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Membrane-Binding and Enzymatic Properties of RPE65

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Abstract

Regeneration of visual pigments is essential for sustained visual function. Although the requirement for non-photochemical regeneration of the visual chromophore, 11-*cis*-retinal, was recognized early on, it was only recently that the *trans* to *cis* retinoid isomerase activity required for this process was assigned to a specific protein, a microsomal membrane enzyme called RPE65. In this review, we outline progress that has been made in the functional characterization of RPE65. We then discuss general concepts related to protein-membrane interactions and the mechanism of the retinoid isomerization reaction and describe some of the important biochemical and structural features of RPE65 with respect to its membrane-binding and enzymatic properties.

Keywords

retinoid isomerase; RPE65; membrane protein; metalloenzyme; retinal pigment epithelium; Leber congenital amaurosis

1. Introduction

1.1. Structure and function of the photoreceptor and pigment epithelial layers of the retina

Vision is a physiological process involved in nearly every aspect of human life. The light-sensing tissue in humans is the retina, which is located in the posterior portion of the eye. The main light-sensitive cells of the retina, called photoreceptor cells, consist of two different types; rod cells for vision under low-illumination conditions and cone cells for color vision in well-illuminated environments (Rodieck, 1998). Visual pigments are the light-sensitive molecules found in photoreceptor cells. Visual pigments consist of a protein moiety called opsin and a vitamin A-derived chromophore called 11-*cis*-retinal that is covalently bound to a Lys side chain amino group of the opsin via a protonated Schiff base linkage (Palczewski, 2006). The covalently bound retinoid is called 11-*cis*-retinylidene. Visual pigments are seven-pass transmembrane proteins that are tightly packed together in pancake-like membrane structures known as disks, which are located in the outer segments of photoreceptor cells (Palczewski, 2006). Adjacent to the photoreceptor outer segments is a monolayer of epithelial cells known as the retinal pigment epithelium (RPE). The RPE transports nutrients to and removes waste products from the photoreceptors, absorbs excess light through melanin granules, phagocytoses fragments of shed outer segments and recycles chromophore for visual pigments. In addition, all-*trans*-retinol is bi-directionally transferred between the systemic circulation and the RPE via a membrane transporter found on the basolateral plasma membrane of RPE cells called

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STRA6 (Kawaguchi et al., 2007; Qtaishat et al., 2003). Therefore, this cell layer is vital for the health of the retina.

1.2. The retinoid (visual) cycle

Light sensation by photoreceptor cells in the retina begins with a photochemical event in which absorption of a single photon causes the geometrical photoisomerization of the pigment 11-*cis*-retinylidene chromophore to an all-*trans* configuration (Palczewski, 2006). This conversion activates the visual pigment allowing it to trigger a cascade of downstream signaling events that lead to the transmission of electrical signals to the visual cortex and the perception of light by the brain. After a brief period of time, signaling by the pigment is terminated by phosphorylation and subsequent binding of a silencing protein known as arrestin (Polans et al., 1996). Following a light stimulus, the pigment is no longer capable of being photoactivated. Thus, a mechanism for the regeneration of light-sensitive pigments, that is the isomerization of all-*trans*-retinylidene back to the 11-*cis* configuration, is essential for continuity of vision (Wald and Brown, 1953) (Fig. 1). At least in vertebrates, the chromophore is not directly reisolomerized while it is bound to opsin. Instead, the labile Schiff base linkage between the chromophore and opsin is hydrolyzed and free all-*trans*-retinal is released in a process known as bleaching (Matthews et al., 1963). all-*trans*-Retinal is subsequently reduced in the photoreceptor cells by NAD(P)H-dependent retinol dehydrogenases (RDHs) to yield all-*trans*-retinol (vitamin A). The vitamin A is transferred to the RPE where it is esterified by lecithin:retinol acyltransferase (LRAT) (Batten et al., 2004)(summarized in (Travis et al., 2007)). It is these retinyl esters that serve as substrates for retinoid isomerization, which occurs in a complex enzymatic reaction involving simultaneous hydrolysis of the ester moiety. The retinoid product of this reaction, 11-*cis*-retinol, is then oxidized to 11-*cis*-retinal and transported back to the photoreceptors where it condenses with opsin to reform a light-sensitive pigment (summarized in (Travis et al., 2007)). This multistep process of converting all-*trans*-retinal to 11-*cis*-retinal is known as the retinoid or visual cycle. A 61 kDa RPE-specific protein called RPE65, because its apparent molecular mass based on SDS-PAGE analysis is 65 kDa, is the enzyme responsible for the light-independent conversion of all-*trans*-retinyl esters, primarily palmitoyl esters, into 11-*cis*-retinol. Although the need for light-independent isomerization activity in the regenerative visual cycle was recognized early, the identification of RPE65 as the responsible enzyme was not straightforward and occurred only recently (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). RPE65 is involved not only for regeneration of rhodopsin but also plays an important role in regeneration of cone opsins and is critical for the health of cone photoreceptors (Jacobson et al., 2007). In contrast to the RPE65-dependent chromophore regeneration pathway for rhodopsin, there is substantial evidence that chromophore for cone opsins is at least partially regenerated through an alternative metabolic pathway involving enzymes located in cone photoreceptor and Müller cells (Mata et al., 2002; Mata et al., 2005; Muniz et al., 2009; Wang et al., 2009). This review summarizes the history, biochemical and structural properties of RPE65 with an emphasis on its mode of membrane binding and enzymatic activity, the former being highly critical for the latter.

2. Characterization of RPE65 function

The first description of RPE65 was published in 1991 when a monoclonal antibody obtained from mice exposed to chicken RPE cell antigens was shown to recognize an RPE-specific protein with an apparent molecular mass of 63 kDa (Sagara and Hirosawa, 1991). Immunocytochemical analysis revealed that the antigen was localized almost exclusively to the smooth endoplasmic reticulum (sER). The protein was not only found in chicken RPE but also in the RPE of a variety of different animals suggesting an important and highly conserved function. Although the amino acid sequence of this protein was not determined, its near-65 kDa apparent molecular mass and localization to sER of the RPE strongly suggest RPE65 was

the protein identified in this study because these are now well-recognized features of this protein. RPE65 is most highly expressed in the RPE but its mRNA and protein have also been reported by one group in cone photoreceptors of some amphibians and mammals (Ma et al., 1998; Znoiko et al., 2002).

RPE65 was initially thought to be a receptor for serum retinol-binding protein (RBP), and in these studies it was referred to as p63 (Bavik et al., 1992; Bavik et al., 1991; Bavik et al., 1993). However, the localization of RPE65 to the sER rather than the basolateral plasma membrane, where the RBP receptors were known to reside (Bok and Heller, 1976; Heller and Bok, 1976), argued against a physiological role for RPE65 as an RBP receptor. Furthermore, the deduced amino acid sequence of RPE, revealed by the cloning and sequencing of RPE65 cDNA (Bavik et al., 1993; Hamel et al., 1993b), did not reveal potential alpha-helical transmembrane segments, which would likely be present in a small-molecule transporter. Therefore, the RBP receptor hypothesis for RPE65 function fell out of favor.

Some light was shed on RPE65 function in 1997 when mutations in the RPE65 gene were found in patients with Leber congenital amaurosis (LCA), a severe retinopathy resulting in early childhood blindness (Gu et al., 1997; Marlhens et al., 1997) (see Cideciyan, 2010; den Hollander et al., 2008) for recent reviews). Additionally, RPE65-null mice were shown to develop early-onset blindness and displayed a metabolic dysfunction characterized by near-complete absence of ocular 11-*cis* retinoids in addition to substantial accumulation of all-*trans*-retinoids in the RPE (Redmond et al., 1998). Together, these observations strongly suggested that RPE65 may play an important role in the metabolic processing of retinoids. In 1997, an ortholog of RPE65 found in plants called viviparous 14 (VP14) was shown to oxidatively cleave carotenoids (Schwartz et al., 1997). The overall sequence homology and absolute conservation of four histidine residues, thought to bind an iron cofactor, between these two proteins strongly suggested that RPE65 too could possess enzymatic activity (Tan et al., 1997).

However, some observations and properties of RPE65 were thought not to be consistent with this hypothesis. In 1998 it was reported that near-complete removal of RPE65 from bovine RPE microsomes by high salt extraction did not substantially impair retinoid isomerization activity of the microsomal fraction (Choo et al., 1998). This effect was not observed in our laboratory, however, as discussed later. Attempts to demonstrate retinoid isomerization activity of purified RPE65 were unsuccessful (Mata et al., 2004). Indeed, solubilization of the RPE microsomal proteins with most detergents resulted in nearly complete loss of retinoid isomerization activity (Barry et al., 1989; Bernstein et al., 1987). Based on the sheer abundance of RPE65 in RPE cells it was argued that the protein might act stoichiometrically, for example, as retinoid-binding protein, rather than enzymatically (Gollapalli et al., 2003). Up until 2005 it was generally thought that RPE65 was a retinyl ester binding-protein or a retinoid chaperone that extracted and presented insoluble retinyl esters to a hypothetical enzyme called isomerohydrolase (Gollapalli et al., 2003; Mata et al., 2004). The perceived action of RPE65 was made even more elaborate by the presentation of the “palmitoylation switch mechanism” hypothesis which proposed a role for light-dependent, reversible palmitoylation of specific cysteine residues in the control of RPE65 membrane binding and retinoid-binding specificity (Xue et al., 2004).

Strong evidence against a pure retinoid binding-function for RPE65 was provided in 2005 when three groups independently demonstrated that coexpression of RPE65 and LRAT in various mammalian cell lines as well as insect cells actually conferred retinoid isomerization activity (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). RPE65 was proposed to be the actual isomerase whereas LRAT was needed to provide retinyl ester substrates *in situ*. Despite the major advance made by these studies, some doubt still lingered regarding the

identity of RPE65 as the isomerase in these assays because whole cells or cell lysates were used rather than purified proteins (Xue et al., 2006). This doubt was largely removed by the demonstration that purified RPE65 possessed retinoid isomerase activity when reconstituted with retinyl ester-containing phospholipid vesicles (Nikolaeva et al., 2009b), even though the observed activity of the reconstituted protein was far below what is physiologically required (Lyubarsky et al., 2005). The study also suggested that RPE65 interactions with an intact phospholipid bilayer are critically important for its activity. The loss of native microsomal retinoid isomerization activity upon perturbation of membrane structure by addition of a wide variety of detergents or by phospholipase A2 (PLA₂) treatment strongly supported this contention (Golczak, 2010).

3. RPE65 membrane binding

3.1. Peripheral versus integral membrane proteins

Before proceeding it is important to describe the different types of membrane proteins and briefly review their properties. Singer and Nicolson first defined two broad classes of membrane proteins, the peripheral and the integral membrane proteins (Singer and Nicolson, 1972). Peripheral membrane proteins typically associate with membranes through metal-dependent or independent electrostatic interactions with lipid head groups, by fatty acid acylation, prenylation, or glycosyl phosphatidylinositol (GPI) anchors or by interactions with integral membrane proteins (Ohlendieck, 1996; Singer, 1974). These proteins can usually be extracted from membranes under relatively mild conditions such as high or low ionic strength, treatment with divalent cation chelators (e.g. EDTA), or by incubation in alkaline carbonate buffers (Penefsky and Tzagoloff, 1971; Singer, 1974). Following extraction from membranes, peripheral membrane proteins are usually stable after removal of the extraction agent and can be handled like typical soluble proteins (Penefsky and Tzagoloff, 1971). By contrast, integral membrane proteins bind to membranes in a much tighter, often irreversible fashion and interact directly with the membrane hydrophobic core. Importantly, the interaction between an integral membrane protein and the lipid matrix is usually of functional significance (Singer, 1974; Tanford and Reynolds, 1976). Quantitative extraction of integral membrane proteins requires the presence of membrane-disrupting concentrations of detergents, and after extraction detergent must remain in the purification buffers to keep the protein soluble (Singer, 1974). The integral membrane proteins can be further subdivided based on their topology with respect to the membrane into monotopic, bitopic or polytopic proteins as described by Blobel (Blobel, 1980). Bitopic and polytopic membrane proteins possess one or multiple membrane spanning domains, respectively, and therefore have portions of their chains located in two topologically distinct spaces. There are numerous examples of both bitopic and polytopic membrane proteins. Monotopic membrane proteins, on the other hand, have hydrophobic segments that extensively interact with the lipophilic core of the membrane but these segments do not span the bilayer. This group can be further subdivided based on the presence or absence of a hydrophobic endoplasmic reticulum (ER) signal peptide sequence. The last distinction is important because different mechanisms of membrane insertion are required for each type (Blobel, 1980). The former class can be cotranslationally inserted into the ER membrane by translocon machinery, whereas the latter must be translated on a free ribosome and then targeted to the correct membrane by a different mechanism such as interaction with another membrane-bound protein. Although monotopic membrane proteins are thought to be much rarer in nature than transmembrane proteins, structures of several of these proteins are now known. The first monotopic membrane protein to have its structure revealed was ovine prostaglandin H2 synthase-1 in the laboratory of Garavito in 1994 (Picot et al., 1994). This microsomal protein is a prototypical example of a monotopic membrane protein targeted to the ER membrane by an N-terminal signal peptide. Subsequently, the structure of a squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* was determined in the Schulz laboratory (Wendt et al.,

1997). The squalene-hopene cyclase is an example of a monotopic membrane protein that lacks an N-terminal signal peptide. A mammalian monotopic membrane protein that is localized to the ER membrane but lacks a signal peptide is lanosterol synthase (Abe and Prestwich, 1995; Thoma et al., 2004). Thus, the absence of potential transmembrane segments and/or an N-terminal signal peptide in the primary amino acid sequence of a protein does not necessarily exclude the possibility that it is an integral membrane protein. In reality, the distinction between peripheral and integral membrane proteins is sometimes difficult to make on the basis of extraction experiments (Ohlendieck, 1996; Penefsky and Tzagoloff, 1971). For proteins that bind via direct interactions to the bilayer, the ratio of polar to non-polar interactions can take on a continuum of values; thus classification of proteins as peripheral or integral membrane can be ambiguous and somewhat arbitrary. Therefore, structural and functional information must also be considered when classifying protein-membrane interactions as peripheral or integral.

3.2. Biochemical studies concerning the interaction of RPE65 with membranes

A fairly large number of studies have investigated the nature of RPE65-membrane interactions, and at least four different proposals have been made (Fig. 2). Localization of RPE65 to the sER was recognized during its initial characterization (Bavik et al., 1991; Hamel et al., 1993a; Sagara and Hirose, 1991). The sER is an unusually abundant organelle in RPE cells where it occupies a large fraction of the cytoplasmic space (Garron, 1963). An abundance of sER is typically found in cells involved in metabolism of lipophilic compounds, consistent with the retinoid processing function of the RPE (Alberts, 2002). As expected from its subcellular location, RPE65 cosediments with microsomal membranes during centrifugation, and this fact can be exploited for its purification (Hamel et al., 1993a; Kiser et al., 2009). Initial studies indicated that RPE65 has many characteristics consistent with its classification as an integral membrane protein. Detergents, especially Triton X-100 and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), were found to be the most effective agents for extracting RPE65 from microsomal membranes (Bavik et al., 1992; Hamel et al., 1993a). Additionally, Triton X-114-solubilized RPE65 partitioned almost exclusively into the detergent-rich fraction after temperature-induced phase separation, a behavior usually observed for integral membrane proteins (Bavik et al., 1992; Bordier, 1981; Golczak, 2010; Hamel et al., 1993a). However, other experiments indicated a peripheral membrane association. Treatment of RPE microsomes with alkaline carbonate solutions extracted significant amounts of RPE65 (Bavik et al., 1992; Golczak, 2010; Yuan et al., 2010). An alkaline pH may disrupt membrane-protein or protein-protein salt bridges by deprotonation of titratable amino groups. It was also reported that exposure of RPE microsomes to neutral pH solutions containing 0.75 M to 1 M KCl released significant amounts of RPE65 (Choo et al., 1998; Hamel et al., 1993b), although we have been unable to corroborate this effect and routinely wash RPE microsomal membranes with 1 M KCl prior to detergent extraction of RPE65 without appreciable loss of membrane-bound RPE65 or retinoid isomerization activity (Kiser et al., 2009). These observations together with the lack of an N-terminal signal peptide and putative transmembrane segments were taken as evidence of a peripheral membrane association for RPE65 (Hamel et al., 1993a; Hamel et al., 1993b; Ma et al., 2001). However some important points argue against this interpretation. First, it has never been shown that the RPE65 released under the aforementioned conditions can be purified while preserving its native structure. Second, it has never been demonstrated that RPE65 remains soluble after removal of these extracting agents as would be expected for a peripheral membrane protein. Instead, it has been observed that RPE65 released by carbonate treatment converts to a particulate form after adjustment of the pH from 11.5 to 6 or after dialysis against phosphate-buffered saline (PBS), pH 7.4 (Golczak, 2010; Penefsky and Tzagoloff, 1971). Additionally, the initial study reporting the KCl-extraction phenomenon also demonstrated that the salt-extracted RPE65 partitioned into the detergent-rich phase during Triton X-114 phase separation just as the detergent-

extracted protein does, indicating that the salt-extracted protein is not a distinct “soluble” form (Hamel et al., 1993a).

Fatty acid acylation, particularly palmitoylation was, for several years, thought to confer RPE65 membrane affinity (Xue et al., 2004). Palmitoylation-dependent membrane association is an attractive hypothesis for a protein like RPE65 that apparently lacks an intrinsic ER localization signal, as palmitoylation is known to influence membrane localization of intracellular proteins (Greaves and Chamberlain, 2007). Initial data supporting this hypothesis was derived from matrix-assisted laser desorption/ionization (MALDI) mass spectrometric measurements of RPE65 (Ma et al., 2001). These measurements indicated the mass of native bovine RPE65 purified from membranes was significantly higher than the calculated value for the unmodified polypeptide chain whereas cytosolic RPE65 had a mass only slightly above the theoretical value. It was also estimated that the ratio of cytosolic to microsomal RPE65 in native bovine RPE is approximately 1:2, indicating the presence of a potentially distinct soluble form of the protein. In 2004, it was reported that RPE65 is reversibly palmitoylated by LRAT on three specific residues, Cys 231, Cys 329 and Cys 330 (Xue et al., 2004). Such palmitoylation was proposed to control RPE65 membrane affinity as well as alter retinoid-binding specificity. Intriguing as this hypothesis was, subsequent studies indicated that it was probably incorrect. Membrane localization of RPE65 in LRAT knockout compared with that of wild-type mice was not substantially different (Jin et al., 2007). Mutation of all three Cys residues to Ala or Ser did not substantially affect RPE65 membrane binding (Jin et al., 2007; Takahashi et al., 2006). Furthermore, mass spectrometric analyses of native, membrane-bound RPE65 that were of higher quality than those originally performed revealed no palmitoylation of Cys residues 231, 329 and 330 (Jin et al., 2007; Yuan et al., 2010). A recently described novel palmitoylation site at Cys 112 (Kiser et al., 2009; Takahashi et al., 2009) was reported to be critical for membrane targeting based on the observation that Ala substitutions at that position led to the RPE65 being expressed in intracellular aggregates (Takahashi et al., 2009). A subsequent study questioned the palmitoylation of Cys 112 and suggested that RPE65 does not undergo any significant post-translational modifications (Yuan et al., 2010). Thus, despite a number of biochemical studies, no clear consensus has emerged regarding RPE65 palmitoylation. However, these studies indicate that RPE65 is not palmitoylated on Cys 231, 329 and 330.

Another potential mechanism for RPE65 binding to membranes is through interactions with other membrane-bound proteins. Initial studies indicated that RPE65 is part of a large molecular weight complex that possibly includes other RPE microsomal proteins (Bavik et al., 1992). Copurification of RDH5, a known integral membrane protein, with RPE65 or its fragments has been observed in several studies (Golczak, 2010; Hemati et al., 2005; Simon et al., 1995; Trudel et al., 2006) and the observed crosslinking between RPE65 and RDH5 provides evidence that the RDH5 catalytic domain is oriented towards the cytosolic space rather than the ER lumen (Golczak, 2010). Protein-protein interactions may help direct RPE65 to the ER and assist in its membrane anchoring. However, the observations that purified RPE65 can bind directly to liposomes (Nikolaeva et al., 2009b; Tsilou et al., 1997) and that it can extract retinyl esters directly from membranes (Mata et al., 2004) indicate that it has inherent membrane affinity. Furthermore, enzymatic activity of purified RPE65 can be restored upon reconstitution with retinyl ester-containing liposomes demonstrating that RPE65 is not dependent on another protein for membrane association and substrate extraction (Nikolaeva et al., 2009b). In accord with this notion, PLA₂ treatment of native RPE microsomal membranes, which loosens the membrane structure through removal of a fatty acid from the sn2 position of phospholipids, significantly impairs retinoid isomerization activity and disrupts the association of RPE65 with other RPE microsomal proteins but does not result in dissociation of RPE65 from the membrane (Golczak, 2010). This observation suggests that RPE65 interacts directly with the membrane in a highly specific manner and that this interaction is critical for its enzymatic activity.

Disruption of the membrane structure could affect the orientation of the retinoid substrate in the membrane such that it cannot be effectively extracted by RPE65. Substrate recognition elements of RPE65 may also be destabilized when the membrane structure is altered.

Interestingly, some RPE65 relatives such as β -carotene dioxygenases are water-soluble proteins (Duszka et al., 1996; Lindqvist and Andersson, 2002) whereas others like certain 9-*cis*-epoxycarotenoid dioxygenases appear to be exclusively membrane bound (Qin and Zeevaart, 1999). The substrates of these enzymes are hydrophobic carotenoids that have essentially no aqueous solubility. Because the active sites are located deep within the carotenoid oxygenases rather than near their surfaces (Kloer et al., 2005) the substrates must be physically removed from the membrane for enzymatic processing. Delivery of substrates in micelles or bilayers is required for their enzymatic processing by these enzymes. Thus, it is likely that these enzymes, regardless of their degree of water solubility, must interact with the micelle or bilayer to extract substrate. The possible structural basis of this interaction is discussed below.

3.3. Structural observations and insights

Crystallographic analysis of RPE65 provided valuable insights into the membrane-binding mechanism of this protein (Kiser et al., 2009). The protein used for this study was solubilized and purified from native bovine RPE microsomes in the presence of octyltetraoxyethylene (C_8E_4), a non-ionic detergent. It was noted that RPE65 prepared in this manner could be stably concentrated to at least 15 mg/ml. The crystal structure revealed a single-domain, 7-bladed beta propeller fold that has the general features of a typical water-soluble protein (Fig. 3A). However, there is one face of the protein where several lipophilic and aromatic residues are clustered. This face is formed by both alpha helical and loop structures enriched in residues known to preferentially interact with headgroups (Ser, Trp, Tyr, Lys and Arg) and the hydrophobic core (Phe, Leu and Ile) of the phospholipid bilayer (Picot and Garavito, 1994) (Fig. 3B). One potentially alpha helical, amphipathic segment composed of residues 109–126 that may contribute to membrane affinity is disordered in the reported *P65* crystal form. The hydrophobic face surrounds the opening of a large channel that leads to the catalytic site of RPE65. Given that RPE65 can directly extract retinyl esters from the lipid bilayer for metabolic processing (Mata et al., 2004; Nikolaeva et al., 2009b) and also considering that these extremely hydrophobic retinyl esters are dissolved in the lipid matrix of the bilayer, it seems reasonable to conclude that this hydrophobic surface of the protein is embedded in the bilayer allowing the protein to gain access to the retinyl ester substrate. This mode of membrane interaction is consistent with many of the biochemical studies mentioned above.

Interestingly, the structure of *Synechocystis* apocarotenoid oxygenase (ACO) also features a hydrophobic patch that is located in structurally equivalent position to that observed in the RPE65 structure, and it was speculated that this patch allows the enzyme to dip into membranes or micelles to extract carotenoid substrates (Kloer et al., 2005). Like the β -carotene oxygenases mentioned above, this enzyme was found to be water soluble. However, the addition of non-ionic detergent to the purification buffers was noted to shift the migration pattern of the enzyme on a gel filtration column from a monomeric species to an apparent trimeric species suggesting that the protein can significantly interact with detergent micelles. The aqueous solubility of this enzyme is easily explained by the fact that the large fraction of its surface is formed by polar or charged residues with the hydrophobic patch being small in comparison. However, the hydrophobic patch confers the ability to transiently interact with membranes or micelles to extract substrate. Biochemically, RPE65 appears to possess a much stronger membrane-binding character than β -carotene or apocarotenoid oxygenases. Structural comparison of RPE65 and ACO indicates that the hydrophobic patch on RPE65 is more extensive than that of ACO, assuming that residues 109–126 of RPE65 also adopt an amphipathic structure with the hydrophobic face being surface exposed. Alignments of the RPE65 and β -carotene

oxygenase amino acid sequences do not reveal major differences between these enzymes in the regions that are predicted to confer RPE65 membrane affinity. Thus, the physical basis for the differential membrane affinity between these members of the CCO family remains unclear.

An alternative mode of RPE65 membrane attachment involving one or two patches of basic residues was proposed on the basis of biochemical experiments indicating that RPE65 interacts with negatively charged phospholipids and requires these lipids to efficiently extract retinyl esters from liposomes (Yuan et al., 2010). One patch consisting of residues 294–298 is located on the bottom side of blade III of the β propeller and is quite distant from the openings to the active site. The other patch consisting of residues 354–359 is located near the opening of a narrow tunnel that leads to the active site. However, this tunnel, described in more detail below, appears too narrow to allow passage of retinoids, and the residues that form this tunnel do not appear to be particularly flexible based on the crystallographic *B*-factors of their constituent atoms. The topography of these patches seems to exclude the possibility that they could work in concert with the aforementioned hydrophobic patch to promote RPE65 membrane binding. As mentioned above, there are basic residues found within the same region as the hydrophobic patch that could promote membrane binding.

The asymmetric unit of the *P*₆₅ crystal form contains an RPE65 dimer that is oriented in parallel, with each hydrophobic surface facing the same side of the dimer. The extensive dimer interface buries a total of $\sim 3100 \text{ \AA}^2$ of surface area, which is about 3 times greater than the next highest buried surface in the crystal. These features suggest that the observed dimer could represent the arrangement of RPE65 on membranes; however, in detergent-containing solution RPE65 behaves as a monomer possibly interacting with a detergent micelle (Kiser et al., 2009). The dimer is packed in the crystal with the hydrophobic surface facing the largest solvent channel in the crystal, an arrangement that may result from the need to accommodate a protein-detergent mixed micelle (Golczak, 2010). This packing arrangement is consistent with a type II membrane protein crystal (Michel, 1983; Ostermeier and Michel, 1997). It is notable that prototypical monotopic membrane proteins, such as prostaglandin H₂-synthase-1 and squalene-hopene cyclase, form similar parallel symmetric dimers and are also packed in the crystal with their hydrophobic surfaces facing solvent channels (Picot et al., 1994; Wendt et al., 1999).

Consistent with biochemical studies on RPE65 palmitoylation (Jin et al., 2007; Takahashi et al., 2006), no evidence of Cys 231, 329 and 330 palmitoylation was observed in the RPE65 crystal structure. In fact, none of these Cys side chains are surface exposed indicating that significant conformational changes would be required for them to become palmitoylated. By contrast, Cys 112 is found on the N-terminal side of a ~ 15 residue-long, potentially amphipathic sequence that surrounds the entrance to the RPE65 active site and may form part of the membrane docking surface (Kiser et al., 2009). Thus, palmitoylation of this residue appears quite feasible from a structural perspective, but the modification could not be confirmed in the crystal structure because Cys 112 is located in a highly disordered and unresolved portion of the protein. The predicted close proximity of this Cys to the membrane suggests it could be subject to non-enzymatic palmitoylation by palmitoyl-CoA present in the sER lipid bilayer (Duncan and Gilman, 1996).

3.4. Is “soluble” RPE65 a distinct form of the protein with functional significance?

Classification of the mode of RPE65 membrane binding is not merely an academic exercise. The presence of a distinct soluble variety of the protein could imply functional differences between the cytosolic and membrane-bound forms and possibly a regulatory mechanism for the visual cycle at the level of RPE65. A function for “soluble RPE65” was proposed in the “palmitoylation switch” hypothesis, namely to bind all-*trans*-retinol present in the cytosol and

present it for esterification by LRAT (Xue et al., 2004); however, subsequent studies have disproved most of its requirements.

During microsome isolation, we find only traces of native bovine RPE65 in the 100,000g supernatant, even though others have reported more significant amounts of the protein (between 1/5 to 1/3 of total RPE65) in this fraction (Jin et al., 2007; Ma et al., 2001). This discrepancy may relate to differences in isolation methodologies employed. Possibly, the cytosolic form of RPE65 is simply an artifact of the isolation procedure. Known integral monotopic membrane proteins, such as the squalene cyclases, can appear soluble after cell homogenation and high-speed centrifugation under certain conditions (Balliano et al., 1992). Instability of this apparently soluble form of the cyclase becomes apparent when one attempts to purify the protein in detergent-free buffers. In these studies it was speculated that the 'soluble' form of the cyclase was actually a non-sedimentable lipoprotein particle generated during expression or cell homogenation. A similar phenomenon may occur during the RPE microsome isolation procedure under certain conditions. We note that no convincing method for purifying cytosolic RPE65 in the absence of detergents has yet been reported.

Monotopic members are unique amongst the integral membrane proteins with respect to distribution of their hydrophobic and hydrophilic residues (Blobel, 1980). In contrast to bitopic and polytopic proteins, monotopic proteins could in principle form finite, small oligomeric assemblies that shield their hydrophobic surfaces from water and consequently cause them to behave like soluble proteins. The recently determined crystal structure of an established monotopic membrane protein from *Aquifex aeolicus*, sulfide:quinone oxidoreductase, indicates that this idea is structurally feasible (Marcia et al., 2009). The asymmetric unit of the crystal contained a complex arranged such that the hydrophobic face of one protomer was shielded by another related by a two-fold rotation. Thus, it might be envisioned that such a complex could behave like a soluble protein in solution because most of the hydrophobic surface is buried. Although the protein used in the aforementioned study was purified in the presence of detergent, another study describing crystallization of a closely-related homolog from a different organism (*Acidithiobacillus ferrooxidans*) with a membrane-binding motif predicted to be highly similar to that of the *Aquifex aeolicus* enzyme did not employ detergent during purification or crystallization (Zhang et al., 2009). It is conceivable that the formation of hydrophobic surface-buried complexes may have facilitated detergent-free purification. At least in principle, it is possible that RPE65 under certain conditions could form similar complexes in solution making the protein appear soluble.

4. RPE65 Enzymology

4.1. Substrates and Products

Originally, an enzymatic activity that produces 11-*cis*-retinol directly from all-*trans*-retinol or 11-*cis*-retinyl esters from all-*trans*-retinyl esters was thought to be responsible for light-independent chromophore regeneration in the eye (Wald, 1968). The bulk of current data now suggest that it is all-*trans*-retinyl esters that are directly converted to 11-*cis*-retinol by RPE65. Identification of the substrate was not trivial though owing to presence of several other retinoid-modifying reactions, such as those catalyzed by acyl transferases and hydrolases, which occur simultaneously with retinoid isomerization in RPE microsomes and can complicate data analysis.

The proposal that retinyl esters rather than retinol were the substrates for the isomerization reaction was made primary on theoretical grounds. It was hypothesized that the energy released by ester hydrolysis could drive the thermodynamically unfavorable *trans* to *cis* isomerization (discussed in more detail below) (Deigner et al., 1989). RPE65-null mice were noted to accumulate large stores of retinyl esters in the RPE, suggesting the metabolic blockade in these

mice is at the retinyl ester-processing step (Redmond et al., 1998). Also, experiments that employed specific LRAT inhibitors, all-*trans*-retinyl bromoacetate or dodecyl chloromethyl ketone, demonstrated that 11-*cis*-retinol generation from all-*trans*-retinol only occurred when retinyl esters were allowed to form (Gollapalli and Rando, 2003; Moiseyev et al., 2003). More recently it was demonstrated in a chemically defined system that retinyl esters are converted to 11-*cis*-retinol in the presence of RPE65 (Nikolaeva et al., 2009b).

However, pulse-chase experiments performed by Stecher and colleagues using radiolabeled all-*trans*-retinol demonstrated that at a given time most of the retinyl esters in the RPE were not able to be enzymatically isomerized (Stecher et al., 1999a). It was speculated that only a small pool of the retinyl esters in the ER membranes were accessible to the isomerase. The observations in this study were nicely explained by the discovery of dynamic retinyl ester-containing lipid bodies, termed retinosomes, in the RPE through the use of two-photon microscopy (Imanishi et al., 2004).

4.2. Thermodynamic and kinetic considerations

As mentioned in the Introduction, the reaction catalyzed by RPE65 is complex, consisting of a geometrical isomerization of a *trans* double bond coupled with an atypical ester hydrolysis. The isomerization reaction is energetically unfavorable both thermodynamically and kinetically. It is well-known that *cis* double bonds are generally higher in free energy than their *trans* counterparts except in certain compounds such as cycloalkenes. In the retinol molecule the difference in free energy between the all-*trans* and 11-*cis* isomers is ~4 kcal/mol (Rando and Chang, 1983). It was proposed that the energy released by ester hydrolysis (~5 kcal/mol) was sufficient to drive the endergonic isomerization (Deigner et al., 1989). However, the fact that 11-*cis*-retinoid binding proteins are required for robust production of 11-*cis*-retinol *in vitro* indicates that product release may be the slow step of the isomerization reaction and that the reaction is actually driven by mass action (McBee et al., 2000; Stecher et al., 1999a; Winston and Rando, 1998).

The retinyl moiety of an all-*trans*-retinyl ester is rigid owing to its conjugated π bond system. Therefore, geometrical isomerization of the ground-state molecule is kinetically highly unfavorable, requiring an activation energy of ~36 kcal/mol (McBee et al., 2000). For the isomerization reaction to occur at physiological temperatures, the activation energy for the process must be reduced, and this is accomplished by temporarily reducing the carbon-carbon bond order of the system. The bond order, defined by equation (1), is approximately equal to 1.5 for the carbon-carbon bonds in the polyene chain of ground state retinol (McQuarrie and Simon, 1997).

$$\text{bond order} = (\text{number of electrons in bonding orbitals} - \text{number of electrons in antibonding orbitals}) / 2 \quad \text{equation (1)}$$

Thus, this equation suggests two mechanisms by which double bond isomerization chemistry can occur: 1) temporary electron removal from bonding p orbitals, or 2) generation of transient antibonding orbitals, e.g. via photochemical processes. As the visual cycle isomerization reaction is known to occur in the absence of light it probably involves the first mechanism.

4.3. Mechanisms of retinoid isomerization

Interestingly, many important characteristics of the isomerase enzymatic reaction mechanism were discovered long before RPE65 was identified as the visual cycle isomerase and before the discovery that all-*trans*-retinyl esters (Gollapalli and Rando, 2003; Moiseyev et al., 2003; Nikolaeva et al., 2009b) rather than all-*trans*-retinol were its substrates. Identification of RPE65 as the visual cycle isomerase combined with recent structural data gathered on it (Kiser

et al., 2009) and a related protein, ACO, (Kloer et al., 2005) have revived interest in the retinoid isomerization mechanism. Initial evidence for a coupled isomerase/hydrolase reaction was derived from experiments showing that both activities were always found together during fractionation (Barry et al., 1989). Isotope labeling studies established that a retinoid in the alcohol oxidation state (retinol or a retinyl ester) is the species that is enzymatically isomerized rather than retinal (Bernstein and Rando, 1986). An intriguing early observation was that the stereochemical configuration of retinoid carbon 15 inverts during the isomerization reaction, indicating breakage of the C15-O single bond at some point during the isomerization reaction (Jang et al., 2000; Law and Rando, 1988) (see Fig. 4A for standard numbering of retinoids). Further isotope labeling studies demonstrated that the oxygen present in the all-*trans*-retinoid substrate is replaced by a different oxygen atom in the 11-*cis* product (McBee et al., 2000), an oxygen atom later found to be derived from water (Kiser et al., 2009).

Two different mechanisms of retinoid isomerization have been proposed. The first mechanism is a dual S_N2' nucleophilic substitution (Fig. 4B). In this reaction an enzyme active site nucleophile, presumably a cysteine sulfur atom, attacks the retinyl C11 atom with simultaneous dissociation of a leaving group from C15 (Law and Rando, 1988). This results in an intermediate that can undergo low-energy rotation about C11-C12. In the second part of this reaction, a nucleophile (presumably hydroxide) attacks C15 resulting in the expulsion of the C11-bound nucleophile and restoration of the conjugated π bond system, now in the 11-*cis* configuration. Based on this mechanism, the isomerase predictably would be highly specific with respect to isomerization of the 11-12 double bond. However, this high specificity is not experimentally observed as discussed below. There are also some chemical considerations that make this mechanism improbable as discussed previously (McBee et al., 2000). The major difficulty is that the first step involves a nucleophile attacking an already electron-rich atom, i.e. C11. S_N2 -type nucleophilic substitutions involving allylic carbons are known to be chemically unfavorable (Smith and March, 2001). Furthermore, this reaction would lead to formation of a highly stable carbon-heteroatom bond, most likely a thioether or ether group, which would be difficult to break in the subsequent step.

The second mechanism involves an S_N1 -type nucleophilic substitution (McBee et al., 2000) (Fig. 4C). Here, the leaving group dissociates first via alkyl-oxygen cleavage to generate a resonance-stabilized carbocation intermediate. Formation of a carbocation intermediate is known to be the slow step of an S_N1 reaction and a good leaving group is required. Although an ester can leave as a relatively stable, resonance-stabilized acyloxy anion, this reaction could be promoted by the interaction of the ester acyl oxygen with a Lewis acid (a proton or metal ion) (McBee et al., 2000). The retinyl carbocation intermediate has a reduced polyene carbon-carbon bond order which results in a substantial decrease in the activation energy needed for *trans* to *cis* isomerization from ~36 to ~18 kcal/mol (McBee et al., 2000). The latter value is consistent with the experimentally determined isomerization activation energy of 17 kcal/mol derived from Arrhenius plots (McBee et al., 2000). Because the carbocation is delocalized, it could be predicted that an isomerase which operates via this mechanism may not be 11-*cis* specific. Indeed, both 11-*cis* and 13-*cis*-retinol are produced by RPE65 and the isomer that accumulates depends on which retinoid-binding proteins are available for product sequestration (McBee et al., 2000; Redmond et al., 2010). After isomerization, attack by a nucleophile on C15 would quench the carbocation and lock the molecule in the *cis* configuration. In an S_N1 mechanism, the strength of an attacking nucleophile is less important compared to ease of leaving group dissociation (Smith and March, 2001). Thus, a water molecule, which would subsequently be deprotonated, rather than hydroxide could well be the nucleophile in this S_N1 reaction. On the basis of this proposed mechanism, a putative carbocation transition state analog, all-*trans*-retinylamine, was developed. This compound was found to be a highly effective blocker of 11-*cis*-retinol production both *in vitro* and *in vivo* (Golczak et al., 2005; Maeda et al., 2006) and was later shown to bind directly to RPE65

(Golczak et al., 2008). The first step of this mechanism, the alkyl-oxygen cleavage, deserves a bit more discussion. In virtually all biochemical ester hydrolysis reactions, it is thought that the reaction proceeds through a tetrahedral intermediate with the oxygen atom of water (or hydroxide) attacking the carbonyl carbon (Nelson et al., 2005). But it is also known from non-biological organic chemistry that cleavage is favored at the alkyl rather than the acyl carbon in S_N1 reactions when the carbocation intermediate can be stabilized by hyperconjugation or by resonance (Smith and March, 2001). The retinyl ester molecule presents such a situation wherein a generated carbocation is stabilized by extensive resonance delocalization. For general S_N1 reactions performed in solution, it is often the case that the chirality of the electrophilic center is racemized; thus this consideration could argue against an S_N1 mechanism for retinoid isomerization because it has been observed that a high percentage of the C15 atoms in the retinoid products undergo inversion of configuration (Jang et al., 2000; Law and Rando, 1988). However, because the reaction occurs in the active site of RPE65, it is easy to imagine that the enzyme only allows water to approach C15 from a specific direction, which would result in the formation of only one enantiomer.

4.4. Substrate specificity

A number of studies have examined the structural features of the retinyl ester molecule that are required for enzymatic isomerization. As the currently proposed mechanisms of RPE65-mediated retinoid isomerization both minimally require the presence of the 13–14 double bond, it was of interest to determine if compounds that were saturated at this position as well as the other positions along the polyene chain could be enzymatically isomerized. Interestingly, saturation of any double bond of the polyene chain blocks RPE65-dependent isomerization indicating a possible role of the entire conjugated double bond system in the reaction (Law et al., 1988). This is a surprising requirement for the mechanism shown in Fig. 4B because the concerted reaction appears to involve only carbon atoms 11 through 15. However, the mechanism shown in Fig. 4C relies on extensive resonance stabilization of a carbocation; thus, the requirement of a fully conjugated double bond system would be expected. Interestingly, the more extensive conjugated double bond system in vitamin A₂ (all-*trans*-3,4-dehydroretinol) does not inhibit or promote its isomerization.

The requirements for the C18, C19 and C20 methyl groups have also been investigated. An initial report indicated that the presence of C18 and C20 methyl groups were not required for isomerization, whereas all-*trans*-9-des-methylretinol could not be isomerized (Canada et al., 1990). A subsequent study on 9 and 13-desmethyl-all-*trans*-retinol showed that these compounds could be isomerized albeit less effectively than all-*trans*-retinol (Stecher et al., 1999b). These studies demonstrated that a mechanism of retinoid isomerization involving proton abstraction from the C18, C19 and C20 methyl groups was highly unlikely.

Fluorinated retinoids have also been prepared and tested for the ability to be isomerized. all-*trans*-19,19,19-trifluororetinol was found not to be enzymatically isomerized (Canada et al., 1990). Similarly, all-*trans*-11-fluororetinol did not undergo enzymatic isomerization (McBee et al., 2000). Based on these results, it was hypothesized that the electron-withdrawing character of the fluoro group could make the formation of a carbocation intermediate more difficult. Thus, these experiments are consistent with the S_N1 nucleophilic substitution mechanism shown in Fig. 4C.

Although it is often stated that retinyl esters are the substrates for RPE65, not all retinyl esters are capable of being isomerized. *In vivo* the most abundant retinyl ester present is retinyl palmitate because a palmitoyl group is most often found in the sn1 position of lecithin, which is used by LRAT as an acyl donor. It has been observed that short esters such as retinyl acetate and retinyl heptanoate are unable to be processed by RPE65 (unpublished data). The specificity for long chain fatty acid esters has not yet been explained. However, shorter esters may orient

incorrectly in the membrane for uptake into the active site of RPE65 or they may not bind properly in the active site because a long saturated hydrophobic chain could greatly contribute to the binding affinity but is missing in these compounds. It is highly unlikely that the fatty acid chain directly participates in the isomerization reaction though.

4.5. The requirement of an iron cofactor

Another interesting property of RPE65-dependent isomerization is its requirement for iron, specifically divalent iron (Moiseyev et al., 2006). The iron requirement of this enzyme was anticipated based on its evolutionary relationship to lignostilbene and carotenoid cleavage oxygenases (CCO) (Redmond et al., 2005; Schwartz et al., 1997; Tan et al., 1997). All members of this family, including RPE65, possess a set of four absolutely conserved histidine residues that directly bind iron. The requirement of iron for RPE65 activity has been well documented by mutating key iron-binding residues (Redmond et al., 2005; Takahashi et al., 2005) and by chelation experiments (Kiser et al., 2009; Moiseyev et al., 2006). Additionally, metal add-back experiments demonstrated that Fe^{3+} , Cu^{2+} , Mg^{2+} and Zn^{2+} were unable to restore RPE65 isomerase activity whereas addition of Fe^{2+} resulted in partial restoration (Moiseyev et al., 2006). For members of the CCO family other than RPE65, iron is thought to activate oxygen for cleavage of carotenoids or lignostilbenes (Borowski et al., 2008; Kloer and Schulz, 2006). However, RPE65 is not known to possess carotenoid-cleavage or other oxygenase activities, so the iron cofactor in this enzyme could be used for some other purpose. One major unresolved mystery of RPE65 enzymology is the requirement of the reduced form of a redox-active metal cofactor for a non-redox reaction. As mentioned above, the rate-limiting, alkyl-oxygen cleavage step of the $\text{S}_{\text{N}}1$ -type mechanism of retinoid isomerization is facilitated by interaction of the carbonyl oxygen of a retinyl ester with an acid (Smith and March, 2001). It is possible that the iron atom either directly interacts with the carbonyl oxygen (Kiser et al., 2009) or binds a water molecule that can subsequently donate a proton to the carbonyl oxygen (Golczak et al., 2005; Redmond et al., 2010). Either of these routes could facilitate ester dissociation, and while both are still hypothetical, there is some structural evidence to support the former mechanism (see below).

4.6. Insights from the RPE65 crystal structure

The crystallographic structure of RPE65 has provided important insights into several aspects of RPE65 enzymology as well as the flow of retinoids in the visual cycle (Kiser et al., 2009). The active site of the RPE65, defined by the bound iron cofactor, is found in an interior cavity of the protein. As the hydrophobic retinyl ester substrates are dissolved in the lipid core of the bilayer, the protein must have a mechanism for physically transferring them from the membrane to the active site. The RPE65 structure reveals a single, hydrophobic tunnel that could serve this purpose. As mentioned above, the entrance of this tunnel is surrounded by several hydrophobic residues that are likely integrated into the hydrophobic core of the membrane in order to provide an energetically favorable passageway for retinoids. The retinyl ester could conceivably be orientated in the membrane such that the retinyl or palmitate moiety enters the active site first. Furthermore, it cannot be totally excluded that the retinyl ester enters the tunnel in a bent conformation with the ester moiety entering first, although the tunnel appears to be too small to accommodate such a conformation. A co-crystal structure of RPE65 with a bound retinyl ester is essential to help resolve this substrate orientation ambiguity and will provide definitive evidence that retinyl esters are the true substrates of RPE65. Owing to the presence of a single passageway of suitable width to the active site, it is likely that 11-*cis*-retinol exits the active site through the same tunnel the retinyl ester substrate entered and diffuses back into the membrane for further processing rather than being transferred directly to a retinoid-binding protein (Fig. 5).

The interior cavity of RPE65 is lined primarily by apolar residues as expected based on the hydrophobicity of its retinoid substrates. Interestingly, a large majority of these lining residues are aromatic hydrophobes (Fig. 6). The aromatic side chains of Phe, Tyr and Trp are known to be good stabilizers of carbocation intermediates because of their ability to form cation- π interactions, and similarly lined active sites have been observed for other enzymes that catalyze reactions that transition through carbocation intermediates (Lesburg et al., 1997; Wendt et al., 1997). Thus, RPE65 appears to have an active site that could support the S_N1 mechanism mentioned above. A recent mutagenesis study supports this hypothesis by demonstrating that the ratio of 11 to 13 *cis* retinoids produced by RPE65 can be altered by substitutions of active site residues, including some aromatics (Redmond et al., 2010). Exposed Cys residues that could act as nucleophiles in an S_N2 -type nucleophilic substitution reaction are notably absent in the interior cavity. But there are a few interestingly positioned Tyr residues that may well have a role in the RPE65 catalytic mechanism. There is a hydrogen bonding network involving Tyr 338, Tyr 239, Glu 148 and His 241 that lines part of the putative substrate entry tunnel and the active site cavity near the iron atom. These residues may interact with or be involved in proton shuttling to and from the retinoid substrate during catalysis. Indeed, the requirement of Glu and Tyr residues in positions 148 and 239, respectively, for RPE65 catalytic activity suggests a functional role for this group of residues in catalytic isomerization (Hanein et al., 2004; Redmond et al., 2010). The hydrophobic cavity of RPE65 potentially could be a favorable binding site for non-physiological lipophilic or amphiphilic molecules such as detergents. A detergent molecule was identified in the active site in one crystal form of the RPE65 homolog ACO, suggesting that such binding may also occur with RPE65 (Kloer et al., 2005). This possibility suggests that competitive binding to the RPE65 active site could be a second mechanism, the first being direct disruption of membrane structure, by which detergents inhibit RPE65 isomerase activity.

In addition to the large hydrophobic tunnel described above, there also exists a smaller, narrow tunnel that enters the protein via a surface far removed from the predicted membrane-binding face. Although, the functional significance of this tunnel is not yet clear, it appears to be potentially large enough to allow passage of small molecules such as water. An improved resolution structure of RPE65 revealed well-ordered water molecules in this tunnel and in the interior cavity of the protein suggesting that the waters might have entered via the small passageway (Golczak, 2010). Thus, water molecules used in the retinoid isomerization reaction may gain access to the substrate by this route. Notably, there is a thin tube of poorly defined electron density leading from the narrow tunnel to a portion of the active site near the iron atom that may originate from several mobile water molecules. A Phe side chain (residue 418) appears to cause bending of this electron density and may restrict the approach of water to the substrate in order to promote its nucleophilic attack on the correct carbon atom, i.e. C15 rather than the carbonyl carbon. Thus, it would be interesting to determine the effects of Phe418Trp or Tyr substitutions on retinoid isomerization activity as these mutants, by potentially restricting water access to the substrate, might result in longer lived reaction intermediates and potentially alternative products.

The iron cofactor of RPE65 is directly coordinated, not by a heme moiety but rather by a set of four His residues (residues 180, 241, 313 and 527) arranged in a distorted, partially-filled, octahedral geometry (Fig. 7). Although the His-Fe-His bond angles indicate that the best description of this geometry is octahedral, it could also be described as trigonal bipyramidal (D_{3h} point group symmetry) depending on ligand interactions in the open coordination sites (e.g. mono vs. bidentate binding). A Val residue (residue 134) side chain located 4.9 Å away from the iron may block access of ligands to one of the open iron coordination sites. Of the four His residues, three (residues 241, 313 and 527) individually form hydrogen bonding interactions with one member of a set of three highly conserved Glu residues (residues 148, 417 and 469), which form the second shell of the iron coordination sphere, whereas the His

180 side chain forms a hydrogen bond interaction with a water molecule (Fig. 7). This general mode of iron coordination is virtually identical to that of ACO, another CCO family member (Kloer et al., 2005). Aside from ACO, the four-His iron coordination motif has been observed in only three other proteins, photosynthetic reaction center (PDB code 5PRC), photosystem II (PDB code 1S5L) and 15-lipoxygenase (PDB code 1LOX); however, these three proteins lack second shell Glu residues. Mutagenesis experiments combined with mutations observed in LCA patients have established that the second shell Glu residues are essential for RPE65 isomerase activity and even highly similar residues such as Asp and Gln are unable to functionally substitute for them (Hanein et al., 2004; Nikolaeva et al., 2009a; Redmond et al., 2005; Takahashi et al., 2005). It might be expected that the negatively charged second shell could help strengthen the Fe-His coordinate bonds by partially deprotonating the δ N atoms of the three His residues. However, the four Fe-His bond lengths are identical, within the resolution of the diffraction data, suggesting that this is not the case. Thus, the function of the second shell is probably to help position the inner shell His ligands correctly and fine-tune the electronic properties of the iron atom for catalysis. Interestingly, there are hydrogen bonding interactions between the second shell Glu residues and residues even more distal to the iron atom, namely Tyr 239, Trp 331 and Arg 44 (Fig. 7). Substitutions at Trp 331 and Tyr 239 were detrimental for RPE65 retinoid isomerization activity (Redmond et al., 2010), whereas Arg44Gln substitutions have been found in patients with LCA (Simovich et al., 2001).

The reported structure of native RPE65, as purified, contained electron density, not accounted for by protein atoms, in its active site (Kiser et al., 2009). This electron density occupies a portion of the putative substrate entry tunnel and appears to interact directly with the iron cofactor, possibly filling one or both of the vacancies in its octahedral coordination sphere. Based on its appearance, the density was suggested to represent a bound fatty acid molecule, possibly derived from an all-*trans*-retinyl ester. If this hypothesis is true, it could mean that iron acts directly to bind and polarize the ester moiety thus promoting its dissociation. Interestingly, for three of the proteins mentioned above that contain four-His iron coordination motifs, a carboxylate or bicarbonate ligand occupies a position similar to the carboxylate moiety of the putative fatty acid molecule found in the active site of RPE65. Confirmation of the identity of this electron density in RPE65 will require additional structures, for example containing a bound all-*trans*-retinyl ester molecule.

4.7. The story of RPE65-dependent retinoid isomerization is not over yet: some unexplained observations

Significant progress has been made in understanding the mechanistic basis of retinoid isomerization; however, there are observations concerning the retinoid isomerization reaction that remain to be explained.

The first is the stimulatory effect of phosphate-containing compounds on the isomerization reaction (Stecher and Palczewski, 2000). ATP has been noted to stimulate the isomerization reaction by 3 to 10 fold. Even non-nucleotide phosphate compounds such as pyrophosphate and tripolyphosphate strongly stimulate the reaction. The effect is not limited to high-energy phosphate compounds because phosphate alone also stimulates the reaction. As there is no obvious role for phosphate compounds in the catalytic mechanism, these compounds could stimulate RPE65 isomerase activity through an allosteric mechanism. Alternatively, they may modulate the interaction of RPE65 with membranes and increase the rate of substrate abstraction, for example.

Another intriguing observation is the rapid loss of RPE65 isomerase activity upon exposure to low concentrations (~ 0.1% v/v) of a variety of alcohols, particularly branched-chain, hydrophobic alcohols. This effect was first noted when ethanol was used as a vehicle to deliver all-*trans*-retinol in the retinoid isomerization reaction. Further investigation revealed that

compounds such as isobutanol cause a profound decrease in isomerase activity without dramatically affecting retinyl ester formation by LRAT (Stecher and Palczewski, 2000). These compounds may inhibit RPE65 isomerase activity by binding inside the hydrophobic active site of the enzyme. This possibility could be addressed by further crystallographic studies.

The kinetics of retinoid isomerization has a peculiar feature that has defied a mechanistic explanation. It has been noted *in vitro* that, even in the presence of a large molar excess of retinyl esters, the 11-*cis*-retinol production versus time curve is not linear but rather hyperbolic (Golczak, 2010). An explanation for this effect is that a critical, as of yet uncharacterized, substrate or cofactor on the pathway from all-*trans* to 11-*cis*-retinol is depleted during the reaction. Alternatively, the enzyme may undergo an inactivation process during the reaction in an *in vitro* setting. Given that RPE65 is dependent on a ferrous iron cofactor for activity, the loss of activity with time may be related to iron oxidation to the trivalent state. It would be interesting to determine the oxidation state of the iron before initiation of the reaction as well as after the reaction has plateaued in order to test this possibility.

A final observation that has been noted a number of times by different investigators is the propensity of RPE65 to associate with other visual cycle proteins, in particular the 11-*cis*-RDH, RDH5. As mentioned above, the presence of RPE65 in a protein complex was noted during its initial characterization (Bavik et al., 1991). RPE65 has been observed to form complexes with RDH5, cellular retinaldehyde-binding protein (CRALBP) and retinal G protein-coupled receptor (RGR) (Golczak, 2010; Hemati et al., 2005; Wu et al., 2006). Interaction with RDH5 seems to be the most robust and the RPE65-RDH5 complex can be trapped by crosslinking on native RPE microsomal membranes (Golczak, 2010). Degradation of microsomal phospholipids via PLA₂ treatment disrupts complex formation between RPE65, RDH5 and RGR and significantly impairs RPE65 activity (Golczak, 2010). It remains to be determined whether the loss of RPE65 activity is a result of disruption of the protein complex or from alterations in the membrane structure *per se* or a combination of both.

It has been suggested that RPE65-RDH5 association ensures efficient transfer of retinoids from RPE65 to RDH5 *in vivo*. Lipid soluble retinoids are transported from one cellular compartment to another by retinoid-binding proteins. In addition to this transport function, binding proteins also protect retinoids from chemical and enzymatic oxidation as well as thermal and light-dependent isomerization. With the notable exception of retinoid isomerization, most of the reactions of the retinoid cycle are energetically neutral. Thus, reaction rates are governed by binding affinities and transfer rates between proteins. Formation of protein complexes can accelerate multistep reactions and this concept may be relevant to the RPE65-RDH5 complex. Humans and mice lacking RDH5 exhibit prolonged dark adaptation suggesting an inadequate rate of chromophore production (Cideciyan et al., 2000; Driessen et al., 2000; Yamamoto et al., 1999). Moreover, over-accumulation of 13-*cis*-retinoids was demonstrated in RDH5-null mice (Jang et al., 2001), which could be a result of production of 13-*cis*-retinol by RPE65, which is not efficiently oxidized but is instead esterified and stably stored in retinosomes. The observed metabolic derangements indicate that elucidation of the physiological complexes involving retinoid cycle proteins is requisite for a full understanding of retinoid cycle biochemistry.

5. Summary and Conclusions

As result of a large body of research by several investigators, it now seems that we have a solid general understanding of RPE65-catalyzed retinoid isomerization. In the visual cycle pathway elegantly outlined by George Wald many years ago, this non-photochemical isomerization step was the one that resisted molecular characterization for the longest period of time. Many peculiar features of RPE65 seem to have been responsible for this difficulty, in particular its

exquisite sensitivity to detergents which prevented purification of the enzyme in an active form. It is now clear that interactions of RPE65 with an intact phospholipid bilayer are critical for its enzymatic activity, a fact not appreciated until quite recently. Although the nature of RPE65 association with membranes appears complex, the bulk of experiments suggest that RPE65 is tightly bound to the membrane through direct interactions with the hydrophobic core. However, hydrogen bonding and electrostatic interactions might also contribute to RPE65 membrane affinity. Considered together with the functional requirement for a phospholipid bilayer and the exposed hydrophobic surface near the active site entrance revealed by the crystal structure, we regard RPE65 as an integral monotopic membrane protein with the understanding that it may not bind to membranes as tightly as transmembrane proteins do under some non-physiological conditions.

Although basic RPE65 enzymology is now generally understood, there are still many mysteries regarding the details of the isomerization reaction. The bulk of available data suggests that the reaction proceeds through a carbocation intermediate involving an alkyl-oxygen cleavage step in an S_N1 -type nucleophilic substitution reaction. It is still not clear what the role of iron is in the catalytic mechanism of RPE65, but it is surely remarkable how similar the iron centers of RPE65 and ACO are, even though they are apparently involved in fundamentally different processes. Exploration of the role of iron in RPE65 enzymology should be a fertile area for future research. Given the structural similarities between RPE65 and ACO, a thorough study of the requirement for oxygen in the isomerization reaction is warranted. The recent development of techniques for reconstituting enzymatically active, recombinant RPE65 together with the knowledge gained from the RPE65 crystal structures should greatly facilitate detailed studies related to the structural and enzymatic properties of this protein.

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Abbreviations used

ACO	apocarotenoid oxygenase
CCO	carotenoid cleavage oxygenase
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CRALBP	cellular retinaldehyde-binding protein
C ₈ E ₄	octyltetraoxyethylene
ER	endoplasmic reticulum
GPI	glycosyl phosphatidylinositol
LCA	Leber congenital amaurosis
LRAT	lecithin:retinol acyltransferase
MALDI	matrix-assisted laser desorption/ionization
PBS	phosphate-buffered saline
PLA ₂	phospholipase A ₂
RBP	retinol-binding protein

RDH	retinol dehydrogenase
RGR	retinal G protein-coupled receptor
RPE	retinal pigment epithelium
RPE65	retinal pigment epithelium-specific 65 kDa protein
sER	smooth endoplasmic reticulum
S _N 1	unimolecular nucleophilic substitution
S _N 2	bimolecular nucleophilic substitution
VP14	viviparous 14

References

- Abe I, Prestwich GD. Molecular cloning, characterization, and functional expression of rat oxidosqualene cyclase cDNA. *Proc Natl Acad Sci U S A* 1995;92:9274–9278. [PubMed: 7568116]
- Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*. 4. Garland Science; New York: 2002.
- Balliano G, Viola F, Ceruti M, Cattel L. Characterization and partial purification of squalene-2,3-oxide cyclase from *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 1992;293:122–129. [PubMed: 1731628]
- Barry RJ, Canada FJ, Rando RR. Solubilization and partial purification of retinyl ester synthetase and retinoid isomerase from bovine ocular pigment epithelium. *J Biol Chem* 1989;264:9231–9238. [PubMed: 2722827]
- Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W, Palczewski K. Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. *J Biol Chem* 2004;279:10422–10432. [PubMed: 14684738]
- Bavik CO, Busch C, Eriksson U. Characterization of a plasma retinol-binding protein membrane receptor expressed in the retinal pigment epithelium. *J Biol Chem* 1992;267:23035–23042. [PubMed: 1331074]
- Bavik CO, Eriksson U, Allen RA, Peterson PA. Identification and partial characterization of a retinal pigment epithelial membrane receptor for plasma retinol-binding protein. *J Biol Chem* 1991;266:14978–14985. [PubMed: 1651317]
- Bavik CO, Levy F, Hellman U, Wernstedt C, Eriksson U. The retinal pigment epithelial membrane receptor for plasma retinol-binding protein. Isolation and cDNA cloning of the 63-kDa protein. *J Biol Chem* 1993;268:20540–20546. [PubMed: 8397208]
- Bernstein PS, Law WC, Rando RR. Biochemical characterization of the retinoid isomerase system of the eye. *J Biol Chem* 1987;262:16848–16857. [PubMed: 3500173]
- Bernstein PS, Rando RR. In vivo isomerization of all-trans- to 11-cis-retinoids in the eye occurs at the alcohol oxidation state. *Biochemistry* 1986;25:6473–6478. [PubMed: 3491624]
- Blobel G. Intracellular protein topogenesis. *Proc Natl Acad Sci U S A* 1980;77:1496–1500. [PubMed: 6929499]
- Bok D, Heller J. Transport of retinol from the blood to the retina: an autoradiographic study of the pigment epithelial cell surface receptor for plasma retinol-binding protein. *Exp Eye Res* 1976;22:395–402. [PubMed: 945179]
- Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* 1981;256:1604–1607. [PubMed: 6257680]
- Borowski T, Blomberg MR, Siegbahn PE. Reaction mechanism of apocarotenoid oxygenase (ACO): a DFT study. *Chemistry* 2008;14:2264–2276. [PubMed: 18181127]
- Canada FJ, Law WC, Rando RR, Yamamoto T, Derguini F, Nakanishi K. Substrate specificities and mechanism in the enzymatic processing of vitamin A into 11-cis-retinol. *Biochemistry* 1990;29:9690–9697. [PubMed: 2271609]

- Choo DW, Cheung E, Rando RR. Lack of effect of RPE65 removal on the enzymatic processing of all-trans-retinol into 11-cis-retinol in vitro. *FEBS Lett* 1998;440:195–198. [PubMed: 9862453]
- Cideciyan AV. Leber congenital amaurosis due to *RPE65* mutations and its treatment with gene therapy. *Prog Retin Eye Res*. 2010 in review.
- Cideciyan AV, Haeseleer F, Fariss RN, Aleman TS, Jang GF, Verlinde CL, Marmor MF, Jacobson SG, Palczewski K. Rod and cone visual cycle consequences of a null mutation in the 11-cis-retinol dehydrogenase gene in man. *Vis Neurosci* 2000;17:667–678. [PubMed: 11153648]
- Deigner PS, Law WC, Canada FJ, Rando RR. Membranes as the energy source in the endergonic transformation of vitamin A to 11-cis-retinol. *Science* 1989;244:968–971. [PubMed: 2727688]
- den Hollander AI, Roepman R, Koenekoop RK, Cremers FP. Leber congenital amaurosis: genes, proteins and disease mechanisms. *Prog Retin Eye Res* 2008;27:391–419. [PubMed: 18632300]
- Driessen CA, Winkens HJ, Hoffmann K, Kuhlmann LD, Janssen BP, Van Vugt AH, Van Hooser JP, Wieringa BE, Deutman AF, Palczewski K, Ruether K, Janssen JJ. Disruption of the 11-cis-retinol dehydrogenase gene leads to accumulation of cis-retinols and cis-retinyl esters. *Mol Cell Biol* 2000;20:4275–4287. [PubMed: 10825191]
- Duncan JA, Gilman AG. Autoacylation of G protein alpha subunits. *J Biol Chem* 1996;271:23594–23600. [PubMed: 8798571]
- Duszka C, Grolier P, Azim EM, Alexandre-Gouabau MC, Borel P, Azais-Braesco V. Rat intestinal beta-carotene dioxygenase activity is located primarily in the cytosol of mature jejunal enterocytes. *J Nutr* 1996;126:2550–2556. [PubMed: 8857516]
- Garron LK. The Ultrastructure of the Retinal Pigment Epithelium with Observations on the Choriocapillaris and Bruch's Membrane. *Trans Am Ophthalmol Soc* 1963;61:545–588. [PubMed: 16693628]
- Golczak M, Kiser PD, Lodowski DT, Maeda A, Palczewski K. Importance of membrane structural integrity for RPE65 retinoid isomerization activity. *J Biol Chem*. 2010
- Golczak M, Kuksa V, Maeda T, Moise AR, Palczewski K. Positively charged retinoids are potent and selective inhibitors of the trans-cis isomerization in the retinoid (visual) cycle. *Proc Natl Acad Sci U S A* 2005;102:8162–8167. [PubMed: 15917330]
- Golczak M, Maeda A, Bereta G, Maeda T, Kiser PD, Hunzelmann S, von Lintig J, Blaner WS, Palczewski K. Metabolic basis of visual cycle inhibition by retinoid and nonretinoid compounds in the vertebrate retina. *J Biol Chem* 2008;283:9543–9554. [PubMed: 18195010]
- Gollapalli DR, Maiti P, Rando RR. RPE65 operates in the vertebrate visual cycle by stereospecifically binding all-trans-retinyl esters. *Biochemistry* 2003;42:11824–11830. [PubMed: 14529294]
- Gollapalli DR, Rando RR. All-trans-retinyl esters are the substrates for isomerization in the vertebrate visual cycle. *Biochemistry* 2003;42:5809–5818. [PubMed: 12741839]
- Greaves J, Chamberlain LH. Palmitoylation-dependent protein sorting. *J Cell Biol* 2007;176:249–254. [PubMed: 17242068]
- Gu SM, Thompson DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A, Murthy KR, Rathmann M, Kumaramanickavel G, Denton MJ, Gal A. Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. *Nat Genet* 1997;17:194–197. [PubMed: 9326941]
- Hamel CP, Tsilou E, Harris E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM. A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. *J Neurosci Res* 1993a;34:414–425. [PubMed: 8474143]
- Hamel CP, Tsilou E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM. Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. *J Biol Chem* 1993b;268:15751–15757. [PubMed: 8340400]
- Hanein S, Perrault I, Gerber S, Tanguy G, Barbet F, Ducroq D, Calvas P, Dollfus H, Hamel C, Loppönen T, Munier F, Santos L, Shalev S, Zafeiriou D, Dufier JL, Munnich A, Rozet JM, Kaplan J. Leber congenital amaurosis: comprehensive survey of the genetic heterogeneity, refinement of the clinical definition, and genotype-phenotype correlations as a strategy for molecular diagnosis. *Hum Mutat* 2004;23:306–317. [PubMed: 15024725]
- Heller M, Bok D. A specific receptor for retinol binding protein as detected by the binding of human and bovine retinol binding protein to pigment epithelial cells. *Am J Ophthalmol* 1976;81:93–97. [PubMed: 943148]

- Hemati N, Feathers KL, Chrispell JD, Reed DM, Carlson TJ, Thompson DA. RPE65 surface epitopes, protein interactions, and expression in rod- and cone-dominant species. *Mol Vis* 2005;11:1151–1165. [PubMed: 16379027]
- Imanishi Y, Batten ML, Piston DW, Baehr W, Palczewski K. Noninvasive two-photon imaging reveals retinyl ester storage structures in the eye. *J Cell Biol* 2004;164:373–383. [PubMed: 14745001]
- Jacobson SG, Aleman TS, Cideciyan AV, Heon E, Golczak M, Beltran WA, Sumaroka A, Schwartz SB, Roman AJ, Windsor EA, Wilson JM, Aguirre GD, Stone EM, Palczewski K. Human cone photoreceptor dependence on RPE65 isomerase. *Proc Natl Acad Sci U S A* 2007;104:15123–15128. [PubMed: 17848510]
- Jang GF, McBee JK, Alekseev AM, Haeseleer F, Palczewski K. Stereoisomeric specificity of the retinoid cycle in the vertebrate retina. *J Biol Chem* 2000;275:28128–28138. [PubMed: 10871622]
- Jang GF, Van Hooser JP, Kuksa V, McBee JK, He YG, Janssen JJ, Driessen CA, Palczewski K. Characterization of a dehydrogenase activity responsible for oxidation of 11-cis-retinol in the retinal pigment epithelium of mice with a disrupted RDH5 gene. A model for the human hereditary disease fundus albipunctatus. *J Biol Chem* 2001;276:32456–32465. [PubMed: 11418621]
- Jin M, Li S, Moghrabi WN, Sun H, Travis GH. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell* 2005;122:449–459. [PubMed: 16096063]
- Jin M, Yuan Q, Li S, Travis GH. Role of LRAT on the retinoid isomerase activity and membrane association of Rpe65. *J Biol Chem* 2007;282:20915–20924. [PubMed: 17504753]
- Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, Sun H. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science* 2007;315:820–825. [PubMed: 17255476]
- Kiser PD, Golczak M, Lodowski DT, Chance MR, Palczewski K. Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. *Proc Natl Acad Sci U S A* 2009;106:17325–17330. [PubMed: 19805034]
- Kloer DP, Ruch S, Al-Babili S, Beyer P, Schulz GE. The structure of a retinal-forming carotenoid oxygenase. *Science* 2005;308:267–269. [PubMed: 15821095]
- Kloer DP, Schulz GE. Structural and biological aspects of carotenoid cleavage. *Cell Mol Life Sci* 2006;63:2291–2303. [PubMed: 16909205]
- Law WC, Rando RR. Stereochemical inversion at C-15 accompanies the enzymatic isomerization of all-trans- to 11-cis-retinoids. *Biochemistry* 1988;27:4147–4152. [PubMed: 3261995]
- Law WC, Rando RR, Canonica S, Derguini F, Nakanishi K. The Necessity of an Intact Polyene for the Biological Isomerization of Vitamin-A. *Journal of the American Chemical Society* 1988;110:5915–5917.
- Lesburg CA, Zhai G, Cane DE, Christianson DW. Crystal structure of pentalenene synthase: mechanistic insights on terpenoid cyclization reactions in biology. *Science* 1997;277:1820–1824. [PubMed: 9295272]
- Lindqvist A, Andersson S. Biochemical properties of purified recombinant human beta-carotene 15,15'-monooxygenase. *J Biol Chem* 2002;277:23942–23948. [PubMed: 11960992]
- Lyubarsky AL, Savchenko AB, Morocco SB, Daniele LL, Redmond TM, Pugh EN Jr. Mole quantity of RPE65 and its productivity in the generation of 11-cis-retinal from retinyl esters in the living mouse eye. *Biochemistry* 2005;44:9880–9888. [PubMed: 16026160]
- Ma J, Xu L, Othersen DK, Redmond TM, Crouch RK. Cloning and localization of RPE65 mRNA in salamander cone photoreceptor cells. *Biochim Biophys Acta* 1998;1443:255–261. [PubMed: 9838153]
- Ma J, Zhang J, Othersen KL, Moiseyev G, Ablonczy Z, Redmond TM, Chen Y, Crouch RK. Expression, purification, and MALDI analysis of RPE65. *Invest Ophthalmol Vis Sci* 2001;42:1429–1435. [PubMed: 11381042]
- Maeda A, Maeda T, Golczak M, Imanishi Y, Leahy P, Kubota R, Palczewski K. Effects of potent inhibitors of the retinoid cycle on visual function and photoreceptor protection from light damage in mice. *Mol Pharmacol* 2006;70:1220–1229. [PubMed: 16837623]
- Marcia M, Ermler U, Peng G, Michel H. The structure of Aquifex aeolicus sulfide:quinone oxidoreductase, a basis to understand sulfide detoxification and respiration. *Proc Natl Acad Sci U S A* 2009;106:9625–9630. [PubMed: 19487671]

- Marlhens F, Bareil C, Griffoin JM, Zrenner E, Amalric P, Eliaou C, Liu SY, Harris E, Redmond TM, Arnaud B, Claustres M, Hamel CP. Mutations in RPE65 cause Leber's congenital amaurosis. *Nat Genet* 1997;17:139–141. [PubMed: 9326927]
- Mata NL, Moghrabi WN, Lee JS, Bui TV, Radu RA, Horwitz J, Travis GH. Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. *J Biol Chem* 2004;279:635–643. [PubMed: 14532273]
- Mata NL, Radu RA, Clemmons RC, Travis GH. Isomerization and oxidation of vitamin A in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight. *Neuron* 2002;36:69–80. [PubMed: 12367507]
- Mata NL, Ruiz A, Radu RA, Bui TV, Travis GH. Chicken retinas contain a retinoid isomerase activity that catalyzes the direct conversion of all-trans-retinol to 11-cis-retinol. *Biochemistry* 2005;44:11715–11721. [PubMed: 16128572]
- Matthews RG, Hubbard R, Brown PK, Wald G. Tautomeric Forms of Metarhodopsin. *J Gen Physiol* 1963;47:215–240. [PubMed: 14080814]
- McBee JK, Kuksa V, Alvarez R, de Lera AR, Prezhdo O, Haeseleer F, Sokal I, Palczewski K. Isomerization of all-trans-retinol to cis-retinols in bovine retinal pigment epithelial cells: dependence on the specificity of retinoid-binding proteins. *Biochemistry* 2000;39:11370–11380. [PubMed: 10985782]
- McQuarrie, DA.; Simon, JD. *Physical chemistry: a molecular approach*. University Science Books; Sausalito, Calif: 1997.
- Michel H. Crystallization of Membrane-Proteins. *Trends in Biochemical Sciences* 1983;8:56–59.
- Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma JX. RPE65 is the isomerohydrolase in the retinoid visual cycle. *Proc Natl Acad Sci U S A* 2005;102:12413–12418. [PubMed: 16116091]
- Moiseyev G, Crouch RK, Goletz P, Oatis J Jr, Redmond TM, Ma JX. Retinyl esters are the substrate for isomerohydrolase. *Biochemistry* 2003;42:2229–2238. [PubMed: 12590612]
- Moiseyev G, Takahashi Y, Chen Y, Gentleman S, Redmond TM, Crouch RK, Ma JX. RPE65 is an iron (II)-dependent isomerohydrolase in the retinoid visual cycle. *J Biol Chem* 2006;281:2835–2840. [PubMed: 16319067]
- Muniz A, Betts BS, Trevino AR, Buddavarapu K, Roman R, Ma JX, Tsin AT. Evidence for two retinoid cycles in the cone-dominated chicken eye. *Biochemistry* 2009;48:6854–6863. [PubMed: 19492794]
- Nelson, DL.; Cox, MM.; Lehninger, AL. *Lehninger principles of biochemistry*. 4. W.H. Freeman; New York: 2005.
- Nikolaeva O, Takahashi Y, Moiseyev G, Ma JX. Negative charge of the glutamic acid 417 residue is crucial for isomerohydrolase activity of RPE65. *Biochem Biophys Res Commun*. 2009a
- Nikolaeva O, Takahashi Y, Moiseyev G, Ma JX. Purified RPE65 shows isomerohydrolase activity after reassociation with a phospholipid membrane. *FEBS J* 2009b;276:3020–3030. [PubMed: 19490105]
- Ohlendorf K. Extraction of membrane proteins. *Methods Mol Biol* 1996;59:293–304. [PubMed: 8798208]
- Ostermeier C, Michel H. Crystallization of membrane proteins. *Curr Opin Struct Biol* 1997;7:697–701. [PubMed: 9345629]
- Palczewski K. G protein-coupled receptor rhodopsin. *Annu Rev Biochem* 2006;75:743–767. [PubMed: 16756510]
- Penefsky HS, Tzagoloff A. Extraction of Water-Soluble Enzymes and Proteins from Membranes. *Methods Enzymol* 1971;22:204–219.
- Picot D, Garavito RM. Prostaglandin H synthase: implications for membrane structure. *FEBS Lett* 1994;346:21–25. [PubMed: 8206152]
- Picot D, Loll PJ, Garavito RM. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 1994;367:243–249. [PubMed: 8121489]
- Polans A, Baehr W, Palczewski K. Turned on by Ca²⁺! The physiology and pathology of Ca(2+)-binding proteins in the retina. *Trends Neurosci* 1996;19:547–554. [PubMed: 8961484]
- Qin X, Zeevaert JA. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc Natl Acad Sci U S A* 1999;96:15354–15361. [PubMed: 10611388]

- Qtaishat NM, Redmond TM, Pepperberg DR. Acute radiolabeling of retinoids in eye tissues of normal and rpe65-deficient mice. *Invest Ophthalmol Vis Sci* 2003;44:1435–1446. [PubMed: 12657577]
- Rando RR, Chang A. Studies on the Catalyzed Interconversions of Vitamin-a Derivatives. *Journal of the American Chemical Society* 1983;105:2879–2882.
- Redmond TM, Poliakov E, Kuo S, Chander P, Gentleman S. RPE65, visual cycle retinol isomerase, is not inherently 11-cis-specific: support for a carbocation mechanism of retinol isomerization. *J Biol Chem* 2010;285:1919–1927. [PubMed: 19920137]
- Redmond TM, Poliakov E, Yu S, Tsai JY, Lu Z, Gentleman S. Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. *Proc Natl Acad Sci U S A* 2005;102:13658–13663. [PubMed: 16150724]
- Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet* 1998;20:344–351. [PubMed: 9843205]
- Rodieck, RW. The first steps in seeing. Sinauer Associates; Sunderland, MA: 1998.
- Sagara H, Hirosawa K. Monoclonal antibodies which recognize endoplasmic reticulum in the retinal pigment epithelium. *Exp Eye Res* 1991;53:765–771. [PubMed: 1783014]
- Schwartz SH, Tan BC, Gage DA, Zeevaart JA, McCarty DR. Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* 1997;276:1872–1874. [PubMed: 9188535]
- Simon A, Hellman U, Wernstedt C, Eriksson U. The retinal pigment epithelial-specific 11-cis retinol dehydrogenase belongs to the family of short chain alcohol dehydrogenases. *J Biol Chem* 1995;270:1107–1112. [PubMed: 7836368]
- Simovich MJ, Miller B, Ezzeldin H, Kirkland BT, McLeod G, Fulmer C, Nathans J, Jacobson SG, Pittler SJ. Four novel mutations in the RPE65 gene in patients with Leber congenital amaurosis. *Hum Mutat* 2001;18:164. [PubMed: 11462243]
- Singer SJ. The molecular organization of membranes. *Annu Rev Biochem* 1974;43:805–833. [PubMed: 4277710]
- Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. *Science* 1972;175:720–731. [PubMed: 4333397]
- Smith, M.; March, J. March's advanced organic chemistry: reactions, mechanisms, and structure. 5. Wiley; New York: 2001.
- Stecher H, Gelb MH, Saari JC, Palczewski K. Preferential release of 11-cis-retinol from retinal pigment epithelial cells in the presence of cellular retinaldehyde-binding protein. *J Biol Chem* 1999a; 274:8577–8585. [PubMed: 10085092]
- Stecher H, Palczewski K. Multienzyme analysis of visual cycle. *Methods Enzymol* 2000;316:330–344. [PubMed: 10800685]
- Stecher H, Prezhdo O, Das J, Crouch RK, Palczewski K. Isomerization of all-trans-9- and 13-desmethylretinol by retinal pigment epithelial cells. *Biochemistry* 1999b;38:13542–13550. [PubMed: 10521261]
- Takahashi Y, Moiseyev G, Ablonczy Z, Chen Y, Crouch RK, Ma JX. Identification of a novel palmitoylation site essential for membrane association and isomerohydrolase activity of RPE65. *J Biol Chem* 2009;284:3211–3218. [PubMed: 19049981]
- Takahashi Y, Moiseyev G, Chen Y, Ma JX. Identification of conserved histidines and glutamic acid as key residues for isomerohydrolase activity of RPE65, an enzyme of the visual cycle in the retinal pigment epithelium. *FEBS Lett* 2005;579:5414–5418. [PubMed: 16198348]
- Takahashi Y, Moiseyev G, Chen Y, Ma JX. The roles of three palmitoylation sites of RPE65 in its membrane association and isomerohydrolase activity. *Invest Ophthalmol Vis Sci* 2006;47:5191–5196. [PubMed: 17122102]
- Tan BC, Schwartz SH, Zeevaart JA, McCarty DR. Genetic control of abscisic acid biosynthesis in maize. *Proc Natl Acad Sci U S A* 1997;94:12235–12240. [PubMed: 9342392]
- Tanford C, Reynolds JA. Characterization of membrane proteins in detergent solutions. *Biochim Biophys Acta* 1976;457:133–170. [PubMed: 135582]
- Thoma R, Schulz-Gasch T, D'Arcy B, Benz J, Aepli J, Dehmlow H, Hennig M, Stihle M, Ruf A. Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. *Nature* 2004;432:118–122. [PubMed: 15525992]

- Travis GH, Golczak M, Moise AR, Palczewski K. Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. *Annu Rev Pharmacol Toxicol* 2007;47:469–512. [PubMed: 16968212]
- Trudel E, Beaufils S, Renault A, Breton R, Salesse C. Binding of RPE65 fragments to lipid monolayers and identification of its partners by glutathione S-transferase pull-down assays. *Biochemistry* 2006;45:3337–3347. [PubMed: 16519528]
- Tsilou E, Hamel CP, Yu S, Redmond TM. RPE65, the major retinal pigment epithelium microsomal membrane protein, associates with phospholipid liposomes. *Arch Biochem Biophys* 1997;346:21–27. [PubMed: 9328280]
- Wald G. Molecular basis of visual excitation. *Science* 1968;162:230–239. [PubMed: 4877437]
- Wald G, Brown PK. The molar extinction of rhodopsin. *J Gen Physiol* 1953;37:189–200. [PubMed: 13109155]
- Wang JS, Estevez ME, Cornwall MC, Kefalov VJ. Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat Neurosci* 2009;12:295–302. [PubMed: 19182795]
- Wendt KU, Lenhart A, Schulz GE. The structure of the membrane protein squalene-hopene cyclase at 2.0 Å resolution. *J Mol Biol* 1999;286:175–187. [PubMed: 9931258]
- Wendt KU, Poralla K, Schulz GE. Structure and function of a squalene cyclase. *Science* 1997;277:1811–1815. [PubMed: 9295270]
- Winston A, Rando RR. Regulation of isomerohydrolase activity in the visual cycle. *Biochemistry* 1998;37:2044–2050. [PubMed: 9485331]
- Wu Z, Bhattacharya SK, Jin Z, Bonilha VL, Liu T, Nawrot M, Teller DC, Saari JC, Crabb JW. CRALBP ligand and protein interactions. *Adv Exp Med Biol* 2006;572:477–483. [PubMed: 17249612]
- Xue L, Gollapalli DR, Maiti P, Jahng WJ, Rando RR. A palmitoylation switch mechanism in the regulation of the visual cycle. *Cell* 2004;117:761–771. [PubMed: 15186777]
- Xue L, Jahng WJ, Gollapalli D, Rando RR. Palmitoyl transferase activity of lecithin retinol acyl transferase. *Biochemistry* 2006;45:10710–10718. [PubMed: 16939223]
- Yamamoto H, Simon A, Eriksson U, Harris E, Berson EL, Dryja TP. Mutations in the gene encoding 11-cis retinol dehydrogenase cause delayed dark adaptation and fundus albipunctatus. *Nat Genet* 1999;22:188–191. [PubMed: 10369264]
- Yuan Q, Kaylor JJ, Miu A, Bassilian S, Whitelegge JP, Travis GH. Rpe65 isomerase associates with membranes through an electrostatic interaction with acidic phospholipid headgroups. *J Biol Chem* 2010;285:988–999. [PubMed: 19892706]
- Zhang Y, Cherney MM, Solomonson M, Liu J, James MN, Weiner JH. Preliminary X-ray crystallographic analysis of sulfide:quinone oxidoreductase from *Acidithiobacillus ferrooxidans*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2009;65:839–842.
- Znoiko SL, Crouch RK, Moiseyev G, Ma JX. Identification of the RPE65 protein in mammalian cone photoreceptors. *Invest Ophthalmol Vis Sci* 2002;43:1604–1609. [PubMed: 11980880]

Figure 1.

Schematic representation of the retinoid (visual) cycle - Vision begins when light ($h\nu$) causes photoisomerization of the 11-*cis*-retinylidene chromophore of ground-state rhodopsin. Subsequently, the Schiff base linkage loses a proton enabling rhodopsin to activate G proteins (i). After remaining active for a short period of time, the isomerized chromophore is released via hydrolysis, generating free all-*trans*-retinal and opsin (ii). The all-*trans*-retinal is enzymatically reduced (iii) and the resultant all-*trans*-retinol is exported from the rod outer segment to the RPE. Here all-*trans*-retinol is metabolized by LRAT to produce all-*trans*-retinyl esters (iv), which can be either stored in retinosomes or further processed. RPE65 is the key enzyme that catalyzes the conversion of all-*trans*-retinyl esters to 11-*cis*-retinol (v). 11-*cis*-Retinol is enzymatically reduced to 11-*cis*-retinal (vi), which is then transported back to the photoreceptor outer segment where it recombines with opsin to form ground-state rhodopsin (vii). Continuous operation of this cycle is what sustains vision under conditions where rods are primarily active.

Figure 2.

Potential mechanisms of RPE65 membrane association - Four potential modes of RPE65-membrane interactions have been proposed based on extraction experiments and other biochemical and biophysical studies. **A)** Anchoring via electrostatic interactions between RPE65 side chains and the charged headgroups of phospholipids. Blue circles indicate positively charged residues and red circles indicate negatively charged phospholipid moieties. This mode of interaction was proposed based on the results of high-salt and carbonate extraction experiments. **B)** Anchoring via covalently attached S-palmitoyl groups. Green circles and lines indicate the cysteine and palmitate moieties, respectively. This mode of interaction was proposed on the basis of mass spectrometry experiments. **C)** Anchoring via direct interactions between hydrophobic side chains (colored brown) and the lipid matrix. The mode of interaction is supported by detergent extraction and phase separation experiments as well as structural and enzymological observations. **D)** Attachment via interactions with other membrane-bound proteins. The observation that RPE65 appears to form complexes with other RPE microsomal proteins might suggest this mode of interaction. A hypothetical transmembrane protein is shown colored orange. Further details and considerations are discussed in the text

Figure 3.

Crystallographic structure of bovine RPE65 - **A)** The chain adopts a seven bladed beta propeller fold with one face covered by a helical cap. The iron cofactor, shown as an orange sphere, is directly coordinated by four histidine residues that are conserved amongst members of the CCO family. The red dashed line indicates a portion of the chain (residues 109–126) that was unresolved in the final electron density maps. The putative membrane-binding surface is facing down in this panel with the horizontal, black dashed line indicating the hypothetical interface between headgroup and core regions of the membrane. **B)** Stereoview of the putative membrane-binding surface of RPE65 - The portion of the protein structure facing down in panel A has been rotated 90 degrees around the horizontal axis and is now projects towards the viewer. Segments of the chain that surround the entrance to the active site are enriched in residues that are favored in interfacial (Arg, Lys, Ser, Trp and Tyr) and lipid core regions (Phe, Leu and Ile) of the membrane. Therefore, these regions probably interact extensively with the membrane including the lipid core, consistent with the need for this enzyme to assess membrane-dissolved retinyl esters. The view is approximately down the substrate entry/product exit tunnel with the iron cofactor shown as an orange sphere. The two green spheres indicate the positions of Phe 108 and Glu 127, which flank the unresolved region of the RPE65 chain.

Figure 4.

Proposed catalytic mechanisms of retinoid isomerization - Two mechanisms for retinoid isomerization have been proposed that account for the experimentally observed loss of the C15-bound oxygen from the all-*trans*-retinyl ester during enzymatic processing. **A)** A retinoid molecule showing the standard numbering scheme. R indicates a saturated hydrocarbon chain. **B)** In this dual S_N2 -type nucleophilic substitution mechanism a protein associated nucleophile (X) (e.g. a Cys side chain sulfur atom) attacks C11 (i) resulting in the loss of the ester moiety via alkyl-oxygen cleavage and the formation of an intermediate (ii) with a freely rotatable C11-C12 single bond. In the second step (iii) another nucleophile, presumably a hydroxide ion, attacks C15 when the intermediate is in a *cis*-like conformation, resulting in loss of the C11-bound protein group and restoration of the conjugated double bond system. **C)** In this S_N1 -type nucleophilic substitution mechanism, the rate-limiting step, i.e. loss of the ester via alkyl-oxygen cleavage, is catalyzed by a Lewis acid (X^+) such as a metal ion or a proton (i). Dissociation of the ester (ii) and the generation of a resonance-stabilized carbocation (iii) lowers the polyene chain carbon-carbon bond order and the activation energy needed for *trans* to *cis* isomerization. After rotation to a *cis*-like conformation, a water molecule, which is either concomitantly or subsequently deprotonated by a base (B), attacks C15, restoring the conjugated double bond system in the 11-*cis* configuration (iv). Dissociation of the ester leaving group regenerates the Lewis acid catalyst (v). In both mechanisms the thermodynamic energy needed for the endergonic isomerization reaction may derive from the ester hydrolysis or from a downstream process such as interaction of the 11-*cis* products with retinoid-binding proteins.

Figure 5.

Proposed flow of retinoids within the RPE - In this scheme, all-*trans*-retinol from the rod outer segments, bound by cellular retinol-binding protein in the RPE cytosol, is presented to LRAT for esterification. The resulting esters diffuse through the membrane to RPE65, which catalytically isomerizes them to 11-*cis*-retinol. Based on the presence of only one suitable tunnel for retinoid passage observed in the RPE65 structure, we propose that the 11-*cis*-retinol product reenters the membrane and diffuses to RDH5. The 11-*cis*-retinal product is taken up either from the membrane or directly from RDH5 by CRALBP and transported to the RPE plasma membrane where it is released from the RPE cell and is transported back to the rod outer segment by an incompletely understood process.

Figure 6.

Stereoview of the RPE65 interior cavity - The internal cavity of RPE65 housing the iron cofactor is lined with several aromatic residues possibly involved in stabilizing a carbocation intermediate of the retinoid isomerization reaction. The blue mesh delineates the boundaries of this cavity. The carbon atoms of Trp, Tyr and Phe residues are colored orange and the iron cofactor is displayed as an orange sphere.

Figure 7.

Stereoview of the RPE65 iron center and surrounding residues - The iron inner coordination sphere consists of four histidine residues arranged in a distorted octahedral geometry. One or two of the remaining sites in the octahedral geometry are filled by non-protein electron density of uncertain origin (not shown but discussed in the text). The Val 134 side chain may block access to one of the iron coordination sites. His residues 241, 313 and 527 hydrogen bond with Glu residues 148, 417 and 469, respectively. Additionally, there are hydrogen bonding interactions between these Glu residues and Tyr 239, Trp 331 and Arg 44, respectively. With the exception of Val 134, all of these residues are known to be critical for RPE65 activity based on data from either from *in vitro* isomerase activity assays or LCA patients with *RPE65* mutations. This arrangement suggests a highly tuned metal-binding site that is extremely susceptible to perturbation by amino acid substitutions. The iron cofactor and a water molecule are shown as brown and red spheres, respectively. Dashed lines indicate interactions, either hydrogen bonds or coordinate bonds, between atoms.