Dual-substrate Specificity Short Chain Retinol Dehydrogenases from the Vertebrate Retina*§

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Retinoids are chromophores involved in vision, transcriptional regulation, and cellular differentiation. Members of the short chain alcohol dehydrogenase/reductase superfamily catalyze the transformation of retinol to retinal. Here, we describe the identification and properties of three enzymes from a novel subfamily of four retinol dehydrogenases (RDH11–14) that display dual-substrate specificity, uniquely metabolizing all-trans- and 9-cis-retinol with C15 pro-R specificity. RDH11–14 could be involved in the first step of all-trans- and 9-cis-retinoic acid production in many tissues. RDH11–14 fill the gap in our understanding of 11-cis retinal and all-trans-retinal transformations in photoreceptor (RDH12) and retinal pigment epithelial cells (RDH11). The dual-substrate specificity of RDH11 explains the minor phenotype associated with mutations in 11-cis-retinol dehydrogenase (RDH5) causing fundus albinopunctatus in humans and engineered mice lacking RDH5. Furthermore, photoreceptor RDH12 could be involved in the production of 11-cis-retinal from 11-cis-retinol during regeneration of the cone visual pigments. These newly identified enzymes add new elements to important retinoid metabolic pathways that have not been explained by previous genetic and biochemical studies.

Retinoids are indispensable light-sensitive elements of vision and also serve as essential modulators of cellular differentiation and proliferation in diverse cell types, including those comprising the epithelium and immune system. Retinoids modulate the growth of both normal and malignant cells through their binding to retinoid receptors. All-trans-retinoic acid signals through specific interactions with the nuclear retinoic acid receptors, whereas its isomer, 9-cis-retinoic acid, is a high affinity ligand of retinoic acid receptors and retinoid X receptors. In the retina, light-dependent photoisomerization of 11-cis-retinylidene to the all-trans-retinylidene moiety of rod and cone photoreceptors is a key reaction that triggers visual sensation (1). Restoration of the visual chromophore occurs through a complex set of reactions, termed the retinoid cycle, in photoreceptor cells and adjacent retinal pigment epithelial cells (RPE)1 (2). Dietary deficiencies in retinoids and retinoid precursors cause visual impairment, developmental abnormalities, and immune deficiency (3, 4).

Transformations of retinoids occur mostly through enzymatic or photochemical reactions, although they readily isomerize non-enzymatically to thermodynamic equilibrium when unprotected by the retinoid-binding proteins (5). The key enzymes involved in retinoid metabolisms are alcohol and aldehyde dehydrogenases that convert retinols to aldehydes and aldehydes to carboxylic acids, respectively. The first oxidation reaction is catalyzed by a large number of enzymes from the SDR superfamily (6, 7) and by classic medium chain alcohol dehydrogenases (8). SDRs are weakly conserved in their primary sequences, with the exception of key residues involved in catalysis, nucleotide recognition, and members of closely related subfamilies. SDRs also display NADP or NAD cofactor preference and, if they are retinol dehydrogenases (RDHs), favor all-trans- or cis-retinol substrates. Some RDH enzymes also catalyze the oxidation of steroids in addition to retinols (9). Localized expression of these enzymes in the photoreceptor and RPE cells, where heavy traffic of diffusible retinoids occurs, strongly suggest that retinols and retinals are their in vivo substrates. The role of specific SDRs in vision has been determined from the biochemical characterization of enzymes isolated from specific compartments of the retina, analyses of retinoid flow in genetically engineered mice, and from associations of human visual dysfunctions with specific disabling mutations in one of the SDR genes, RDH5 (for review, see Ref. 2).

The present study was undertaken to resolve the discrepancy between biochemical and genetic analyses of the RDH activity responsible for 11-cis-retinal and 9-cis-retinal production. An enzyme encoded by the RDH5 gene (10, 11), 11-cis-RDH, was proposed to be responsible for both 11-cis-retinal and 9-cis-retinal production due to its relaxed substrate specificity (12–15). However, the disruption of this gene in a mouse model led to uninterrupted production of 11-cis-retinal (16, 17) and a lack of any embryonic abnormalities. Furthermore, patients with fundus albinopunctatus who also have a disabling mutation in 11-cis-RDH were unaffected. The present results are consistent with the notion that enzymatic activity alone is insufficient to account for the phenotype (18). The enzyme(s) involved in this process remains to be elucidated. The identification of RDH11–14 could provide further insights into the role of these enzymes in retinoid metabolism.

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the RDH5 gene still show efficient production of the chromophore, albeit with slower kinetics (18, 19). Here, we characterized members of a novel subfamily of SDRs cloned from the retina that display novel properties of dual cis- and all-trans-retinol substrate specificities. The recognition of both types of isomers studied by stereospecific substrates unravels a novel mode of substrate recognition. The RDH11 transcript has been previously identified as one that exhibits increased expression on exposure to androgens in the LNCaP prostate cancer cell line (20) and is proposed to be involved in the metabolism of retinoids (21).

**MATERIALS AND METHODS**

**Cloning of Human RDH11–14**—Full-length RDHs were amplified by PCR from human (RDH11–13) or mouse (RDH14) retina cDNA libraries using primers FH497 (5′-GAGATGGTTGAGCTCATGTTC-3′) and FH498 (5′-GTAGTCTATTTGGAGGCC-3′) for RDH11, FH500 (5′-ac-gatgcggtctggaagtc-3′) and FH501 (5′-ACATGCTGCTACCTTG-GGACTG-3′) for RDH12, FH502 (5′-AGACGCTACCTTG-GGACTG-3′) and FH503 (5′-ATGAGGCGTACCTTG-GGACTG-3′) for RDH13, and FH504 (5′-ACGATGCTGCTACCTTG-GGACTG-3′) for RDH14.
Fig. 2. Immunolocalization of RDH11 in bovine and monkey retina. A, specificity of anti-RDH5 (lane 1) and anti-RDH11 (lanes 2–5) antibodies. Lanes 1 and 2, bovine RPE; lane 3, bovine ROS; lane 4, S9 cell lysate expressing recombinant RDH11; lane 5, S9 cell lysate; lane 6, purified RDH5-His<sub>6</sub>. B–D, immunofluorescence localization of RDH11 in monkey retina. B, control bright field image of monkey retina. C, RDH11 immunolabeling is predominant in the RPE cell layer of monkey retina. D, addition of purified peptide (0.5 μg/ml) abolishes RDH11 immunoreactivity. E–G, immunofluorescence localization of RDH11 in bovine retina. E, control bright field image of bovine retina. F, RDH11 immunolabeling is predominant in the RPE cell layer of bovine retina. G, addition of purified peptide (0.5 μg/ml) abolishes RDH11 immunoreactivity. Bar, 50 μm.

H, co-localization of RDH11 and glial fibrillary acidic protein in the inner retina. The bovine retina section was double-labeled with antibodies to glial fibrillary acidic protein and RDH11. Arrows show the colocalization of glial fibrillary acidic protein (green) and RDH11 (red) in Müller cells. Bar, 50 μm. OPL, outer plexiform layer; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve-fiber layer.

For immunohistochemistry, retinal sections were blocked for nonspecific labeling by incubating in 1.5% normal goat serum in PBS buffer (136 mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) for 15 min at room temperature. Sections were incubated with purified anti-RDH11 monoclonal antibody or anti-RDH13 serum overnight at 4°C. Controls were prepared by absorbing the antibodies with an excess amount of RDH11 peptide (0.5 μg/ml) or purified RDH13 (2 μg/ml). Sections were rinsed in PBS and incubated with indocarbocyanine (Cy3)-conjugated goat anti-mouse IgG. Sections were then rinsed in PBS and mounted in 50 μl of 2% 1,4-diazabicyclo-2,2,2-octane in 90% glycerol to slow photobleaching. Sections were analyzed under a confocal microscope (Zeiss LSM510). Bright field images were captured with Nomarski optics (NIKON).

Preparation of Anti-RDH11, -RDH13, -RDH14, and -RDH5—The antibody against RDH11 was generated against CHVAWVSVQARNE-TIAR-CONH<sub>2</sub> peptide (21). Bacterially expressed and purified RDH13 and RDH14 were used to immunize BALB/c mice to obtain anti-RDH13/14 antisera. Mouse anti-RDH5 monoclonal antibodies were raised against RDH5-His<sub>6</sub> purified from RDH5-His<sub>6</sub>-infected S9 cells (24).

Retinoids—All reactions involving retinoids were carried out under dim-red light conditions. Retinoids were stored in N,N-dimethylformamide under argon at −80°C. Retinoids were purified by normal phase HPLC (Beckman Instruments, Ultrasphere-Si, 4.6 mm × 250 mm) with 10% ethyl acetate, 90% hexane at a flow rate of 1.4 ml/min using an HP1100 with an on-line diode-array detector and HP Chemstation A.08.03 software.

Preparation of Proteins—Fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing Co., Inc., Stonewood, WA). ROS membranes were isolated from bovine retina using the sucrose gradient centrifugation method (25). RPE microsomes were prepared as described previously (26). Expression of RDH5 with a His<sub>6</sub> tag at the carboxyl terminus in S9 cells was reported previously (24). Horse liver alcohol dehydrogenase (Sigma/Aldrich) was purified on a Mono Q H5/5 (Amersham Biosciences) column equilibrated with 10 mM BTP, pH 7.3, using a linear gradient from 0 to 500 mM NaCl over 60 min at a flow rate of 0.7 ml/min. The horse liver alcohol dehydrogenase fraction (eluted at 1–3 min, 0.6 mg/ml) containing the highest dehydrogenase activity was collected and concentrated by ultrafiltration (Millipore Centricon 30) before use.

Purified RDH5-His<sub>6</sub>. Not RDH13, or mouse RDH14 was transferred as a PCR-TOPO vector and linearized with appropriate endonucleases. Antisense and sense RNA probes (0.9–1 kb) were synthesized by run-off transcription from the SP6 or T7 promoter with digoxigenin-UTP, as recommended in the manufacturer’s protocol (Roche Molecular Biochemicals).
activity when assayed with pro-\(R\) [4-\(3H\)]NADH, and all-trans-retinal or 11-cis-retinal was used in further studies (24). \(\beta\)-Thylactin dehydrogenase (Sigma/Aldrich) was dialyzed against 10 mM BTP, pH 7.3, 0.1 M NaCl before use.

Preparation of Pro-\(R\) [4-\(3H\)]NADH, Pro-\(S\) [4-\(3H\)]NADH, Pro-\(R\) [4-\(3H\)]NADPH, and Pro-\(S\) [4-\(3H\)]NADPH—The preparation of pro-\(R\) [4-\(3H\)]NADH was accomplished by utilizing the pro-\(R\)-specific enzyme yeast alcohol dehydrogenase (Sigma) to reduce NAD with 1-\(3H\)-labeled ETOH (American Radiolabeled Chemicals, Inc.) as previously described (16). Syntheses of pro-\(S\) [4-\(3H\)]NADH and pro-\(S\) [4-\(3H\)]NADPH were carried out with \(\beta\)-glutamic dehydrogenase (Sigma), NAD(P)(H) (Sigma), and \(L\)-\(2.3\)-glutamic dehydrogenase (PerkinElmer Life Sciences) as previously described (19). Synthesis of pro-\(R\) [4-\(3H\)]NADPH was prepared with \(\beta\)-glutamic dehydrogenase, [4-\(3H\)]NADP, and \(\beta\)-glutamic acid, as described previously (24). The product was purified on a Mono Q HR 5/5 column equilibrated with 10 mM BTP, pH 7.3, using a linear gradient from 0 to 500 mM NaCl over 60 min at a flow rate of 0.7–1 ml/min. Concentrations of NADH and NADPH (pH 7.4) were determined using \(\epsilon = 6,220\) at 340 nm, and concentrations of NAD and NADP (pH 7.4) were determined using \(\epsilon = 18,000\) at 260 nm (27).

Preparation of Pro-\(R\), S-9-cis-[15-\(3H\)]retinol, Pro-\(R\), S-11-cis-[15-\(3H\)]retinol, and Pro-\(R\), S-all-trans-[15-\(3H\)]retinol and Their Corresponding 15-\(3H\)-Labeled Retinols—Pro-\(R\), S-9-cis-[15-\(3H\)]retinol, pro-\(R\), S-11-cis-[15-\(3H\)]retinol, and pro-\(R\), S-all-trans-[15-\(3H\)]retinol were prepared by the reduction of their respective retinals with \([\text{H}]\text{NADH}\), (PerkinElmer) as described before (24). [15-\(3H\)]Retinol was synthesized by \(\text{MnO}_2\) oxidation of the corresponding pro-\(R\), S-9-cis-[15-\(3H\)]retinol as previously described (24).

Syntheses of Stereospecific 15-\(3H\)-Labeled Retinols—Table I summarizes the syntheses of various stereospecific 15-\(3H\)-labeled retinols by different dehydrogenases. pro-\(R\) and pro-\(S\) designations were used for 15-\(3H\)-labeled retinols produced by the enzyme for which the stereospecificity is known (24).

Assay for RDH Activity—Activities of RDHs (recombinant SF9 cells suspended in 20 mM BTP, pH 7.4, 0.25 mM \(n\)-dodecyl-\(\beta\)-maltoside, 1 mm DTT, 1 \(\mu\)M leupeptin, 10 \(\mu\)M NAD and NADP (0.9–1.81 mg/ml) were assayed by monitoring the production of either [15-\(3H\)]retinol (reduction of retinal) or [4-\(3H\)]NADPH (oxidation of retinol) (24, 28). RDH activities were measured using the phase partition assay (29) or HPLC assays as described (24).

Preparation of RDH5 Affinity Column—Monoclonal anti-RDH5 antibody was purified on a protein-A column, and then the purified antibody was coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) following the manufacturer’s procedures.

Purification of RDH5 and RDH5-His6 from Bovine RPE Microsomes and RDH5-His6-transfected SF9 Cells, Respectively—RPE microsomes (1.3 ml, 5 mg/ml) were solubilized with 5 mM \(n\)-dodecyl-\(\beta\)-maltoside in the presence of 20 mM BTP, pH 7.4, 1 mM DTt, and 1 \(\mu\)M leupeptin (buffer A) with 20 mM NaCl and NADP for 80 min on ice. The solubilized mixtures were centrifuged at 71,000 × g for 40 min, and the supernatant was loaded onto the monoclonal anti-RDH5 antibodies Sepharose 4B (~0.6 ml of gel) equilibrated with buffer A. The column was then washed with 12 ml of the same buffer, and RDH5 was eluted by 45 mM sodium citrate, pH 3.0, 5 mM \(n\)-dodecyl-\(\beta\)-maltoside, and 1 mM DTt, and the fraction was immediately neutralized with 1.35 × Tris-HCl, pH 8.8, to pH 6–7. The purification of RDH5-His6, from the transfected SF9 cells (~1.5 ml of cell pellets) was carried out similarly except using solubilization buffer at a final volume of 6 ml and 1 ml of gel.

RDH Assays with Sepharose-Antibody-bound RDHs—Sepharose-antibody-bound RDH activities and substrate specificities were carried out by monitoring the production of [15-\(3H\)]retinol (reduction of retinal) (24). The reaction mixture (150 \(\mu\)l) contained MES (final concentration, 70–74 mM, pH 5.5), DTT (1 mM), pro-\(S\) [4-\(3H\)]NADH (26 \(\mu\)M), or pro-\(S\) [4-\(3H\)]NADPH (26 \(\mu\)M), 20–25 \(\mu\)l of Sepharose-antibody-bound RDH gel suspension (suspended in 2× volumes of buffer A) in the presence or absence of NAD(P)H (520 mM), and 2 \(\mu\)l of retinal (120–140 \(\mu\)M) substrate stock was added last to initiate the reaction. The reaction was incubated at 37 °C for 50 min then terminated with 400 \(\mu\)l of methanol and 100 \(\mu\)l of 1 M NaCl and extracted with 500 \(\mu\)l of hexane. Radioactivity was measured in the organic phase by scintillation counting.

RESULTS

Initial screening of prostate short chain dehydrogenase/reductase I (PSDR1) expression, an enzyme cloned by Nelson and co-workers (20) from prostate epithelium, reveals that this enzyme is also expressed in the eye (data not shown). Therefore, the name PSDR1 was changed into RDH11 to reflect its broader expression.

**RDH11–14 Sequence Analyses and Gene Structures**—Nucleic acid and protein sequence databases were searched with the RDH11 cDNA sequence (identical to the sequence of the PSDR1I gene product (20)) using Blast. This search identified full-length cDNA clones that show homology to RDH11 and encode RDH12 (first deposited by T. Isogai and under accession number AF054835), RDH13 (expressed sequence tag (EST) deposited by R. Strausberg and under accession number BE376147), and RDH14 (also named PAN2 and deposited by Z. Krozowski under accession number AF237952). The analysis of these cDNAs shows open reading frames of 316, 331, and 336 amino acids for RDH12, RDH13, and RDH14, respectively, encoding proteins of ~35, ~36, and ~37 kDa. RDH11 shares 79% similarity with RDH12 and ~60% similarity with RDH13 and RDH14. RDH12, RDH13, and RDH14 share ~60% similarity among themselves (Fig. 1, A and B).

These proteins contain two motifs highly conserved among SDRs, the cofactor-binding site (GXXXGXXG) and catalytic residues (YXXXX). SDRs contain a motif at the amino terminus consisting of \(\beta\)-strand A, \(\alpha\)-helix B, \(\beta\)-strand B, and \(\alpha\)-helix C (part of the \(\beta\)A-\(\beta\)-B-\(\alpha\)-C-\(\beta\)-C-\(\beta\)-D-\(\beta\)-D that forms the Rossman fold), which interacts with the adenosine monophosphate moiety of the cofactor. The residues present at the junctions \(\beta\)A-\(\beta\)B and \(\beta\)B-\(\alpha\)C are thought to be important in selectivity for NAD(H) versus NADP(H). For favorable interaction with NADP(H), positively charged residues are present at the \(\beta\)A-\(\beta\)B junction (glycine-rich motif) and/or at the beginning of \(\alpha\)-helix C (7). For all RDH11–14, no charged amino acids are present in
the Gly-rich motif (GANTGIG for RDH11–13 or GANSGLG for RDH14), and there are positively charged residues present at the β-α-C junction (RDVEK, RDVLK, RDMEK, RDRARA for RDH11, -12, -13, and -14, respectively), suggesting a preference for NADPH/NADH (Fig. 1A).

The tissue distribution of RDH11–14 was deduced from the array of ESTs displayed in databases corresponding to these RDHs. RDH11 was reported to be expressed abundantly in prostate tissue but also in eye, kidney, pancreas, liver, testis, heart, and brain (20). In addition, ESTs corresponding to RDH11 were also found in libraries from eye, skin, and muscle. ESTs matching RDH12 were identified in multiple tissues, most of them from eye, but also some from kidney, brain, skeletal muscle, and stomach. RDH13 ESTs were obtained mostly from eye, pancreas, placenta, and lung. Many ESTs from brain, kidney, pancreas, and placenta correspond to RDH14.

Genomic clones were identified by GenBank™ data base searches with the coding sequences of the RDHs. Clone Hs14_10185 contains both entire human RDH11 and RDH12 genes. The RDH11 gene is located ~30 kb from the RDH12 gene in the 3′-RDH11-5′ 5′-RDH12-3′ orientation. This gene clone originates from chromosome 14 at q23.3. These genes are located at the locus for a recessive blinding disease, Leber’s congenital amaurosis 3 (LCA3) (www.sph.uth.tmc.edu/Retnet). Clone AC011476.7, obtained from chromosome 19 at q13.42, contains the complete RDH13 gene. Clone Hs2_16082 contains the RDH14 gene and originates from chromosome 2 at p24.1. Comparison of the RDH cDNAs with these genomic clones solved the gene structures. The gene structures of RDH11, RDH12, and RDH13 are almost identical and are interrupted by six introns. The intron/exon junctions of RDH11 and RDH12 are at the same positions, whereas intron 6 of RDH13 is positioned 35 amino acids upstream compared with RDH11-13, which is located at the same position as intron 3 of RDH11–13, and is a relatively small gene (~5 kb compared with 15–18 kb for the RDH11-13) (Fig. 1C). This gene structure is different from other SDR superfamily RDHs expressed in the eye (30–32).

Localization of RDH11–14 in the Eye—A monoclonal antibody specific for RDH11 did not cross-react with RDH5, a prominent enzyme present in the RPE as shown by immunoblotting (Fig. 2A). The lack of cross-reactivity is apparent because RDH5 and RDH11 have different molecular masses. Strong immunoreactivity was detected in bovine and monkey RPE cells, an albino mouse retina was examined in this study (Fig. 3, A and B, left). To visualize the chromogenic signal in RPE cells, an albino mouse retina was examined in this study (Fig. 3B). In albino mouse (BALB/c) retina (Fig. 3B, left), signals were not observed in the RPE cell layer. As a negative control, the sense RDH12 RNA probe did not produce significant hybridization signals in monkey or mouse retina (Fig. 3, A

Fig. 4. Immunolocalization of RDH13 in human and monkey retina. A, specificity of anti-RDH13 antibodies. Lane 1, SF9 cell lysate expressing recombinant RDH11; lane 2, SF9 cell lysate expressing recombinant RDH12; lane 3, SF9 cell lysate expressing recombinant RDH13; lane 4, SF9 cell lysate expressing recombinant RDH14; lane 5, monkey retinal homogenate; lane 6, human retinal homogenate. B–D, immunofluorescence localization of RDH13 in human retina. B, bright field image of human retina. C, RDH13 immunolabeling is predominant in the photoreceptor inner segments of human retina. Weak signals were observed in the inner plexiform layer and inner nuclear neurons proximal to the outer plexiform layer. D, addition of purified RDH13 (2 μg/ml) abolishes RDH13 immunoreactivity. E–G, immunofluorescence localization of RDH13 in monkey retina. E, bright field image of monkey retina. F, RDH13 immunolabeling is predominant in the photoreceptor inner segments of monkey retina. Weak signals were observed in inner plexiform layer and inner nuclear neurons proximal to the outer plexiform layer. Bar, 50 μm. G, addition of purified RDH13 (2 μg/ml) abolishes RDH13 immunoreactivity. H, localization of RDH13 (red) in monkey retina. The cone sheath was simultaneously visualized by fluorescein-conjugated peanut agglutinin (green). Inset, higher magnification image. Arrows show the localization of RDH13 (red) in cone inner segments surrounded by the cone sheath (green). Asterisks indicate the localization of RDH13 in rod inner segments. Bar, 50 μm. RPE, retinal pigment epithelium; OS, photoreceptor outer segments; INL, photoreceptor inner segments; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve-fiber layer.
and B, right). No specific anti-RDH12 antibodies have been generated so far.

Antibodies recognizing RDH13 (Fig. 4A) labeled inner segments of the photoreceptor cells of human and monkey retina (Fig. 4, C and F). Weak signals were observed in a small population of inner nuclear neurons and the inner plexiform layer. Higher magnification images localized RDH13 expression to inner segments of rod and cone photoreceptors (Fig. 4H, inset). This immunoreactivity is specific as it was blocked by purified RDH13 protein. RDH14 yielded hybridization signals in the photoreceptor nuclear layer, and this enzyme appears to be expressed at low levels in the eye, although RDH14 immunolabeling was clearly observed in the bovine cone and ROS with a weaker signal in Müller cells (Supplemental Fig. 1).

**RDH Activity of RDH11, RDH12, and RDH14**—RDH11 catalyzed the reduction of all-trans-retinal and its 9-cis-, 11-cis-, and 13-cis-retinal isomers. The activity was observed in the presence of NADPH and with Sf9 insect cell membranes only when Sf9 cells were transfected with the cDNA encoding RDH11 (Fig. 5). The products were clearly identified by the characteristic spectrum for each retinol isomer and a retention time that was similar to authentic standards (Fig. 5). This analysis avoids problems associated with the isomerization among retinols during incubation or sample handling. The activity toward 13-cis-retinal was the lowest of the retinoid substrates tested and was only detected using high sensitivity HPLC analysis. The summary of the product conversion is illustrated in Fig. 6A. RDH12 and RDH14 have very similar properties to those of RDH11 (Fig. 6, B and C). However, RDH13, expressed in insect cells (Fig. 4), displayed no RDH activity. The double specificity exhibited by RDH11, RDH12, and RDH14 toward cis- and all-trans-retinoids makes these enzymes unique among short chain RDHs.

RDH11, RDH12, and RDH14 demonstrate a clear specificity for the pro-S hydrogen on C4 of NADPH (shown only for one enzyme, Fig. 6D) and the pro-R hydrogen on C15 of all highly active retinols (Fig. 6E). These properties resemble those of the photoreceptor dehydrogenase, prRDH (24, 31), and not those of the RPE enzyme RDH5 (16, 24), which is active toward the pro-S position of both substrates. The results also suggest that these enzymes catalyze the reaction in both directions, NADPH/retinols ↔ NADP/retinoids. RDH11, RDH12, and RDH14 show equal utilization of 11-cis-retinal and all-trans-retinal when these substrates are present at equal concentrations in the same mixture (data not shown), a property that suggests similar efficiency toward both substrates. No steroid dehydrogenase activity was detected for RDH11, RDH12, and RDH14. The activity of the RDH11–14, photoreceptor prRDH,
and RDH5 was potently inhibited by retinoic acids (for example, 9-cis-retinoic acid, \(K_I\) for RDH11, recombinant CRBP1 (Supplemental Table 1, prRDH), and CRALBP (Supplemental Table 2, RDH5).

Co-purification of RDH5 and RDH11 and Expression of RDHs in Immortal ARPE19 Cells—When RDH5 was purified from RPE membranes using anti-RDH5 monoclonal antibody affinity chromatography, NAD(H)-dependent (RDH5) and NADP(H)-dependent (RDH11) enzymes were also isolated based on immunoblotting and retinol activity profiles (Fig. 7A). The activity was suppressed by diluting \([4-\text{3H}]\)NADH with NADH and NADPH. Because the anti-RDH5 antibody did not cross-react with RDH11 (Fig. 2A), these results suggest that both enzymes may form a larger oligomeric structure and/or interact with each other. However, when RDH5 was expressed and purified from insect cells using anti-RDH5 monoclonal antibody affinity chromatography, only NADH- and cis-retinoid preferable properties were observed (Fig. 7B). Qualitatively, the stereospecificity of the mixture of RDHs isolated from RPE membranes (Fig. 7A) matched the sum of stereopreferences toward retinals of RDH5 (Fig. 7B) and RDH11 (Figs. 5 and 6) or the sum of stereo-preferences of RDH5 (Fig. 7B) and the remaining activity in RPE membranes derived from rdh5/H11002/H11002 mice (Fig. 7C). This suggests that the enzyme responsible for oxidation of 11-cis-retinol in these membranes is RDH11.

As with many enzymes involved in retinoid metabolism, the expression of RDH11 and RDH5 is lost in ARPE19, an immortalized RPE cell line (Supplemental Fig. 2A), although other functions of retinal epithelium are preserved. These cells also lack RDH activity toward retinals (Supplemental Fig. 2B). These findings are not due to a secondary effect caused by a lack of other retinoid-processing enzymes because transfecting these cells with the RDH11 or 12 cDNAs restores RDH activity. This observation supports the hypothesis that RDH11 is involved in 11-cis-retinol oxidation in the RPE (Supplemental Fig. 2C).

**DISCUSSION**

**Dual-substrate Specificity, Dual Responsibility; Mechanistic Considerations and Physiological Implications—**Studies of stereochemical transformations catalyzed by RDH enzymes in native tissues and in heterologous expression systems provide important insights detailing physiological substrates, cofactors, and potential complementary enzyme activities. Here, we demonstrated that RDH11–14 catalyze hydrogen transfer from the pro-S \(C_4\) position of NADPH but not of NADH. This specificity is also observed in the eye, one of the most active tissues in retinoid metabolism (2, 24). For all convergently evolved RDH enzymes from the SDR superfamily, only one conformational orientation of dinucleotides within the binding site has been observed. This is consistent with the structural conservation and rigidity of the Rossmann fold (33–37). However, the binding of hydrophobic substrates
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Fig. 7. Enzymatic activities and RDH11 immunoreactivity of affinity column-purified of RDH5. A, RDH5 from bovine RPE microsomes was purified as described under “Materials and Methods.” The assay with pro-S [4-3H]NAD(P)H and geometric isomers of retinals in the presence or absence of NADPH was carried out for 50 min at 37 °C using the phase partition assay as described under “Materials and Methods,” and the amount of [15-3H]retinol isomer was quantified. B, RDHS-His6 from transfected Sf9 cells was purified as described under “Materials and Methods.” The assay was carried out for 50 min at 37 °C using phase partition assay as described under “Materials and Methods.” Indicated is the amount of the corresponding [15-3H]retinol isomer formed in pmol/min. Inset, immunoblot of the purified fraction on anti-RDH5 antibody column probed with specific anti-RDH5 (lane 1) and RDH11 antibodies (lane 3). Lane 2 is a 33-kDa molecular marker. C, stereospecificity toward retinal in RPE membranes derived from rdh5−/− mice (16). Another function of RDH11, along with RDH5, might be to produce 11-cis-retinal from 11-cis-retinol. RDH5 is responsible for the majority of RDH activity in RPE membranes. In humans, mutations in this gene are associated with fundus albipunctatus, a disease expressed by delayed dark adaptation of both cones and rods. Detailed analyses of rdh5−/− mice have identified only a minor phenotype manifested by the accumulation of 13-cis-retinoids. These studies indicate that another enzyme is responsible for the production of 11-cis-retinal under bleaching conditions (16). The RDH(s) responsible for the production of 11-cis-retinal in RPE membrane from rdh5−/− mice display pro-S hydrogen specificity for NADP and utilize 9cis- and 11-cis-retinal but not 13-cis-retinal (16). Furthermore, pro-R stereospecificity toward 11-cis-retinol with NADP was observed in bovine RPE (24). The pro-R stereospecificity toward the retinols is a rare property among SDRs. These results are in perfect agreement with the properties of RDH11 determined in the experiments reported here (Figs. 5–7). Therefore, it is highly probable that RDH11 is the missing enzyme of the RPE in vertebrates as no other known RDH exhibits similar properties (Supplemental Table 3).

RDH12 is expressed in photoreceptor cells, although RDH14 appears to be a minor enzymatic component of ocular retinoid metabolism based upon its level of expression. The substrate specificity of RDH12 indicates that it functions in a fashion complementary to previously identified RDHs (31, 32) involved in the production of all-trans-retinol from all-trans-retinal. We hypothesize that RDH12 might be the key enzyme in the formation of 11-cis-retinol from 11-cis-retinol during regeneration of the cone visual pigments (for review, see Ref. 2).

Bliss reports that the equilibrium constant for reduction of all-trans-retinal is 10−2 to 10 between pH 6.6 to 9.4, respectively (40). Depending on the layer of the retina, the ratio of NADP to NADPH is between 4 to 1 and 1.5 to 1, whereas the ratio of NAD to NADPH could be as high as 300 to 1 (41). Therefore, NAD-dependent enzymes will be mostly oxidizing RDHs, whereas NADP-dependent enzymes will have the ability to catalyze reactions in both directions depending on the retinol/retinal ratio.

Involvement of RDH11−14 in Retinoid Transformation and All-trans- and 9-cis-Retinoic Acid Production—The RDH5 gene product has been implicated in the production of 9-cis-retinal before this aldehyde is further oxidized to 9-cis-retinoic acids (12, 14). However, disabling mutations in men and mice produce no embryonic developmental abnormalities (16–19). In this report, we provide unequivocal evidence that RDH11−14 display high activity toward 9-cis- and all-trans-retinol. The unique substrate utilization of all-trans- and cis-retinoids com-
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Fig. 8. Isomeric specificity of the retinoid cycle reactions in the vertebrate retina. Light causes the isomerization of rhodopsin chromophore, 11-cis-retinal, to all-trans-retinal, which is next reduced in the reaction catalyzed by all-trans-retinal-specific RDH(s). This dehydrogenase activity utilizes pro-S [4-Ha] of NADPH and does not bind NADH to generate pro-R [15-Ha]all-trans-retinol. Next, pro-R [15-Ha]all-trans-retinol or its derivative is isomerized with the inversion of the C15 prochiral methylene hydroxyl group configuration, specifically generating pro-S [15-Ha]11-cis-retinol isomer. Pro-S [15-Ha]11-cis-retinol isomer is then oxidized by RDH5 activity (resulting in the loss of pro-S [15-Ha]) or by RDH11 (resulting in the loss of pro-R [15-Ha]) to 11-cis-retinal with concomitant generation of pro-S [4-Ha]NADPH or pro-S [4-Ha]NADP to complete the cycle (modified version from (24)).

References


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