Molecular Characterization of a Novel Short-chain Dehydrogenase/Reductase That Reduces All-trans-retinal*

(Received for publication, April 28, 1998, and in revised form, June 3, 1998)

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The reduction of all-trans-retinal in photoreceptor outer segments is the first step in the regeneration of bleached visual pigments. We report here the cloning of a dehydrogenase, retSDR1, that belongs to the short-chain dehydrogenase/reductase superfamily and localizes predominantly in cone photoreceptors. retSDR1 expressed in insect cells displayed substrate specificities of the photoreceptor all-trans-retinol dehydrogenase. Homology modeling of retSDR1 using the carbonyl reductase structure as a scaffold predicted a classical Rossmann fold for the nucleotide binding, and an N-terminal extension that could facilitate binding of the enzyme to the cell membranes. The presence of retSDR1 in a subset of inner retinal neurons and in other tissues suggests that the enzyme may also be involved in retinol metabolism outside of photoreceptors.

Vitamin A and its metabolites are active participants in a number of important physiological processes (see Fig. 1). All-trans-retinol is the precursor of other naturally occurring retinoids and appears to be indispensable in reproduction (1). All-trans-retinal is an intermediate in the production of all-trans- and perhaps 9-cis-retinoic acids, which act as hormones, affecting important biological processes such as morphogenesis and differentiation through their interaction with ligand-gated transcription factors (2). In most tissues, retinals do not accumulate in high concentrations, perhaps because of their chemical reactivity and potential toxicity. However, the visual system is unique in that its function is based on retinals and as a consequence, they accumulate in extraordinarily high concentrations, up to 3 mM in the outer segment of photoreceptor cells (3). 11-cis-Retinal is the chromophore for all known visual pigments; however, this isomer is not found in other tissues in significant amounts. Thus, metabolism of 11-cis-retinal and its photoproduct all-trans-retinal, in particular, are of prime importance in the visual system.

In the retina, light isomerizes 11-cis-retinal, activates rhodopsin, and initiates the process of phototransduction by which the visual sensation is produced. The product of photoisomerization, all-trans-retinal, enters into a series of reactions that regenerate the 11-cis-configuration and the native visual pigment. At any given level of illumination, a steady state is established in which the visual pigment photoisomerization rate is equal to and opposed by the rate of visual pigment regeneration (4, 5). Thus, phototransduction and regeneration reactions are irrevocably linked in a cyclical process called the visual cycle (6).

The regeneration reactions begin with the NADPH-dependent reduction of all-trans-retinal. The enzyme catalyzing this reaction, all-trans-retinol dehydrogenase (RDH),1 plays an important role in photoreceptor physiology that is only beginning to be understood. RDH catalyzes the rate-limiting reaction of the visual cycle in rodent rod photoreceptors (7) and plays an important role in phototransduction as the final step in the quenching of photoactivated rhodopsin (8, 9). Furthermore, the activity of RDH controls the level of all-trans-retinal in the retina. This retinoid is responsible, in part, for setting the level of sensitivity of the visual system (10) and may play a role in retinal pathology. All-trans-retinal has been shown to be a constituent of the major fluorescent component of lipofuscin (Fig. 1), a pigment that accumulates in retinal pigment epithelium (RPE) during aging and in pathological conditions (12).

The reactions and enzymes of phototransduction have been thoroughly characterized at a molecular level (12); however, the molecular details of the visual cycle remain poorly characterized. The amino acid sequence is known for only one enzyme of the cycle, 11-cis-retinal dehydrogenase (13). The remaining reactions have been characterized only as enzymatic activities in membrane preparations (14, 15). In the present study, we report the molecular cloning of a cDNA expressing a short-chain dehydrogenase/reductase (retSDR1), and demonstrate that this enzyme is highly abundant in cone outer segments. Expression of retSDR1 in other cells of the retina, and in other tissues, suggests that it may be involved more generally in retinoid metabolism. The molecular characterization of retSDR1 will open the way for further studies of this visual cycle enzyme, and of its potential role in retinal cone dystrophies.

1 The abbreviations used are: RDH, all-trans-retinol dehydrogenase; HPLC, high performance liquid chromatography; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; kb, kilobase; ROS, rod outer segment; HSD, hydroxysteroid dehydrogenase; RPE, retinal pigment epithelium; OS, outer segment; SDR, short-chain dehydrogenase/reductase; MES, (2-N-morpholino)ethanesulfonic acid.

*This work was supported in part by National Institutes of Health NEI Grants EY02317, EY01730, and EY09339; by an award from Research to Prevent Blindness, Inc.; and by an award from the Royalty Research and Development Foundation of the University of Washington. 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.

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**Materials**—All-trans retinal was obtained from Sigma, [3H]NaBH₄ was from NEN Life Science Products, and 11-cis-retinal was a gift from the National Eye Institute. Retinoids were purified by HPLC (16).

**DNA Sequence Analysis**—A search of the EST data base with primer FH28 (5'-GGCCTGGTCAACAATGCTGG-3') was performed in the GenBank data base with FASTA from the GCG package. Amino acid sequence alignments were generated with PILEUP.

**Cloning of Human retSDR1**—Total RNA was isolated from human retinal tissue, obtained from the Lions Eye Bank at the University of Washington, using the Ultraspec RNA isolation system (Biotecx, Inc.) and reverse transcribed with oligo(dT) (Life Technologies, Inc.). Rapid amplification of cDNA ends (RACE) was performed to amplify the 3'-end of the selected EST cDNA using the Marathon cDNA amplification kit (CLONTECH) and the Expand high fidelity PCR system (Boehringer Mannheim). 5'-RACE was primed with an internal gene-specific primer (FH43: 5'-TCCAAGAACTGGCCCAGGGTGGT-3', a gene-specific primer) and the Marathon adaptor primer (AP1) (CLONTECH). Samples were heated at 95 °C for 5 min and amplified for 40 cycles at 94 °C for 30 s, 68 °C for 4 min. A secondary PCR reaction was carried out using the AP2 Marathon adapter primer and a nested gene-specific primer (FH42: 5'-ATGGCGTGGAAACGGCTGGG-3') as described above. The 5'-end of the selected EST cDNA was amplified from a human retina cDNA library with primers FH43 (5'-TCCAAGAACTGGCCCAGGGTGGT-3', a gene-specific primer) and Agt10S. After heating at 95 °C for 5 min, the reactions were cycled 5 times through 94 °C for 30 s, 72 °C for 4 min; 5 times through 94 °C for 30 s, 70 °C for 4 min; and 30 times through 94 °C for 5 s, 68 °C for 4 min. Two amplification products for each PCR were cloned into pCR™2.1 vector (TA cloning kit, Invitrogen) and sequenced by dye-deoxy-terminator sequencing (ABI-Prism, Perkin-Elmer).

**Cloning of Bovine retSDR1**—Total RNA was isolated from bovine retinas using the Ultraspec RNA isolation system (Biotecx, Inc.). cDNA used in the PCR was prepared by reverse transcription with oligo(dT) (Life Technologies, Inc.). 5'-RACE PCR was carried out using 0.5 μl of cDNA and the Expand high fidelity PCR system (Boehringer Mannheim) with a gene-specific primer FH43 (5'-TCCAAGAATGGCCCAGGGTGGT-3') and the Marathon adaptor primer (AP1) (CLONTECH). Samples were heated at 95 °C for 5 min and amplified for 5 cycles at 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 4 min and for 35 cycles at 94 °C for 30 s, and 68 °C for 4 min. A secondary PCR amplification was carried out using the AP2 Marathon adapter primer and primer FH43, for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and 25 cycles at 94 °C for 30 s, and 68 °C for 3 min. 3'-RACE was performed with primers FH42 (5'-ATGGCGTGGGAAGCCGGCTGGG-3') and the Marathon adaptor primer (AP1) (CLONTECH) followed by a secondary PCR amplification carried out using the AP2 Marathon adapter primer and a nested gene-specific primer FH41 (5'-CGGNCCGGAGAGGGTGTTG-3') using the same PCR conditions described for 5'-RACE. Two amplification products for each PCR were cloned into pCRTM2.1 vector (TA cloning kit, Invitrogen) and sequenced by dye-deoxy-terminator sequencing (ABI-Prism, Perkin-Elmer).

**Figure 1.** Diagram illustrating the biological roles of isomers and oxidation products of all-trans-retinol (vitamin A). LRAT, lecithin: retinol acyltransferase; RDH, retinol dehydrogenase.
Cloning of Mouse retSDR1—The mouse cDNA sequence was amplified from a λZAPII both oligo(dT)- and random-primed mouse retinal cDNA library (obtained from Dr. W. Baehr, University of Utah) in two overlapping fragments. The 5' end of fragment was amplified with primers FH100 and FH59 (5'-TCATGTCCTCAGTACTCATGAACTT-3', gene-specific primer) and FH100 (5'-TTGTGATAGCTCTACTATAGGCGG-3', covering T7 primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; and for 5 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary nested PCR amplification was carried out using primers FH100 and FH50 (5'-ATGGTCTCTTGTGCTGTCAG-3') for 5 cycles at 94 °C for 30 s, 64 °C for 30 s, and 68 °C for 1 min and for 25 cycles at 94 °C for 30 s, and 68 °C for 1 min. The 3'end was amplified with primers FH100 and FH50 (5'-TCGGGACTTGTCGCGGGAGAACGTCC-3', gene-specific primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and for 30 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary PCR amplification was performed with primers FH100 and FH58 (5'-ATCGGCCACTTCCTGCGGGG-3', nested gene-specific primer) for 25 cycles at 94 °C for 30 s, and 72 °C for 2 min. Two amplification products for each PCR were sequenced by dideoxy-terminator sequencing (ABI-Prism, Perkin-Elmer). 

Expression of Human retSDR1 in Escherichia coli—The full-length 1.4-kb human retSDR1 cDNA was generated by cloning the 5'end of human retSDR1 cDNA library (obtained from Dr. W. Baehr, University of Utah) in two overlapping fragments. The 5'end of fragment was amplified with primers FH100 and FH59 (5'-TCATGTCCTCAGTACTCATGAACTT-3', gene-specific primer) and FH100 (5'-TTGTGATAGCTCTACTATAGGCGG-3', covering T7 primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and for 5 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary nested PCR amplification was carried out using primers FH100 and FH50 (5'-ATGGTCTCTTGTGCTGTCAG-3') for 5 cycles at 94 °C for 30 s, 64 °C for 30 s, and 68 °C for 1 min and for 25 cycles at 94 °C for 30 s, and 68 °C for 1 min. The 3'end was amplified with primers FH100 and FH50 (5'-TCGGGACTTGTCGCGGGAGAACGTCC-3', gene-specific primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and for 30 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary PCR amplification was performed with primers FH100 and FH58 (5'-ATCGGCCACTTCCTGCGGGG-3', nested gene-specific primer) for 25 cycles at 94 °C for 30 s, and 72 °C for 2 min. Two amplification products for each PCR were sequenced by dideoxy-terminator sequencing (ABI-Prism, Perkin-Elmer) after subcloning into pCR™ T1.2 vector (TA cloning kit, Invitrogen). 

Cloning of Mouse retSDR1—The coding sequence for retSDR1 was amplified from a λZAPII both oligo(dT)- and random-primed mouse retinal cDNA library (obtained from Dr. W. Baehr, University of Utah) in two overlapping fragments. The 5' end of fragment was amplified with primers FH100 and FH59 (5'-TCATGTCCTCAGTACTCATGAACTT-3', gene-specific primer) and FH100 (5'-TTGTGATAGCTCTACTATAGGCGG-3', covering T7 primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and for 5 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary nested PCR amplification was carried out using primers FH100 and FH50 (5'-ATGGTCTCTTGTGCTGTCAG-3') for 5 cycles at 94 °C for 30 s, 64 °C for 30 s, and 68 °C for 1 min and for 25 cycles at 94 °C for 30 s, and 68 °C for 1 min. The 3'end was amplified with primers FH100 and FH50 (5'-TCGGGACTTGTCGCGGGAGAACGTCC-3', gene-specific primer) for 5 cycles at 94 °C for 30 s, and 72 °C for 3 min; for 5 cycles at 94 °C for 30 s, and 70 °C for 3 min; and for 30 cycles at 94 °C for 30 s, and 68 °C for 3 min. A secondary PCR amplification was performed with primers FH100 and FH58 (5'-ATCGGCCACTTCCTGCGGGG-3', nested gene-specific primer) for 25 cycles at 94 °C for 30 s, and 72 °C for 2 min. Two amplification products for each PCR were sequenced by dideoxy-terminator sequencing (ABI-Prism, Perkin-Elmer) after subcloning into pCR™ T1.2 vector (TA cloning kit, Invitrogen). 

The coding sequence for retSDR1 was amplified from this plasmid with PCR primers FH48 (5'-GATGGTGATGGTGATGTGTCCGCCCTTTGAAAGTGTT-3') and FH50 (5'-XbaI-HindIII from pFR415 covering the human retSDR1 coding sequence was cloned between the XbaI and HindIII sites of pFastBac1 expression vector (Life Technologies, Inc.). The expression cassette was then inserted into the baculovirus shuttle vector (bacmid) by transposition. S9 insect cells were transfected with the recombinant bacmid using cationic liposome-mediated transfection (CellFECTIN reagent, Life Technologies, Inc.) according to the manufacturer's protocol. The coding sequence for 11 cis-retinol dehydrogenase was amplified from bovine retina cDNA by PCR with primers FH51 (5'-GATGGTGATGGTGATGTGTCCGCCCTTTGAAAGTGTT-3') and FH52 (5'-GGCTGGCGGAGAAAGCTCC-3') performed in BL21(DE3) pLysS after induction with 0.1 mM isopropyl-1-thiogalactopyranoside (IPTG) were harvested by centrifugation at 1200 × g, 72-96 h after infection. Variable expression and activity levels were observed between different preparations. The reasons for these differences were not investigated.

Expression of Truncated Human retSDR1 in Insect Cells—An ATG and Ndel restriction site were introduced upstream of the amino acid sequence of retSDR1. The truncated retSDR1 cDNA was then transfected into insect cells following the same procedure as for the full-length retSDR1. Following the transfer of the cDNA, BmNPV polyhedra were harvested by centrifugation at 1200 × g, 72-96 h after induction. The peptide was coupled to a carrier protein and used for immunization as described previously (21). Anti-retSDR1 and anti-11-cis-retinol dehydrogenase reacted specifically with the dehydrogenase used for immunization.

In Situ Hybridization—Samples of bovine, monkey and human retina were processed with sense and antisense digoxigenin-labeled riboprobes as described previously (21).
Reduction of All-trans-retinal

Immunocytochemistry—The anterior segments of bovine, monkey, and human eyes were removed and the eye cups immersed in 4% paraformaldehyde, 0.13 M sodium phosphate, pH 7.4, at 4 °C for 6 h. For immunoperoxidase staining, the whole mount retinal sections were processed as described by Milam et al. (22). For confocal microscopy, agarose-embedded retinal sections (100 μm) were processed as described previously (22, 23).

Fluorescence in Situ Hybridization—A 1.5-kb cDNA probe covering human retSDR1 was labeled with biotin-11-dUTP by nick translation (Life Technologies, Inc.). Metaphase chromosome preparations from lymphocytes of a human male were obtained using 75 mM KCl as a hypotonic buffer and methanol-acetic acid (3:1, v/v) as fixative. The hybridization was carried out as described previously by Edelhoff et al. (24). Hybridization signals were detected using a detection system from Vector Laboratories. After incubation with goat anti-biotin antibody, the slides were rinsed in modified 2× SSC, 0.1% Tween 20, and 3× SSC followed. The hybridization signals were visualized by fluorescence microscopy, using a dual band pass filter (Omega).

**RESULTS**

Molecular Cloning of Human retSDR1—The enzymatic properties of retinol dehydrogenase in ROS extracts, such as molecular weight, sensitivity to thiol-reactive reagents, and solubility, suggested that the enzyme was likely to be a member of the short-chain dehydrogenase/reductase (SDR) superfamily. A DNA sequence (~40 base pairs) (see “Experimental Procedures”) corresponding to a conserved domain among retinol dehydrogenases was used to search for homology in an expressed sequence tag (EST) data base. One EST obtained from a human retinal cDNA library (W22782 generated by J. Macke, P. Smallwood, and J. Nathans) was similar to 11-cis-retinol dehydrogenase (AF103513); 11-cisRDH, 11-cis-retinol dehydrogenase (Q92781); 9-cis-retinol dehydrogenase (AF030513); BDH, 9-β-hydroxybutyrate dehydrogenase precursor (Q02338); 17βHSD1, estradiol 17-β-dehydrogenase 1(P14061); PGDH, 15-hydroxyprostaglandin dehydrogenase (P15428); and retSDR1 cloned in this study from human (h), bovine (b) and mouse (m).

**Fig. 2.** Primary structure analysis of retSDR1 proteins. A, alignment of the deduced amino acid sequences of human, bovine and mouse retSDR1. The divergent amino acids between retSDR1 from three species are boxed in gray. The amino acids conserved among about 70% of the members of the SDR family are boxed; those strictly conserved in SDR family are boxed in black. Arrowheads show the amino acids important for cofactor specificity. Residues shown under the sequences are part of the domains important for enzymatic activity and cofactor binding. B, dendrogram of related SDRs. The tree was plotted based on progressive pairwise sequence alignment performed in GCG using PILEUP. Distance along the horizontal axis is proportional to the difference between sequences. The abbreviations used are: 11βHSD1, corticosteroid 11-β-dehydrogenase isozyme 1 (P28845); RODH1, RODH2, and RODH3, retinol dehydrogenase type 1 (U18762), 2 (U33500), and 3 (U33501), respectively; CRAD, cis-retinol/androgen dehydrogenase (AF030513); MLCR, 11-cis-retinol dehydrogenase (Q92781); 9-cis-retinol dehydrogenase (AF030513); BDH, 9-β-hydroxybutyrate dehydrogenase precursor (Q02338); 17βHSD1, estradiol 17-β-dehydrogenase 1 (P14061); PGDH, 15-hydroxyprostaglandin dehydrogenase (P15428); and retSDR1 cloned in this study from human (h), bovine (b) and mouse (m).
the Kozak consensus sequence for translation initiation (28). Bovine and mouse cDNAs were cloned following the same strategy, and sequences are shown in Fig. 2A. The amino acid sequences of human, bovine and mouse retSDR1s are 94–98% identical (Table I). This high homology is absent in the 5′- and 3′-untranslated regions of bovine, human, and mouse retSDR1 (data not shown).

Amino Acid Sequence of retSDR1—The retSDR1 sequence contains all the motifs present in the SDR superfamily of enzymes including the invariant XXXX motif (amino acids 188–192), the catalytic Ser-175, the highly conserved Ser before Lys-192 residue, the highly conserved nucleotide binding motif TGXXXGXG (44–51), and the conserved Gly, Asp, Gly, Leu, Asn, Ala, Asn, Gly, Ile, Leu, Val, and Pro at sequence positions 62, 96, 115, 120, 123, 124, 147, 168, 170, 202, 212, and 219, respectively. The presence of K-TEK at positions 71–74 is consistent with the specificity of this enzyme for NADPH (29).

Tissue Distribution of retSDR1 mRNA—Tissue distribution of retSDR1 mRNA was assessed by Northern blot analysis of several human tissues and bovine retina. A transcript of ~1.8 kb is present in placenta, lung, liver, kidney, pancreas, and retina but was not detected in brain. The size of the human retSDR1 detected by blotting is similar to that of the partial (perhaps lacking fragments of untranslated regions) isolated cDNAs (1.401 for human, 1.478 for mouse, and 1.460 for bovine retSDR1). A transcript of smaller size (~1.4 kb) was observed in heart and skeletal muscle (Fig. 3). This transcript could arise from tissue-specific polyadenylation of the retSDR1 mRNA or splicing of the retSDR1 pre-mRNA, because a similar hybridization pattern was observed with the probe derived from the 3′-untranslated region of retSDR1 (data not shown). The presence of the retSDR1 mRNA, and not a cross-reacting transcript, in other tissues was also supported by a homology search of EST data bases, which revealed sequences identical to retSDR1 in several mouse and human tissues. retSDR1 was not detected, however, by Western blotting with specific monoclonal anti-retSDR1 antibody (described below) at 10 μg of membranous proteins loaded per lane from bovine brain, mammary gland, heart, skeletal muscle, liver, kidney, testis, adrenal gland, RPE, retina, or rod outer segments (data not shown). The failure of mAb to react with membrane components of outer segments (OS) preparations is not surprising, considering that only about 10% of the photoreceptors in mammalian retinas are cones, that the method of isolation of OS is preferential for rods, and that retSDR1 is a minor component related to cone opsins. Even antibodies to cone opsins fail to detect the proteins in preparations of mammalian OS (data not shown). Thus, the expression level must be low in these tissues or restricted to a subset of cells or cellular compartments.

Immunocytochemical Localization of retSDR1—A monoclonal antibody was raised against bacterially expressed retSDR1-His6 protein, and a hybridoma cell producing mAb A11 was cloned. The mAb A11 reacted with retSDR1 expressed in E. coli and in insect cells and did not cross-react with other SDRs, such as expressed 11-RDH (results not shown). Immunostaining with mAb A11 was used to determine the cellular localization of retSDR1 in bovine retina. The antibody reacted intensely with cone but not rod outer segments when examined by immunoperoxidase (Fig. 4A). A similar result was obtained by indirect immunofluorescence (Fig. 4B), which revealed intense labeling of cone outer segments, diffuse labeling of the remainder of the cone photoreceptors, and no labeling of rod outer segments. All cone types were immunoreactive as determined by double labeling immunofluorescence with cone-type specific markers (data not shown). Both methods also showed immunoreactivity with a subset of somata localized in the inner nuclear layer. Similar results were obtained with sections of human and monkey retina. The restricted distribution of retSDR1 to cone and not rod photoreceptors is a strong indication of the specificity of the antibody because rods contain an RDE activity (10, 34, 36, 37) and outnumber cones in bovine retina by about 10 to 1. At present, we do not know whether reactivity of the mAb A11 with a subset of neurons within inner nuclear layer represents the presence of the retSDR1 within these cells or cross-reactivity with another SDR. These cells have not been identified.

In Situ Hybridization—Specific hybridization with digoxigenin-labeled sense and antisense riboprobes was used to provide...
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**Fig. 4. Localization of retSDR1 protein and mRNA in bovine retina.** A and B, immunoperoxidase. Blocks of fixed tissue were processed with specific mAb (A) or specific mAb preabsorbed with antigen (B), and then stained with immunoperoxidase reagents and sectioned at 5 μm. Note the intense immunoreactivity of the cone outer segments (open arrows) and the lack of staining of rod outer segments. Note also the staining of a subset of somata within the inner nuclear layer (arrowheads). C and D, confocal microscopy of retina stained with specific mAb (C) or mAb preabsorbed with antigen (D). Note the intense labeling of cone outer segments (open arrows), and the lighter staining of the rest of the cone photoreceptors, including the synaptic triad. Labeled somata are also evident within the inner nuclear layer (arrowheads). Sections of 100 μm were employed. E and F, in situ hybridization with digoxigenin-labeled antisense (E) and sense (F) riboprobes. Cone ellipsoids (arrow) appear as negative images within the heavily labeled inner segment layer. Staining of somata within the ganglion cell layer was noted with both the sense and antisense probes and is likely to be an artifact of the procedure. Sections of 5 μm were used. OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 50 μm.

Further indication of the bovine retinal cell type expressing retSDR1. The antisense riboprobe produced an intense hybridization signal in a band corresponding to photoreceptor inner segments (Fig. 4E). Cone ellipsoids, which are regions of the photoreceptor rich in mitochondria, appeared as negative images within the layer of intense hybridization. Somata located in the inner nuclear layer also displayed an intense hybridization signal, similar to the pattern seen with antibody staining. A third type of hybridization signal was seen in the ganglion cell layer; however, this signal was also produced by the sense riboprobe and is likely to be an artifact (30). No specific staining of the photoreceptor layer was observed with the sense riboprobe (Fig. 4F). Similar results were obtained with monkey and human retinas.

**Enzymatic Activity of Expressed retSDR1—**Human retSDR1 and 11-cis-retinol dehydrogenase were expressed in insect cells and assayed for RDH activity. The expression of both enzymes was confirmed by Western blot analysis with mAb A11 (anti-retSDR1) and polyclonal anti-serum specific for a peptide derived from the 11-cis-retinol dehydrogenase sequence (data not shown). Membranes from insect cells infected with virus containing 11-cis-retinol dehydrogenase reduced 11-cis-retinal, but not all-trans-retinal (Fig. 5A). Membranes from insect cells infected with virus without RDH sequences (bacmid) displayed little activity (Fig. 5A), however, above that found in the absence of retinal. Similar studies revealed that NADH could not substitute for NADPH in the reduction of all-trans-retinal (results not shown).

ROS and RPE membranes utilized the pro-4S proton of NADPH (Fig. 5B) and, after 20 min, more than 75% of all-trans-retinal generated from bleached rhodopsin and 11-cis-retinal added to the reaction mixture were converted to all-trans-retinol and 11-cis-retinol, respectively. 11-cis-Retinol dehydrogenase and retSDR1,2 expressed in insect cells, also displayed pro-4S specificities (Fig. 5B), yielding 11-cis-retinol (not shown) and all-trans-retinol (Fig. 5C), respectively. The basal activity in the bacmid control was abolished, suggesting that this low activity in the insect cell membranes resulted

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2 The N terminus is important for the stability of retSDR1, because when the dehydrogenase lacking residues 2–35 was expressed in insect cells, the protein was not detectable by Western blotting (data not shown), despite high levels of mRNA (as determined by Northern blotting).
from a dehydrogenase activity with pro-4R specificity.

Potential 17β-HSD, 11β-HSD, and 3α-HSD activities were assayed with selected steroids using membranes from insect cells infected with virus containing the retSDR1 sequence or control cells. Membranes containing retSDR1 did not exhibit HSD activities above those of the control cells toward these selected steroids (Fig. 5D). In contrast, in control experiments, liver microsomes displayed 5 times higher 17β-HSD activity than insect cell membranes (data not shown).

Chromosomal Location of the retSDR1 Gene—The retSDR1 gene was mapped to human metaphase chromosomes by FISH using a 1.4-kb human cDNA probe. Of 117 cells examined, 79 (68%) showed signals on both chromatids of chromosome 1 at band p36.1 (Fig. 6). The localization of a STS (WI-16826) assigned to position 54.6 cR (a measure of distance that is analogous to centimorgans but depends on the radiation dose) from a dehydrogenase activity with pro-4R specificity. A microsomal membrane fraction was isolated and tested for retinol dehydrogenase activity with all-trans- and 11-cis-retinal and a chemically prepared mixture of [3H]pro-4S-NADPH and [3H]pro-4R-NADPH for 10 min as described under “Experimental Procedures.” Membranes from insect cells infected with the vector virus (no RDH sequence, bacmid) were included as controls. [3H]Pro-4S-NADPH (20 μM) was used to determine the nucleotide specificities. The initial reduction rate was determined from measurements of the dehydrogenase activity at 0, 3, 5, and 15 min. For ROS membranes, all-trans-retinal was generated by bleaching rhodopsin (50 μM); for RPE membranes, exogenous 11-cis-retinal (100 μM) was added; for 11-cis-retinol dehydrogenase (11-cis-RDH) and retSDR1 expressed in insect cells, 11-cis-retinal (100 μM) or all-trans-retinal (100 μM) was added, respectively; and for bacmid control, all-trans-retinal (100 μM) was added. C, HPLC analysis of the product generated by expressed retSDR1 in the presence of all-trans-retinal. Retinols were separated using 10% ethyl acetate in hexane on a Silica 5U column (Alltech; 250 mm × 2.1 mm) at a flow rate of 0.3 ml/min, and retinols were detected at 325 nm. The radioactive profile (dotted line) represents a sample produced in the RDH assay by retSDR1 expressed in insect cells, and the elution profile of standard retinols is shown as a solid line. D, steroids as substrates for retSDR1. The assay was carried out as described under “Experimental Procedures.” 17β-HSD activity was assayed by following the reduction of β-estradiol to estrone, 4-androstene-3,17-dione to testosterone, and 5α-androstan-17β-ol-3-one to 5α-androstan-3α, 17β-diol; 11β-HSD activity was assayed by following the reduction of 11-dehydrocorticosterone to corticosterone, and 3α-HSD activity was assayed by following the reduction of 5α-androstan-3,17-dione to androsterone. Controls included reduced forms of steroids and sample without steroids. The data are an average of three independent measurements. retSDR1 does not exhibit significant enzymatic activity toward these selected steroids.

**FIG. 5.** Specificity of retSDR1 and 11-cis-retinol dehydrogenase expressed in Sf9 insect cells. A, retinal stereospecificity. A microsomal membrane fraction was isolated and tested for retinol dehydrogenase activity with all-trans- and 11-cis-retinal and a chemically prepared mixture of [3H]pro-4S-NADPH and [3H]pro-4R-NADPH for 10 min as described under “Experimental Procedures.” Membranes from insect cells infected with the vector virus (no RDH sequence, bacmid) were included as controls. B, nucleotide specificity. [3H]Pro-4S-NADPH (20 μM) was used to determine the nucleotide specificities. The initial reduction rate was determined from measurements of the dehydrogenase activity at 0, 3, 5, and 15 min. For ROS membranes, all-trans-retinal was generated by bleaching rhodopsin (50 μM); for RPE membranes, exogenous 11-cis-retinal (100 μM) was added; for 11-cis-retinol dehydrogenase (11-cis-RDH) and retSDR1 expressed in insect cells, 11-cis-retinal (100 μM) or all-trans-retinal (100 μM) was added, respectively; and for bacmid control, all-trans-retinal (100 μM) was added.

DISCUSSION

Over a century ago, Boll and Kühne (31, 32) reported the progressive change in colors from red to yellow to colorless that occurred in a frog retina exposed to light. Decades later, Wald and colleagues (6, 33) established the molecular foundations for these color changes as the photosomerization of opsin-bound 11-cis-retinal to all-trans-retinal (red to yellow) and reduction of all-trans-retinal to all-trans-retinol (yellow to colorless). Subsequently, it was established that the reduction of all-trans-retinal is catalyzed by a membrane bound dehydrogenase of ROS (34), whose required cofactor is NADPH (35), and that the enzyme is highly specific for all-trans-retinal (36, 10). Although several detergents were reported to solubilize the enzyme, reports of purification of the enzyme did not lead to its molecular characterization (37). In retrospect, it is apparent that the low abundance of RDH relative to opsin and peripherin/ROM, proteins present in orders of magnitude greater amounts, confounded the identification of RDH in enriched preparations. The difficulties are magnified even more with biochemical approaches to identify cone enzymes. However, identification of an SDR sequence motif in an EST data base from human retina led to cloning of a full-length cDNA encompassing this EST and generation of molecular probes (antibodies and riboprobes) and allowed demonstration of RDH activity by the expressed protein, localization of its mRNA to photoreceptor inner segments, and localization of the protein to cone photoreceptor outer segments.

**Amino Acid Sequence and Structure of retSDR1**—A number
of enzymes have been postulated to be members of the SDR superfamily based on the occurrence of several invariant sequence motifs rather than on their overall amino acid sequence homology, which is low (25–40%) (27, 38). These SDRs catalyze diverse oxidation/reduction reactions of selected stereoisomers of hydrophilic aldehydes and ketones. Crystal structures have been determined for members of this superfamily, and a similar core structure is clearly evident in each. The sequence of retSDR1, translated from the cDNA sequence, contains the sequence motifs shared by members of this superfamily, including the invariant XXXK active site motif, which is not present in the medium-chain alcohol dehydrogenase or aldo-keto reductase superfamilies, and the highly conserved nucleotide binding motif (TGXXXGXXG). Other dehydrogenases catalyzing the interconversion of retinol and retinal have been shown to be members of the SDR superfamily, including three isozymes of liver all-trans-retinol dehydrogenase (39, 40), and 11-cis-retinol dehydrogenase of RPE (13). retSDR1 is a member of the subfamily (as defined in Fig. 2B) of SDRs that includes 11-cis-RDH, liver retinol dehydrogenases I–III, CRAD, the steroid metabolizing dehydrogenases, and 15-hydroxyprostaglandin dehydrogenase. Members of the SDR family transfer the pro-4S hydrogen atom of NAD(P)H, while aldo-keto reductases, and medium-chain alcohol dehydrogenases transfer the pro-4R atom (41, 42), and retSDR1 transfers the pro-4S hydrogen (Fig. 5B). The specificity of hydrogen transfer of this enzyme had been addressed previously (43) with [3H]NADH; however, this dinucleotide is not a substrate for RDH (10).

A model of retSDR1 was constructed (Fig. 7A) employing the two crystal structures of ternary complexes of SDR with NADPH (see "Experimental Procedures"). The region 35–302 (shown in red) comprises two domains of different topology. The N-terminal region (residues 35–204) of retSDR1 has some amino acid identity with other α/β doubly wound enzymes that bind NADP (shown as a gray stick-and-ball model). Our simulations showed that the N-terminal α/β unit has the characteristics of the dinucleotide binding fold ("Rossmann fold") of lactate dehydrogenase (44). The phosphate moiety of NADP has hydrogen bonding contacts with side chains of Arg-56, Arg-39, and Thr-40 in mouse carbonyl reductase (25), on which structure the current model has been built. Thus, the environment of the phosphate moiety is conserved between the two enzymes. The conservation in this region is not observed between retSDR1/carbonyl reductase and 17β-hydroxysteroid dehydrogenase (26), even though all of these enzymes utilize NADP as a substrate. 11-cis-Retinol dehydrogenase utilizes either NAD or NADP and lacks the Arg-71-Thr-72 motif in corresponding positions. The C-terminal α-helical region is responsible for binding all-trans-retinol (shown as a black stick-and-ball model). Carboxyl reductase is specific for a secondary alcohol while all-trans-retinol is a primary alcohol. It appears that replacement of V190 by M230 fills the cavity in which the secondary β carbon is placed and provides tight contacts between the retinol and the enzyme. Other residues in retSDR1 involved in the contacts with the retinol molecule are: Leu-177 (the corresponding positions in carbonyl reductase are given in parentheses; Val-138), Ile-182 (Phe-143), His-221 (Val-181), Leu-241 (Leu-200), Glu-244 (Arg-203), and Leu-282 (Tyr-241). The environment of the retinol molecule is shown in Fig. 7B.

The model of the N-terminal extension (amino acids 1–34) (shown in yellow on one side and gray on the other) is highly speculative, but of great interest, and provides a working hypothesis for further experimental studies of how the enzyme is anchored to the membranes or how it interacts with itself or other proteins. The N terminus is unique among SDRs, and does not correspond to any known signal peptide. Its predominantly hydrophobic character (except for Lys-22) suggests that it interacts with cellular membranes. This region appears to be also necessary for the stability of retSDR1 in the insect cells. The two positively charged regions Lys-4–Arg-5, and Lys-32-Leu-33-Arg-34 are likely to be involved in the interaction with the phosphate rich layer of the membranes. The N-terminal extension shows a strong preference for a β-conformation (as depicted in Fig. 7A), in which the side chain of Lys-22 could serve as the transmembrane anchor, 43Å from the Arg/Lys-rich region on the other side of the membranes. Within the β-structure, Pro-12 and Pro-30 would be close to the membrane face and adjacent on different strands. In these positions, the...
strands form a twist, because Pro residues cannot form H-bonds. Although it is generally thought that transmembrane segments of proteins are in α-helical conformations due to the requirement for main chain hydrogen bonding (45), the alternative model presented here is worth experimental scrutiny. The main chain hydrogen bonding in the β-structure may be formed as a result of oligomerization (tetramerization) of retSDR1 or interaction with other proteins anchoring the dehydrogenase to specific regions of the cone cell.

Physiological Relevance—The signal transduction cascade in photoreceptor cells is characterized by expression of enzymes and proteins specific to photoreceptors or to a small number of related neurons. From Northern analysis, retSDR1 mRNA is present not only in the retina (Fig. 4), but also is clearly evident in a number of other tissues. This widespread distribution of retSDR1 is borne out by EST data bases from several tissues in which fragments of the retSDR1 sequence are present. It is possible that retSDR1 acts as a generic all-trans-retinol dehydrogenase in many tissues, ensuring that deleterious levels of all-trans-retinal do not accumulate. Perhaps expression in photoreceptor cells evolved hand-in-hand with the evolution of a phototransduction cascade based on production of all-trans-retinal.

In that light, it is not unexpected that several tissues express the message for retSDR1. retSDR1 may also be involved in other reduction/oxidation reactions throughout the body (including the retina). A similar situation is evident for 11-cis-retinol dehydrogenase (13) in that Northern analysis provides evidence for high level expression of mRNA encoding the enzyme in RPE, whereas a search of EST data bases reveals fragments of the sequence in many tissues (Ref. 46 and data not shown), even though 11-cis-retinal is unique to the visual system.

The relatively broad substrate specificity of SDRs has generated uncertainty regarding their physiological substrates in some tissues. For instance, rat and human hepatic retinol dehydrogenases (RODH1) have recently been shown to oxidize 5α-androstane-3α,17β-diol to dihydrotestosterone as efficiently as they oxidize retinol (47). However, bleaching of the visual pigment in photoreceptor cells is known to produce all-trans-retinal within the outer segments in concentrations approaching 3 mM (48, 49). Thus, the conclusion that retSDR1 is involved in reduction of all-trans-retinal in vivo is relatively secure given the large amounts of this substrate generated within the compartment in which the enzyme is localized. retSDR1 appears to lack HSD activity, as tested with the model steroid compounds.

Cone photoreceptors are known to regenerate bleached visual pigments more rapidly than rods. For instance, the time constant for regeneration of human cone visual pigments is 150 s and that of human rods is 400 s (4–5). We observed that cone outer segments label heavily with anti-retSDR1 (mAb A11), and that the enzyme is not detectable in rod outer segments, suggesting that the amount of retSDR1 is much greater in cones. This raises the intriguing hypothesis that the difference in the regeneration rates of rods and cones may be related, in part, to the amount of retSDR1 in the outer segments. However, another idea, which we favor, is that a homologous enzyme is responsible for the reduction in all-trans-retinal in rods.

In summary, we have demonstrated that a novel member of the short-chain dehydrogenase/reductase superfamily is abundant in cone outer segments and that the enzyme is possibly responsible for reduction of all-trans-retinal in the visual cycle. The amount of retSDR1 in cone outer segments appears to be much greater than in rod outer segments, suggesting an explanation for the faster regeneration of bleached visual pigments observed in cone vision. These results provide the basis for a more detailed study of the control, mechanisms, and role in retinal pathologies of this important enzyme. So far, no retinal disease-specific mutations have been found that map to chromosome 1 at band p36.1, where the retSDR1 gene is located. However, due to the potential involvement of the dehydrogenase in cone retinoid metabolism, retinal diseases characterized by retarded (cone) pigment regeneration appear to be worth testing for mutations in the retSDR1 gene. In addition, age-related macular degeneration and diseases characterized by accumulation of lipofuscin and drusen, may result from the mutations in the retSDR1 gene.
Acknowledgments—We thank Dr. Christine M. Disteche and S. Thomas (University of Washington, Department of Pathology) for chromosomal localization of the retSDR1 gene, Dr. Hartmut Stecher for HPLC analyses, D. C. Possin for assistance with images, and J. P. Van Hooser for critical reading of the manuscript.

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