

The visual cycle retinol dehydrogenase: possible involvement in the 9-*cis* retinoic acid biosynthetic pathway

C.A.G.G. Driessen*, H.J. Winkens, E.D. Kuhlmann, A.P.M. Janssen, A.H.M. van Vugt, A.F. Deutman, J.J.M. Janssen

Department of Ophthalmology, University of Nijmegen, Philips van Leydenlaan 15, 6525 EX Nijmegen, Netherlands

Received 2 March 1998; accepted 17 April 1998

Abstract The 11-*cis*-retinol dehydrogenase (11-*cis*-RoDH) gene encodes the short-chain alcohol dehydrogenase responsible for 11-*cis*-retinol oxidation in the visual cycle. The structure of the murine 11-*cis*-RoDH gene was used to reinvestigate its transcription pattern. An 11-*cis*-RoDH gene transcript was detected in several non-ocular tissues. The question regarding the substrate specificity of the enzyme was therefore addressed. Recombinant 11-*cis*-RoDH was found capable of oxidizing and reducing 9-*cis*-, 11-*cis*- and 13-*cis*-isomers of retinol and retinaldehyde, respectively. Dodecyl- β -1-maltoside used to solubilize the enzyme was found to affect the substrate specificity. This is the first report on a visual cycle enzyme also present in non-retinal ocular and non-ocular tissues. A possible role in addition to its role in the visual cycle is being discussed.

© 1998 Federation of European Biochemical Societies.

Key words: Retinoid metabolism; Retinol dehydrogenase; Substrate specificity; Visual cycle; Retinoic acid

1. Introduction

Vision is mediated by the visual pigments present in the outer segments of the photoreceptor cells. Phototransduction is initiated by the capture of a photon and subsequent isomerization of 11-*cis*-retinaldehyde to all-*trans*-retinaldehyde. All-*trans*-retinaldehyde is converted into all-*trans*-retinol by all-*trans*-retinol dehydrogenase. All-*trans*-retinol is transported to the retinal pigment epithelium (RPE) where it is esterified and subsequently isomerized. It can remain esterified and stored as a 11-*cis*-retinyl ester [1,2] or hydrolyzed and further oxidized to form 11-*cis*-retinaldehyde [3] which is transported back to the photoreceptor cells and linked to the apoprotein, thereby completing the visual cycle.

The oxidation of 11-*cis*-retinol is catalyzed by an oxidoreductase called 11-*cis*-retinol dehydrogenase (11-*cis*-RoDH) [4–6]. A full-length cDNA encoding bovine 11-*cis*-RoDH was isolated [7,8]. Northern blot analysis using RNA isolated from several ocular and non-ocular tissues suggested that the gene encoding 11-*cis*-RoDH is transcribed only in the RPE. However, upon immunohistochemical analysis using a monoclonal antibody (designated 21-C3/AV) directed against

11-*cis*-RoDH, reactivity was also observed to a number of other ocular epithelial cells in addition to smooth muscle tissue of the small arteries in kidney, liver and heart [9]. It was hypothesized that the monoclonal antibody recognizes a shared epitope on (an) other antigen(s), most likely (a) dehydrogenase(s) also belonging to the family of short chain alcohol dehydrogenases. In this report, however, the presence of 11-*cis*-RoDH gene transcripts in both ocular and non-ocular tissues is shown. Immunoprecipitation was used to demonstrate that the apparent molecular weight of the antigens recognized in bovine RPE and kidney are identical. Based on these observations we concluded that the 11-*cis*-RoDH gene is also transcribed in other ocular and non-ocular tissues.

As we know of no reports on the presence of 11-*cis*-retinoids in tissues other than the retina, we decided to determine the substrate specificity of 11-*cis*-RoDH. The present study will show that the enzyme is also capable of oxidizing 9-*cis*- and 13-*cis*-retinol. This observation provides an explanation for the presence of the enzyme outside the retina.

2. Materials and methods

2.1. Isolation of murine 11-*cis*-RoDH genomic clones and Southern blot analysis

Cosmid clones were isolated from a mouse strain 129/SvJ genomic DNA library using the bovine 11-*cis*-RoDH cDNA as a probe [8]. Three cosmid clones were isolated and designated MGRDH1/CD, MGRDH4/CD and MGRDH5/CD.

Genomic DNA was isolated from mouse strain 129/SvJ liver tissue. Genomic DNA (10 μ g) was digested with *Kpn*I, *Hind*III, *Pst*I, *Eco*RI, *Bam*HI or *Sst*I, electrophoresed on a 0.8% agarose gel and transferred to Hybond-N⁺ (Amersham, Buckinghamshire, UK). As a probe a cDNA encompassing exon IV and V of the murine 11-*cis*-RoDH gene was used.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

First strand cDNA was synthesized using 1 μ g total RNA, 2 μ g random hexamer primers (Boehringer, Mannheim, Germany), 200 units Superscript RNase H⁻ Reverse Transcriptase (Gibco Life Technologies, Breda, The Netherlands), 625 μ M of each dNTP, 5 mM DTT in the first strand buffer supplied with the reverse transcriptase. The PCR reaction contained 0.5 μ g of first strand cDNA, 250 μ M of each dNTP, 2.5 mM MgCl₂, 0.2 μ M of each primer, and 1.5 unit AmpliTaq polymerase (Perkin-Elmer-Cetus, Branchburg, NY). Several gene specific primers were used (Table 1). Cycling conditions: initial 5 min incubation at 94°C, 50 s at 94°C, 1.45 min at 55°C and 3 min at 72°C for 35 cycles and finally 10 min at 72°C. PCR products were cloned into PCRII (Invitrogen, Leek, The Netherlands).

2.3. Immunoprecipitation

Bovine kidney tissue (50 g) was homogenized in 50 mM NaP_i buffer (pH 7.3) and centrifuged at 17 000 \times g for 30 min. The supernatant was subsequently centrifuged at 90 000 \times g for 2.5 h. The microsomal pellet was extracted with 20 mM dodecyl- β -1-maltoside (DoM) in 40 mM NaP_i (pH 7.3) for 3 h at room temperature. Immunoprecipitation was performed using 2 mg kidney protein extract, 100 μ l mono-

*Corresponding author. Fax: (31) (24) 3540522.
E-mail: c.driessen@ohk.azn.nl

Abbreviations: RPE, retinal pigment epithelium; 11-*cis*-RoDH, 11-*cis* retinol dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction; DoM, dodecyl- β -1-maltoside; Sf9, *Spodoptera frugiperda*; 9cRDH, 9-*cis*-retinol dehydrogenase; Bps, binding proteins; CRalBP, cellular retinal binding protein; LRAT, lecithin:retinol acyltransferase

clonal antibody 21-C3/AV culture supernatant [9] and 1.5 mg protein A Sepharose (Pharmacia Biotech, Roosendaal, The Netherlands) in a final volume of 500 μ l binding buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.02% Na₂S₂O₅, 10 mM DoM). After 3 h at 4°C the reaction mixture was centrifuged (13 000 \times g, 5 min). The pellet was washed 3 times with 1 ml binding buffer. Precipitated proteins were subsequently dissolved in 50 μ l sample buffer (final concentrations: 50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 100 mM dithiothreitol, 10% sucrose). As a negative control the immunoprecipitation was also performed in the absence of 21-C3/AV. For gel analysis 10 μ l of each sample was electrophoresed on a 13.7% SDS-polyacrylamide-tricine gel [10]. Gels were either stained with Coomassie blue R-250 or blotted to nitrocellulose. Blots were subsequently incubated with 21-C3/AV.

2.4. Substrate specificity

Substrate specificity was tested using a modification of a method described previously [8]. Briefly, 11-*cis*-RoDH containing protein extracts were either derived from recombinant baculovirus infected *Spo-doptera frugiperda* (Sf9) cells or freshly isolated bovine RPE cells. Recombinant virus infected Sf9 (4 days post infection) or bovine RPE cells were harvested by centrifugation (4000 \times g, 10 min, 4°C). Cell pellets were resuspended and lysed upon homogenization (Potter-Elvehjem-tube) in 5 mM Tris-HCl, 10 mM NADH, 20% glycerol, 0.15 M NaCl, pH 7.4. NADH was replaced by NAD⁺ when retinoid oxidation was studied. Homogenized cell suspensions were kept on ice during experiments. All further manipulations were performed in a nitrogen atmosphere under dim red light (Schott-Jena, RG 645 cut-off filter). We used 60- μ l aliquots (=enzyme solution) to mix with 60 μ l substrate solution. For retinaldehyde reduction the substrate solution consisted of the retinaldehyde isomer in 0.2 M acetate buffer, 5% methanol, 1 mM NADH, pH 5.0. When retinol oxidation was studied the substrate solution consisted of 0.1 M Tris-HCl, 5% methanol, 1 mM NAD⁺, pH 8.5. Enzyme activity was determined by measuring the velocity of substrate conversion over a 6-min time period at 37°C. The reaction was started by mixing the substrate and enzyme solution. At definite time intervals 25- μ l aliquots were taken and frozen in dry ice/ethanol. Retinaldehyde decrease/increase was determined by the thiobarbituric acid method of Fütterman and Saslow [11]. Substrates tested were: all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinaldehyde and all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinol. Except for 11-*cis*-retinaldehyde, a kind gift of Dr. W.J. de Grip (Department of Biochemistry, University of Nijmegen, The Netherlands), retinaldehydes were purchased from Sigma. Retinaldehyde stock solutions were prepared in hexane and stored in a nitrogen atmosphere at -80°C in the dark. Retinols were synthesized from their corresponding retinaldehydes by NaBH₄ reduction [12]. Enzyme activity was measured immediately following NaBH₄ reduction. Retinoid samples were checked routinely by UV/VIS spectroscopy (Lambda Bio 10 spectrophotometer, Perkin Elmer). Spectroscopical data were used to determine the retinoid concentration and λ_{max} . The latter was compared to those reported previously for retinaldehyde and retinol isomers [12].

3. Results

3.1. The murine 11-*cis*-RoDH gene

The murine 11-*cis*-RoDH gene is composed of five exons

Table 1
Primers used for RT-PCR analysis

Gene	Reference	Species	Exon	Sequence
Rhodopsin	[37]	Bovine	2/3	5'-gtcggctgtgtccaggtac-3'
			5	5'-gccacagcagagagtgtg-3'
RPE-65	[38]	Bovine	12	5'-cttggcttgaatcactttgt-3'
	[39]		14	5'-tgaacagtcacatgacaaagtg-3'
11- <i>cis</i> -RoDH	[7] [8] [13]	Bovine	4	5'-ttcttccgaacccctgtgac-3'
			5	5'-ccagagcagcttggcatccc-3'
			2	5'-atgtggctgcctctgtcttct-3'
		Mouse	4	5'-ttcttccgaacccctgtgac-3'
			5	5'-ccagagcagcttggcatccc-3'

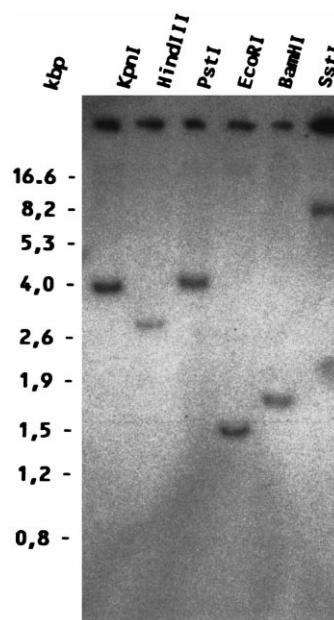


Fig. 1. Southern blot analysis. A Southern blot containing 10 μ g murine genomic DNA digested with *Kpn*I, *Hind*III, *Pst*I, *Eco*RI, *Bam*HI or *Sst*I. The blot was hybridized with a cDNA probe encoding exon IV and V of the murine 11-*cis*-RoDH gene. The sizes of marker DNA are shown on the left.

separated by four introns. The first intron is situated in the 5' non-coding sequence, 35 nucleotides upstream the start codon. The other three introns fall in codons 104, 190 and 245, respectively. A polyadenylation site was found 197 nucleotides downstream the TGA stop codon. Sequence data from murine 11-*cis*-RoDH cDNA clones obtained by RT-PCR confirmed the positions of all exon/intron boundaries and previously determined exon sequence data obtained from cloned genomic DNA fragments. RNase protection assays showed the presence of six putative transcription initiation sites, 163, 164, 174, 175, 187 and 188 nucleotides before the translation start site. The nucleotide sequence mentioned above is deposited in the GenBank/EMBL databases under accession number X97752.

The open reading frame showed 83.9% identity with the bovine nucleotide sequence. The deduced amino acid sequence showed 86.2% identity with the bovine [7] and 88% with the human [13] 11-*cis*-RoDH sequences.

Southern blot analysis was used to determine the copy number of the gene in the murine genome. Selected enzymes recognize a site within intron III and a second site at the

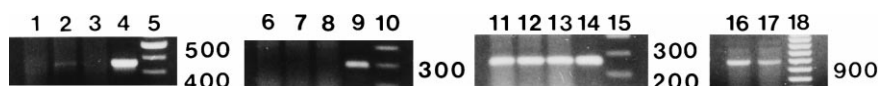


Fig. 2. Tissue specificity of 11-*cis*-RoDH. RT-PCR products were analyzed on a 0.8% TBE-agarose gel. The position of a 100-bp DNA size marker is shown in lanes 5, 10, 15 and 18. RT-PCR products obtained with opsin primers and bovine RNA isolated from liver (1), kidney (2), cornea (3) and retina (4); with RPE65 primers and bovine RNA from liver (6), kidney (7), cornea (8) and RPE (9); with primers in exons IV and V of the 11-*cis*-RoDH gene and bovine RNA from liver (11), kidney (12), cornea (13), RPE (14); with primers in exon II and exon V of the 11-*cis*-RoDH gene and bovine RNA from kidney (16) and cornea (17).

3'-end of exon V, except for *Sst*I and *Bam*HI. The latter cuts 30 bases upstream of the TGA stop codon in exon V whereas the former cuts 1.8 kb upstream of exon I. Restriction digests of murine genomic DNA were hybridized with a cDNA encoding exons IV and V of the murine gene (Fig. 1). Based on the results we conclude that the murine 11-*cis*-RoDH gene is a single copy gene.

3.2. Tissue specificity

Tissue specificity was tested on total RNA isolated from Swiss mouse eyes and liver using an upstream primer in exon IV and a downstream primer in exon V of the murine 11-*cis*-RoDH gene. A PCR product was detected in both samples. Upon sequence analysis, the liver derived PCR product showed 100% identity with the murine 11-*cis*-RoDH sequence. The experiment was repeated using bovine total RNA isolated from liver, kidney, cornea, neural retina and RPE. Primers detecting opsin or RPE65 gene transcripts were included as controls. Both genes were reported to be retina specific [14,15]. A PCR product was obtained in all tested tissues using 11-*cis*-RoDH specific primers (Fig. 2, lanes 11, 12, 13 and 14). The RPE65 transcript was detected only in RPE derived total RNA (Fig. 2, lane 9). The opsin transcript was detected only in neural retina derived total RNA (Fig. 2, lane 4). The complete translated region of 11-*cis*-RoDH cDNA was obtained from both bovine kidney and cornea RNA (Fig. 2, lanes 16 and 17). The obtained cDNA sequences were found to be completely identical to the bovine 11-*cis*-RoDH cDNA sequence.

Monoclonal antibody 21-C3/AV [9] was used for immunohistochemical analysis on frozen cross-sections of bovine liver, kidney, intestine and heart. In liver 21-C3/AV reacted with smooth muscle cells of blood vessels in the portal field (Fig. 3, panel A). Reactivity was also found with smooth muscle cells of small arteries in kidney (Fig. 3, panel C), small intestine (Fig. 3, panel E) and heart (Fig. 3, panel G). In small intestine additional staining was found in the muscularis mucosae and muscularis propria (Fig. 3, panel E). Reactivity with ocular tissues has been described previously [9].

Immunoprecipitation was used for purification of 11-*cis*-RoDH from a bovine microsomal kidney protein extract. The immunoblot revealed a specific protein band of approximately 36 kDa (Fig. 4, lane 5) which comigrates with 11-*cis*-RoDH detected in a bovine RPE derived protein sample (Fig. 4, lane 4). When 21-C3/AV was omitted from the precipitation reaction no 36-kDa protein band was detected (Fig. 4, lane 6). A number of non-specific protein bands were detected in both samples (Fig. 4, lane 5 vs. lane 6). These bands originate from coprecipitated 21-C3/AV (Fig. 4, lane 5) and/or bovine Ig (Fig. 4, lanes 5 and 6) that are also recognized by the peroxidase labelled second antibody. The band displaying the highest apparent molecular weight most likely originates from non-reduced intact immunoglobulins (Igs) while the

smaller bands represent a light chain Ig (± 33 kDa), a heavy chain Ig (± 60 kDa) and one complexed heavy and light chain Ig (± 93 kDa). All four bands were also detected in the absence of 21-C3/AV during protein blot incubation, whereas the 36-kDa band detected in lane 5 was not (data not shown).

3.3. Substrate specificity

Substrate specificity of 11-*cis*-RoDH was determined using the baculovirus expression system. Protein extracts and homogenized Sf9 cell suspensions obtained from recombinant virus infected cells were incubated with: all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinaldehyde and retinol. Both enzyme mediated oxidation and reduction were studied (Table 2). Substrate concentrations at which the velocity of the enzyme-catalyzed reaction is independent from the substrate concentration (V_{max}) were determined prior to determining the enzymes sub-

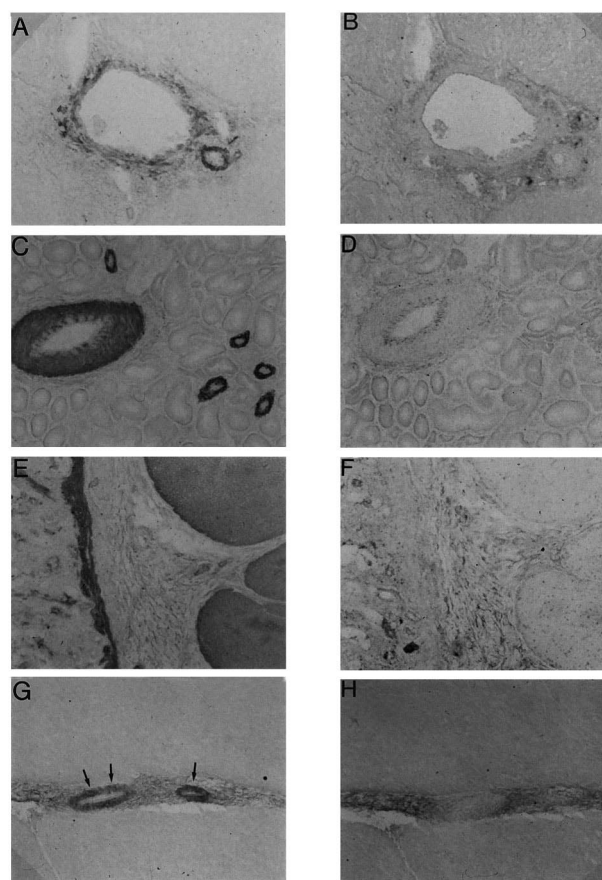


Fig. 3. Staining pattern of 21-C3/AV in non-ocular tissues. 21-C3/AV reacts with smooth muscle cells of the small arteries in liver (A), kidney (C), small intestine (E) and heart (G). In small intestine 21-C3/AV was also found to react with the muscularis mucosae and the muscularis propria (E). Background staining was observed when sections were incubated without 21-C3/AV (B,D,F,H).

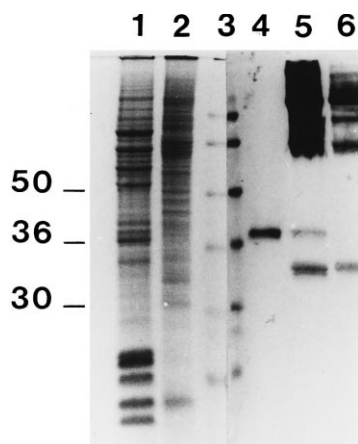


Fig. 4. Immunoprecipitation. The following protein samples were loaded for PAGE and Western-blot analysis: 20 μ g RPE derived protein (lane 1), 12.5 μ g kidney microsomal protein (lane 2), 0.5 μ g RPE derived protein (lane 4), 21-C3/AV immunoprecipitated protein from a kidney microsomal protein sample (lane 5) and kidney-derived precipitated proteins in the absence of 21-C3/AV (lane 6) and electrophoresed on a 13.7% SDS-polyacrylamide-tricine gel. Samples loaded in lanes 1–2 were stained with Coomassie blue R-250. Samples loaded in lanes 4–6 were transferred to nitrocellulose and probed with 21-C3/AV. The antibody stains a specific band of 36 kDa in the protein sample obtained by an immunoprecipitation of a bovine kidney microsomal fraction in the presence of 21-C3/AV. A prestained marker was run in lane 3. At the left molecular weight markers are indicated in kDa.

strate specificity. To compare the V_{\max} values for the substrates mentioned in Table 2, 0.6 mM of each retinoid was used. No decrease or increase of the retinaldehyde isomers was detected in the absence of recombinant 11-*cis*-RoDH (i.e. wild-type virus infected derived protein extracts and cell homogenates, data not shown). Recombinant 11-*cis*-RoDH reduces 11-*cis*- and 13-*cis*-retinaldehyde at a rate of 57 nmol/min/mg protein (Table 2, cell homogenate). Also 9-*cis*-retinaldehyde is reduced efficiently (56 nmol/min/mg). RPE cell homogenates reduce 9-*cis*- and 13-*cis*-retinaldehyde at a rate that is 142% and 185% compared to 11-*cis*-retinaldehyde reduction. Oxidation reactions were also studied. Recombi-

nant 11-*cis*-RoDH oxidizes 13-*cis*-retinol at a rate of 17 nmol/min/mg protein. The oxidation velocity of 9-*cis*- and 11-*cis*-retinol, however, was 2-fold higher (38 nmol/min/mg). Recombinant protein extracts did not oxidize all-*trans*-retinol. Total RPE cell homogenates also oxidized 9-*cis*-, 11-*cis*- and 13-*cis*-retinol. RPE mediated oxidation of 9-*cis*-retinol (26 nmol/min/mg), however, is 2.5-fold higher than 13-*cis*-retinol oxidation and 6.5 times higher than 11-*cis*-retinol oxidation.

In most cases the data obtained from homogenized RPE cell samples agree with those obtained from recombinant virus infected Sf9 cells. Observed differences are likely to be caused by the different amounts of 11-*cis*-RoDH present in both homogenates and by the presence of additional retinoid processing enzymes in RPE derived samples. This probably also explains the large difference in the 11-*cis*-retinol oxidation velocity observed between Sf9/baculovirus and RPE derived samples. The fact that this difference is much larger than that observed for the 9-*cis*- and 13-*cis*-retinol oxidation velocity suggests the presence of higher concentrations of 11-*cis*-retinoid processing enzymes in the RPE.

Retinoid reduction and oxidation was also studied using DoM-solubilized protein samples (Table 2, protein extract). DoM solubilization clearly affects the substrate-specificity of 11-*cis*-RoDH. For example, DoM-solubilized recombinant 11-*cis*-RoDH reduced 9-*cis*-retinaldehyde at a rate that is approximately 20% of that observed for both 11-*cis*- and 13-*cis*-retinaldehyde, whereas in homogenized samples derived from the same culture the reduction velocity for all three isomers is almost equal. This effect is even more pronounced in RPE derived protein extracts. The 13-*cis*-retinaldehyde reduction velocity is 8.5 times higher compared to 9-*cis*-retinaldehyde reduction (622 vs. 73 nmol/min/mg). In RPE derived cell homogenates this ratio is only 1.5.

4. Discussion

4.1. Expression pattern and substrate specificity

Previously a cDNA clone encoding bovine 11-*cis*-RoDH was isolated [7,8]. Upon Northern blot analysis a transcript

Table 2
Substrate specificity

Oxidation (nmol/mg/min)

	Retinal pigment epithelium		Sf9/baculovirus	
	Cell homogenate	Protein extract	Cell homogenate	Protein extract
all- <i>trans</i> -retinol	0	0	0	1 \pm 0
9- <i>cis</i> -retinol	26 \pm 3	18 \pm 1 ^a	38 \pm 3	21 \pm 2 ^a
11- <i>cis</i> -retinol	4 \pm 0	nd	38 \pm 2	nd
13- <i>cis</i> -retinol	10 \pm 1	16 \pm 1	17 \pm 1	14 \pm 2

Reduction (nmol/mg/min)

	Retinal pigment epithelium		Sf9/baculovirus	
	Cell homogenate	Protein extract	Cell homogenate	Protein extract
all- <i>trans</i> -retinal	22 \pm 3	nd	16 \pm 3	nd
9- <i>cis</i> -retinal	116 \pm 6	73 \pm 16	56 \pm 1	44 \pm 5
11- <i>cis</i> -retinal	81 \pm 22	nd	57 \pm 3	204 \pm 6
13- <i>cis</i> -retinal	150 \pm 16	622 \pm 37	57 \pm 3	186 \pm 16

The data are the means \pm S.D. from three separate and independent experiments.

^aOxidation velocity as measured over the first 2 min of the 6-min time interval (see also Section 2). In contrast to the other measurements, 9-*cis*-retinol oxidation was not linear over the time interval. No 9-*cis*-retinal synthesis was observed during the last 4 min of the 6-min measuring time. nd, not determined.

was detected only in the RPE. Hence, the protein was thought to be RPE specific. However, when data on the murine 11-*cis*-RoDH gene structure became available to us, the expression pattern of the 11-*cis*-RoDH gene was re-examined as we had previously observed 21-C3/AV reactivity outside the RPE [9].

The 11-*cis*-RoDH gene is composed of five exons. Members of the short-chain alcohol dehydrogenase family are known to have closely related isoforms derived from separate genes. Southern blot data obtained on the murine gene, however, exclude the existence of a second nearby homologous gene. Mertz and coworkers [16] described the cloning of a 9-*cis*-retinol dehydrogenase (9cRDH). Alignment of the human 9cRDH (GenBank/EMBL databases, accession no. U89717) to the human 11-*cis*-RoDH protein sequence (GenBank/EMBL databases, accession no. U43559) [13], displays 97% homology at amino acid level. Alternative splicing of exon II was studied because 7 out of 8 observed differences between the two human amino acid sequences are encoded by this exon. No indication for the presence of an alternative exon II within the murine 11-*cis*-RoDH gene was found. Hence, in mice the existence of a second closely related isoenzyme is unlikely. It is therefore likely that both this report and Mertz and coworkers [9] describe the same sequence. This idea is also supported by the observed 11-*cis*-RoDH substrate specificity.

RT-PCR analysis with primers positioned in separate exons was used to detect 11-*cis*-RoDH gene transcripts outside the RPE. RT-PCR products derived from the 11-*cis*-RoDH transcript were detected in all tissues tested. To exclude the possibility that RT-PCR analysis detected only low level background transcriptional activity of the 11-*cis*-RoDH gene, it was also necessary to demonstrate the presence of the encoded protein in a non-ocular tissue. As an antigen source for immunoprecipitation bovine kidney tissue was used because this tissue showed the most intense staining upon immunohistochemical analysis. Western blot analysis detected a specific 36-kDa precipitated protein using 21-C3/AV. In a bovine RPE derived protein sample a protein with the same apparent molecular weight was detected. Hence we conclude that, contrary to what was previously reported, 11-*cis*-RoDH is not RPE specific.

Enzymes involved in the biosynthesis of 11-*cis*-retinoid isomers are thought to be specifically expressed in retinal tissues. Therefore, finding 11-*cis*-RoDH expressed in non-retinal ocular and non-ocular tissues raised the question regarding its role in the general retinoid metabolism. Our data and those presented by others [6,7] show that both 11-*cis*-retinol and 11-*cis*-retinaldehyde are good substrates, whereas all-*trans*-isomers appear to be poor substrates. In addition to 11-*cis*-retinol, 9-*cis*- and 13-*cis*-retinol and the respective aldehydes are also substrates for the enzyme. Suzuki and coworkers already showed that partially purified 11-*cis*-RoDH from bovine RPE also oxidized and reduced the 13-*cis*-isomers of retinol and retinaldehyde [6]. No reactivity with the 9-*cis*-isomers of these retinoids was observed. However, in their experiments they used Emulphogene BC-720 or Triton X-100 to solubilize and purify 11-*cis*-RoDH. Purified 11-*cis*-RoDH was subsequently used to study the substrate specificity of the enzyme. From our own experimental data it is obvious that the use of detergent dramatically affects the substrate specificity. In the absence of detergent, the recombinant 11-*cis*-RoDH mediated 9-*cis*-, 11-*cis*- and 13-*cis*-retinaldehyde reduction velocity is

almost equal. In the presence of DoM the 11-*cis*- and 13-*cis*-retinaldehyde reduction is 4.5 times faster than 9-*cis*-retinaldehyde reduction. In bovine RPE derived protein extracts 13-*cis*-retinaldehyde reduction velocity is even 10 times higher compared to 9-*cis*-retinaldehyde reduction velocity. In some experiments 1% DoM was replaced by 1% Triton X-100. Similar differences were observed between cell homogenates and 1% Triton X-100 protein extracts (data not shown). Hence Triton X-100, like DoM, stimulates 13-*cis*- and 11-*cis*-retinaldehyde reduction, whereas 11-*cis*-RoDH mediated 9-*cis*-retinaldehyde reduction is not affected by the use of detergent. In their experiments Suzuki and coworkers also used bovine RPE homogenates to study RPE mediated 9-*cis*-retinaldehyde reduction [6]. Their substrate solution, however, contains 1.2% Tween-80 and in view of the data presented this is likely to have affected the substrate specificity.

Recently Chai and coworkers [17] reported on the cDNA cloning of a murine *cis*-retinol/3 α -hydroxysteroid short-chain dehydrogenase. The deduced amino acid sequence of this protein shows 51% identity to the murine 11-*cis*-RoDH sequence. CHO cells were used for in vitro biosynthesis of the encoded alcohol dehydrogenase. Cell homogenates were able to oxidize 9-*cis*-, 11-*cis*- and 13-*cis*-retinol at a rate of 9.9, 13 and 0.8 nmol/min/mg protein, respectively. No enzyme activity with all-*trans*-retinol was detected. Hence at least two proteins seem to play a role in 9-*cis*-retinol oxidation. The importance in 9-*cis*-retinol oxidation of both proteins needs to be further addressed.

4.2. Functional implications

The finding that 9-*cis*-retinoic acid binds to a subset of receptors known as RXR-receptors [18] has provoked many to study its biosynthesis mode. Studies regarding 9-*cis*-retinoic acid formation have been carried out with liver microsomes [19]. These studies did not show the presence of a retinoic acid specific isomerase, however, bovine liver membranes were found to isomerize all-*trans*-retinoic acid into both 9-*cis*- and 13-*cis*-retinoic acid non-enzymatically. Additionally 9-*cis*-retinoid biosynthesis from all-*trans*-retinol and all-*trans*-retinaldehyde was not influenced by the presence of bovine liver microsomal proteins. In kidney 9-*cis*-retinol appears to be 10% of the all-*trans*-retinol content [20]. Rat liver cytosolic retinaldehyde dehydrogenases have been shown to oxidize both all-*trans*- and 9-*cis*-retinaldehyde [21] and retinoic acid binding protein binds both all-*trans*- and 9-*cis*-retinoic acid [22]. Several all-*trans*-retinol dehydrogenases have been cloned [23–25]. Liver microsomal retinol dehydrogenases are reported to convert neither 9-*cis*- nor 13-*cis*-retinol into their respective aldehydes (for review see [26] and references therein). Biochemical pathways resulting in the formation of 9-*cis*-retinoic acid could include oxidation of 9-*cis*-retinol. As such, enzymes capable of 9-*cis*-retinol oxidation could be part of this biosynthetic pathway.

In the RPE 11-*cis*-RoDH is thought to catalyze 11-*cis*-retinol oxidation. In vitro, however, the reverse reaction, i.e. 11-*cis*-retinaldehyde reduction, is much faster. However, the present study does not take into account the role of retinoid binding proteins (BPs). BPs play an important role in directing the retinoid metabolism in vivo [27–31]. Enzymes responsible for retinol esterification and retinaldehyde oxidation are also important effectors of the retinoid metabolism. In the retina, for example, the cellular retinal binding protein

(CRalBP) and the isomerase could be responsible for directing the retinoid metabolism. CRalBP was shown to bind 11-*cis*-retinol and 11-*cis*-retinaldehyde with high affinity and specificity [32]. The isomerase processed neither 9-*cis*- nor 13-*cis*-retinol [33]. CRalBP was furthermore shown to retard lecithin:retinol acyltransferase (LRAT) catalyzed esterification and to stimulate 11-*cis*-RoDH mediated 11-*cis*-retinol oxidation [3]. Absence of CRalBP and the isomerase and presence of BPs and retinaldehyde dehydrogenases in non-retinal ocular and non-ocular tissues with different retinoid binding specificities could make 11-*cis*-RoDH catalyze the first enzymatic step needed for retinoic acid formation in these tissues.

Retinoic acid biosynthesis in the retina has been studied [34,35] and was found to be light mediated [36]. Most of the retinoic acid in the adult retina is synthesized by the RPE. In addition to all-*trans*-retinoic acid, high concentrations of 13-*cis*-retinoic acid (40–60%) have been detected [34]. However, lack of a clear biological function and non-enzymatic 13-*cis*-retinoid formation from all-*trans*-retinoids [19] make it difficult to address questions regarding the biological significance of retinol dehydrogenase mediated 13-*cis*-retinol oxidation.

Acknowledgements: The authors would like to thank Dr. W.J. de Grip and J.W.H. Janssen for helpful discussions. This work was supported by grants from the following foundations: Haagse Oogheekundig Fonds, Rotterdamse Vereniging Blindenbelangen and Stichting Blindenhulp.

References

- [1] Saari, J.C. and Bredberg, D.L. (1990) *Methods Enzymol.* 190, 156–163.
- [2] Rando, R.R., Bernstein, P.S. and Barry, R.J. (1991) *Prog. Retinal Eye Res.* 10, 161–178.
- [3] Saari, J.C., Bredberg, D.L. and Noy, N. (1994) *Biochemistry* 33, 3106–3112.
- [4] Lion, F., Rotmans, J.P., Daemen, F.J.M. and Bonting, S.L. (1975) *Biochim. Biophys. Acta* 384, 283–292.
- [5] Zimmerman, W.F., Lion, J.P., Daemen, F.J.M. and Bonting, S.L. (1975) *Exp. Eye Res.* 21, 325–332.
- [6] Suzuki, Y., Ishiguro, S. and Tamai, M. (1993) *Biochim. Biophys. Acta* 1163, 201–208.
- [7] Simon, A., Hellman, U., Wernstedt, C. and Eriksson, U. (1995) *J. Biol. Chem.* 270, 1107–1112.
- [8] Driessen, C.A.G.G., Janssen, A.P.M., Winkens, H.J., van Vugt, A.H.M., de Leeuw, A.L.M. and Janssen, J.J.M. (1995) *Invest. Ophthalmol. Vis. Sci.* 36, 1988–1996.
- [9] Janssen, J.J.M., Janssen, A.P.M. and van Vugt, A.H.M. (1994) *Invest. Ophthalmol. Vis. Sci.* 35, 189–198.
- [10] Schagger, H. and Jagow von, G. (1987) *Anal. Biochem.* 166, 368–379.
- [11] Futterman, S. and Sallow, L.D. (1961) *J. Biol. Chem.* 236, 1652–1657.
- [12] Hubbard, R., Brown, P.K. and Bownds, D. (1971) *Methods Enzymol.* 18, 615–653.
- [13] Simon, A., Lagercrantz, J., Bajalica-Lagercrantz, S. and Eriksson, U. (1996) *Genomics* 36, 424–430.
- [14] Zack, D.J., Bennet, J., Wang, Y., Davenport, C., Klaunberg, B., Gearhart, J. and Nathans, J. (1991) *Neuron* 6, 187–199.
- [15] Bavik, C.O., Levy, F., Hellman, U., Wernstedt, C. and Eriksson, U. (1993) *J. Biol. Chem.* 268, 20540–20546.
- [16] Mertz, J.R., Shang, E., Piantedosi, R., Wei, S., Wolgemuth, D.J. and Blanner, W.S. (1997) *J. Biol. Chem.* 272, 11744–11749.
- [17] Chai, X., Zhai, Y. and Napoli, J.L. (1997) *J. Biol. Chem.* 272, 33125–33131.
- [18] Heyman, R.A., Mangelsdorf, D.J., Dydi, J.A., Stein, R.B., Eichele, G., Evans, R.M. and Thaller, C. (1992) *Cell* 68, 397–406.
- [19] Urbach, J. and Rando, R.R. (1994) *Biochem. J.* 299, 459–465.
- [20] Labrecque, J., Dumas, F., Lacroix, A. and Bhat, P.V. (1995) *Biochem. J.* 305, 681–684.
- [21] El Akawi, Z. and Napoli, J.L. (1994) *Biochemistry* 33, 1938–1943.
- [22] Newcomer, M.E., Pappas, R.S. and Ong, D.E. (1993) *Proc. Natl. Acad. Sci. USA* 90, 9223–9227.
- [23] Chai, X., Boerman, M.H.E.M., Zhai, Y. and Napoli, J.L. (1995) *J. Biol. Chem.* 270, 3900–3904.
- [24] Chai, X., Zhai, Y., Popescu, G. and Napoli, J.L. (1995) *J. Biol. Chem.* 270, 28408–28412.
- [25] Chai, X., Zhai, Y. and Napoli, J.L. (1996) *Gene* 169, 219–222.
- [26] Napoli, J.L. (1996) *FASEB J.* 10, 993–1001.
- [27] Posch, K.C., Boerman, M.H.E.M., Burns, R.D. and Napoli, J.L. (1991) *Biochemistry* 30, 6224–6230.
- [28] Posch, K.C., Burns, R.D. and Napoli, J.L. (1992) *J. Biol. Chem.* 267, 19676–19682.
- [29] Napoli, J.L., Posch, K.C. and Burns, R.D. (1992) *Biochim. Biophys. Acta* 1120, 183–186.
- [30] Boerman, M.H.E.M. and Napoli, J.L. (1995) *Biochemistry* 34, 7027–7037.
- [31] Napoli, J.L. (1997) *Cell Dev. Biol.* 8, 403–415.
- [32] Saari, J.C. and Bredberg, D.L. (1987) *J. Biol. Chem.* 262, 7618–7122.
- [33] Bernstein, P.S., Law, W.C. and Rando, R.R. (1987) *J. Biol. Chem.* 262, 16848–16853.
- [34] McCaffery, P., Posch, K.C., Napoli, J.L., Gudas, L. and Dräger, U.C. (1993) *Dev. Biol.* 158, 390–399.
- [35] Dräger, U.C. and McCaffery, P. (1997) *Prog. Ret. Eye Res.* 16, 323–351.
- [36] McCaffery, P., Mey, J. and Dräger, U.C. (1996) *Proc. Natl. Acad. Sci. USA* 93, 12570–12574.
- [37] Nathans, J. and Hogness, D.S. (1983) *Cell* 34, 807–814.
- [38] Nicoletti, A., Wong, D.J., Kawase, K., Gibson, L.H., Yang-Feng, T.L., Richards, J.E. and Thompson, D.A. (1995) *Hum. Mol. Genet.* 4, 641–649.
- [39] Hamel, C.P., Tsilou, E., Pfeffer, B.A., Hooks, J.J., Detrick, B. and Redmond, T.M. (1993) *J. Biol. Chem.* 268, 15751–15757.