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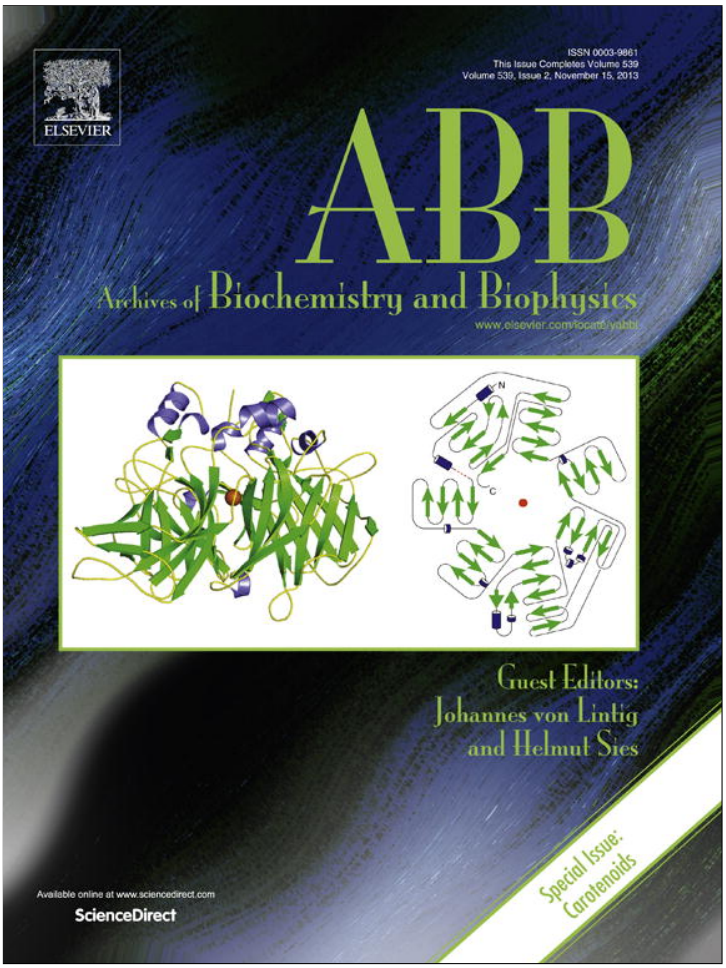

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Cis-retinoids and the chemistry of vision

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ABSTRACT

We discuss here principal biochemical transformations of retinoid molecules in the visual cycle. We focus our analysis on the accumulating evidence of alternate pathways and functional redundancies in the cycle. The efficiency of the visual cycle depends, on one hand, on fast regeneration of the photo-bleached chromophores. On the other hand, it is crucial that the cyclic process should be highly selective to avoid accumulation of byproducts. The state-of-the-art knowledge indicates that single enzymatically active components of the cycle are not strictly selective and may require chaperones to enhance their rates. It appears that protein–protein interactions significantly improve the biological stability of the visual cycle. In particular, synthesis of thermodynamically less stable 11-*cis*-retinoid conformers is favored by physical interactions of the isomerases present in the retina with cellular retinaldehyde binding protein.

Introduction

The biological function of vision requires complex photochemical and biophysical processes in which retinoids (vitamin A, and its multiple chemical derivatives) play a major role as primary photon detectors. In the evolution of eukaryotic organisms these light-sensitive processes have been selected and organized in the retina as a part of the central nervous system. Retina is a highly complex tissue covering the back of the eye, formed by supportive structures such as the retinal pigment epithelium (RPE[1]) and the Müller glial cells, and five different types of neurons (ganglion cells, amacrine cells, bipolar cells, horizontal cells and photoreceptor cells) involved in light-processing [1].

Persistent vision requires efficient supply of the photon detector molecules that are consumed upon the absorption of light and a series of biochemical transformations that reconstitute the photo-detectors from the consumed molecules. This process is termed visual cycle [2]. In the visual cycle, at one end 11-*cis*-retinal constitutes the prototypical photo-active detector molecule, while all-*trans*-retinal represents its light-insensitive counterpart. Most of the biochemical steps of the visual cycle take place in RPE or in the Müller cells and only few steps in the photoreceptor cells themselves [3]. Recent studies evidence an alternate regeneration pathway in the Müller glia cells for specifically supplying cone cells with 11-*cis*-retinal [4].

The visual cycle of rod cells is very well established in the literature (Fig. 1). Detection of light occurs when 11-*cis*-retinal is covalently bound as protonated Schiff base to rhodopsin GPCR. After photoabsorption and consequent isomerization, the protonated Schiff base is hydrolyzed and released as all-*trans*-retinal. This species is reduced to all-*trans*-retinol by multiple membrane bound dehydrogenases (including RDH8 and RDH12) in the photoreceptor cells [5]. All-*trans*-retinol is then transported into the RPE either by interphotoreceptor retinol binding protein (IRBP), if released to the cytosol of rod outer segment, or by ABCA4 transporter, if released to the intra-discal lumen. In RPE, all-*trans*-retinol is passed onto lecithin retinol acyl-transferase (LRAT) by cellular retinol-binding protein (CRBP). LRAT acylates the alcohol group yielding all-*trans*-retinyl ester. This ester is concomitantly hydrolyzed and isomerized by the same enzyme (RPE65) in complex with other enzymes and/or retinoid binding proteins to form 11-*cis*-retinol [6–8]. The highly light sensitive 11-*cis*-retinol is then taken up by cellular retinaldehyde binding protein (CRALBP) chaperoning its oxidation to 11-*cis*-retinal by *cis*-specific retinol dehydrogenase (RDH5) [5]. The freshly formed 11-*cis*-retinal is again taken up by CRALBP, transported to the apical membrane of RPE and transferred back to the photoreceptor cells via IRBP, where it is eventually reattached to opsin to reconstitute functional rhodopsin [5].

In rod visual cycle, RPE65 balances the action of rhodopsin, catalyzing the *trans*- to *cis*- back-isomerization of the chromophore [3,6–8]. In the cone/Müller glial cells a fully detailed

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[1] *Abbreviations used:* CRALBP, cellular retinaldehyde binding protein; CRBP, cellular retinol-binding protein; DES1, *trans*- to *cis*-retinol isomerase; GPCR, G-protein coupled receptor; HPLC, high pressure liquid chromatography; IRBP, interphotoreceptor retinol binding protein; LCA, Leber Congenital Amaurosis; 11β-HSD1, 11β-hydroxysteroid dehydrogenase; LRAT, lecithin retinol acyl-transferase; PCR, polymerase chain reaction; RLBP1, gene coding for CRALBP; RPE, retinal pigment epithelium; RA, retinoic acid; RAR, nuclear retinoic acid receptor; RBP, retinol-binding protein; RDH5, *cis*-specific retinol dehydrogenase; RDH8, *trans*-retinol specific dehydrogenase; RPE65, retinal pigment epithelium-specific 65 kDa protein; RXR, nuclear retinoic acid receptor; SDR, short-chain dehydrogenase; STRA6, cell-surface receptor of retinol-binding protein; WT, wild type.

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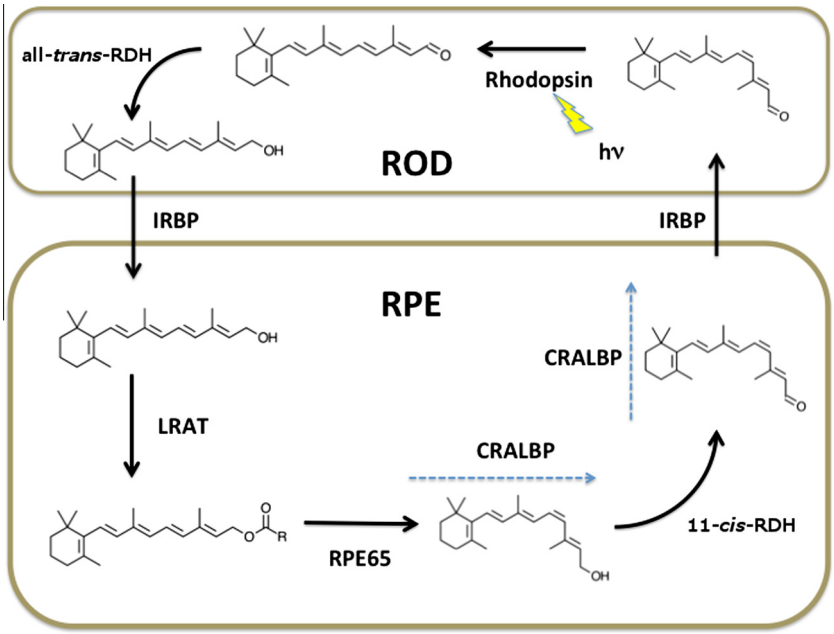

Fig. 1. Visual cycle in the Rod-RPE system. Simplified scheme of the Rod-RPE visual cycle. The main retinoid species entering into the cycle, as well as the principal protein components responsible for their transformation, are reported.

characterization of the visual cycle is still debated [9,10]. The evidence for an alternate pathway has been first evidenced in cone-dominated chicken retinas [11,12]. Recently, DES1, a *trans*- to *cis*-retinol isomerase enzyme expressed in the Muller glial cells, has been identified and characterized [13].

For both visual cycles *in vivo* experiments report globally high substrate specificity toward 11-*cis*-retinal as the active chromophore. Specifically, past experimental determination of retinoid isomers in the mouse eye has shown the presence of other *cis*-forms (i.e. 9-*cis* and 13-*cis*) only in negligible quantities [13,14] (Fig. 2). This established paradigm set the initial idea that the various proteins involved in the cyclic process are specific for either 11-*cis* or all-*trans*-retinoids, so that no other isomers are produced/transduced in chemically relevant concentrations in the eye. However, independent experiments targeting single components of the visual cycle have been depicting a more complex framework.

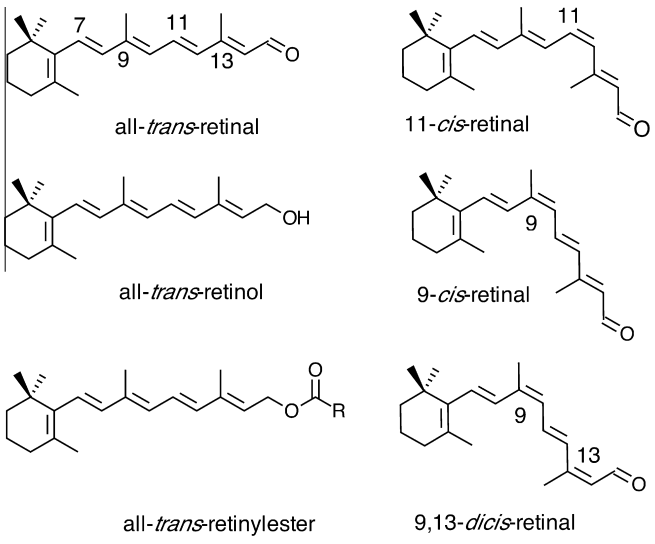

Fig. 2. Chemical structures of the retinoids discussed in the text.

Since several decades rhodopsin was shown to bind and to be photochemically active in complex with retinaldehydes other than 11-*cis*. In particular, opsin bound to 9-*cis*-retinal and 9–13-*dicis*-retinal (called isorhodopsin-I and II) were shown to provoke action potentials *in vivo* in various animal models [15,16]. In more recent times, distinct components of the visual cycle (RPE65, RDH5, CRALBP, DES1) were reported to exhibit specificity for alternate *cis*-conformations [6,13,17,18]. These findings include evidence for the in vitro formation of relevant quantities of 13-*cis* and 9-*cis*-retinol, at least comparable to those of 11-*cis*-retinol, by RPE65 [6]. Also the recently identified DES1 isomerase produces in vitro and *in vivo* mixtures of *cis*-retinols [13]. Other components in RPE, like RDH5 and CRALBP, show catalytic activity and good binding affinity respectively for 9-*cis*-retinol/al substrates [17,18]. All these recent findings suggest that either retinoids other than 11-*cis* or all-*trans* may be effectively produced but efficiently processed in the eye or that *in vivo* co-expression and interaction of distinct visual cycle components enhances selectivity towards 11-*cis* conformation.

In this short review, we shall discuss recent progress on the (bio)chemistry of retinoids in the visual cycle. In particular we will address new findings about retinoid conformations other than 11-*cis* or all-*trans*. Because of their better characterization, we shall focus mostly on proteins expressed in the rod-RPE cell system, with some cone-Müller associated examples also being presented. This review does not intend to be an exhaustive work about the visual cycle and its components, or on retinoid (bio)chemistry. For more extended analyses on these topics, reference to other works (for example: [4,19–22]) may be envisaged.

General retinoid metabolism

Biological processing of retinoids requires multiple extra- and intracellular binding proteins able to recognize and traffic their different chemical and isomeric forms. Evolution of retinoid-specific binders is driven by the need for facilitating diffusion of poorly soluble substrates and also by the necessity for sequestration of

highly reactive intermediates. These proteins accompany all biochemical processes associated to retinoids (such as uptake, biosynthesis, gene expression, embryonal development, visual cycle) and therefore are not solely expressed in the retina.

Retinoids in vertebrates are primarily provided by β-carotene (pro-vitamin A) present in many food sources. β-Carotene is cleaved and oxidized into short-chain retinoids in the small intestine and in the liver [23]. Physiologically, the canonical uptake of retinoids into the cell proceeds via the specific cell-surface receptor STRA6 [24,25] when bound to serum retinol-binding protein RBP [26,27]. Inside cells, all-*trans*-retinol and its primary oxidation product all-*trans*-retinal are associated with isoforms of the 15 kDa cellular retinol-binding protein (CRBP-I and CRBP-II). Cytosolic CRBPs function as intracellular retinoid chaperones by binding all-*trans* and 9-*cis*-geometric isomers of retinol and retinal, but not retinoic acid (RA), with high affinity [28]. The two CRBP isoforms have different functions and expressed in different tissues. Holo-CRBP-I facilitates binding of its ligand all-*trans*-retinol to LRAT for esterification. CRBP-II is specifically expressed in the absorptive cells of the small intestine where it has a role in the initial processing of retinol from food [29].

Biosynthesis of the most oxidized RA involves reversible dehydrogenation of retinol into retinal, catalyzed by membrane-bound, short-chain retinol dehydrogenases/reductases, followed by irreversible dehydrogenation of retinal by soluble retinal dehydrogenases [30,31]. RA binds to retinoic acid-binding proteins CRABP-I and CRABP-II. While CRABP-I increases rates of retinoic acid-metabolizing enzymes, CRABP-II delivers RA to the RAR nuclear receptor in a direct collision process [32]. It is established that the biologic activity of all-*trans* and 9-*cis* geometric isomers of RA is based on high affinity interactions with the RAR [33] and RXR [34] subfamilies of nuclear RA receptors, respectively.

Many retinoid-binders and retinoid-processing enzymes are active with 9-*cis*-retinoids [30,32]. Dietary 9-*cis*-β-carotene can act as one source for 9-*cis*-retinoids by oxygenase-mediated cleavage into 9-*cis*-retinal and all-*trans*-retinal [35]. The fact that chow-fed wild-type mouse liver contains 9-*cis*-retinol indicates that 9-*cis*-retinal is reduced into 9-*cis*-retinol, esterifies and transported from the intestine. All this implies that 9-*cis*-retinol is a naturally taken up retinoid in vertebrates. In addition Rotenstreich et al. have shown that 9-*cis*-β-carotene can reverse retinal dystrophy in man [16]. However, it has also been reported that the dietary use of 9-*cis*-β-carotene is insufficient to rescue vision in mouse models of Leber Congenital Amaurosis [36]. It was shown that the photoreceptors of LRAT$^{-/-}$ mice essentially lacked any 9-*cis*-retinal. This implicates that retinyl-ester formation is an essential step for 9-*cis*-retinoid accumulation in the eye when feeding 9-*cis*-β-carotene. These findings also indicate that the major source of 9-*cis*-retinal in the eye may not be directly associated with dietary *cis*-carotenoids stored in the liver [36].

Biochemistry of cis-retinoids in the visual cycle

In the visual cycle there are essentially five groups of proteins associated each to a different function. These are photoreceptors, transferases, isomerases, dehydrogenases and chaperones. In this chapter we discuss proteins belonging to these groups, for which association to both 11-*cis* and other *cis*-geometries have been characterized.

Rhodopsin

Rhodopsin is a 40 kDa membrane protein responsible for photon capture and initiation of signal transduction in the visual cycle. It consists of trans-membrane helix bundle (opsin) covalently bound to 11-*cis*-retinaldehyde chromophore. Opsin belongs to the G-protein coupled receptor (GPCR) superfamily. The prototypical fold of GPCRs was determined thanks to crystallization of rhodopsin in the year 2000 [37–39]. The crystallographic data revealed the presence of seven transmembrane helices encapsulating the chromophore in a tight cavity and an extended loop region responsible for the transducin binding and activation. Current knowledge of vertebrate rhodopsin mostly refers to the rod-dominated bovine retina while little is known about the corresponding cone photoreceptors.

Rhodopsin is the best-characterized protein of the visual cycle with several crystal structures capturing different states of the photoisomerization process [40–45]. The photo-isomerization of 11-*cis*-retinal in rhodopsin has been broadly investigated by several computational groups [46–48]. The isomerization process involves transition through several structurally and spectroscopically well-characterized states of the protein [49–55]. After the initial photoexcitation, the protein reaches the photorhodopsin-state (after about 200 fs), then bathorodopsin (few ps), the blue-shifted intermediate and lumi-rhodopsin (ns) to finally evolve into meta-rhodopsin-I (μs) and then the G-protein activating conformation meta-rhodopsin II (ms) [56].

Several studies suggest that rhodopsin aggregation occurs in vivo and affects the mechanism of G-protein activation [57–62]. In 2010 molecular dynamics simulations of rhodopsin dimers predicted allosteric signaling between the photoactivated subunit and the transducin binding-site of the partner [63] (Fig. 3, panel A).

In the visual cycle, opsin acts as a terminal sink by covalently binding 11-*cis*-retinal as its natural cofactor. In the literature, opsins bound to 9-*cis*-retinal (isorhodopsin) or 9,13-*dicis*-retinal (isorhodopsin II) were also described to be functional, though having quantum yields half than 11-*cis* bound rhodopsin [64,65] (Fig. 4). In that report, the absorption efficiency of the different isoforms of rhodopsin originating from bovine rods was assessed. In particular, photosensitivity of the pigments was determined by photobleaching experiments using 540 nm light, while extinction coefficients were determined by UV/VIS spectroscopy. The activity of the isorhodopsin I and II isoforms was later confirmed by both, *ex vivo* and *in vivo* experiments. Specifically, it could be proven that explanted skate retinas partially regained sensitivity after bleaching when treated with 9,13-*dicis*-retinal [15]. More recently, experiments on retinas of long-term dark-adapted RPE65$^{-/-}$ mice reported accumulation of endogenous isorhodopsin up to 5% of the total chromophores of wild-type animals [66]. The crystal structure of squid isorhodopsin has been recently reported [45]. In 2008 Olivucci showed that rhodopsin and isorhodopsin relax along a common excited-state potential energy valley starting from opposite ends [67]. More recently, Ishida and coworkers showed that rhodopsin isomerizes faster and more efficiently than isorhodopsin, thanks to constraint pathways that lead to relaxation of the electronic excited state through conical intersection [68].

RPE65

The primary function of RPE65 in the visual cycle is the production of 11-*cis*-retinol by hydrolysis and isomerization of all-*trans*-retinylesters. Early immuno-cytochemical studies [69] identified a RPE-specific 63 kDa membrane protein in smooth endoplasmatic reticulum of the chicken retina being highly conserved in other animals. Bavik et al. made the initial characterization of RPE65 [70,71]. In 1993, its primary sequence was defined [72,73]. However, assignment of its function was only achieved 12 years later by three independent groups [6–8]. They showed that RPE65 is the light-independent *trans*- to *cis*-isomerization component of the RPE. It was demonstrated that expression of RPE65 is essential to the health of cone photoreceptors implicating its more general

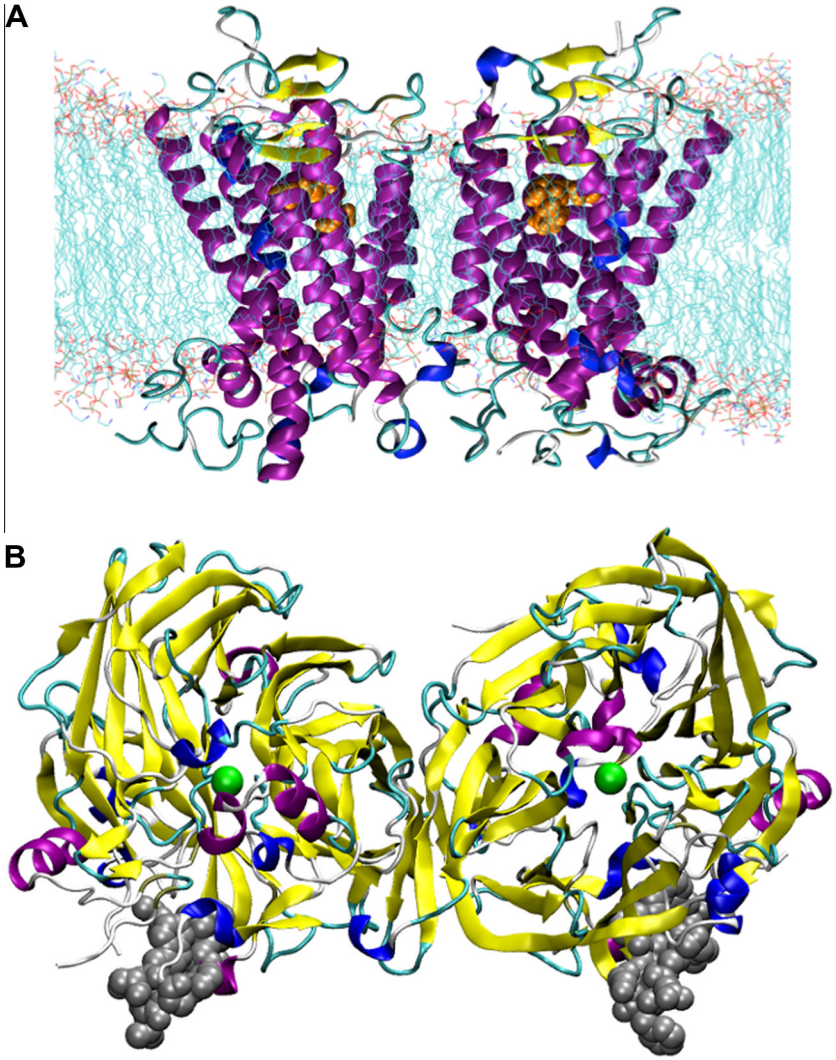

Fig. 3. Structurally characterized homo-dimeric structures in the visual cycle. Panel A: computational model of the rhodopsin dimer in the dark state from Ref. [63]. The proteins are represented in cartoon, the 11-*cis*-retinal in orange spheres. The phospholipid bilayer embedding the dimer in the MD simulation is shown in lines. Panel B: crystallographic structure of RPE65 dimer as reported in Ref. [78]. The Green spheres show the binding site of the catalytic Fe(II) ion. Gray spheres highlight the lipid binding region. 3D structures were drawn using the VMD software [128]. The proteins are colored according to their secondary structural elements: magenta for α-helices, blue for 3,10-helices, yellow for β-sheets, white/cyan for coil/loops.

role in the biology of the retina [74,75]. Since 2009, Palczewski and coworkers have published multiple crystallographic structures of bovine RPE65. These structures revealed the presence of a catalytic iron-center responsible for the hydrolysis-isomerization reaction [76–78]. Very recently, a set of structures of RPE65 in different lipid like environments gave evidence that the biologically active unit may be an RPE65 dimer [78] (Fig. 3, panel B). Nonetheless, ultimate proof for this may come from experiments in solution.

Despite in vivo evidence for highly selective back-isomerization into 11-*cis*-retinal, independent groups have reported over the last decade that RPE65 can catalyze conversion to 13-*cis*-retinol in vitro [79,80]. Recently, Redmond and coworkers reported that modulation of specifity can occur by point mutations of aromatic residues in the binding cavity of the substrate} [80,81]. Most recent experiments in a single-cell model of rod-visual cycle highlight that RPE65 has the propensity to produce 9-*cis*-retinol [82]. The leaky isomerase activity of RPE65 seems to be under the control of physically interacting protein partners, specifically lecithin:retinol acyltransferase (LRAT), CRALBP and RDH5. It has been reported that besides all-*trans*-retinol also the 11-*cis*- and 9-*cis*-isomers can be esterified by LRAT *in vitro* to yield the corresponding retinyl esters. Nonetheless, the rate of 11-*cis*-retinol esterification is six fold slower than that of all-*trans*-retinol. The capability of esterifying *cis*-retinols can be associated to storaging functions in the presence of excess concentrations [79,83,84]. In particular CRALBP is strictly required to selectively produce 11-*cis*-retinol in RPE microsomes [21,83]. Aberrant accumulation of 13-*cis*-retinylesters in retinosomes of RDH5$^{-/-}$ mice indicates that RPE65 is also controlled by the expression of the partner dehydrogenase [85,86].

DES1

While RPE65 is known since 2005 [6–8] as the key isomerase responsible for the *trans*- to *cis*- conversion in RPE, the existence of an alternate pathway in strong-light conditions has been postulated [87–91]. Very recently, dihydroceramide desaturase-1 (DES1) [92,93] has been identified as retinol isomerase in Müller cells [13]. DES1 is a protein bound to the endoplasmatic reticulum [94,95] and acts via a free radical mechanism [95].

With respect to RPE65, DES1 in vitro shows even less selectivity towards the products. In fact the enzyme yields 11-*cis*, 9,13-*dicis*, 9-*cis* and 13-*cis*-retinols from all-*trans*-retinol in ratios close to

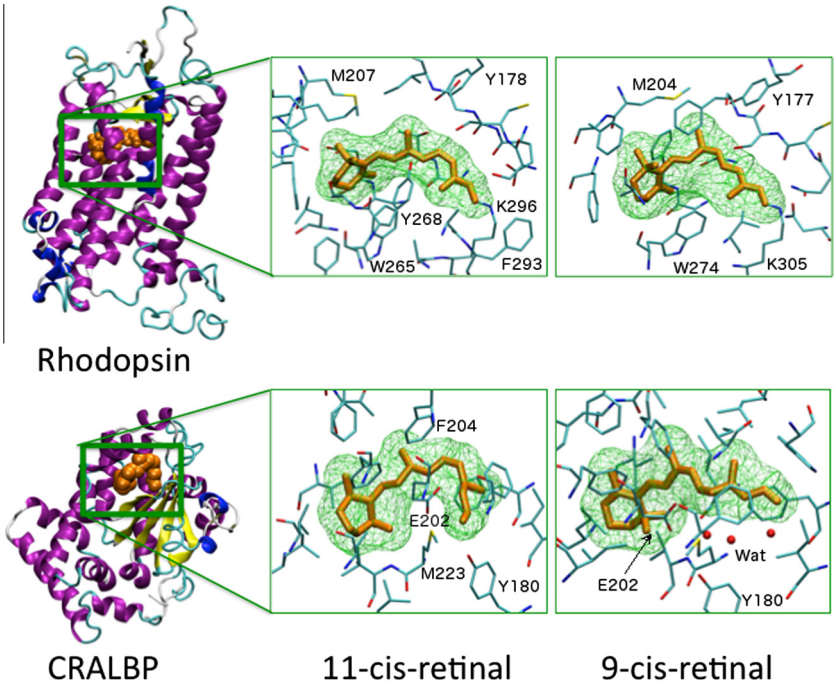

Fig. 4. Binding of 11-*cis* and 9-*cis*-retinal to rhodopsin and CRALBP. Comparison between the crystallographic structures of the binding cavities of rhodopsin and CRALBP loaded with 11-*cis*-retinal and 9-*cis*-retinal, respectively (PDB codes: 1JFP, 2PED; 3HY5, unpublished). Retinal is depicted in thick licorice. Residues forming the binding cavity are drawn in thin licorice. 3D structures were drawn using the VMD software [128].

the thermodynamically equilibrated free retinoid samples. In particular Travis and coworkers report 11-*cis*:9,13-*dicis*:9-*cis*:13-*cis* ratios of 1:78:158:388 by DES1 [13], while incubation of all-*trans*-retinyl-palmitate in the presence of I_2 catalyst [96] showed ratios equal to 1:40:109:231. Detection of DES1 mRNA by quantitative real-time PCR could demonstrate that DES1 is expressed at fivefold higher level in chicken retinas than in mouse or cow retinas. This implies that DES1 is more abundantly expressed in cones than in rods. The *in vitro* low selectivity of DES1 might be regulated, *in vivo*, by the presence of interacting protein factors [13]. In particular the same authors reported a threefold increase of 11-*cis*-retinol production, together with a 1.4-fold increase of 9-*cis*-retinol and concomitant reduction of 13-*cis*-retinol in co-immuno-precipitates of DES1 and CRALBP. The in vivo significance of DES1 was proven by experiments partially rescuing the physiological phenotype of the RPE65$^{-/-}$ mouse model. In particular, Travis and coworkers forced DES1 overexpression by gene therapy using recombinant adenovirus. Co-injection of all-*trans*-retinol into the vitreal space yielded threefold increased levels of 9- and 11-*cis*-retinal, and consequent partial rescue of functional photosensitivity in these mutant mice.

CRALBP

11-*cis*-retinoids are the dominating photoactive pigments in the visual cycle. The 11-*cis*-conformation is thermodynamically unfavorable compared to other *cis*-isomers [96]. Therefore proteins in the visual cycle must carefully select for this geometry. 11-*cis*-retinal and also its precursor 11-*cis*-retinol both associate with cellular retinaldehyde binding protein (CRALBP) in several cell types of the eye. CRALBP was first described in bovine retina as a soluble retinoid carrier by Futterman et al. [97]. Human CRALBP is a 36.4 kDa protein encoded by the gene RLBP1 [98]. In a 1987 published study by Saari and Bredberg [99], CRALBP was identified as high affinity binder for 9-*cis*-retinal, 11-*cis*-retinal and 11-*cis*-retinol. The same study showed that CRALBP does not bind to either 13-*cis* or all-*trans*-retinal. Measured affinities of the protein for the different compounds in the literature report the following K_d values: 11-*cis*-retinal, K_d = 20 nM [18]; 9-*cis*-retinal, K_d = 53 nM [18]; and 11-*cis*-retinol, K_d = 60 nM [99]. CRALBP enters the visual cycle by stimulating isomerase activity of RPE65 [83,100]. Then it facilitates binding of 11-*cis*-retinol into RDH5 [18,101]. Finally it chaperones translocation of 11-*cis*-retinal from RDH5 through the cytoplasm out of the cell. Essentially, CRALBP provides vectorial retinal flux, hence increases the efficiency of the process [3]. Characterization of gene mutations of human CRALBP could show that this protein is essential for efficient dark adaptation in rods and cones [102]. Accordingly, experiments on Rlbp1$^{-/-}$ mice revealed a profound delay in regeneration of rhodopsin associated with accumulation of all-*trans*-retinylesters [103]. An additional function of CRALBP is given by its capability of protecting *cis*-ligands from premature isomerization [99].

The crystal structures of both WT CRALBP and its Bothnia disease mutant R234W in complex with the endogenous ligands 11-*cis*-retinal or 9-*cis*-retinal were recently solved [82,104]. The structures revealed a tight hydrophobic binding-pocket, which allows sequestration of bent retinoids. The particular shape of the pocket is sufficient to explain lack of affinity for more elongated conformations as those of 13-*cis* and all-*trans*-retinoids. Combined crystallographic analysis and molecular dynamics simulations confirmed the presence of alternate binding geometries for 11-*cis* and 9-*cis*-retinal in CRALBP. Specifically, the binding of 9-*cis*-retinal is characterized by a residual micro-solvation pattern, absent when 11-*cis*-retinal binds to CRALBP [82,105] (Fig. 4). Incubation in the absence of light of CRALBP:9-*cis*-retinal complexes revealed secondary activity as thermal isomerase yielding 9,13-*dicis*-retinal. The same experiments reported strong modulation of the isomerase activity, associated with subtle variations in the micro-solvation pattern, for the Bothnia disease mutant R234W [82,105].

RDH5

The dehydrogenase activity in the visual cycle is most probably highly redundant and expressed by multiple dehydrogenase-enzymes [85]. Nonetheless, the major flow of retinol oxidation is

attributed to RDH5 [85,106–108]. RDH5 is a short-chain dehydrogenase (SDR) of the endoplasmatic reticulum involved in the oxidation of 11-*cis*-retinol along the visual cycle. It was first identified in bovine retina [109,110]. Orthologous sequences with high sequence similarities have been identified in human and in mouse genomes. The sequences were originally labeled with different acronyms such as 9cRDH, RDH4, 11-*cis*-RoDH or cRDH [111–114], but then eventually re-conducted to one single enzyme called RDH5 [115].

The family to which RDH5 belongs includes different members expressed in multiple tissues. Their function is mostly associated to regulation of retinoid and androgen biosynthesis as well as to gene expression. In these functions the major substrates besides the androgens are 9-*cis*, 11-*cis* and/or all-*trans*-retinol.

The crystal structure of RDH5 has not been solved yet. Within the SDR family close homologs have been structurally elucidated [116,117]. Among these, 11β-hydroxysteroid dehydrogenase (11β-HSD1) is of particular interest for the peculiar multicomponent feature of its biologically active unit. In fact, it has been proposed that the homo-dimeric complex evidenced in vitro, may be able to tetramerize in vivo, forming functional channels coupling different lipid compartments [117]. The close primary and secondary structure similarities between 11β-HSD1 and RDH5 apart from the C-terminal region suggest that the latter may be functional as a multimer as well (Fig. 5).

In the visual cycle, the specificity of the RDH5 enzyme has converged towards similar high affinities for 9- or 11-cis-retinoids and decreased activity towards all-*trans*-retinol. In an initial investigation, Napoli and co-workers measured the activity of human RDH5 by HPLC purification of the products at different time points, reporting identical oxidation rates for 9-*cis* and for 11-*cis*-retinol (V_{max}/K_M = 3.5 nmol min^{-1} mg^{-1} mM^{-1}) [17]. In the same year, Blaner and co-workers [112] reported substrate conversions for mouse and human RDH5 (called cRDH in the paper) enzymes. Despite the very close homology of the sequences (87.1% of identity), the reported rates in the two species show significant differences in both the absolute values, and in the relative ratio between different *cis*-retinoids. Specifically, values of 204 and 74 pmol min^{-1} (mg protein)$^{-1}$ for 9-*cis*-retinol in human and in mouse respectively were measured. The corresponding rates for 11-*cis*-retinol were 510 and 60 pmol min^{-1} (mg protein)$^{-1}$. In both cases, the enzymes showed significantly lower activities with all-*trans*-retinol.

More recently Crabb and coworkers reported binding constants and oxidation rates for recombinant human RDH5 in the presence or absence of recombinant human CRALBP by tritium labeling and extraction of the products [18,118]. These newer measurements for the free enzyme were characterized by a V_{max}/K_M ratio fivefold larger than that by the group of Napoli [17]. The presented data indicate a profound effect of binding partner proteins onto the activity of RDH5 with an increase of V_{max}/K_M from 15.3 to 57.2 nmol min^{-1} mg^{-1} mM^{-1}. The data from Blaner and Crabb suggest that both, the enzymatic activity and the substrate specificity of RDH5 might be significantly influenced by subtle conformational changes due to either, local mutations in the primary structure or to steric restraints imposed by interacting protein partners [18,115]. Genetic studies originally revealed a role of RDH5 in 11-*cis*-retinal production [119]. In depth investigations on the RDH family of proteins by the Palczewski group have uncovered RDH5 as the major responsible for RDH activity in RPE membranes [120]. Nonetheless, the loss of RDH5 function in patients with fundus albipunctatus may be partially compensated by other dehydrogenases. A good candidate for it might be RDH10, which was found to be co-localized with RPE65 and CRALBP in the RPE [107]. In recent times, electrophysiological studies by Niwa et al. showed that RDH5 mutation induce simultaneously cone and rod dysfunction in Fundus Albipunctatus [121]. This implies that RDH5 is also involved in cone visual cycle. In any case, particular care must be taken in interpreting species-specific dehydrogenase activities, because the different genomes may translate into dehydrogenase networks with significantly altered phenotypes.

Discussion and perspectives

Retinoids are involved in several biochemical processes in superior animals. For example, they play a crucial role in embryo development, cellular growth, or gene expression [122]. In the visual cycle, they act as photon-capture systems as Schiff's bases covalently bound to opsin proteins in photoreceptor cells [20].

In the body, retinoids are functional in three different redox states, namely retinol, retinaldehyde and retinoic acid [31]. The most common isomerization states processed in the body usually are all-*trans* or 9-*cis*, which correspond to the thermodynamically most stable ones [96]. The visual cycle process coevolved a set of enzymes able to select the less stable 11-*cis*-retinoid conformation. The evolutionary pressure leading to 11-*cis* biochemistry was most probably associated to the better photo-absorption efficiency for 11-*cis*-retinal over other *cis*-isomers. In fact, the quantum yield of 11-*cis*-retinal in rhodopsin is twice as large as those of 9-*cis*-retinal or 9,13-*dicis*-retinal [65,123].

Recently opsin paralogs of the GPCR family have been reported in the pineal gland (pinopsin) and in the ganglion (melanopsin) with non-image forming functions [124,125]. These proteins are functional in regulating biological clocks and circadian rhythm. Melanopsin is expressed in vision cells of cephalochordate invertebrates, putatively representing an early stage in evolution of the visual cycle [126]. In this GPCR, affinity and functionality in both 11-*cis* and 9-*cis*-retinal loaded species has been reported [127].

Despite 11-*cis*-retinal is the major pigment involved in the visual cycle, different experiments showed that cone-dominated retinas contain significant amounts of 9-*cis*-retinal, and that rod-dominated retinas loaded with 9,13-*dicis*-retinal are still functional [15]. The photoactive form of opsin in complex with 9-*cis*-retinal (Isorhodopsin I) has been structurally characterized [45]. Quantum calculations showed subtle differences in the isomerization process, putatively responsible for the lower efficiency compared to rhodopsin [67,68].

The presence of active, less efficient chromophores in intense light photoreceptors could be evolutionarily favored by a corresponding elevation of the photo-bleaching threshold. This comes at the cost of respective increase of the scotopic threshold for cones, which is complemented by the presence of dim-light photoreceptor cells (rods). It appears that there might be a functional 9-*cis*-retinol gradient from RPE to the cones, as evidenced in RPE65$^{-/-}$ mice models, implying direct substrate exchange across the compartments of the retina [22,66].

Proteins involved in the visual cycle and interacting with *cis*-retinoids seem to possess relatively low geometric specificity toward their substrates. This feature is shared by both proteins that are not solely expressed in the retina (like chaperones or dehydrogenases), and proteins exclusively associated to the visual cycle. For example, apart from its function in the visual cycle, RDH5 may participate in the production of 9-*cis*-retinal for synthesis of 9-*cis*-retinoic acid [31]. This is supported by reports of RDH5 expression in several retinoid-responsive adult tissues outside the pigment epithelium [17,111,114] and expression in mouse embryos [113]. It is particularly striking that both RPE65 and DES1, the two known isomerases of the visual cycle, produce in vitro and in the absence of interacting partners, significant amounts of 13-*cis*-retinol [6,13]. This isomer has no known biological function, and is not detected in vivo. Several evidences on multiple components of the visual cycle highlight that *cis*-retinoid selectivity

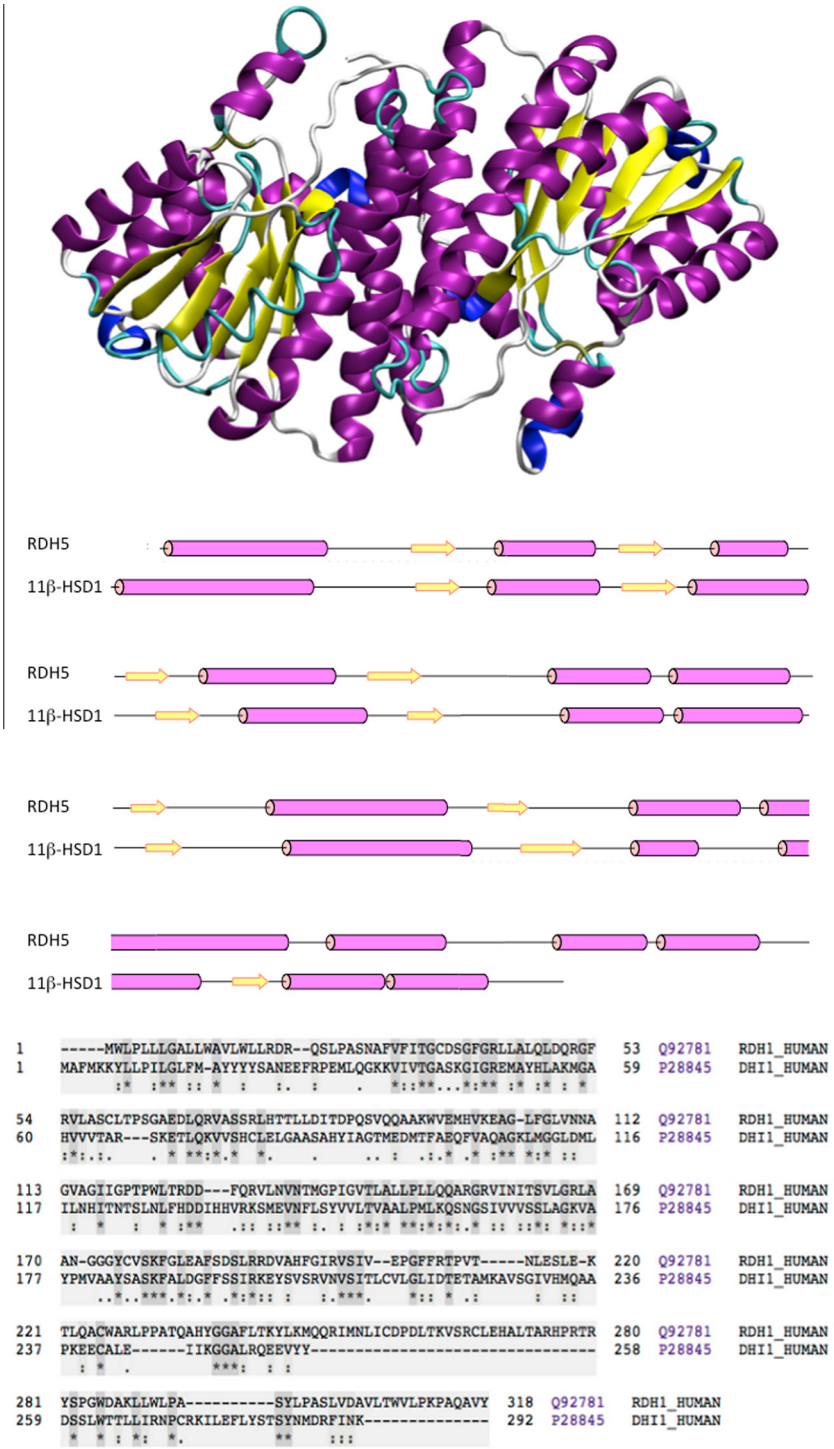

Fig. 5. Dimeric structure of RDH5 homologous 11β-hydroxysteroid dehydrogenase (11β-HSD1). The upper panel shows the three-dimensional structure of the dimer of 11β-HSD1 as from Ref. [117] (PDB code: 1XU7). The middle panel reports a schematic picture of the secondary structure similarity between the sequences of RDH5 and 11β-HSD1. The secondary structure predictions were obtained from the Protein Sequence Analysis Workbench web-server (PSIPRED; http://bioinf.cs.ucl.ac.uk/psipred/). 3D structures were drawn using the VMD software [128]. The lower panel reports the sequence alignment of the RDH5 and 11β-HSD1 primary structures carried out with the online UniProt alignment tool (http://www.uniprot.org/align/).

occurs upon complexation of these same components with interacting protein partners. In particular, CRALBP provides geometric specificity to the visual cycle by selecting for 11-*cis* and 9-*cis* conformations, thus quenching down production of 13-*cis*-retinol. This is done by physically binding to RPE65 or DES1 and extracting only those catalytic products compatible with the steric restraints of its binding-pocket [13,78,83]. CRALBP also affects the rates of both, substrate binding to, and product release from RDH5, thus enhancing the relative concentration of 11-*cis*-retinal over both, 9-*cis*-retinal, and *cis*-retinols in RPE [18]. The action of CRALBP on both, isomerase and dehydrogenase enzymes, enhances the retinoid flux from all-*trans* to photoactive *cis*-retinaldehyde isoforms. Covalent binding of the produced *cis*-retinoids to opsin constitutes the final driving force for the reconstitution of the pigments [19]. CRALBP

seems to be the crucial factor for correct selection of the different retinoid isoforms in the eye. The complexity of the action of CRALBP in retinoid biology has been further evidenced by the recent discovery of its secondary activity as thermal isomerase [82]. This function is present only when 9-*cis*-retinal is bound, yielding 9,13-*dicis*-retinal. On the contrary it is silent when bound to 11-*cis*-retinal. The relevance of this function in retinoid metabolic pathways remains to be ascertained.

The state-of-the-art knowledge of the visual cycle strongly points at the essential contribution of protein–protein interactions for the stability of this system. However, the contribution of each single protein component in putative retinoid-processing complexes has not been studied in depth yet. Improvement of knowledge of such systems would afford a broad system-biological approach. The newest progresses in proteomics and lipdomics combined to metabolomics studies can aid in the near future to the understanding of the eye's function. Given the non-linear behavior of the enzymes in the visual cycle, it is mandatory to assess their functionality in the presence of interacting partners. Future integrated studies combining high-resolution imaging and crystallographic techniques to bioinformatic and computational approaches may lead to the determination of three-dimensional models of the functional complexes of the visual cycle.

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