1. Introduction

As succinctly summarized by Wolf, lack of vitamin A (all-trans-retinol) was recognized by ancient Egyptians as causing a visual deficiency involving the retina and cornea that could be cured by eating liver. One of the symptoms of vitamin A deficiency is night blindness or nyctalopia (from Greek νυκτ—from night; and άλαος—blindness), recognized by ancient Greeks, including Hippocrates, as affecting the retina. In 1913 McCollum showed that “fat-soluble factor A” was essential for growth of a rat colony (reviewed in ref 3). The treatment of factor A-deficiency included liver or liver extracts, but later in 1930 Moore found that yellow pigment (carotene) was a good substitute for this therapy. A major breakthrough occurred in 1931 when the chemical structures for β,β-carotene and retinol (its all-trans isomer now known as vitamin A) were determined by Karrer and colleagues. But, it was Wald who discovered that retinol derivatives constitute the chemical basis of our vision, a contribution subsequently recognized by a Nobel Prize award in 1967. In 1950−1960, a variety of vitamin A metabolic...
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Figure 1. Mevalonate pathway for the synthesis of IPP. Two molecules of acetyl-CoA are joined together to form acetoacetyl-CoA (i) in a reaction catalyzed by thiolase with the release of free CoA (CoASH). A third molecule of acetyl-CoA is added by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to form HMG-CoA (ii). Compound ii is reduced by HMG-CoA reductase in an NADPH-dependent manner to form (R)-mevalonate (iii), which is the rate-limiting step of the pathway. Compound iii is sequentially phosphorylated by mevalonate kinase and phosphomevalonate kinase to form (R)-mevalonate-5-diphosphate (iv). Finally, iv is decarboxylated by mevalonate-5-diphosphate decarboxylase in an ATP-dependent manner to form IPP (v). Coloring of oxygen atoms is intended to assist in tracking of the chemical origin of the carbon skeleton. P, phosphoryl group; OPi, inorganic phosphate.

Figure 2. MEP (nonmevalonate) pathway for the synthesis of IPP and DMAPP. First, D-glyceraldehyde-3-phosphate (i) is condensed with pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DOXP, ii) catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) using a thiamine diphosphate cofactor with the loss of CO2. ii is isomerized and reduced by DOXP-isomerase (IspD) in an NADPH-dependent manner to form 2C-methyl-D-erythritol-4-phosphate, which is then conjugated with CTP to form 4-diphosphocytidyl-2C-methyl-D-erythritol (iv) in a reaction catalyzed by 2C-methyl-D-erythritol cytidylyltransferase (IspD). iv is then phosphorylated to form 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (v) by 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (IspE). v is then reduced with cleavage of the cyclodiphosphate moiety to form 1-hydroxy-2-methyl-2,4-butenyl diphosphate (vi) by the iron-sulfur enzyme IspG using ferrodoxin as a cofactor. Finally, vi is further reduced by a second iron–sulfur protein IspH, giving a mixture of IPP (vii) and dimethylallyl diphosphate (DMAPP, viii). The red colored oxygen atom is intended to assist in tracking of the chemical origin of the carbon skeleton. P, phosphoryl group; OPi, inorganic pyrophosphate.

transformations, including oxidation/reduction and esterification, were elucidated by Olson,7–20 Goodman,21–32 Chytil and Ong,33–41 and Norum and Blomhoff.42–57 The discovery that one set of these metabolites, namely retinoic acids, plays a key role in the nuclear regulation of a large number of genes added a notable dimension to our knowledge of gene expression. This mechanism is also a critical player in the successful healing of corneal wounds,58 a second manifestation of vitamin A deficiency recognized earlier.

Further progress in understanding the multiple physiological roles of retinoids has been made in recent years, due mainly to the successful application of modern scientific technology. Examples include enzymology combined with structural biology, in vivo imaging based on retinoid fluorescence, improvements in analytical methods, generation and testing of animal models of human diseases with specific pathogenic genetics, genetic analysis of human conditions related to changes in vitamin A metabolism, and pharmacological approaches to combat these diseases.

In this review we focus on the involvement of retinoids in supporting vision via light-sensitive rod and cone photoreceptor cells in the retina. We begin with a brief description of isopentenyl diphosphate (IPP) biosynthesis, which is essential for carotenoid (C40 isoprenoid) production. Certain of these colored compounds, such as lutein, are deposited in our retina’s macula, appearing as a “yellow” spot. Other carotenoids containing at least one unmodified β-ionone ring (represented by β,β-carotene and cryptoxanthin) serve as precursors of all-trans-retinal. Many different compounds can be generated from this monocylic diterpenoid, which contains a β-ionone ring and polyene chain with a C15 aldehyde group. Among the numerous enzymatic activities that contribute to retinoid metabolism, polyene trans/cis isomerization is a particularly fascinating reaction that occurs in specialized structures of the retina based on a two cell system comprised of retinal photoreceptor cells and the retinal pigment epithelium (RPE). A specific enzyme system, called the retinoid (visual) cycle, has evolved to accomplish retinoid isomerization that is required for visual function in vertebrates. Individual enzymes of this pathway harbor secrets about the molecular mechanisms of this chemical transformation. Malfunctions of these processes or other pathological reactions often precipitate severe retinal pathologies. This review attempts to balance contributions that have been published over the past decades and does not intend to replace the views of investigators with different perspectives of retinoid chemistry in the eye.59–85

2. CHEMISTRY OF ISOPRENOIDS

In animals, carotenoids and retinoids must be acquired through the diet, as they cannot be synthesized de novo. These compounds are involved in critical functions of many organs in addition to their vital involvement in vision.86

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2.1. Mevalonate and Nonmevalonate Pathways

Naturally occurring carotenoids are all synthesized from two basic 5-carbon precursors: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). A multitude of additional compounds, including steroids, ubiquinones, and chlorophylls, are also synthesized from these isoprene precursors and are thus collectively referred to as isoprenoids or terpenoids. Until the early 1990s it was believed that isoprenoid precursors were synthesized exclusively through the mevalonate pathway, a series of enzymatically catalyzed reactions in which three molecules of acetate, in the form of acetyl-coenzyme A (acetyl-CoA), are condensed and modified by reduction, phosphorylation, and decarboxylation to generate IPP (Figure 1). Research in eubacteria and plants then revealed a second metabolic route for IPP synthesis referred to as the methylenetetritol 4-phosphate (MEP) or nonmevalonate pathway, which utilizes the triose derivative, 3-glyceraldehyde 3-phosphate along with pyruvate as starting materials.

Following condensation of these two molecules, a methyl isomerization reaction occurs that converts the initially linear carbon chain into an isopentyl linkage. Subsequent reduction, CDP transfer, phosphorylation, and reduction serve to eliminate hydroxyl functional groups and introduce a diphosphate to generate IPP as well as DMAPP in about a five to one ratio. In many cases, a particular organism encodes the enzymes for only one of these two metabolic pathways in its genome. Metazoans, fungi, and archaea rely exclusively on the mevalonate pathway for isoprenoid biosynthesis, whereas cyanobacteria and algae make sole use of the MEP pathway. Depending on the species, eubacteria and protozoa can employ either of the two pathways in a mutually exclusive fashion. However, most eubacteria utilize the MEP pathway. In higher plants, most cytosolic IPP and DMAPP are derived from the mevalonate pathway via enzymes encoded by genomic DNA. By contrast, these isoprene compounds are synthesized through the MEP pathway in plastids of plants as a consequence of the evolutionary relationship of plastids to cyanobacteria. However, mixing of cytosolic and plastid isoprene precursors has been reported. Most carotenoids consumed by humans are synthesized from MEP-derived IPP and DMAPP.

IPP and DMAPP differ only in the position of an alkene double bond and are thus classified as geometric isomers (Figure 3). Enzymes known as IPP isomerases (IDI) catalyze the reversible isomerization of IPP into DMAPP. For organisms that solely use the mevalonate pathway for isoprene biosynthesis, IDIs are essential for production of DMAPP because only IPP is generated through this pathway. Although both IPP and DMAPP are produced via the MEP pathway, IDIs are still required to generate the proper IPP/DMAPP ratio for isoprenoid biosynthesis.

Two structurally unrelated enzymes, called IDI type 1 and IDI type 2, can each carry out the reaction. Type 1 IDIs were the first to be characterized and rely on an active site Cys residue as well as divalent cations for their catalytic function. Biochemical and crystallographic analyses of type I IDI indicate that an active site, metal-binding Glu residue transfers a proton to the alkene π bond of IPP, generating a tertiary carbocation or carbocation-like intermediate. Abstraction of a proton from C2 of the isoprene skeleton by an active site Cys residue regenerates the alkene forming DMAPP. Isotope labeling studies demonstrated that the proton transposition occurs in an antarafacial manner, a mechanism consistent with the placement of catalytic groups in the active site of type I IDIs. Type II IDIs are a more recently discovered class of enzymes that catalyze the same reaction as type I IDIs but are structurally and evolutionarily unrelated. In contrast to type I IDIs, type II enzymes rely on flavin mononucleotide (FMN<sub>red</sub>) and NADPH as well as divalent cations as cofactors to perform catalysis. The dependence on these nucleotide cofactors was somewhat surprising given that IPP to DMAPP isomerization does not involve net redox changes. Free radical mechanisms involving transient abstraction of a hydrogen atom as well as protonation–deprotonation (carbocation intermediate) mechanisms have been proposed as a means to effect the double bond shift. The involvement of carbocation intermediates turns out to be a major theme in the enzymatic isomerization of isoprenoids.

2.2. Carotenoid Biosynthesis

The biosynthesis of carotenoids from IPP and DMAPP begins with the condensation of one molecule of DMAPP with three molecules of IPP catalyzed by the enzyme geranylgeranyl diphosphate synthase to form geranylgeranyl diphosphate (GGPP) (Figure 4). In some plant species, geranyl diphosphate is synthesized first by the enzyme geranyl diphosphate synthase and then elongated by the addition of two IPP molecules in a GGPP synthase-catalyzed reaction. These enzymes belong to the prenyl transferase family, which use divalent cations, such as Mg<sup>2+</sup> or Mn<sup>2+</sup>, to carry out the condensation of isoprenoid precursors. Mechanistically, the divalent cation polarizes the diphosphate moiety of DMAPP to facilitate its dissociation with consequent formation of an electrophilic carbocation intermediate. Proper positioning of IPP in the active site facilitates a nucleophilic attack of the electrophilic carbocation intermediate. This reaction again features a carbocation or carbocation-like intermediate that reacts with a second GGPP to from a cyclopropylcarbonyl diphosphate (prephytoene diphosphate). The colorless, C40 tetraterpenoid bound electrons on C1 of the isoprene carbocation, resulting in formation of a new carbon–carbon single bond. Deprotonation of the diphosphate-containing unit results in C1–C2 π bond formation, allowing chain elongation to continue in the presence of appropriate enzymes. Two molecules of GGPP are then joined in a head to head fashion to form phytoene in a reaction catalyzed by the enzyme phytoene synthase, which is the first committed step in the synthesis of carotenoids. This reaction again features a carbocation or carbocation-like intermediate that reacts with a second GGPP to form a cyclopropylcarbonyl diphosphate compound (prephytoene diphosphate). This compound breaks down with loss of pyrophosphate and a proton to produce 15-cis-phytoene. The colorless, C<sub>40</sub> tetraterpenoid product is then subjected to a series of desaturation and isomerization reactions that culminate in the production of all-trans-lycopene, the immediate precursor of β,β-carotene. In bacteria, a single enzyme called carotene desaturase (CrtI) is responsible for conversion of phytoene into lycopene. In plants, two desaturase enzymes called phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS) and two isomerases called ξ-carotene isomerase and carotenoïd isomerase (CrtI)
together convert phytoene into lycopene.\textsuperscript{87} Interestingly, CrtIso shares significant sequence homology with the carotene desaturase (CrtI) as well as a mammalian enzyme known as retinol desaturase (RetSat).\textsuperscript{63,125} Lycopene, a compound with a red hue conferred by a set of 11 conjugated double bonds, is transformed into $\beta,\beta$-caroten eb y an enzyme known as lycopene-$\beta$-cyclase.\textsuperscript{126,127} Although not a reaction that involves net redox changes, the cyclization performed by lycopene-$\beta$-cyclase is dependent on an NAPDH cofactor.\textsuperscript{128} In keeping with the general theme of terpenoid isomerization, lycopene cyclization reactions also occur through carbocation intermediates.\textsuperscript{129}

### 2.3. Retinoid Metabolism in Vertebrates

Mammals efficiently utilize both preformed vitamin A in the form of all-trans-retinyl esters and pro-vitamin A carotenoids (mainly $\beta,\beta$-carotene) to sustain the body pool of vitamin A.\textsuperscript{130} Unlike retinyl esters, which are hydrolyzed to retinol in the small intestine followed by rapid absorption by enterocytes, uptake of carotenoids is mediated and regulated by scavenger receptor class-B type-I (SR-BI).\textsuperscript{131,132} Upon absorption by enterocytes, $\beta,\beta$-carotene undergoes oxidative cleavage catalyzed by $\beta$-carotene monooxygenase I (BCMO I), yielding two molecules of all-trans-retinal (ii). Retinal can reversibly combine with an amino group to form a retinyl imine (Schiiff base) (iv). Retinal is also subject to oxidation and reduction to form retinoic acid (iii) and retinol (vitamin A) (v), respectively, the latter in a physiologically reversible manner. Retinoic acid can be converted into several conjugated and/or oxidized derivatives, some of which exert biological effects. Retinol also can be converted into several derivatives including retro-retinoids, saturated retinols, and phosphate conjugates. Retinol is also reversibly esterified to produce retinyl esters (vi), the main storage form of vitamin A in the body.
lyzed by ββ-carotene 15,15′-monoxygenase (BCMO1). This symmetric split of the carotenoid produces two molecules of all-trans-retinal that subsequently enter vitamin A metabolic pathways (Figure 5).

The diverse functions of retinoids are carried out by a few physiologically active metabolites of all-trans-retinol that are produced by enzymatic modification of the functional groups of this vitamin and geometric isomerization of its polyene chain. Oxidation of all-trans-retinal catalyzed by retinaldehyde dehydrogenases (RALDHs) leads to formation of all-trans-retinoic acid, a ligand for nuclear retinoic acid receptors (RARs) that, coupled with retinoid X receptors (RXRs), bind to retinoic acid response elements on the promoter region of target genes and regulate their transcription. Because all-trans-retinoic acid formation is irreversible, an excess of this active retinoid can be cleared only by its further conversion to more polar metabolites through oxidation by cytochrome P450 (CYP26) and/or glucuronidation by UDP-glucuronosyl transferases (UGTs). An alternative metabolic pathway of all-trans-retinal leads to formation of 11-cis-retinal, a visual chromophore that couples to rod and cone opsins to form photosensitive pigments. The thermodynamically unfavorable isomerization of the 11−12 double bond does not occur at the aldehyde level in vivo. Instead, all-trans-retinal is first reduced to all-trans-retinol by short-chain dehydrogenase/reductase (SDR) or alcohol dehydrogenase enzymes. Subsequent esterification of all-trans-retinol, mainly by lecithin/retinol acyltransferase (LRAT), provides both a major storage form of retinoids in the body and a direct substrate for RPE65-dependent enzymatic isomerization of the retinoid polyene chain. Multiple additional endogenous retinoid metabolites are derived from all-trans-retinol. These include all-trans-
13,14-dihydroretinol produced by saturation of the double bond by RetSat. Other examples are retro-retinoids, such as anhydroretinol and 14-hydroxy-4,14-retro-retinol. Though the molecular identities of enzymes involved in the production of retro-retinoids in vertebrates are currently unknown, these metabolites have been shown to regulate lymphocyte proliferation.149–151 Transfer of a phospho group onto all-trans-retinol results in formation of retinyl monophosphate that has been detected in the liver of rodents.152,153 Glycophospholipids consisting of a retinol moiety linked by a phosphodiester bond to mannos or galactose were postulated to function in the transfer of sugar units onto proteins to form some glycoproteins.154

3. ISOMERIZATION OF RETINOIDS

Retinoids are reactive compounds that readily isomerize. Here we take a close look at their important chemical transformations.

3.1. Geometric Isomers of Retinoids

Progress in understanding vitamin A metabolism would not be possible without development of adequate analytical methods that allow separation, detection, and quantification of retinoids. Such methods have gradually advanced since the discovery of vitamin A about 100 years ago. A complication is that retinoids exist in several geometrical configurations with differently modified functional groups (Figure 6A). Lipophilic compounds soluble in organic solvents, including retinol and its esters, were initially separated by thin-layer chromatography on alumina- and silica-based stationary phases.155,156 However, introduction of modern high performance liquid chromatography (HPLC) techniques in the early 1970s together with standardized commercially available compact stationary phase columns enabled precise, reproducible analyses of vitamin A metabolites and, most importantly, determination of their isomeric composition.157–160 Today retinoids can be separated under numerous chromatographic conditions (summarized in refs 160 and 161) optimized for normal and reverse-phase columns. Selection of the most appropriate methodology depends on the chemical properties of the particular retinoid as well as the source of the biological sample. The eye exhibits an especially complex retinoid composition. The highest resolution method for separating retinyl esters, retinal, and retinol as well as their isomers present in the eye is normal phase HPLC (Figure 6B).161 The strength of an analyte’s interaction with the silica stationary phase depends not only on its functional groups but also on steric factors and the structure of the molecule. This feature is especially important for separation of molecules that are chemically similar but physically different, e.g. geometric isomers. Moreover, this methodology provides unique flexibility in tuning chromatographic conditions by adjusting the polarity of the mobile phase, routinely composed of hexane and ethyl acetate. Highly hydrophobic hexane simplifies the tissue homogenate extraction procedure and greatly reduces sample complexity without sacrificing overall analytical performance.162

Retinoid detection, identification, and quantification are simplified by their characteristic spectral properties (Figure 6C and D). The conjugated polyene chain contributes to relatively strong absorption at ultraviolet (UV) and visible (Vis) wavelengths. Thus, absorbance maxima as well as the overall shape of the spectra provide valuable information about the number of conjugated double bonds and allow identification of the isomeric states of the compound. UV/Vis detection offers a limit of retinoid quantification at a low picomolar range through most photodiode array detectors and provides excellent linearity over a wide range of concentrations (2–1500 pmol) (Figure 6C). A complementary analytical method allowing precise molecular identification and quantification of retinoids is mass spectrometry (MS). The greatest advantage of MS coupled to HPLC is sensitivity in the low femtomolar range. Moreover, modern tandem mass spectrometry offers definitive molecular identification based on the induced fragmentation pattern of the precursor ion (Figure 6D).

3.2. Retro- and anhydro-retinols

In the 1920s two groups164,165 made the discovery that vitamin A-containing solutions, in the presence of Bronsted and Lewis acids, undergo a color change from pale yellow (λmax ~ 325 nm) to brilliant blue (λmax ~ 600 nm). The blue color is semistable with an effective lifetime of up to ~3 min when chloroform is used as a solvent.166 This color change, when initiated using antimony trichloride as the Lewis acid, is known as the Carr–Price reaction and was a primary means of retinol detection and quantification prior to the advent of chromatographic methods.167 Investigation into the mechanism of the reaction underlying the color change revealed that the hydroxyl moiety of retinol readily combines with acids (e.g. protons or antimony), converting it into a good leaving group.168 Hydroxyl dissociation generates a short-lived retinyllic cation that can undergo proton elimination to form anhydroretinol (Figure 7).

Anhydroretinol can then undergo further chemical reactions to generate relatively long-lived, blue-color species.168 In addition to anhydroretinol, direct protonation of the 13,14 retinol double bond followed by proton elimination at C4 leads to formation retro-retinol (Figure 7). Generation of the retinyllic cation can also be accomplished by flash photolysis of retinyl acetate via heterolytic carbon–oxygen bond dissociation169 or by reactions of protons, released by radiolytic pulses, with retinol or retinyl acetate.170 The special location of the retinol hydroxyl group with respect to the conjugated polyene chain makes it a much better leaving group than is typical for regular aliphatic alcohols. Upon dissociation, the resulting retinyllic cation is stabilized by extensive delocalization along the polyene chain with a half-life on the nanosecond time scale.169,171 Hydroxyl dissociation can also be facilitated by its conjugation with an electron-withdrawing group, for example a sulfo moiety, as occurs catalytically via the enzyme retinol dehydratase. This
member of the sulfotransferase family is responsible for the catalytic conversion of retinol into anhydroretinol. In this reaction, a sulfo moiety is first transferred from 3′-phosphoadenosyl-5′-phosphosulfate (PAPS) onto the retinol hydroxyl group to produce retinyl sulfate. Sulfate then readily dissociates, generating a resonance-stabilized carbocation that is quenched by proton loss from carbon 4 of the ionone ring to yield anhydroretinol (Figure 8).

**Figure 8.** Production of anhydroretinol from all-trans-retinol catalyzed by retinol dehydratase. First, a sulfo group is transferred to all-trans-retinol (i) to form retinyl sulfate (ii). Loss of sulfate then generates a carbocationic intermediate (iii) that, in the confines of the enzyme active site, preferentially rearranges with loss of a proton to form anhydroretinol (iv).

### 3.3. Chemical/photochemical isomerization of retinoids

Spectroscopic properties of retinoids can be directly related to their conjugated double bond system. Mobile \( \pi \) electrons of polyenes are delocalized over the entire molecule, resulting in resonance stabilization of the compound. Thus, polyene single bonds display some double-bond characteristics contributing to their preferred planar conformation. The most stable conformation for retinoids is all-trans. Alternative geometrical isomers introduce some steric hindrance that increases the conformational energy compared to the all-trans isomer. Although some retinal isomers introduce only mild steric clashes, e.g., between 12H and 15H in 13-cis or between 8H and 11H in the 9-cis conformation, other isomers such as 11-cis and 7-cis constitute examples of severely hindered isoprenoids in which the planar conformation of the polyene chain cannot be sustained. To eliminate the strain, a twist is introduced that further contributes to the increased conformational energy of the retinoid.

**Cis/trans** isomerization of retinals does not occur efficiently in the dark at room temperature. However, an equilibrium between retinoid isomers can be introduced chemically or photochemically (Figure 9). The radical-mediated, iodine-catalyzed isomerization of polyenes is a three-step process that involves binding of an iodine atom to a carbon, an internal rotation, and then detachment of the iodine. Because iodine atoms are easily formed thermally from I\(_2\), retinoid isomerization can be studied independently of light. Starting from all-trans-retinal, the composition of retinal isomers at equilibrium was consistent among multiple reports that included all-trans, 13-cis, 9-cis, 11-cis, and 9,13-di-cis isomers in ratios of about 0.62, 0.23, 0.11, 0.001, and 0.04, respectively. The same results were obtained when all-trans-retinol or its palmitoyl ester was used as the starting material. Interestingly, kinetic studies revealed that the 9,13-di-cis isomer was a favored intermediate in the isomerization of 9-cis-retinal, whereas 13-cis-retinal was directly converted into the all-trans conformation without the contribution of any other isomeric intermediates. Consequently, different isomers lead to a diverse retinoid composition in the equilibrium state. Nevertheless, 11-cis-retinal was never preferably formed upon I\(_2\) or acid-catalyzed isomerization. In contrast, early work by Wald indicated that photolysis of dilute ethanolic solutions of all-trans-retinal resulted in the formation of about 50% cis-retinals, with an exceptionally high contribution of 11-cis-retinal representing 25% of the mixture. Since then, quantum yields for trans/cis and cis/trans photoisomerization upon light excitation of retinal have been studied under a variety of conditions demonstrating both isomer and solvent dependence. The quantum yields for all-trans-retinal were considerably lower in polar (0.1–0.2) than in nonpolar (0.4–0.7) solvents.

The influence of solvents on the efficiency and composition of isomers at photoequilibrium has been attributed to solvent-dependent shifting of the \( \pi^* \) and \( \pi \pi^* \) excited states. For example, in hexane light illumination led to almost exclusive formation of the 13-cis isomer whereas increased solvent polarity contributed to efficient production of 11-cis, 9-cis, and 7-cis-retinals in 0.19, 0.06, and 0.005 ratios, respectively. Many studies were dedicated to elucidate the mechanism(s) of photoisomerization, particularly the potential role of singlet and triplet excitation states of retinals that could provide a mechanistic explanation for early stages of rhodopsin-mediated visual excitation. Although the directly formed singlet excited state had a major role in the photoisomerization of either all-trans or 13-cis-retinal, in the...
case of 11-cis-retinal and 7-cis-retinal, isomerization occurred also after an intersystem crossing to an excited triplet state, so the triplet state was responsible for up to 50% of the observed isomerization with the other half of isomerized retinoid arising from the singlet state. Because the triplet state of chromophore bound to rhodopsin has never been spectroscopically observed, it is not clear whether these observations can be translated into a biologically relevant model. Nevertheless, these early studies on retinoid isomerization clearly showed that the mixture of isomers obtained by any of the available methods did not recapitulate the equilibrium observed in living tissue. Thus, they indirectly facilitated a shift in research focus toward the potential role of enzymes and specific binding proteins in maintaining the composition of retinoid isomers in vivo.

3.4. Dihydroretinol

As mentioned above, retinoid isomers can be directed toward equilibrium by free radicals via addition—elimination processes which impart transient disintegration of the double bond prior to an internal rotation. By analogy, reduction of a selected double bond that allows free rotation followed by its desaturation could represent an alternative route to polyene isomerization (Figure 10). Although the conjugated double bond system of retinoids is resistant to chemical reduction, this scenario can be effectively completed in vivo. Cyanobacterial and plant CrtIso catalyze the isomerization of \((7Z,9Z,9^\prime Z,7^\prime Z)\)-tetra-cis-lycopene (prolycopene) to all-trans-lycopene. This enzyme-mediated isomerization employing redox cofactors occurs through a reversible saturation—desaturation reaction of the cis-double bonds. Animal homologues of the plant enzyme are inactive toward prolycopene. Instead, the mouse CrtIso-related enzyme accepts all-trans-retinol as a substrate in carrying out the saturation of the retinoid 13-14 double bond. However, in contrast to plant CrtIso, this reaction is not accompanied by isomerization of the retinoid, yielding (R)-all-trans-13,14-dihydroretinol as a final product (Figure 11). Consequently, this animal enzyme has been named retinol saturase or RetSat. The enzymatic activity of RetSat does not contribute to visual chromophore regeneration in the eye but rather influences processes involving peroxisome proliferator-activated receptor γ (PPARγ) activity, which regulates lipid accumulation in mice.

4. PHOTORECEPTOR CELLS AND PIGMENTED HELPER CELLS

To place retinoid chemical transformations in response to light in a proper context, we must review the evolution of the visual system. Ancient photoreceptors composed of light-sensing cells (proto-eyes) mediated phototaxis with the assistance of pigmented cells, precursors of the retinal pigment epithelium (RPE) in the retina of the eye. The proto-eye could likely detect directionality of light and could also be considered a precursor of a primitive circadian clock. As living organisms evolved over time, the cell numbers, complexity, and functional capability of the light-sensing organ increased. For example, whereas Drosophila melanogaster eye has 10 different cells, that number has grown to 400 in humans, with a parallel development of retinal circuitry. Darwin wrote "Reason tells me, that if numerous gradations from a simple and imperfect eye to one complex and perfect can be shown to exist, each grade being useful to its possessor, as is certainly the case; if further, the eye ever varies and the variations be inherited, as is likewise certainly the case and if such variations should be useful to any animal under changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, should not be considered as subversive of the theory."

A large number of light sensitive molecules are needed to increase the probability of photon capture. These light-sensitive molecules must also couple to a receptor protein capable of initiating the required biochemical chain of events. Such 30–60 kDa proteins were given the name “opsins,” and in bacteria they function as cation and anion pumps, whereas in higher organisms they are coupled to G proteins and therefore are called G protein-coupled receptors (GPCRs). In every case, the chromophores are retinals bound to opsin via a Schiff base (retinylidene) group with a transmembrane domain (TMD) Lys residue. Because opsins are membrane proteins, they are densely packed into specific regions of the cell to prevent their random diffusion. For example, in bacteria they form 2-dimensional crystalline membrane patches, whereas invertebrate opsins occupy cellular membrane protrusions called microvilli of rhabdomeric photoreceptor cells (Figure 12). A different solution is employed by vertebrate photoreceptors which are modified ciliary elongated protrusions called outer segments (OS). Rhodopsin and ciliary photoreceptors also employ fundamentally different mechanisms for regeneration of their visual pigments following light stimulation. Light-activated rhodopsin opsin can be restored to their ground state by absorption of a second, lower energy photon in a process known as photoreversal. Light activated ciliary opsin, on the other hand, do not undergo photoreversal but instead rely on biochemical regeneration of the visual chromophore via the retinoid (visual) cycle. Notably, there are exceptions to these typical routes of visual chromophore regeneration, as has been described, for example, in Drosophila. Rods and cones are the two morphologically distinct types of ciliated photoreceptor cells found in human retina (Figure 12). Cones are further divided based on the opsin they express, such as short-wavelength responsive cells (S cones that express blue opsin with an absorption peak at 420–440 nm), medium-wavelength responsive cells (M cones that express so-called “green” opsin with an absorption peak at 530–540 nm), and long-wavelength responsive cells (L cones...
that express “red” opsin with a maximum absorbance at 560−580 nm)\(^6\).84,235–241

4.1. Structure of the Mammalian Retina

The vertebrate eye evolved to utilize key optical principles along with the physicochemical properties of retinoids. First, light is focused by passing through two proteinaceous biological lenses at the front of the eye, namely the cornea and lens.\(^{242}\)

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**Figure 12.** Comparison of photoreceptor structure between invertebrates represented by *Drosophila* and vertebrates represented by man. Invertebrates utilize a rhabdomeric photoreceptor cell whereas vertebrate photoreceptors are modified ciliary cells. Notably, a few invertebrates, such as Amphioxus, employ ciliary photoreceptors.

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**Figure 13.** Structure of the mammalian retina. The retina consists of several layers of neuronal cells. The innermost photoreceptor layer is embedded in an epithelial monolayer, known as the RPE. Electron micrographs show stacks of membranous disks within ROS (left) and COS (right), which contain visual pigments and the associated phototransduction machinery. The leftmost and rightmost electron micrographs display the bacillary structure of the outer segments and their interaction with the RPE, respectively. A portion of this figure is reproduced with permission from ref 235. Copyright 2009 Elsevier.
Then the focused wave of photons is absorbed by photoreceptors of the retina (Figure 13), a highly layered tissue, where rod and cone photoreceptors constitute more than 75% of the cells. Detailed phylogenetic analyses across species demonstrated that the last common ancestor of all jawed vertebrates evolved ∼400 million years ago with a key role for transcriptional master regulator paired box gene 6 (PAX6) in controlling development of eyes and other sensory organs.243–245 Vision is initiated by absorption of light by photoreceptors, with rod cells acting as photon counters because under dim illumination they can respond to a single photon.246,247 The extreme photosensitivity of the 11-cis-retinylidene chromophore is modulated by the chemical environment of the opsin chromophore binding pocket, yielding different visual pigments that respond maximally to light at different wavelengths, causing the so-called “opsin shift” that allows us to discern differences in color.235,236 Forced twisting of the polyene backbone away from its normally planar configuration induced by the binding pocket as well as the electrostatic environment surrounding the chromophore are major factors that influence its absorbance maximum.248 The key role of binding pocket electrostatics in spectral tuning of the retinylidene chromophore has been vividly demonstrated in a rationally engineered cellular retinol-binding protein (CRBP).250

There are similarities and differences between rods and cones. Rod cells are more light-sensitive than cones but saturate at relatively low levels of light. At the light level at which rods reach saturation, cones generate measurable responses but have an extremely high photon saturation threshold.242 The number and types of cones that collect photons in ambient light differ dramatically, from less than 1% in rats to more than 95% in the ground squirrel.251,252 Both rod and cone cells feature the same structural design. The most distal portions, called rod/cone outer segments (ROS/COS), are in close contact with the RPE cell layer. These ciliary structures connect with the soma via an inner segment endowed with a high density of mitochondria that supply energy to the highly metabolically active photoreceptor. The soma in turn connects with synaptic termini via inner fibers (Figure 13). The specialized photoreceptor cilia contain visual pigments responsible for absorption of light and house all phototransduction proteins needed for amplification and quenching of the light signal. Here there is yet another difference between rods and cones. ROS are made up of a stack of individualized disks surrounded by the ROS plasma membrane, whereas COS differ by having a series of invaginations continuously connected with the COS plasma membrane.

The structure of ROS is better known than that of COS because more advanced methods were developed for its isolation233,234 including mouse ROS, which can be altered genetically.255–258 ROS structure has recently been reviewed.259,260 In short, a mouse ROS with a length of ∼24 μm and a diameter of ∼1.2 μm contains about 600–800 membranous stacked disks which increase the density of rhodopsin available for photon absorption. ROS extend up to the apical part of the RPE and are tightly enveloped by microvilli of RPE cells that increase the contact area of these two cell types to facilitate transfer of substances, including retinoids.

4.2. Structure of the RPE

Without RPE cells, our vision would not be sustainable. The RPE is a monolayer of highly polarized, quasi-hexagonal, epithelial cells. The apical membrane of RPE cells lies adjacent to the photoreceptor OS, whereas the RPE cell basal surface faces Bruch’s membrane.260–262 Contact with ROS is maintained by a highly elaborate network of microvilli visible under the electron microscope (Figure 14) which in some species changes shape and elongates when the eye is exposed to light. About 20–40 photoreceptor cells project toward a single RPE cell. In humans, this ratio depends on the location of these cells in the eye because the gradient of rod/cone cells and also slight differences in the dimension of photoreceptors dictates their packing density. In the periphery, the rod to RPE cell ratio is 29 whereas, in the fovea, the cones to RPE cells ratio is 23.263 Functions of the RPE cell layer are diverse,59,264 but from the perspective of this review, two roles are the most critical. The first function of the RPE is to facilitate photoreceptor cell renewal. To provide optimal signal amplification, membranes of ROS and COS contain high levels of unsaturated lipids.265 These lipids are prone to oxidation in the presence of light, (photo)reactive retinal, and high oxygen tension266,267 which are physiological conditions encountered in the retina. Photochemical reactions induced by light in transparent retinal tissue require a delicate balance between protein, lipid, and metabolite renewal and damaged component disposal, which when disturbed can lead to rapid and massive retinal degeneration. Impressively, postmitotic rod and cone photoreceptor cells undergo a daily regeneration process wherein ∼10% of their OS volume is shed, subsequently phagocytosed by adjacent RPE cells268 and replaced with newly formed outer segments. Thus, RPE cells dispose of but also accumulate an immense amount of oxidized cellular debris. Indeed it was estimated that each RPE cell phagocytoses hundreds of thousands of OS disks over a human lifetime.269 Several potentially toxic byproducts are condensation compounds
derived from all-trans-retinal. Dysfunction of such processes as phagocytosis, lysosomal degradation, and removal of waste products by the RPE can lead to severe retinopathies, including age-related macular degeneration (AMD)\textsuperscript{270−274}.

As a second major function, the RPE expresses key metabolic enzymes required for production of the visual chromophore, 11-cis-retinal, and thus comprises an integral part of the retinoid cycle.\textsuperscript{59,63−65,68,69,71,83,278,279,284−287} In the first step of this metabolic pathway, LRAT, through its ability to catalyze the formation of retinyl esters that are readily sequestered by aggregation into lipid droplets called retinosomes, changes the mass action ratio to favor retinoid uptake from both photoreceptors and the choroidal circulation.\textsuperscript{278,282,283} In addition to LRAT, another critical enzyme called RPE65 (retinoid isomerase) catalyzes the conversion of all-trans-retinyl esters into 11-cis-retinol. This cis-retinol is oxidized and sent back to ROS/COS to re-form photoactive visual pigments. Diffusion likely suffices for this process because the chromophore forms, especially in rods, a highly stable covalent complex with opsin, thereby driving the transfer of retinoids to photoreceptors.

5. TRANSFORMATION OF RETINOIDS WITHIN THE EYE

The photosensitive active retinoid, 11-cis-retinal, is produced in the RPE and delivered to the photoreceptors.\textsuperscript{59,64,68,69,71,83,278,279,284−287} It has been postulated that additional but less understood transport processes take place between Müller cells and cone photoreceptors.\textsuperscript{35,285,288} Because retinols and retinyl esters (but not retinals) are intrinsically fluorescent, their transformation can be followed by fluorescence induced by two-photon excitation.\textsuperscript{278,282,283,289−293} Further development of this method guarantees improved understanding of retinoid flow at the subcellular level.

5.1. Canonical Retinoid (Visual) Cycle

The initial discovery of a light-sensitive pigment in the retina is generally attributed to a German physiologist by the name of Böll, who, in ca. 1876, observed the “purple red color of the
bacillary (i.e. rod) layer of the retina” while dissecting the retina of a frog kept in the dark just prior to the procedure.\cite{1,294} The red color of frog rod cells had actually been noted some 25 years earlier by Müller, who incidentally first described retinal Müller cells,\cite{295} but the color was attributed to hemoglobin and not further explored.\cite{1} Böll made the key observation that the red hue of the retina was fleeting, gradually transforming to a yellow color and then fading over the course of several seconds, leaving the tissue colorless. Böll insightfully inferred that this light-induced bleaching of the retina must be reversed when the animal was maintained in the dark.

A second German physiologist by the name of Kühne greatly expanded on Böll’s research by showing that a photobleached retina regained its red color when placed in contact with the RPE and stored in the dark.\cite{296,297} This critical experiment demonstrated that at least two different tissues were required for the bleach-regeneration cycle proposed by Böll. Kühne used bile salts, which he had on hand from his experiments on digestion, to solubilize the red pigment and then used the preparation to demonstrate a correspondence between its absorption spectrum and the spectral sensitivity of the retina to light, firmly establishing the red substance as the visual pigment of rod cells. Kühne named the pigment “sehpurpur” or “visual purple” and later referred to it as “rhodopsin.”\cite{297}

Despite the rapid progress made by Böll and Kühne on the mechanism of light perception, the field was essentially dormant for the next 50 years. In the 1930s the task of identifying the molecular components of the visual system was taken up by Wald and his colleagues. Among his many achievements, Wald discovered that the chromophore imparting a red color to rhodopsin was a vitamin A-derived compound called 11-cis-retinal. Moreover, he found photons striking this chromophore could induce a change in its configuration from an 11-cis, through a series of photo-intermediates, to an all-trans state, and that this photochemical reaction, which initiates the series of retinal color changes observed by Böll and Kühne, represents the first step in the sensing of light by the eye.\cite{6} He also showed that, following photoactivation, rhodopsin decomposes into its protein and retinal components. Wald was the first to lay out a general scheme of chemical reactions, termed the visual or retinoid cycle, which underlies visual perception and regeneration.\cite{6} Dowling, a student of Wald, through detailed measurements of retinoid flow between photoreceptors and RPE during light exposure and dark adaption, established that the retinal liberated from photoactivated rhodopsin is rapidly taken up by the RPE and esterified. During dark adaption, the flow of retinoids proceeds in a reverse manner from the RPE to photoreceptors for rhodopsin regeneration to occur. These findings firmly established the role of the RPE in visual chromophore regeneration.\cite{298} This visual cycle scheme was refined over the years, and the enzymes responsible for catalyzing the individual reactions were identified. Our current understanding of the retinoid (visual) cycle is summarized in Figure 15.

5.2. Cone Visual Cycle

In addition to the canonical visual cycle, several lines of evidence indicate that cone photoreceptors might have access to a special source of visual chromophore not available to rods\cite{205} (Figure 16). For example, after equal levels of bleaching, cones dark-adapt faster than rods by a factor of ∼10.\cite{299} In contrast to rods, cones operate in bright light without saturating, implying that the rate of visual chromophore delivery to these two cell types must be substantially different.\cite{300,301} Additionally, studies have found that the canonical visual cycle is too slow to provide enough visual chromophore to maintain cone light responsiveness under bright light conditions.\cite{302} The distribution of retinoids in cone-dominant retinas is substantially different from that of rod-dominant species, with an abundance of 11-cis-retinyl esters found in the retina as opposed to the stores of all-trans-retinyl esters in the RPE.\cite{303,304} Three enzymatic activities, namely isomerase, 11-cis- and all-trans-retinyl ester synthase, and retinol dehydrogenase/reductase, are associated with membrane fractions from chicken neural retina.\cite{305} Cultured primary Müller cells from chickens were found to convert all-trans-retinol added to the media into 11-cis-retinol and 11-cis-retinyl esters, suggesting that this cell type could be a site of visual chromophore production in vivo.\cite{303} Moreover, the 11-cis-retinol-binding protein, cellular retinaldehyde-binding protein (CRALBP), is known to be expressed in Müller cells, providing additional support for the above hypothesis.\cite{306,307} Although significant progress has been made in elucidating this alternative pathway, most of the responsible enzymes have not yet been molecularly characterized. A candidate protein responsible for the alternative retinoid isomerization (all-trans-retinol to 11-cis-retinol) activity found in chicken retinas was identified as dihydroceramide desaturase-1 (DES-1), a member of the integral membrane hydroxylase/desaturase enzyme family that contains an eight-His-coordinated di-iron active site.\cite{308,309} This enzyme is a sphingolipid Δ4-desaturase that converts dihydroceramide into ceramide, with the latter being a more active signaling molecule.\cite{310,311} Interestingly, DES-1 produces mainly 9-cis- and 13-cis-retinoids rather than 11-cis-retinoids from all-trans-retinol, which is unexpected given the abundance of 11-cis-retinoids in the chicken retina.\cite{308} Further research is required to determine the physiological relevance of DES-1 to the synthesis of a visual chromophore.

5.3. Retinoid-Containing Structures: Retinosomes and Retinal Condensation Products Found in Healthy and Diseased Eyes Imagined by Two-Photon Microscopy (TPM)

Once released from ROS, all-trans-retinol is reduced to all-trans-retinyl which then diffuses into the RPE. There, this alcohol is esterified by fatty acid in a reaction catalyzed by
LRAT. As highly hydrophobic substances, in part because of the physicochemical properties of their long fatty acid esters, these retinoid ester products then coalesce into lipid droplets termed retinosomes, which are found in vertebrate RPE. These esters can be minor or major components of lipid droplets that along with phospholipids total about 160 molecular species including other fatty acid esters, triglycerides, cholesterol, cholesterol esters, and various proteins. Retinosomes are reminiscent of lipid droplets in other tissues that store other hydrophobic substances. The highly efficient enzymatic activity of LRAT traps retinol delivered from the photoreceptors or from the circulation, whereas retinosomes are absent in the eyes of Lrat−/− mice deficient in retinyl ester synthesis. The lipid core content of retinosomes appears to be homogeneous. Retinosomes are reminiscent of lipid droplets in other tissues that store other hydrophobic substances. The highly efficient enzymatic activity of LRAT traps retinol delivered from the photoreceptors or from the circulation, whereas retinosomes are absent in the eyes of Lrat−/− mice deficient in retinyl ester synthesis. The lipid core content of retinosomes appears to be homogeneous. Retinosomes recruit proteins such as caveolin-1, perilipins PLIN1-3, sterol carrier protein-2, structural proteins, chaperone proteins, and redox enzymes. We postulated that retinosomes in RPE cells perform functions similar to those of lipid droplets in other types of cells, suggesting that they are rather dynamic tissue-specific organelles that change their composition in response to fatty acid, cholesterol, and all-trans-retinol availability. Because retinoids are naturally fluorescent, the study of retinosomes could provide important insights into the formation and metabolism of lipid droplets in general, especially because these structures are accessible for real time TPM imaging in vivo. Capitalizing on the intrinsic fluorescence of all-trans-retinyl esters, noninvasive TPM revealed that retinoids are elongated structures approximately 8 μm long and 1 μm wide with their long axis oriented perpendicularly to the RPE basal surface (Figure 17). Retinyl esters in retinosomes accumulate in Rpe65−/− mice lacking retinoid enzymatic isomerization. Retinosomes are located close to the RPE plasma membrane and are essential components for 11-cis-retinal production. The RPE also accumulates other retinoids, including retinal condensation products. These metabolites also can be detected by TPM, and since they are characterized by unique spectral properties, the intracellular accumulation and distribution of these compounds can be monitored independently from other retinoids (Figure 17). Resulting from inadequate reduction/clearance of all-trans-retinal, they are often detected as small 1 μm deposits scattered throughout RPE cells. These condensation products accumulate most prominently in mice lacking the ABCA4 transporter and retinol dehydrogenase 8 (RDH8) (Figure 17). They also increase with age because they are delivered from photoreceptors (at least the first steps of condensation reaction occur in ROS) by daily phagocytosis. Some investigators consider these retinoid condensation products as major contributors to retinal dysfunction in Stargardt and age-related macular degeneration (AMD) diseases. Others propose that these biomarkers are merely indicative of inadequate clearance of all-trans-retinal,
6. PROTEINS AND ENZYMES OF THE RETINOID CYCLE

Here we describe the molecular properties of proteins involved in retinoid transformation in the retina including the light receptor rhodopsin.

6.1. Rhodopsin

At the center stage of phototransduction is rhodopsin, the most extensively studied GPCR. Rhodopsin is the main component of disk and plasma membranes of ROS, accounting for 90 and 75% of their protein content. In disk membranes, the density of rhodopsin translates into about 50% of the entire volume or surface area.61,66,79,225,226,233,321–324 Thus, it is not surprising that the expression level of rhodopsin dictates the size of the ROS.259,325

Although rhodopsin is not uniformly distributed throughout disks,84,328 its local high density within each disk allows efficient absorption of light. Corresponding visual pigments in cone cells of frog retina are so dense that they form crystalline structures.329 Physiologically, the minimal building block of rhodopsin is a dimer in which only one monomer is activated under normal lighting conditions330–335 (reviewed in refs 336–338).

The fundamental photochemical reaction of our visual system is isomerization of 11-cis-retinylidene to all-trans-retinylidene (Figure 18). The Schiff base between 11-cis-retinal and Lys296 of opsin is protonated to allow a spectral shift to longer wavelengths (at least in rhodopsin, green and blue pigments). It is remarkable that a ligand, retinal, which is just slightly larger than tryptophan, when photoisomerized, causes a reliable change in the conformation of rhodopsin to its activated form that couples with G protein. This activation is accomplished through geometric cis/trans isomerization of the chromophore,182 deprotonation of the Schiff linkage,339 and reorganization of water molecules within the TMD of this receptor.333,340–342

Photoactivation of rhodopsin causes conformational changes that provide a binding site for the rod G protein called transducin.343,344 No high resolution structure of the complex is yet available, but lower resolution methods have been informative. On the basis of structural mass spectrometry techniques, we found that the transition of ground state rhodopsin to its photoactivated state causes a structural relaxation that then tightens upon transducin binding.333

Using affinity chromatography, we trapped and purified the photoactivated rhodopsin–transducin complex. Scanning transmission electron microscopy demonstrated about a 221 kDa molecular weight for this complex. A 22 Å structure was calculated from projections of negatively stained photoactivated rhodopsin–transducin complexes. The determined molecular envelope accommodated two rhodopsin molecules together with one transducin heterotrimer, indicating a heteropentameric structure for the photoactivated rhodopsin–transducin complex.351 The dimeric structure of rhodopsin in the complex was confirmed using succinylated concanavalin A as a labeling probe.353 Recently, we used the retinoid chromophores, 11-cis-retinal, 9-cis-retinal, and all-trans-retinal to monitor each dimeric rhodopsin monomer within a stable complex with transducin. We found that each of the dimeric rhodopsin monomers contributed differently to the pentameric complex, indicating a functional distinction between rhodopsin monomers in their oligomeric form.332 For a more detailed description of the activation events, recent reviews are available (refs 66,79, and 346–349).

Two other important questions need to be answered about the rhodopsin cycle. First, how is the chromophore released and how do opsins recombine with 11-cis-retinal to regenerate rhodopsin and cone visual pigments? Mechanistically, more information is available for rhodopsin than for cone pigments. Key residues in rhodopsin’s active site are Lys296, Glu113, and Glu181 (Figure 19).60,139,226,227,240,350–355 The 11-cis-retinylidene bond is protonated, and Glu113 is the counterion of this linkage. A counterion is essential, as positively charged groups are extremely rare in the TMD of membrane proteins. Upon illumination of rhodopsin (λmax = 500 nm), the chromophore undergoes geometrical isomerization.356,357 Next, rapidly formed and decaying intermediates have been detected before Meta I is observed (λmax = 478 nm). Though the difference in absorption results from relaxation of the chromophore, only small changes in the protein moiety take place at this stage358–360 and it is believed that a switch in the counterion occurs from Glu113 to Glu181.324,361–364 Further relaxation...
combined with Schiff base deprotonation results in a signaling form called Meta II (\(\lambda_{\text{max}} = 382\) nm)\(^{343}\) which then interacts with G protein-coupled receptor kinase 1 and arrestin.\(^{60,62,239,365,366}\) Especially in cold blooded vertebrates, a prominent fraction of Meta I relaxes into Meta III (\(\lambda_{\text{max}} = 465\) nm), the difference in the latter’s light absorption derived from an \textit{anti} to \textit{syn} thermal isomerization of the Schiff base double bond.\(^{367}\) From both Meta II and Meta III, a protonated carbinol ammonium ion is formed before \textit{all-trans}-retinal is released from opsin. When regeneration with \textit{11-cis}-retinal subsequently occurs, formation of the Schiff base requires polarization of the carbonyl group of \textit{11-cis}-retinal and deprotonation of the Lys296 side chain amine as well as exclusion of water from the active site (Figure 19). Regeneration and recombination of \textit{11-cis}-retinal with opsin restores the dark state condition needed for subsequent photon absorption. Although there is convincing evidence for the roles proposed for Glu113 and Glu181, independent verification of this switch and the whole mechanism of chromophore regeneration is still lacking.

How the chromophore migrates into and out of opsin remains an open question. Taking into account the hydrophobic nature of retinal, does it dissociate into the lipid bilayer or the cytoplasm for the reduction reaction, or could it enter...
the intradiscal space where condensation products of retinal start forming? Similar to other GPCRs, which in addition to orthosteric-binding sites contain other well-defined allosteric ligand-binding sites, rhodopsin also has two other retinoid-binding sites within opsin in addition to the retinylidene pocket (site I). Site II is called an entrance site, and the exit site (site III) is occupied by retinal after its release from site I. The crystal structure of opsin, an opsin structure with another retinoid bound, and mutagenesis studies all suggest an escape and entrance route for retinal.

6.2. Retinol Dehydrogenases (RDH’s)

An integral part of the retinoid cycle is the interconversion of retinals and retinols. Enzymes that catalyze this process can be classified into three major protein families: cytosolic alcohol dehydrogenases (ADH’s) that belong to a medium-chain dehydrogenase/reductase family and selected members of the aldo-keto reductase family (AKR’s) and microsomal RDH’s that represent the short-chain dehydrogenase/reductase (SDR) group. However, only RDH’s contribute to vision-related metabolism of retinoids. The universal redox carriers for these reactions are the dinucleotide cofactors NAD(H) and NADP(H). In ADH’s and RDH’s they are bound by a Rossmann fold, a classic structural element composed of 6 to 7 parallel β-strands flanked by 3–4 α-helices present in these enzymes (Figure 20). This structural motif contains a Gly-rich segment (TGXXXGXG) responsible for its structural integrity and binding of the diphosphate portion of the nucleotide cofactors. An acidic residue binding to the 2′ and 3′ hydroxyls of the adenine ribose and located downstream of the Gly-rich motif confers NAD(H) specificity, whereas NADP(H) binding is dictated by the presence of a basic residue within the Gly-rich segment. By contrast, AKR’s do not contain the canonical Rossmann fold. Preferable NADPH binding occurs within the characteristic (β/α)7 motif of this protein family. Despite their conserved mode of cofactor binding, ADH and RDH families of oxidoreductases reveal diverse protein domain architectures and mechanisms of catalysis. ADH’s depend on a catalytic Zn atom bound in the active site, which electrostatically stabilizes the substrate’s oxygen and thus increases the acidity of the alcohol proton. In contrast, SDR’s show a Tyr-based catalytic center with adjacent Ser and Lys residues. Here, the deprotonated phenolic group of Tyr initially forms a hydrogen bond with the alcohol hydroxyl group and the deprotonated Tyr residue acts as a catalytic base to extract a proton from the substrate’s hydroxyl group. The hydride ion, extracted from the substrate, can be directly transferred to position 4 of the nicotinamide ring. In addition to the interaction with the nicotinamide ring of the cofactor, the reaction intermediate is stabilized by the hydroxyl group of an adjacent serine residue (Figure 20). Because the phenolic group of the Tyr side chain has a pKa value around 10, the ε-amino group of Lys is needed to convert tyrosine to tyrosinate (pKa 7.6), to facilitate catalysis at neutral pH. Additionally, a Lys residue forms hydrogen bonds to both the 2′- and 3′-hydroxyl groups of the cofactor’s ribose moiety and thus enforces a proper orientation of the nicotinamide ring to allow a proton transfer. Recently, an Asn residue was shown to stabilize the position of this catalytic Lys via a conserved water molecule. Thus, the final sequence of the catalytic tetrad of SDR’s is composed of Asn, Ser, Tyr, and Lys residues (Figure 21). The catalytic mechanism of AKR’s, in principle, is similar to that found in SDR’s with an active site

Tyr residue and an assisting Lys residue facilitating the deprotonation of the Tyr hydroxyl group.

The RDH’s represent a microsomal SDR group with an overall sequence similarity of at least 30% (Figure 21) although the catalytic core of these enzymes reveals a much higher homology with nearly identical folding. Despite these similarities, the mode of membrane binding and membrane topology of specific RDH’s is a matter of controversy. Based on biochemical studies, RDH1 is anchored in ER membranes with the catalytic domain facing the cytoplasm. The N-terminal residues are essential for the membrane localization and topology of this enzyme, whereas the C-terminus was postulated to be involved in stabilization of the protein’s membrane orientation. In contrast to this model, RDH12 was reported to be a glycoprotein carrying an endoglycosidase H-sensitive sugar modification, which suggests a luminal orientation of this enzyme. Yet other models indicate that the hydrophobic stretch of the catalytic domain in RDH1 and RDH4 can contribute to membrane binding. An interesting case is RDH8, which localizes to ROS/COS. Efficient transport to the ROS/COS is mediated by a signaling sequence at the C-terminus of this enzyme whereas membrane
anchoring is achieved by fatty acylation of conserved Cys residues.389 Although all RDH's identified in vertebrates can utilize retinol or retinal, some have wide substrate specificities, and retinoids are not their preferred physiological substrates.374,390 For example, RDH1, RDH3, RDH4, RDH6, and RDH7 reveal 25−60 times higher affinities for androgens than all-trans-retinol.391−393 In fact only RDH5, RDH8, RDH10, RDH11, RDH12, RDH13, RDH14, and retSDR1 were proven to be expressed in the retina or RPE, and their roles have been studied in the context of the visual (retinoid) cycle.68,287 Based on their preferred substrate geometry and role in the retinoid cycle, this group of enzymes can be classified into all-trans and 11-cis-retinol dehydrogenases. RDH8 and RDH12 belong to the first class, whereas RDH5, RDH11, and RDH10 act on 11-cis-retinoids. Functions of the two remaining enzymes, RDH13 and RDH14, have yet to be adequately assigned.394 Reactions catalyzed by RDH's are fully reversible. In a test tube, the net direction of retinoid interconversion depends on the oxidation state of the provided cofactor and the ratio between the concentrations of substrate and product. However, in more complex in vivo systems, the direction of the enzymatic reaction is determined by enzyme specificity for binding either NAD(H) or NADP(H). Under physiological conditions the ratio between NAD/NADH is close to 1000;287 thus, RDH's that bind this cofactor can contribute significantly only to retinol oxidation. In contrast, the ratio of NADP to its reduced form is about 0.005,395 such that enzymes utilizing this dinucleotide reduce retinal to retinol.

The physiological role and significance of particular RDH's in vitamin A homeostasis, retinoic acid signaling, and visual chromophore regeneration has recently been extensively reviewed68,287,396 and thus will not be described here. However, it is worth noting that, in addition to genetic and biochemical identification of RDH's, many of these enzymes were characterized in vivo in the past few years.397−400 These studies not only provided detailed information about the physiological function of many RDH's but also led to the development of animal models to investigate their roles in human pathological conditions, including retinal degenerative diseases.401

6.3. Lecithin/Retinol Acyl Transferase (LRAT)
LRAT is the main enzyme that catalyzes retinyl ester formation in most tissues,147,402,403 with the exception being adipocytes that instead exhibit acyl-CoA-dependent transferase activity of a protein (acyl-CoA:retinol acyltransferase or ARAT) yet to be identified.404 Consequently, LRAT is critical for uptake and storage of retinoids in peripheral tissues, including RPE cells where it plays a pivotal role in providing substrate for visual chromophore regeneration via the enzymatic activity of RPE65.147,148,405 Localized in the endoplasmic reticulum (ER), LRAT is a 25 kDa bitopic integral membrane protein with a single membrane-spanning helix localized at the C-terminus and a potential membrane-interacting N-terminal domain.407 The C-terminal domain is critical for post-translational targeting of retinyl palmitate to LRAT and its subsequent uptake into the ER lumen where it is processed by a series of enzymes including LRAT, acyl-CoA synthetase, and acyl-CoA:cholesterol acyltransferase before delivery to the plasma membrane.408
the enzyme to the endoplasmic reticulum (ER) in a cytosolic TMD recognition complex-dependent manner. On the basis of its amino acid sequence and predicted tertiary structure, LRAT is classified as a member of the NlpC/P60 thiol peptidase protein superfamily (Figure 22). Besides LRAT, there are seven genes in the human genome that encode proteins belonging to the NlpC/P60 family: two neurological sensory proteins (NSE1-2) and five H-ras-like tumor suppressors (HRASLS1-5). The common feature of LRAT and HRASLS proteins is a 6 amino acid sequence that contains a conserved catalytic Cys residue (NCCHFV). Although the structure of LRAT has yet to be determined, recently solved structures of two LRAT-like proteins, human HRASLS2 and HRASLS3, provide important insights into the molecular organization of this enzyme family. Hydrophobic C-terminal membrane-anchoring sequences are colored green. The crystallographic structure of human HRASLS3 is shown on the right with the carbon atoms of the catalytic triad colored orange. The enzyme aligns and structure of LRAT-like acyltransferase enzymes. A protein sequence alignment of all LRAT-like proteins encoded in the human genome is displayed on the left, showing conserved His and Cys residues (orange) that constitute the catalytic triad of this enzyme family. The enzyme catalyzes the formation of retinyl esters by transferring an acyl group directly from the sn-1 position of phosphatidylcholine (PC) onto all-trans-retinol. As a consequence of its structural relationship to thiol peptidases, LRAT adopts an analogous catalytic strategy whereby the deprotonated Cys161 serves as a nucleophile attacking the carbonyl carbon of an ester bond in the lipid substrate, both forcing the carbonyl oxygen to accept a pair of electrons and transforming the sp²-hybridized carbon into an sp³-hybridized tetrahedral intermediate (Figure 23). Collapse of this intermediate results in transient acylation of the protein by formation of a thioester bond at the Cys161 sulfhydryl group. Concomitantly, 1-hydroxy-2-acyl-sn-glycero-3-phosphocholine (Lyso-PC) is liberated as a first product of the reaction. Deprotonation of the hydroxyl group of retinol then permits decomposition of the thioester intermediate and transfer of the acyl group from the enzyme onto retinol to form retinyl esters. Several lines of evidence support this LRAT model. Initially, a role for Cys161 and His60 in catalysis was derived from site-directed mutagenesis studies where replacement of either of these two amino acids abolished retinyl ester formation. Recently, the proposed mechanism was proved by trapping the catalytic intermediate in the absence of an acyl acceptor and directly detecting the covalent thioester protein modification by mass spectrometry. An identical enzymatic mechanism holds for other LRAT-like proteins. Importantly, despite their highly conserved catalytic domains,
HRASLS proteins cannot employ all-trans-retinol as an acyl acceptor and they lack specificity toward the sn-1 ester cleavage site as well. Instead, they catalyze both lipid hydrolysis and acyl transfer onto a variety of enzyme-specific substrates, such as lyso-phospholipids or phosphatidylethanolamine (PE). These fundamental differences in enzymatic activity do not arise from changes in the catalytic mechanism but rather are determined by subtle modifications in the primary sequence and structure of these proteins. Despite recent advances in understanding the principles of catalysis, several critical questions remain, including the evolutionary and structural basis for adaptation of all-trans-retinol processing by LRAT.

6.4. Acyl-CoA/Retinol Acyltransferase

Early studies concerning the esterification of all-trans-retinol revealed two independent enzymatic activities that led to formation of retinyl esters. In addition to lecithin-dependent acyl transfer facilitated by LRAT described above, profound acyl-CoA-dependent activity has been reported to exist in a variety of tissues, including small intestine, liver, adipocytes, skin, testis, and retina. In contrast to LRAT, which efficiently utilizes phospholipids as acyl donors, acyl-CoA/retinol acyltransferase (ARAT) requires a preactivated acyl moiety coupled to coenzyme A (Figure 24).

ARAT has never been purified or cloned. However, studies of LRAT-deficient mice indicate that the intestinal absorption of vitamin A decreased to 50% that of wild-type animals challenged with a physiologic dose of retinol. On the basis of this evidence, ARAT may contribute to retinyl ester formation within the intestine and thus facilitate retinoid uptake and packaging into chylomicrons. A distinct role of acyl-CoA-dependent retinoid esterification has been proposed for the retina. On the basis of enzymatic studies, retinas isolated from cone-dominant species such as ground-squirrel and chicken revealed retinoid isomerase activity that, in contrast to RPE65, converts all-trans-retinol directly into 11-cis-retinol. This enzymatic reaction is thermodynamically driven by secondary esterification of newly produced 11-cis-retinol in a palmitoyl-CoA-dependent manner. Studies involving primary cultures of chicken Müller cells indicate that inner retinal ARAT enzymatic activity is associated with this cell type. Interestingly, the retinal ARAT preferably synthesized 11-cis-retinyl esters distinguishing it from intestinal all-trans-ARAT and suggesting the existence of two or more separate enzymes responsible for acyl-CoA-dependent all-trans-retinol esterification. Intestinal acyl-CoA-diacylglycerol acyltransferase 1 (DGAT1) has been shown to catalyze formation of retinyl esters in an acyl-CoA-dependent manner in vitro. However, later detailed in vivo studies
indicated that DGAT1 deficiency did not cause a decline in retinol esterification but rather markedly reduced postprandial plasma triglyceride and retinyl ester excursions by inhibiting chylomicron secretion.434

Figure 25. Structural alignment of CCO family members. (A) Iron-binding His residues are highlighted in orange, and second sphere Glu residues are highlighted in blue. (B) Structural superposition of CCO members of known structure (RPE65, orange; ACO, blue; VP14, pink). These enzymes adopt a 7-bladed β-propeller fold (blades labeled with Roman numerals) with a helical cap on the top face of the propeller that houses the active site and membrane-binding domain (curved, dashed line), which surrounds the active site entrance indicated by a yellow-green arrow. The iron cofactor located at the center of the propeller is coordinated by four conserved His residues (green). The two views in panel B differ by a 90° horizontal rotation.
6.5. Retinoid Isomerase (RPE65)

Our understanding of RPE65 biochemistry has changed dramatically since the last Chemical Reviews article covering the visual cycle.\footnote{435} Once thought to function solely as a retinoid-binding protein, RPE65 has now been conclusively identified as the retinoid isomerase of the canonical retinoid cycle.

6.5.1. History and Functional Characterization. RPE65 was identified in the early 1990s as a conserved, developmentally regulated, RPE-specific, microsomal membrane protein with an apparent molecular mass of 65 kDa.\footnote{436,438} \textit{Rpe65}\textsuperscript{−/−} mice, generated in the Redmond laboratory, exhibited early onset blindness and ocular retinoid abnormalities consisting of a lack of 11-cis-retinoids and overaccumulation of all-trans-retinyl esters, indicating an important physiological role for RPE65 in the visual cycle.\footnote{439} Furthermore, human RPE65 mutations were shown to cause Leber Congenital Amaurosis (LCA), a recessive, severe childhood blindness disease.\footnote{440,441} These two important findings established a key role for RPE65 in retinal physiology.

At about the same time, in the seemingly unrelated field of plant biology, a carotenoid cleavage enzyme from maize called viviparous 14 (VP14), involved in the production of abscisic acid from 9-cis-violaxanthin,\footnote{442} was identified and shown to possess significant sequence homology to RPE65.\footnote{443} Despite the clear relationship of RPE65 to an established enzyme family, efforts to demonstrate either carotenoid oxygenase\footnote{155} or retinoid isomerase activity\footnote{444,445} for purified RPE65 were initially unsuccessful, leading to the conclusion that RPE65 was not an enzyme but instead a retinoid-binding protein. Through visual cycle complementation experiments using unbiased RPE cDNA libraries\footnote{405} or candidate gene approaches,\footnote{446,447} RPE65 was finally identified as the visual cycle retinoid isomerase in 2005, about 12 years after the gene was first cloned. This delay in identifying the catalytic function of RPE65 was primarily due to its extreme sensitivity to detergents that are required for its solubilization and purification.

6.5.2. Evolution. RPE65 is evolutionarily related to carotenoid cleavage oxygenases (CCOs), a group of enzymes that catalyze the oxidative cleavage of various carotenoids and apocarotenoids as well as certain other olefin-containing compounds, such as lignostilbenes.\footnote{443,447} However, RPE65 is the black sheep of this family, as it is not known to possess such oxygenase activity.\footnote{133} Sequence identity between RPE65 and other CCO members varies from \textasciitilde{}20 to 40%, but all of these enzymes contain an absolutely conserved set of four His and three Glu residues that are involved in binding of a required iron cofactor.\footnote{448,449} (Figure 25). RPE65 is found only in vertebrates and can usually be discriminated from other CCOs on the basis of its characteristic chain length of 533 (±1–2) residues and high (≥70%) sequence conservation. Additionally, sequence alignments have revealed several positions where residue type can be predictive of whether a protein likely has RPE65 activity.\footnote{40,450} The steps in RPE65 evolution from the true CCOs remain unclear, but this process was undoubtedly critical for the establishment of the vertebrate visual cycle.\footnote{451}

6.5.3. Structure. The crystal structure of RPE65, determined by Kiser in 2009 in the Palczewski laboratory, has provided a basis for understanding the substantial biochemical data obtained for this enzyme as well as structural features that distinguish it from true CCOs.\footnote{152} (Figure 26). The basic fold is a seven-bladed \textit{β}-propeller capped on one face by a group of \textit{α}-helices. This same fold was also found for a cyanobacterial apocarotenoid oxygenase (ACO) enzyme, thus confirming that the entire CCO family shares a similar three-dimensional structure\footnote{453} (Figure 25). The propeller is sealed within blade VII in a "Velcro" manner via interactions between the first and last strands of the core propeller fold. An iron cofactor is located on the propeller axis directly coordinated by the four conserved His residues mentioned above. Retinyl esters gain access to the iron center through a tunnel that runs along the interface between the helical cap and the top face of the \textit{β}-propeller (Figure 27). The tunnel terminates within the protein interior, suggesting that the substrate uptake and product egress pathways are the same. By contrast, the structures of two \textit{bota fide} CCOs, ACO, and VP14 show two pathways to the iron center, possibly reflecting the need for the two products of the cleavage reaction to be released into different cellular compartments.
compartments (i.e., membrane vs cytosol). In all crystal forms reported to date, RPE65 forms a dimeric assembly primarily mediated by an extension to the β-propeller structure (Figure 26). The other structurally characterized CCOs lack this extension and consequently are not dimeric (Figure 25).

6.5.4. Expression and Membrane Binding. RPE65 is expressed almost exclusively in the RPE. It localizes to the abundant smooth ER of the RPE where other retinoid processing enzymes such as LRAT and RDH5 are found. Independent studies have shown that RPE65 can associate with RDH5, possibly forming a functional complex. Our understanding of the interaction of RPE65 with the ER membrane has evolved considerably. RPE65 was once thought to be peripherally anchored to the ER by reversible post-translational palmitoylation. Although palmitoylation of a particular Cys residue (position 112 in the bovine sequence) might promote membrane attachment, recent biochemical studies indicate that RPE65 has substantial integral membrane protein character even though it has no transmembrane spanning segments.

Inspection of the RPE65 crystal structure reveals a surface enriched in residues capable of interacting with the lipophilic core as well as headgroups of a phospholipid membrane indicating a monotopic mode of membrane attachment (Figure 26). This surface surrounds and helps form the entrance to the active site tunnel, allowing the enzyme to extract hydrophobic retinoids from the membrane. The structure of RPE65 determined in the presence of native microsomal phospholipids revealed that these membrane-binding residues undergo major conformational changes upon phospholipid removal by detergents, consistent with prior biochemical data showing that RPE65 adopts structurally and functionally different conformations in its membrane-bound versus detergent-solubilized states. These findings provide a structural explanation for the well-known inhibitory effects of detergents on RPE65 activity. The membrane-interacting regions are arranged in parallel (i.e., on the same side) in the RPE65 dimer, allowing them to reinforce each other in anchoring the protein to the membrane (Figure 26). This hydrophobic patch is conserved in all CCOs reported to date, indicating that it is a general structural feature of these enzymes, which allows the extraction of hydrophobic substrates from lipid membranes (Figure 25).

6.5.5. Active Site and Iron Cofactor Binding. The active site cavity of RPE65 is lined primarily by nonpolar side chains, including several aromatics (Tyr, Phe, and Trp residues), consistent with the hydrophobic nature of the retinyl ester substrate (Figure 27). The relatively narrow width of the tunnel indicates that a retinyl ester would have to enter the active site in an extended conformation. A complex structure between RPE65 and an intact retinyl ester has not yet been experimentally determined, so the orientation of retinyl ester entry remains uncertain. However, modeling studies suggest that the retinoid part of the retinyl ester enters first. Like other CCOs, RPE65 contains a ferrous iron cofactor within its active site directly coordinated in a distorted octahedral or trigonal bipyramidal fashion by His residues 180, 241, 313, and 527 (bovine and human sequence numbering) with Fe−N bond lengths between 2.1 and 2.2 Å. A second sphere of highly conserved and functionally important Glu residues 148, 417, and 469 hydrogen bond with the N̂ atoms of His residues 241, 313, and 527, respectively. The open iron coordination sites (i.e., sites not occupied by protein ligands) contain electron density that has been attributed to a bound fatty acid molecule coordinating the iron through its free carboxylate moiety based on crystallographic and extended X-ray absorption spectroscopy (EXAFS) data. Notably, iron−carboxylate interactions have been observed in a number of proteins containing the 4-His iron-binding motif, including photosystem II, 15-lipoxygenase, and the photosynthetic reaction center.

The functional importance of several RPE65 active site residues has been probed either directly through site-directed mutagenesis or indirectly by identifying RPE65 mutations that give rise to type II (RPE65-associated) LCA. Mutations in the iron-coordinating His residues, the second sphere Glu residues, and even a third sphere of residues that form hydrogen bonding interactions with the second-sphere Glu residues, either abolish or greatly reduce RPE65 activity. In general, any change in active site amino acid composition is detrimental for activity. Interestingly, mutations of a few residues in close proximity to but not directly binding the iron cofactor, namely Phe103, Thr147, Tyr338, and Phe526, can alter RPE65 product specificity by changing the ratio of 11-cis to 13-cis-retinol isomers produced (Figure 27) (vide infra). Moreover, even wild-type RPE65 in native membranes produces some 13-cis-retinol.

7. MECHANISMS OF RETINOID ISOMERIZATION

The heart of the retinoid cycle is the isomerization reaction. The reaction catalyzed by RPE65 consists of two steps: a trans/cis alkene bond isomerization and an ester bond cleavage. Because of this dual activity, RPE65 is frequently referred to as an isomerohydrolase, although it is not hydrolytic water that mediates this process.

7.1. Acyl versus O-Alkyl Cleavage in the Hydrolysis of Esters

The ester cleavage reaction catalyzed by RPE65 is not an ordinary ester bond hydrolysis whereby attack of water on the acyl carbon generates a tetrahedral intermediate that collapses to form a carboxylic acid and an alcohol (Figure 28). Instead, the ester dissociates by cleavage of the O-alkyl bond. Owing to this distinction, we prefer to refer to the process as ester "cleavage" rather than "hydrolysis." This unusual reaction was identified by using various isotopically labeled retinols as substrates for the isomerization reaction. It was found that a stereochemical inversion of carbon 15 occurs and that the 15-hydroxy oxygen is lost and replaced by bulk water-derived oxygen during the isomerization. Because 11-cis-retinol is thermodynamically less stable than all-trans-retinol by ~4 kcal/mol, it has been argued that ester cleavage, which typically releases ~5 kcal/mol of free energy, could be used to drive the
isomerization reaction. The requirement of CRALBP and other binding proteins for robust 11-cis-retinol production by RPE65 indicates that product release could be rate limiting. The unusual ester cleavage reaction instead seems to be more important in overcoming the ∼36 kcal/mol activation energy barrier of the double bond isomerization. The retinoid polyene chain is relatively rigid, owing to favorable continuous π-orbital overlap. Thus, in order for isomerization of these double bonds to occur at physiological temperatures, the carbon–carbon bond order must be temporarily lowered.

7.2. Binuclear Nucleophilic Substitution Mechanism for Retinoid Hydrolysis/Isomerization

Taking into account these biochemical data, three different mechanisms of RPE65-dependent retinoid isomerization have been proposed. The first, proposed by Rando and colleagues, involves the attack of an enzyme-associated nucleophile on C11 of the retinyl ester with simultaneous ester dissociation and shuffling of the double bonds (Figure 29). The enzyme-linked retinoid intermediate could undergo low-energy rotation around the 11–12 single bond to a cis-like conformation. Attack of water or hydroxide on C15 would lead to a reshuffling of the double bonds and dissociation of the retinoid–enzyme covalent intermediate locking the retinoid in an 11-cis configuration. This mechanism, which can be classified as a dual $S_2^1$ nucleophilic substitution reaction, predicts a high degree of 11-cis-retinol product specificity for RPE65. However, RPE65 does not exhibit such specificity and can readily produce 13-cis or all-trans-retinol, depending on the reaction conditions and retinoid-binding proteins used for the assay.

Structurally, RPE65 also does not possess a suitable nucleophile such as a Cys residue in its active site.

7.3. Unimolecular Nucleophilic Substitution Mechanism for Retinoid Isomerization

A second mechanism, postulated by Palczewski and colleagues, proposes a key role for a carboxylation intermediate in the isomerization reaction (Figure 30). In this reaction involving an $S_2^1$ nucleophilic substitution mechanism, ester dissociation via O-alkyl cleavage removes electrons from the polyene, thereby generating a retinyl cation with reduced carbon–carbon bond order allowing bond rotation to take place. Quantum mechanical calculations indicate that the activation energy of trans/cis isomerization at the 11–12 bond is reduced to about 18 kcal/mol, consistent with the experimentally determined energy of activation. The aromatic residues lining the active site cavity of RPE65 are ideal for stabilizing carbocation intermediates. Following rotation of the 11–12 bond, likely under steric influence by the enzyme active site, water or hydroxide attacks C15, quenching the carboxylation with the 11–12 double bond in a cis configuration. The interior cavity of RPE65 indeed has a curved shape that can accommodate cis retinoid isomers (Figure 27). Importantly, this mechanism allows for the generation of alternative isomers such as the experimentally observed 13-cis-retinol. As discussed earlier, retinyl cation formation from retinol or retinyl esters is facilitated by Bronsted or Lewis acids. The iron-coordinated fatty acid observed via crystallographic and XAS data suggests that the RPE65 iron cofactor could promote catalysis by coordinating the ester moiety and facilitating its dissociation.

This mechanism thus bears substantial similarity to the well-characterized Carr–Price type reactions described earlier, with the exception that 11-cis-retinol rather than anhydroretinol is generated in the RPE65 active site.

7.4. Radical Cation Mechanism for Retinoid Isomerization

A third mechanism, recently proposed by Redmond and colleagues, postulates a role for a retinyl radical cation intermediate in the isomerization reaction (Figure 31). This proposal is based on the finding that certain radical spin-trap compounds, for example N-tetra-butyl-α-phenylnitrone (PBN) can inhibit RPE65-mediated isomerization in an uncompetitive manner. In this proposal, removal of a single π electron from the polyene by an unknown electron acceptor generates a retinyl radical cation intermediate that can undergo double bond rotation to form cis isomers followed by quenching of the radical cation to form an 11-cis-retinyl ester. Ester cleavage then occurs to produce 11-cis-retinol. Reversal of the isomerization and ester cleavage steps in relation to the carboxylation mechanism was proposed based on the finding that all-trans-retinyl ester could not easily be accommodated in the RPE65 active site whereas 11-cis- and 13-cis-retinyl esters could. However, more research is required to develop the mechanistic details of this scheme.
8. RETINOID-BINDING PROTEINS RELEVANT TO RETINOID TRANSPORT TO THE EYE AND THE VISUAL CYCLE

Because retinoids quickly equilibrate between membranes by passage through the aqueous phase, part of their transport likely takes place by passive diffusion. But this process could be facilitated by ATP-dependent transporters such as ABCA4 or the channel-like property of STRA6. Finally retinoids are readily oxidized and isomerized; thus, a set of binding proteins evolved to protect them from these undesirable reactions as well as defend surrounding molecules from retinoid (photo)-reactivity. STRA6, a key transporter involved in retinol transfer between the RPE and choroidal circulation, and its homologue RBPR2 involved in retinol uptake by the liver will be reviewed in detail in another article in this partial thematic issue of Chemical Reviews. Thus, they will not be discussed here.

8.1. Retinoid-Binding Proteins Involved in the Retinoid Cycle

The hydrophobic nature of retinoids limits their ability to diffuse in aqueous environments. This property presents a barrier to retinoid transport from storage sites to sites of utilization as well as between cellular membranes. Retinoid-binding proteins overcome this barrier by reversibly binding and sequestering retinoids away from water in internal cavities, greatly facilitating their diffusion. A number of retinoid-binding proteins have been characterized. Here, the discussion focuses on those binding proteins involved in transport of retinols and retinals.

When required by the body, vitamin A liberated from its hepatic storage sites in Stellate cells by retinyl ester hydrolysis is complexed with a 21 kDa retinoid-binding protein belonging to the lipocalin family called retinol-binding protein 4 (RBP4) to form holo-RBP4. The main structural feature of holo-RBP4, determined crystallographically in the early 1980s, is an eight-stranded β-barrel core containing a single retinol-binding site (Figure 32). all-trans-Retinol is oriented in the binding pocket with its hydroxyl group facing the solvent and the retinyl moiety snugly bound by a number of nonpolar residues in a highly complementary fashion, explaining the high binding specificity toward the all-trans isomer. Holo-RBP4 circulates in the plasma in complex with a second protein called cellular retinol-binding protein (CRBP), a member of the intracellular lipid-binding protein family, this compact 15 kDa protein is evolutionarily unrelated to RBP4. Two isoforms of the protein, CRBP I and CRBP II with ~50% sequence identity depending on the species, exhibit differential tissue expression. CRBP I is expressed in many tissues, including the eye, whereas CRBP II expression is restricted to the intestine. The structure of CRBP, similar to that of RBP4, features a β-barrel fold that houses the retinoid-binding site. However, the orientation of retinol in the binding pocket is reversed in relation to that in holo-RBP4 with the β-ionone nearest the mouth of the pocket and the hydroxyl group hydrogen bonding with a Glu residue at the base of the cavity. An important role for CRBP in all-trans-retinol trafficking in the retina has been demonstrated in CRBP−/− mice, which show defective transport of all-trans-retinol from photoreceptors to ER membranes of the RPE. This is evidenced by accumulation of all-trans-retinol in the retina and reduced formation of retinyl esters in the RPE.

Two retinoid-binding proteins are specifically expressed in the eye. The first of these is an intracellular-binding protein CRALBP that displays a binding preference for 11-cis-retinoids. CRALBP is expressed predominantly in two retinal locations, the RPE and Müller glial cells, where it plays a key role in visual chromophore regeneration by binding 11-cis-retinoids, protecting them from esterification or from photo- or thermal isomerization and facilitating their intracellular transport.
Figure 13. Structure and function of ABCA4. (A) Two-dimensional topology diagram of ABCA4. Positions of the Walker A motifs are indicated by blue dashed lines within the CDs. Glycosylation sites are marked with red stars, and an intramolecular disulphide bridge is indicated by S–S. ECD, exocytotic domain; CD, cytoplasmic domain. (B) Electron microscopic structure of ABCA4 and its dimensions relative to a ROS disk rim. TMDs, transmembrane domains. (C) Structural differences in ABCA4 in the absence and presence of ATP. (D) Role of ABCA4 in the visual cycle and pathology of elevated all-trans-retinal. ABCA4 flips the all-trans-retinal–PE complex, a product of all-trans-retinal (red line structure) condensation with PE (black line structure with blue sphere indicating the headgroup), to the outer leaflet of the disk membrane, allowing dissociation of the complex and subsequent reduction of all-trans-retinal to all-trans-retinol, which then reenters the visual cycle (left). Retinal pathology is observed in mice lacking ABCA4 and RDH8 activities due to accumulation of all-trans-retinal and its lipid adducts (right).

The main retinoids associated with CRALBP isolated from bovine retina. It can also effectively bind 9-cis-retinoids as well but not 13-cis-retinoids. The gene encoding CRALBP, RLB1, was cloned in 1988 and was localized to human chromosome 15. CRALBP is one of the founding members of the CRAL-TRIO protein family, members of which share a common three-dimensional architecture. The crystallographic structure of the CRALBP–11-cis-retinal complex has been determined, revealing a curved binding pocket with high shape complementarity to 9-cis and 11-cis retinoid isomers, consistent with its ligand-binding preferences. The binding pocket completely shields the retinoid from solvent. Bound 11-cis-retinal is found in a 6-s-trans, 11-cis, twisted 12-s-cis configuration. The near perfect cis configuration of the 11–12 double bond imposed by the retinoid-binding pocket is likely important to prevent unwanted photoisomerization and thermal isomerization. A patch of basic amino acid residues on the protein’s surface probably mediates the interaction of the protein with acidic phospholipids, which induce dissociation of the bound retinoid. Mutations in the CRALBP gene are associated with several retinal diseases, including retinitis pigmentosa, Newfoundland rod-cone dystrophy, fundus albipunctatus, and Bothnia retinal dystrophy.

The second eye-specific retinoid-binding protein is a soluble lipoglycoprotein called interphotoreceptor-binding protein (IRBP). This large, 136 kDa protein is produced by photoreceptors and secreted into the interphotoreceptor matrix, where it is the most abundant extracellular protein. Unlike other binding proteins that contain a single retinoid-binding site, IRBP has at least three high affinity sites. The protein can also bind several isomeric forms of retinol and retinal but has a preference for all-trans and 11-cis-retinoids. IRBP can also bind a number of nonretinoid, hydrophobic ligands, but the physiological significance of this capability is not currently understood. The retinoid-binding preferences and localization of IRBP indicate that it participates in the retinoid cycle by transporting retinoids between photoreceptors, RPE, and Müller cells. Interestingly, IRBP knockout mice do not exhibit acute problems with vision or dark adaptation, and no human disease has been attributed to mutations in the gene encoding IRBP. The protein consists of four homologous “modules” generated by gene duplication. Full-length IRBP, as observed by negative stain electron microscopy, adopts a rod-shaped structure that is flexible and undergoes major conformational changes in the presence of retinoids. The crystal structure of an isolated module from X. laevis IRBP has been determined, revealing a two-domain architecture. Domain A adopts a ββα-spiral fold whereas domain B forms an ααα-sandwich. Although a retinoid-bound structure has not yet been obtained, two candidate-binding sites have been identified through molecular modeling: one in the hinge region connecting the two domains and a second in domain B. Notably, domain B of the IRBP module shares structural similarity with the ligand-binding domain of CRALBP.

8.2. Structure and Function of ATP-Binding Cassette Transporter Member 4 (ABCA4)

The metabolism of vitamin A in the eye involves a complex interplay between over twenty different proteins. The flow of retinoid substrates and products between components of the cycle depends on specific binding proteins and transporters. The retinal-specific ATP-binding cassette transporter, ABCA4, plays a special role in this process. ABCA4, a member of the
ABCA transporter subfamily, is predominantly expressed in the outer segments of photoreceptor cells where it is located in the rims of rod disk membranes and cone incisures. As indicated by numerous biochemical studies and the phenotype of Abca4−/− mice, the main function of this transporter is to accelerate the clearance of all-trans-retinal from ROS/HO.401,518

Human ABCA4 is an integral membrane protein with 2273 residues that form two homologous but nonidentical parts. Each carries six membrane-spanning helices that constitute a TMD, a soluble cytoplasmic domain (CD) that hosts a canonical nucleotide-binding site (NBD) with Walker A and Walker B motifs characteristic of ATP-processing enzymes, and an exocytoplasmic (intradiscal) domain (ECD) (Figure 33). The overall topological model for ABCA4 is supported by multiple glycosylation sites identified in both ECD domains, whereas CD1 hosts multiple phosphorylation sites. The structure of native bovine ABCA4 has been determined by negative stain electron microscopy to a resolution of 18 Å, revealing the overall shape of the molecule. On the basis of the accepted model of ATP-driven transport across a lipid bilayer, binding of a molecule to be transported increases the affinity of NBDs for ATP. The nucleotide then induces a conformational change of NBDs that come in close contact to form a dimer with the two nucleotide molecules positioned at its interface. This movement is translated onto a TMD that induces the translocation of the substrate molecule across the lipid membrane. Such conformational changes have indeed been observed by electron microscopy and hydrogen-deuterium exchange. Subsequent hydrolysis of ATP and dissociation of ADP prompt the separation of NBDs, returning the transporter to its initial state and completing the cycle. In the absence of substrate, ABC transporters undergo cycles of slow ATP hydrolysis by individual NBDs, resulting in a basal ATPase activity. Although numerous lipids stimulate ATP hydrolysis by ABCA4, all-trans-retinal or its phosphatidylethanolamine (PE) conjugate, N-retinylidene-PE (N-ret-PE) are the preferred substrates. The topology of this protein in combination with the logic of the visual cycle suggest that ABCA4 acts as an importer, an assumption recently confirmed experimentally. Interestingly, ABCA4 still remains the only known example of an importer among eukaryotic ABC transporters.

The importer activity of ABCA4 has consequences for vitamin A metabolism in photoreceptors (Figure 33C). Decay of photoactivated rhodopsin results in hydrolysis of the opsin-retinylidene Schiff base bond followed by subsequent release of all-trans-retinal into the disk membrane. The next step in the visual cycle is reduction of newly liberated all-trans-retinal to all-trans-retinol by all-trans-RDHs (mainly RDH8 in mouse retina), which associate with the cytoplasmic side of the disk bilayer. The relatively high hydrophobicity of all-trans-retinal allows its rapid partition between the inner and outer leaflets of disk membranes, ensuring its accessibility to RDHs. However, the high chemical reactivity of aldehydes also leads to spontaneous formation of all-trans-retinal adducts with primary and secondary amines. In the biological membrane environment, all-trans-retinal reacts predominantly with PE to form N-ret-PE that, similar to phospholipids, cannot freely flip between the inner and outer leaflets of the lipid bilayer. In a test tube, the formation of N-ret-PE in a mixture of chloroform/methanol (2:1) occurs with a bimolecular rate constant of about $3.75 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$ while the rate of its hydrolysis is about $7.9 \times 10^{-6} \text{s}^{-1}$. This reversible reaction is highly dependent on the presence of water. The time scale for this process in vivo remains to be elucidated. Upon a 45% bleach of rhodopsin in wild-type mice, about 24% of released all-trans-retinal was detected in the form of a conjugate with PE. Thus, the main role of ABCA4 could be aiding the movement of N-ret-PE to the cytoplasmic side of disk membranes. Because the Schiff base bond in N-ret-PE is susceptible to hydrolysis, the resulting all-trans-retinal eventually can be metabolized to all-trans-retinol. Taking into account the partition of retinals into the lipid bilayer and a relatively slow rate of ATP hydrolysis by ABCA4 equivalent to three enzymatic cycles per second, one can assume that only a fraction of the total all-trans-retinal is processed by ABCA4. Nevertheless, the activity of ABCA4 turns out to be essential for lowering all-trans-retinal concentrations below the threshold that could cause photoreceptor toxicity.

Figure 34. Mechanism of A2E formation from all-trans-retinal and phosphatidylethanolamine (R-NH$_2$).
8.3. Condensation Reactions of Retinal

Delayed clearance of all-trans-retinal from the photoreceptors after light exposure can have dramatic pathophysiological consequences. In cultured ARPE-19 cells derived from human RPE cells, exposure to 10 μM all-trans-retinal caused profound cytotoxicity after less than 1 min of incubation by inducing a Ca2+-driven apoptotic cell death pathway.318 Because rhodopsin concentration in ROS reaches 4 mM,257 bleaching of just 0.5% of the total amount could potentially generate toxic levels of all-trans-retinal if this retinoid is not efficiently removed from the retina. The mechanism of all-trans-retinal toxicity involves the generation of superoxide radical, singlet oxygen, and peroxides when irradiated with UVA or blue light.279 In addition, all-trans-retinal can stimulate increased levels of reactive oxygen species in a NADPH oxidase-dependent manner.313,314 Unless quickly dissipated, such reactive oxygen species can cause oxidative damage to lipids and proteins that compromise photoreceptor structure and function.533 Yet another consequence of elevated all-trans-retinal concentrations is the accelerated formation and accumulation of bis-retinoid lipofuscin chromophores within retina and RPE.314,401,520,534,535 Although formation of the Schiff base adduct of all-trans-retinal with PE is fully reversible, reaction of N-ret-PE with a second molecule of all-trans-retinal can initiate a cascade of irreversible nonenzymatic conversions that lead to the production of fluorescent dietretinal compounds, including pyridinium bisretinoid (A2E) (Figure 34) and retinaldehyde dimer (RALdi) (Figure 35). The common precursor in the biosynthesis of these fluorophores is protonated N-ret-PE, that undergoes spontaneous tautomerization via an H-shift [1,6] generating phosphatidyl analogs of enamine.536,537 The subsequent reaction with a second molecule of all-trans-retinal can occur through amine condensation followed by σ-electrocyclization to generate a reduced form of A2E, phosphatidyl-dihydropyridine bisretinoid (A2PE-H2) (Figure 34). Alternatively, the nucleophilic carbon 20 of the N-ret-PE tautomer can react with carbon 13 of all-trans-retinal via [1,4] conjugate addition. Consequent Mannich condensation then leads to the RALdi precursor (Figure 35).534 The strong kinetic isotope effect observed in RALdi synthesis as compared to that found for A2E supports this reaction mechanism.537,538 Aromatization of the dihydropyridine in A2PE-H3 eliminates two hydrogens to yield phosphatidyl-pyridinium bisretinoid (A2PE) whereas the RALdi precursor spontaneously rearranges to eliminate the amino group of PE (Figures 34 and 35, respectively). In the final stage of A2E biosynthesis, its immediate precursor, A2PE, is either hydrolytically cleaved by enzymatic action of phosphodiesterase, which can occur in ROS before internalization by the RPE, or undergoes enzyative acid-catalyzed hydrolysis inside RPE phagosomes.314 As a result of incomplete lysosomal degradation, these byproducts of retinal metabolism accumulate in the RPE as residual bodies called lipofuscin. These bodies provide a useful fluorescent marker for lipofuscin quantification in the living eye, which can serve as long-lasting evidence of excessive past accumulation of all-trans-retinal.539–541 Indeed, the primary fluorescent components of lipofuscin are all-trans-retinal conjugates such as A2E and RALdi.542 A2E was shown to be further metabolized by horseradish peroxidase,543 but there is yet no evidence that this process occurs in vivo. Lipofuscin granules contain several chromophores absorbing UV, blue, and green light whereas A2E acts as an acceptor of energy from their photoexcited states and dissipates that energy mainly by thermal deactivation and partly by emitting fluorescence.544 Considering that lipofuscin granules contain potent photosensitizers, quenching of excited states of lipofuscin photosensitizers by A2E may play a protective antioxidant role. Yet, A2E also exhibits some minor photoactivity and several experiments in vitro demonstrated that A2E and its oxidation products can be cytotoxic to RPE cells and trigger complement activation and inflammatory signaling.317 It remains to be established whether or not these deleterious properties of A2E are physiologically relevant.

The connection between delayed all-trans-retinal clearance, lipofuscin buildup, and retinal degeneration is exemplified by Stargardt disease, a human condition in which juvenile macular degeneration is caused by mutations in both alleles of the ABCA4 gene.547,548 Consequently, retinal samples collected from these patients revealed elevated levels of N-retinylidene-PE and overaccumulation of A2E in the RPE.529 Because the cytotoxicity of retinal conjugates has been widely accepted, it is believed that the precipitating cause of retinal degeneration in Stargardt patients is the deterioration of RPE cells responsible for maintenance of photoreceptors.546 However, recent studies of Abca4+/−Rdh8+/− double knockouts mice that closely recapitulate human retinal pathological conditions indicate that all-trans-retinal itself may play a decisive role in light-induced photoreceptor degeneration.518,520,531

Figure 35. Mechanism of all-trans-retinal dimer formation from all-trans-retinal and phosphatidylethanolamine (R-NH₂).
9. ABERRATIONS IN THE RETINOID CYCLE AND HUMAN RETINAL DISEASES

Studies of mutations in the retinoid cycle genes can teach us about the structure–function of key visual proteins and enzymes as well as the cell biology of rod photoreceptor cells, one of our most metabolically active neurons. Most of the genes encoding retinoid cycle enzymes are associated with retinal disease (Figure 36).64,76,77,285,547–552 For example, point mutations in the rod opsin gene are the most common cause of autosomal dominant retinitis pigmentosa (RP). The most frequent mutation and the first identified as causing blindness is P23H,553 accounting for 10% of human cases of autosomal dominant RP. Only a few mutations, including severe truncation of the opsin gene554 and the c.448G > A (p.E150K) mutation,555,556 are inherited in an autosomal recessive manner. RDH12, LRAT, and RPE65, when inactivated by mutations, cause juvenile forms of blindness called LCA. For RPE65 the severity of disease has been associated with the degree of residual enzymatic activity.274,281,557 Mutations in the transporter ABCA4 cause accumulation of condensation products of all-trans-retinal.520 Here, lack of a functional transporter causes Stargardt disease,545,558,559 a juvenile form of macular degeneration, whereas some other changes were demonstrated to be associated with AMD.560,561 Mutations in other genes, such as those encoding RDH5 or IRBP, are associated with slowly progressive retinal degeneration.562–565

This relationship between genetic mutations and retinal disease also provides information about the physiological role of these proteins in the chemistry of the retinoid cycle, as revealed by many animal model studies. Such generated animal models are also required for testing possible remedies for these blinding diseases.566 In some cases the treatment is highly pertinent because of the significant number of patients involved and the socioeconomic cost of their associated blindness. In other cases, the small number of affected individuals decreases the likelihood of commercially developing a possible treatment. But animal studies are still vital for those afflicted and also may lead to breakthroughs applicable to more common retinal diseases.

Natural and genetically altered animal models have been used to investigate the effects of retinoid supplementation in treatment. Thus, blockage in production of 11-cis-retinal was overcome with oral delivery of another photosensitive but chemically more stable retinoid, 9-cis-retinal (Figure 36)567–571 (reviewed in ref 76). Retinylamine and other primary amines were employed to buffer the toxic effect of all-trans-retinal either by inhibiting the visual cycle or by chemically trapping an excess of all-trans-retinal in the form of a Schiff base when it could not be effectively cleared from photoreceptor cells by reduction to all-trans-retinol (Figure 36).318,572,573 The toxicity of all-trans-retinal could be a major contributor to retinal degeneration in several human diseases, including Stargardt disease.318,401,570 Moreover, there are also experimental therapies that utilize carotenoids, retinoid precursors that comprise an integral part of human macula.574 The therapeutic landscape for inherited and acquired retinal diseases is rapidly evolving. Just a few years ago, it was difficult to imagine strategies for treating these diseases with practical pharmacological approaches. But today, many solid scientific findings provide hope that these chronic diseases will become manageable.
10. FINAL CONCLUSIONS: THE RETINOID CYCLE AND WHOLE BODY RETINOID METABOLISM

Vitamin A must be adequately distributed within the body to maintain the biological function of retinoids in the peripheral tissues and the production of visual chromophore in the eye. Transport of vitamin A is facilitated by RBP4, and its cellular receptor, STRA6, functionally couples with LRAT via CRBP. Recent studies by von Lintig and co-workers suggest that ocular vitamin A uptake is favored over other peripheral tissues in vitamin A deficient states.495 In contrast to other cell types, an overdose of all-trans-retinol does not result in excessive accumulation of retinyl esters in the RPE.567,575 Although the pivotal role of STRA6 and LRAT in vitamin A uptake is widely recognized, the mechanisms that govern “buffering” of vitamin A within the eye still remain unknown. This also applies to the potential role of light in the regulation of retinoid metabolism, especially the rate of the retinoid (visual) cycle. Despite early work showing that retinal G protein-coupled receptor, expressed in the RPE, provides light-dependent modulation of all-trans-retinyl ester synthesis and degradation as well as influences RPE65 activity in mice,576 the molecular mechanism of light-induced stimulation of retinoid isomerization remains an intriguing biological mystery.

A classic unsolved problem is how retinyl esters traffic from lipid storage droplets in the retina to the ER and RPE65. It is still unknown if retinyl palmitate is transported intact or needs to undergo hydrolysis followed by re-esterification by LRAT in the ER close to RPE65 to sustain robust retinoid isomerization. Yet another aspect related to the organization of vitamin A metabolism in the eye is the putative close interaction of proteins involved in this process. Despite several reports indicating interaction of particular proteins, the isomerization complex containing RPE65, RDH5, LRAT, or CRALBP has not been purified or reconstituted in vitro. Determining the molecular details of all-trans-retinol processing proteins at the atomic level through structural biology represents a complementary approach to biochemical studies. Until recently, progress in this field has been marked only by NMR or crystal structures of soluble retinoid-binding proteins such as CRBP1, IRBP, and CRALBP.507,577,578 The structure of RPE65452,453 definitely provided an incentive for obtaining structures of other membrane associated proteins including LRAT and RDHs.

Further structural studies of RPE65 and other CCOs with the goal of obtaining high resolution complexes with retinoids and related compounds will help resolve lingering issues concerning the mechanism of the retinoid isomerization reaction. Spectroscopic approaches, which are becoming more feasible with the advent of improved expression and purification methods, will also be of great utility in unraveling the fine details of the bioinorganic chemistry of this enzyme family.

Analysis of the isomeric composition of retinoids in rod- and cone-dominant retinas reveals striking differences.579 Significant amounts of 11-cis-retinyl esters and 11-cis-retinol in ground squirrel and chicken retinas not observed in rod-dominant mice, rat, or cow are associated with alternatives to RPE65 and LRAT enzymatic activities responsible for formation of these retinoids.302,305,430 These findings suggest the existence of an unconventional cone-specific visual chromophore regeneration pathway. Though supported by biochemical data, this pathway remains only a concept without molecular identification of its protein components. Thus, efforts to clone or purify enzymes involved in cone regeneration currently represent an exciting challenge. Newly generated 11-cis-retinol is needed to reform light-sensitive visual pigments. Thus, another challenging question in studies of the rhodopsin (and other visual pigments) cycle is to determine how the chromophore enters and exits the binding pocket.

In short, recent decades have witnessed an improved understanding of the retinoid cycle and retinoid metabolism in general. Indeed, it would be improper to treat the retinoid cycle as a separate entity because it is so dependent on the metabolism of the whole organism (Figure 37). The coming

Figure 37. Key proteins involved in the transport of retinol from the liver to target tissues. Retinol travels in the circulation bound to RBP. In turn, RBP complexes with a transthyretin (TTR) tetramer, which prevents filtration of RBP across the glomeruli of the kidney. Holo-RBP can dissociate from the TTR tetramer and bind to the retinol membrane transporter, STRA6. all-trans-Retinol is picked up on the cytoplasmic side of STRA6 by CRBP, which shuttles the retinoid to the ER of the RPE. There it is esterified by LRAT to form all-trans-retinyl-esters, which are either used as substrates for visual chromophore production or stored in lipid bodies known as retinosomes: aROL, all-trans-retinol; aRE, all-trans-retinyl ester.

years will certainly bring further molecular characterization of these processes from approaches involving combinations of human molecular genetics, characterization of newly generated animal models, advanced imaging techniques, and structural studies.

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**Notes**

M.G. and K.P. are inventors of one or more of patents (7,706,863; 8,324,270; 7,951,841; 8,338,394; and pending 20120295895) submitted by University of Washington and Case Western Reserve University and briefly discussed in the paper. K.P. is CSO of Polgenix Inc. and Visum Inc., developing further some of these technologies.

**Biographies**

Philip D. Kiser was born in 1980 in Olney, IL. He received a Pharm.D. degree from the St. Louis College of Pharmacy in 2005. During pharmacy school he performed research in the laboratories of Dr. Eric Barker at Purdue University and Dr. Thomas Baranski at Washington University. He then entered graduate school in the Department of Pharmacology at Case Western Reserve University (CWRU), where he earned a Ph.D. in molecular pharmacology in 2010 under the guidance of Prof. Krzysztof Palczewski. He then joined the Department of Pharmacology as an instructor in 2011, following a brief postdoctoral appointment. His primary research interests pertain to the biochemistry and structural biology of retinoid and carotenoid metabolism. He is also involved in teaching pharmacology to undergraduate, graduate, and professional students at CWRU. In his free time, he enjoys playing and listening to music and fixing things around the house with his three sons.

Krzysztof Palczewski, Ph.D., has been Chair and John H. Hord Professor of Pharmacology at Case Western Reserve University since 2005. Before that he was professor of Ophthalmology, Pharmacology and Chemistry at the University of Washington, Seattle. Dr. Palczewski’s notable scientific achievements span the fields of vertebrate vision, structural biology, and pharmacology, including the translation of some of his basic discoveries into treatments for human blinding diseases. He is author/coauthor of scientific publications involving topics such as the following: structures of rhodopsin, retinoid isomerase RPE65, and other retinal proteins; transmembrane signaling; the “retinoid” or visual cycle; animal models of human blinding diseases; and retinal imaging techniques. Palczewski is recipient of several prestigious awards, including the following: the Jules and Doris Stein Research to Prevent Blindness Professor award; the Cogan Award from the Association for Research in Vision and Ophthalmology; the Knight’s Cross of the Order of Merit of the Republic of Poland award; The Roger H Johnson Macular Degeneration Award; and The Friedenwald Award. He also is one of four recipients of the 2012 Award from Foundation for Polish Science, the highest recognition available to Polish scientists living outside that country. Professor Palczewski also serves on the editorial boards of several well recognized biochemical journals and National Institute of Health scientific panels appropriate to his field.

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**Symbols and Abbreviations**

A2E pyridinium bisretinoid

A2PE phosphatidyl-pyridinium bisretinoid

University, Cleveland, OH, Dr. Golczak has focused on vitamin A metabolism, especially the enzymatic pathway called the retinoid (visual) cycle, that leads to regeneration of the visual chromophore required for sight. Currently, he holds an Instructor position in the Department of Pharmacology at Case Western Reserve University. His main interests are the elucidation of molecular mechanisms of vitamin A homeostasis in the eye and the development of small molecule-based therapeutic strategies against light-induced retinal degeneration.
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