer vector and cotransfected with linearized
BaculoGold DNA into Sf9 cells. The recombinant virus was
amplified and used to produce recombinant RoDH-4. The iden-
tity of the band was confirmed by Western blotting using poly-
clonal antiserum raised against partial RoDH-4. Our attempts to
solubilize the recombinant enzyme and purify it from the
membranes using Triton X-100 led to complete inactivation
of the enzyme. Thus, intact membranes were used for kinetic
characterization of RoDH-4.

Human RoDH-4 was most similar to the mouse cis-retinol/
androgen dehydrogenase (CRAD) (6) and rat all-trans-retinol
dehydrogenases RoDH-1, RoDH-2, and RoDH-3 (3, 4). Both
CRAD and RoDH-2 were recently found to oxidize the 3α-hydrox-
ysteroids 3α-adiol and androsterone to dihydrotestosterone and
androstanedione, respectively (6, 18). Thus, we tested whether
RoDH-4 could function as a 3α-hydroxysteroid dehydrogenase.
RoDH-4-containing microsomes were incubated with tritiated

19780

**Human Microsomal NAD+−dependent Sterol/Retinol Dehydrogenase**

3

17

34

min, respectively. All-

all-

trans

trans

furan (70:168:12). The flow rate was 1 ml/min. Under these conditions,
3α-adiol and androsterone in the presence of NAD⁺ or NADP⁺. The product of 3α-adiol oxidation comigrated with dihydrotestosterone on alumina oxide thin layer plate (18). The product of androsterone oxidation comigrated with androstanedione. RoDH-4 also catalyzed the reverse reaction, reduction of tritiated dihydrotestosterone in the presence of NADH, similar to the human 3α-hydroxysteroid dehydrogenase characterized by Biswas and Russell (18). No activity was detected in the microsomes from mock-transfected Sf9 cells, or in the controls without cofactor that were included with each experiment.

The apparent Km values for oxidation of 3α-adiol and androsterone were determined with saturating concentration of NAD⁺, and the Km value for the reduction of dihydrotestosterone was determined with saturating NADH. All three steroids exhibited Km values of under 1 mM (Table I), similar to RoDH-1 and CRAD. The Vmax values of RoDH-4 were in the range of nmol/min/mg of microsomes (Table I). The apparent Km values for void.
cofactors NAD\(^+\) and NADP\(^+\) were determined with saturating androsterone, and the \(K_m\) values for NADH and NADPH were determined with saturating dihydrotestosterone (Table II).

RoDH-4 oxidized all-trans-retinol and 13-cis-retinol to corresponding retinaldehydes (Fig. 4). The reaction products were analyzed by HPLC. Microsomes that contained RoDH-4 oxidized all-trans-retinol to all-trans-retinal (Fig. 4, A and B) in the presence of NAD\(^+\). No activity was detected in the absence of cofactor or with microsomes isolated from mock-transfected cells. The reaction rate increased linearly with the amount of enzyme in the reaction mixture (Fig. 4A). About 400 pmol of all-trans-retinaldehyde were produced from 10 \(\mu\)M all-trans-retinol by 1.9 \(\mu\)g of RoDH-4-containing microsomes in 30 min. More product was formed in the presence of NAD\(^+\) compared with NADP\(^+\) from both all-trans-retinol (not shown) and 13-cis-retinol (Fig. 4C), consistent with the cofactor preference of RoDH-4 determined in the experiments with steroids. Thus, human RoDH-4 is similar to rat RoDH-1 in that it also recognizes all-trans-retinol as a substrate. However, unlike the rat enzyme, human RoDH-4 prefers NAD\(^+\) over NADP\(^+\) and is likely to function in the oxidative direction in vivo. Human RoDH-4 is also different from the NAD\(^+-\)dependent mouse CRAD, which is specific for cis-retinols and does not oxidize all-trans-retinol. Hence, RoDH-4 is clearly a new isoenzyme with a distinctively different primary structure and catalytic properties.

We tested whether stereoisomers of retinol can inhibit oxidation of androsterone, because both retinol and steroids were substrates for RoDH-4. Both all-trans-retinol and 13-cis-retinol acted as competitive inhibitors of androsterone oxidation (Table III). Most all-trans-retinol is bound to CRBP in liver cells. RoDH-1 recognizes CRBP-bound retinol as substrate (3); therefore, it was very interesting to see whether retinol bound to CRBP could act as efficiently as free retinol in inhibiting steroid oxidation. Surprisingly, the apparent \(K_i\) value for CRBP-retinol inhibition was almost the same as the \(K_m\) value for free retinol (Table III). Apo-CRBP alone had no effect on the reaction. The almost identical \(K_i\) values for the free and bound all-trans-retinol suggest that CRBP binding does not alter the affinity of RoDH-4 for all-trans-retinol.

Other inhibitors of RoDH-4 activity toward steroids included citral and acyclic isoprenoids. The concentration of androstene in these experiments was at the \(K_m\) value of 0.14 \(\mu\)M. 20 \(\mu\)M citral (3,7-dimethyl-2,6-octadienal) inhibited RoDH-4 activity with androsterone about 50% (Table IV). Some of the acyclic isoprenoids, perillyl alcohol, geraniol, farnesol, and geranyl geraniol, were even more potent inhibitors of steroid oxidation (Table IV). Ethanol at 100 \(\mu\)M had no effect on RoDH-4 activity with androsterone.

**DISCUSSION**

We have cloned and characterized a new short chain dehydrogenase/reductase, RoDH-4, which represents the first human microsomal enzyme capable of oxidizing all-trans-retinol to all-trans-retinaldehyde and the second human enzyme to oxidize 3a-hydroxysteroids with NAD\(^+\) as the preferred cofactor. The primary structure of RoDH-4 is about 70% identical to the rodent retinol/sterol dehydrogenases.

### Table I

| Substrate         | \(K_m\) (\(\mu\)M) | \(V_{max}\) (nmol/min/mg) |
|-------------------|-------------------|--------------------------|
| Androsterone      | 0.14 ± 0.02       | 2.6 ± 0.1                |
| Androstane-diol   | 0.22 ± 0.06       | 6.9 ± 0.5                |
| Dihydrotestosterone | 0.80 ± 0.17     | 10.0 ± 0.7               |

### Table II

| Cofactor | \(K_m\) (\(\mu\)M) |
|----------|--------------------|
| NAD\(^+\) | 0.13 ± 0.01       |
| NADP\(^+\) | 190 ± 17          |
| NADH     | 0.10 ± 0.01       |
| NADPH    | 25 ± 4            |

All kinetic data were obtained in 90 mM potassium phosphate, pH 7.3, 40 mM KCl at 25 °C. \(K_m\) values for androsterone and androstane-diol were determined with saturating 1 \(\mu\)M NAD\(^+\), for dihydrotestosterone with saturating 0.5 mM NADH, for NAD\(^+\) and NADP\(^+\) with saturating 1 \(\mu\)M androsterone, and for NADH and NADPH with saturating 10 \(\mu\)M dihydrotestosterone.

Currently, four types of retinol-oxidizing short chain dehydrogenases/reductases can be distinguished. The first type are the NADP\(^+-\)preferring isoenzymes that utilize free and CRBP-bound all-trans-retinol in rat (RoDH-1 and RoDH-2) (3, 4) and mouse (RoDH-1 and RoDH-2) (6). The second type is the NAD\(^+-\)dependent CRAD, recently cloned from mouse (6). CRAD has 80–85% sequence identity to RoDHs, and it oxidizes 9-cis-retinol and 11-cis-retinol but not all-trans-retinol. The third type is the eye-specific retinol dehydrogenase in retinal pigment epithelium that recognizes only 11-cis-retinol (2). The fourth type shares 95% amino acid sequence identity with the eye 11-cis-retinol dehydrogenase but apparently prefers 9-cis-retinol as substrate and is found in multiple tissues (5). The last two cis-retinol dehydrogenases prefer NAD\(^+\) over NADP\(^+\), and have only 50 to 55% identity with RoDHs and CRAD.

Recently, a cDNA for the first human RoDH-like short chain dehydrogenase/reductase has been isolated from the human prostate cDNA library by screening with rat RoDH-1 cDNA (18). Analysis of the substrate specificity of the prostate RoDH-like enzyme, transiently expressed in embryonic kidney 293 cells, showed that it oxidizes 3a-hydroxysteroids efficiently and exhibits higher affinity for NAD\(^+\) than for NADP\(^+\). Whereas reductive NADP\(^+-\)dependent 3a-hydroxysteroid dehydrogenases have been described, the prostate RoDH-like dehydrogenase represents the first known oxidative NAD\(^+-\)dependent 3a-hydroxysteroid dehydrogenase. Whether it is also active on retinols was not reported. This prostate 3a-hydroxysteroid dehydrogenase has only 62% amino acid sequence identity with human RoDH-4 reported in this study and is less similar to retinol-oxidizing RoDH isoenzymes (61–63% amino acid sequence identity) than RoDH-4 (72% identity).

Similar to the prostate RoDH-like steroid dehydrogenase, RoDH-1 and CRAD were found to oxidize the 3a-hydroxysteroids 3a-adiol and androsterone to dihydrotestosterone and androstane-dione, respectively (3, 6). We tested whether the new human RoDH-4 was active against steroid alcohols. As expected, human RoDH-4 oxidized 3a-adiol to dihydrotestosterone and androsterone to androstenedione in the presence of either NAD\(^+\) or NADP\(^+\). Accordingly, 3a-dihydrotestosterone was reduced to 3a-adiol in the presence of NADH or NADPH. The catalytic efficiency of RoDH-4 in the reductive direction was about 2.5 times lower than in the oxidative direction.

The nonphosphorylated cofactors NAD\(^+\) and NADH were clearly preferred by RoDH-4: the apparent \(K_m\) value for NAD\(^+\) was almost 1500 times lower than that for NADP\(^+\) (Table III). Similarly, the apparent \(K_m\) value for NADH was 250 times lower than that for NADPH.

The \(K_m\) values of RoDH-4 for steroid substrates (under 1 \(\mu\)M) were in the same range as those of other microsomal dehydrogenases (3, 6). The reported \(V_{max}\) values (nmol/min/mg of cell lysates) for steroid dehydrogenases transiently expressed in eukaryotic cells varied depending on the transfection efficiency.
The catalytic efficiencies were about twice higher for oxidizing short chain dehydrogenases/reductases for the presence of membrane-spanning domains using programs HELIXMEM (23), RAOARGOS (24), and SOAP (25, 26). These programs predict the existence of membrane-associated helices in a protein sequence, evaluate the hydropathic index along the sequence, and predict whether a membrane protein is peripheral or integral. Although the number and exact location of the membrane-spanning domains predicted by the different programs differed somewhat, all retinol-oxidizing short chain dehydrogenases/reductases were classified as integral membrane proteins (Fig. 5A). Comparative analysis of the primary structures of the isoenzymes suggested that RoDH-4 contains four potential transmembrane segments: the N-terminal segment 1 (amino acids 1–21), the two closely positioned central segments 2 and 3 (amino acids 105–125 and 130–150), and the C-terminal segment 4 (amino acids 288–309) (Fig. 5, boxed). The hydrophobic N-terminal segment 1 is followed by the two highly conserved arginines at positions 19 and 21. The hydrophobic domain 3 is also followed by two conserved arginines at positions 156 and 158. The topological rules for membrane proteins (Fig. 5A). Comparative analysis of the primary structures of the isoenzymes suggested that RoDH-4 contains four potential transmembrane segments: the N-terminal segment 1 (amino acids 1–21), the two closely positioned central segments 2 and 3 (amino acids 105–125 and 130–150), and the C-terminal segment 4 (amino acids 288–309) (Fig. 5, boxed). The hydrophobic N-terminal segment 1 is followed by the two highly conserved arginines at positions 19 and 21. The hydrophobic domain 3 is also followed by two conserved arginines at positions 156 and 158. The topological rules for membrane proteins.
A clear tendency for highly charged internal loops to remain on the cytoplasmic side (27). The two neighboring hydrophobic segments, 2 and 3, which are connected by a short loop, can be inserted according to a helical hairpin mechanism (28).

Based on the analysis of the primary structure of RoDH-4, we propose a model of transmembrane insertion of RoDH-4 in the microsomal membrane (Fig. 5B). According to this model, the two loops between the hydrophobic segments will remain on the cytosolic side. Because the concentration of NAD$^+$ in the cytosol greatly exceeds that of NADH, RoDH-4 will likely function as a dehydrogenase, not reductase, in the cells. Interestingly, the cofactor binding consensus sequence is located on the N-terminal loop and is highly conserved among short chain dehydrogenases/reductases. The substrate binding consensus sequence is located on the less conserved C-terminal loop, which is consistent with the different substrate specificity of the isoenzymes. Based on our model of transmembrane insertion of RoDH-4, the cofactor binding domain will face the cytosol, and RoDH-4 will function in the oxidative direction.

This model is consistent with our observation that CRBP-retinol inhibits oxidation of androsterone catalyzed by RoDH-4 with the same efficiency as free retinol, suggesting that both forms of retinol have equal access to the active site. CRBP is a cytosolic protein, and this is consistent with the suggestion that

---

**FIG. 5. Alignment of retinol-oxidizing short chain dehydrogenases/reductases and a proposed model of their transmembrane insertion.** The amino acid sequences of rat RoDH-1 and RoDH-2, mouse CRAD, and human 11-cis-retinol dehydrogenase (11-cis) were obtained from GenBank$^\text{TM}$. The putative transmembrane domains are boxed.
the active site of RoDH-4 faces cytosol. Several microsomal enzymes were found to metabolize bound forms of retinoids with the same catalytic efficiency as free. For example, the intestinal retinal reductase does not distinguish between free and CRBP-II-bound all-trans-retinal (29). It exhibits similar affinity (0.78 μM for free and 0.46 μM for bound) and almost identical rates (~300 pmol/min/mg of microsomal protein) with both forms of retinal. In the pigment epithelium of the eye, 11-cis-retinaldehyde reductase is about equally efficient in reducing free 11-cis-retinal and 11-cis-retinal complexed with cellular retinal-binding protein (30).

Citril is often used to inhibit oxidation of retinol and retinaldehyde to retinoic acid in cell culture experiments (31). Human keratinocytes incubated in the presence of 100 μM citral for 4 h show 75% reduction in retinoic acid synthesis from all-trans-retinol (32). In our experiments, 20 μM citral inhibited RoDH-4 45%. This also distinguishes RoDH-4 from RoDH-1, which is not inhibited by citral.

RoDH-4 can oxidize the carbon 3 alcohol group on 3α-adiol, producing dihydrotestosterone, a powerful androgen with high affinity for androgen receptor. RoDH-4 is the second isoform of human NAD⁺-dependent 3α-hydroxysteroid dehydrogenases; it shares 62% sequence identity with the first isoform, human RoDH-like 3. Dihydrotestosterone is inactivated by NADPH-dependent cytochrome P450 (33). Geraniol and farnesol are present in the essential oils of caraway, and dill. The enzymes that oxidize perillic acid, are more potent inhibitors of tumor cell proliferation than is perillyl alcohol (36). The enzymes that oxidize acyclic isoprenoids to their corresponding acids may function as effective inhibitors of RoDH-4.

Acknowledgments—We thank Dr. Ting-Kai Li, the Principal Investigator on NIAAA Grant AA02342 and the Preceptor on the Research Career Development Award (to N. Y. K.) for his continuing support and guidance. Dr. William F. Bosron and Robert A. Harris for helpful discussions and critical reading of the manuscript, and Natividad DuManal for technical assistance with retinol assays and HPLC analysis.

REFERENCES

1. Jörnvall, H., Persson, B., Krook, M., Attia, S., Gonzalez-Duarte, R., Jeffery, J., and Chosh, D. (1995) Biochemistry 34, 6003–6012
2. Simon, A., Hellman, U., Wernstedt, C., and Eriksson, U. (1995) J. Biol. Chem. 270, 1107–1112
3. Chai, X., Boerman, M. H. E. M., Zhai, Y., and Napoli, J. L. (1995) J. Biol. Chem. 270, 3900–3904
4. Chai, X., Zhai, Y., Popescu, G., and Napoli, J. L. (1995) J. Biol. Chem. 270, 28408–28412
5. Mertz, J. R., Shang, E., Piantedessi, R. W., Wei, S., Wolgemuth, D. J., and Blaner, W. S. (1997) J. Biol. Chem. 272, 11744–11749
6. Chai, X., Zhai, Y., and Napoli, J. L. (1997) J. Biol. Chem. 272, 33125–33131
7. Mangelsdorff, D. J., Umesono, K., and Evans, R. M. (1994) in The Retinoids: Biochemistry, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) 2nd Ed, pp. 319–350, Raven Press, Ltd., New York
8. Napoli, J. L., and Race, K. R. (1987) Arch. Biochem. Biophys. 255, 95–101
9. Posch, K., and Napoli, J. L. (1992) Biochem. Pharmacol. 43, 2296–2298
10. Boleda, M. D., Saubi, N., Farres, J., and Parés, X. (1993) Arch. Biochem. Biophys. 15, 85–90
11. Chytil, F., and Ong, D. E. (1987) Annu. Rev. Nutr. 7, 321–335
12. Chai, X., Zhai, Y., and Napoli, J. L. (1996) Gene 169, 219–222
13. Duester, G. (1996) Biochemistry 35, 12221–12227
14. Veech, R. L., Eggleston, L. V., and Krebs, H. A. (1969) Biochem. J. 115, 609–619
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16. Sherman, D. R., Lloyd, R. S., and Chytil, F. (1967) Proc. Natl. Acad. Sci. U. S. A. 64, 3209–3213
17. Ong, D. E., and Chytil, F. (1978) J. Biol. Chem. 253, 828–832
18. Hiebsch, S. C., and Russell, D. W. (1997) J. Biol. Chem. 272, 15959–15966
19. Segel, I. H. (1976) Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems, pp. 18–159, John Wiley & Sons, New York
20. Marquart, D. W. (1963) J. Soc. Ind. Appl. Math 11, 431–441
21. Blumenthal, M. H. E. M., and Napoli, J. L. (1995) Arch. Biochem. Biophys. 321, 432–441
22. Lee, M.-A., Kim, C.-I., and Lieber, C. S. (1987) Arch. Biochem. Biophys. 259, 241–249
23. Eisenberg, D., Schwarz, E., Komarynow, M., and Wall, R. (1984) J. Mol. Biol. 179, 125–142
24. Rao, M. J. K., and Argos, P. (1986) Biochim. Biophys. Acta 869, 197–214
25. Kyte, J., and Doolittle, R. F. J. (1982) Mol. Biol. 157, 105–132
26. Klein, P., Kanaeisha, M., and DeLisi, C. (1985) Biochim. Biophys. Acta 815, 468–476
27. Gafvelin, G., Sakaguchi, M., Andersson, H., and von Heijne, G. (1997) J. Biol. Chem. 272, 6119–6127
28. Cao, G., Cheng, S., Whiteley, P., von Heijne, G., Kuhn, A., and Dalby, R. E. (1994) J. Biol. Chem. 269, 26998–26903
29. Kakizad, B. P., and Ong, D. E. (1988) J. Biol. Chem. 263, 12916–12919
30. Saari, J. C., and Bredberg, L. (1982) Biochim. Biophys. Acta 716, 266–272
31. Connor, M. J., and Smit, M. H. (1987) Biochem. J. 244, 489–492
32. Kurlandsky, S. B., Xiao, J.-H., Dauel, E. A., Voorhees, J. J., and Fisher, G. J. (1994) J. Biol. Chem. 269, 32821–32827
33. Russell, D. W., and Wilson, J. D. (1997) Cancer Chemother. Pharmacol. 35, 31–37